THE EFFECT OF PATERNAL HEAT STRESS ON THE

DEVELOPMENT OF PREIMPLANTATION EMBRYOS

IN THE MOUSE

By

Bi-ke ZHU

(B.Ag. Sci. and M. Ag. Sci. Univ. North-Western Agriculture, CHINA)

A Thesis Submitted to the University of Adelaide in Fulfilment of

the Requirements for the Degree of Doctor of Philosophy

Department of Animal Science

The University of Adelaide

Australia

July 2000

Table of Contents

Chapter 1   Literature Review

iv

Chapter 4 The Effect of Paternal Heat Stress on Development In Vitro of Preimplantation Embryos in the Mouse

Chapter 5 Effects of Paternal Heat Stress on the Development In Vivo of Preimplantation Embryos in the Mouse

Chapter 6   Protein Profiles of Control-sired and Heat-sired Preimplantation Embryos

Abstract

Paternal heat stress has been reported not only to damage spermatogenesis, endocrine and biochemical functions of testicular tissues, but also to affect the development of preimplantation, implanting and post-implantation embryos in both animals and humans (Setchell, 1998). However, the relationship between the development of preimplantation embryos and the extent of damage of germ cells during heating has not been considered in detail and the molecular mechanisms underlying the effect of paternal heat stress on the development of embryos have not been investigated. These issues form the focus for the studies presented in this thesis. All work was undertaken in mice, using F1 C57/CBA progeny.

Preliminary investigations were undertaken to examine the effects of three media (CZB, KSOM and HCO_3HTF), volume and type of oil, and frequency of observation on the development in vitro of mouse zygotes in order to determine an optimised in vitro culture system. The results showed that a CZB medium was better than KSOM and HCO_3HTF media; the volume of paraffin oil applied had the potential to affect the development of preimplantation embryos depending on the type of culture media; paraffin oil was better than mineral oil in protecting zygotes in culture and finally, repeated observation (every 24h) did not affect the development of embryos to the blastocyst stage.

To investigate the effects of paternal heat stress on the development in vitro of preimplantation embryos in the mouse, normal female mice were superovulated using PMSG/hCG and mated either to an untreated (control) male mouse or to one that had been exposed for 24h to an ambient temperature of $36° \pm 0.1°C$ and $62 \pm 0.4\%$ relative

humidity, between 3 and 42 days previously. Putative zygotes were collected from the oviducts of mated mice, 25-28h after hCG injection, and cultured in vitro. Embryo development was evaluated at 24h intervals for up to 120h. Paternal heat stress accelerated the development of some zygotes, but retarded the developmental capacity of others during the initial 24h of culture and also of later stages during 48h-120h of culture. The proportion of zygotes that developed normally during 24-120h was significantly reduced by paternal heat stress of sires 7-35 days before mating. Development was comparable to control levels when sired by males at days 3 or 42 after heating. Maximum impairment to development occurred in embryos sired by males at days 14 and 21 after heating. The number of non-developing, abnormal and dead embryos significantly increased by day 7, and reached the highest levels by days 14 and 21, then recovered to the control levels by day 42 after heating. Furthermore, whilst all stages of embryo development were affected by paternal heat stress, the development of embryos from the zygote to 2-cell stage appeared to be most severely affected. Consequently, 4-cell to morula stages and morula to blastocyst stages also demonstrated substantial impairment.

To examine the effect of paternal heat stress on the development in vivo of embryos, synchronized female mice were mated either to a control male mouse or to one that had been exposed at 7, 21 or 35 days previously, for 24 h to an ambient temperature of 36 ± 0.3°C and 66 ± 5.6% relative humidity. Embryos were collected from the oviducts of mice at 14 to 16h, 34 to 39h or 61 to 65h after mating or from the uterus at 85 to 90h after mating. Morphology and stage of development of embryos was examined visually and the number of cells within blastocysts was also determined using bisbenzimide-propidium iodide staining. Paternal heat stress retarded the development of embryos. Paternal heat stress 7 days before mating reduced the proportions of embryos developing to 8-cell to

morulae, hatched blastocysts, and total blastocysts. The number of inner cell mass (ICM) and trophectoderm (TE) cells within blastocysts was also reduced. Paternal heat stress 21 days before mating reduced the proportion of 2-cell and 8-cell to morula embryos with no embryos developing to blastocysts. There were also increases in the number of zygotes and abnormal embryos. Paternal heat stress 35 days before mating decreased the proportion of 2-cell embryos, expanded blastocysts and also reduced the number of ICM and TE cells in the blastocysts.

To explore the effect of paternal heat stress on protein patterns of embryos, superovulated mice were caged with a control male mouse or one that had been exposed at 7, 14 or 21 days previously for 24h to an ambient temperature of $36 \pm 0.3°C$ and $62 \pm 2.7\%$ relative humidity. To investigate protein patterns, 2-cell embryos and morulae of zygotes, sired 7, 14 and 21 days after heating of the male were collected, 20 and 64h after culture in vitro in CZB medium and processed for one-dimensional gel electrophoresis and silver staining. The results did not suggest any significant differences in protein profiles between control- and heat-sired 2-cell embryos and between control- and heat-sired morulae. Following that, to study protein synthesis, two-cell embryos, 4-cell embryos, morulae or blastocysts of zygotes, sired 7, 14 and 21 days after heating of the male were collected, 18, 39, 63 or 87h respectively after culture in vitro in CZB medium, and then cultured for 3h in 50μl CZB medium containing 200 μCi/ml of $[^{35}S]$-methionine. Embryos were washed three times and processed for one-dimensional gel electrophoresis and autoradiography. No significant differences in protein synthetic patterns were detected for embryos sired 7 or 14 days after paternal heat stress, but heat stress 21 days before mating did significantly alter protein patterns of 2-cell and abnormal embryos. To extend these investigations, 2-cell embryos and morulae of zygotes, sired 21 days after heating of the male were collected, 18 and 63h

after culture in vitro in CZB medium, then cultured for 3h in 50μl CZB containing 500μCi/ml of [^{35}S]-methionine, and finally processed for two-dimensional gel electrophoresis and phosphoimaging. The results indicated that paternal heat stress significantly influenced protein profiles of 2-cell embryos and morulae. A significant number of proteins were not synthesized in heat-sired embryos at this stage, and others ran at a different pI to that observed in control-sired embryos.

The present results demonstrate that a single episode of paternal heat stress as little as 7 days before mating significantly reduces the development of preimplantation embryos, but this can be recovered by day 42 after heating. Paternal heat stress 21 days prior to mating has the most significant impact on retarding the development of 2-cell embryos and morulae, and this is associated with significant changes in the patterns of protein synthesis by these embryos. These results also support previous work suggesting that sperm from the epididymis as well as germ cells in the testis are susceptible to damage from heat stress, with both spermatids and spermatocytes being most the vulnerable germ cells within the testis.

On the basis of the work presented in this thesis, it is proposed that the model used can now be applied to further investigate the molecular mechanisms underlying the effects of paternal heat stress on the development of preimplantation embryos. More detailed investigation of the changes identified in specific proteins, and the genes regulating the expression of these proteins are clearly warranted.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Bi-ke Zhu.

Acknowledgement

Preface

Some aspects of the work presented in this thesis have been reported elsewhere.

Abstracts

Zhu, B; Walker, SK; Setchell, BP and Maddocks, S (1999) The effect of paternal heat stress on development in vitro of preimplantation embryos in the mouse. Proc. Aust. Soc. Reprod. Biol., **31**, 62.

Zhu, B; Setchell, BP and Maddocks, S (2000) Heat-induced paternal effects on embryo development in vivo and in vitro in mice. Abstracts of The First European Congress of Andrology, Italy, Int. J. Androl., **23** (suppl 1), 39.

Yaeram, J; Zhu, B; Setchell, BP and Maddocks, S (2000) Whole body heat-stress of male mice impairs paternal fertility and embryo development in normal females. The International Congress on Animal Reproduction, Stockholm, Sweden.

Papers

Zhu, B; Walker, SK; Oakey, H; Setchell, BP and Maddocks, S (2000) The effect of paternal heat stress on development in vitro of preimplantation embryos in the mouse. Reproduction, Submitted.

Chapter One

Literature Review

This review will focus mainly on fertilization, the development of preimplantation embryos and implantation in the mouse, but with reference also to other animals and humans. The effects of heat-stress on male reproduction will be also reviewed.

1.1 Fertilization

1.1.1 Introduction

Fertilization is a complex process whereby haploid male and female gametes (the sperm and ovum, respectively) unite to form a diploid zygote with the potential to produce a complete individual. It includes the following processes: 1) the maturation of the sperm and the ovum before fertilization; 2) the encounter of the sperm and the ovum; 3) the penetration of the ovum by the sperm; 4) the formation of the male pronucleus (from the sperm) and the female pronucleus (from the ovum); 5) the growth and development of the pronuclei; 6) the breakdown of nuclear envelopes and finally, 7) the union and pairing up of the two chromosome groups and the production of a zygote. The essential feature of fertilization lies in the mingling of paternal and maternal chromosomes, which will constitute the genetic material of the new individual and restore the diploid chromosome number.

Mammalian fertilization has been studied in the laboratory for more than a century. The many steps that lead to the union of male and female gametes have been examined in greater detail more recently with the introduction of experimental protocols such as in vitro fertilization and molecular biology. However, compared with in vivo fertilization, in vitro fertilization can result in high incidences of polyspermy (20%—84%) in the pig (Niwa, 1993), a longer gestation period, an increased incidence of abortions and a higher perinatal

mortality in cattle and sheep (Behboodi et al., 1995; Hasler et al., 1995; Sinclair et al., 1995; Kruip and den Dass, 1997), and low birth weights in humans (MRC, 1990). This suggests that there are some mechanisms operating in normal in vivo fertilization that are not so well supported with in vitro procedures, but many of these processes remain unknown. Therefore, this section will concentrate on recent progress in understanding the main processes involved in mouse fertilization, including the transport of male gametes in the female tract, sperm capacitation and the acrosome reaction, activation of the ovum and the formation of pronuclei.

1.1.2 the transport of male gametes in the female reproductive tract

Mouse semen is generally deposited in the uterus (Zamboni, 1972). The sperm are transported to the site of fertilization (lower ampulla) by their own motility and by contractions of the myometrium while seminal, uterine and oviductal fluids are also important for sperm transport in the female reproductive tract.

The distribution of mouse sperm varies within sites of the female reproductive tract. The average number of sperm per ejaculate is 50 million, but the number of sperm in the ampulla of the oviduct is below 100 (Harper, 1994). The reasons for this are discussed below: 1) the establishment of sperm reservoirs at the uterotubal junction and within the isthmus significantly influences the population of sperm at the site of fertilization. In the lower isthmus, the sperm are stored by two possible mechanisms, namely, immobilization and adherence of sperm to the epithelial walls (Suarez, 1987); 2) the female reproductive tract might also actively select sperm reaching the site of fertilization. Excluded sperm are not necessarily non-fertilizing, but are probably less able to negotiate the female reproductive tract. One example of selection is the exclusion of presumed diploid sperm

2

from the mouse oviduct (Krzanowska, 1974) resulting in very few such sperm fertilising ova in vivo. However, diploid mouse sperm appear to be very fertile in vitro (Maudlin and Fraser, 1978) and amount to approximately 1% of fertilising sperm (Fraser and Maudlin, 1979); 3) Most "vanguard" sperm are dead and most pass into the peritoneal cavity; 4) Endocrine changes could regulate sperm transport. In an ovulatory cycle, Graafian follicles secrete increasing concentrations of oestradiol as they undergo growth and maturation. High levels of oestradiol lead to oedema of the mucosa and increased tonicity of the reproductive tract, resulting in partial promotion of α-adrenergic receptors in the autonomic nervous system (Hunter, 1988). As a consequence, the patency of the isthmus is reduced, thereby impeding sperm migration. On the other hand, during the interval between the luteining hormone (LH) surge and ovulation, steroid secretion shifts critically from oestradiol to progesterone predominance (Short, 1972). In addition, the concentrations of follicular relaxin and prostaglandins of the E series increase in the reproductive tract. An overall consequence of these specific endocrine changes is a reduced tonicity and an increased patency of the isthmus, thus increasing migration of sperm.

The release of sperm from the isthmic reservoirs into the site of fertilization could be triggered by the arrival of ova, their immediate investments and/or follicular fluid. A more challenging question is how the live sperm/ova ratio can be 1:1 or below 1 at the site of fertilization (Zamboni, 1972; Hunter, 1996). Three levels of control over the number of sperm activated and released from the functional reservoir in the caudal region of the isthmus have been proposed. Firstly, a countercurrent transfer of ovarian follicular progesterone from the ovarian vein into the tubal branch of the ovarian artery could be involved (Hunter, 1996). The concentration of progesterone so transferred would be proportional to the number of preovulatory follicles and thus to the number of ova to be shed and would act progressively to reduce sperm binding to the endosalpinx of the caudal

isthmus. Differential timing of the release of sperm from epithelial binding may be a crucial means of achieving the initial low sperm/ova ratio. The second control mechanism could be by means of molecular messages derived from the mucified oocyte-cumulus complex shortly before and after the time of ovulation. Third, reorientation of sperm trajectories by molecular gradients within the cumulus cell mass may direct competent sperm to those oocytes as yet unpenetrated (Hunter, 1996). Together these differing levels of control would impose low sperm/ova ratios during the initial stages of fertilization, thus probably preventing polyspermy fertilization.

1.1.3 Sperm capacitation

Although sperm that have matured in the epididymis are capable of moving actively, they do not have the immediate ability to fertilise ova since these sperm can not be capacitated. Sperm capacitation involves physiological and biochemical (functional) changes that render the sperm competent to fertilise after residing in the female tract or in vitro medium for some period of time.

1.1.3.1 The time of sperm capacitation

The accurate time for sperm capacitation is not completely known. However, based on the time of penetration of a spermatozoon into an ovum, the capacitated times reported for several species are shown in Table 1.1.

The time of sperm capacitation in the female reproductive tract varies according to the species. This could be related to different sites (vagina or uterus) of semen deposition, the

4

time of transport and the time between insemination and ovulation in the female reproductive tract.

Table 1.1 The time of sperm capacitation in the female tract

Species	Sites of semen deposition*	Time of capacitation (h)	Authors	Year
Rabbit	Vagina	5 - 6	Chang	1955
			Adams and Chang	1962
Rat	Uterus	2 - 3	Austin and Braden	1956
			Noyes	1953
Mouse	Uterus	1 - 2	Whittingham	1968
			Iwamatsu and Chang	1969
Hamster	Uterus	2 - 3	Strauss	1956
			Chang and Sheaffer	1957
Ferret	Uterus	3.5	Chang and Yanagimachi	1963
Pig	Cervix and uterus	2	Hunter and Dziuk	1968
Sheep	Vagina	1.5	Mattner	1963
Cattle	Vagina	6 - 7	Austin	1969
Monkey	Vagina	3	Marston and Kelly	1968
Man	Vagina	6 - 31	Edwards et al.	1969
			Bavister et al.	1969

* From Anderson (1977).

In addition to the uterus and the fallopian tube, capacitation can also occur in sites such as the anterior chamber of the eye, the colon and the bladder (Noyes et al., 1958), the isolated vagina (Bedford, 1970) and oestrous uteri from other species (Bedford and Shalkovsky, 1967; Hamner and Sojka, 1967).

1.1.3.2 Mechanism of sperm capacitation

Sperm capacitation has been shown to be correlated with changes in sperm plasma membrane fluidity, intracellular ion concentrations, metabolism and motility (Yanagimachi, 1994). Cellular and molecular events during capacitation are summarised in Fig 1.1.
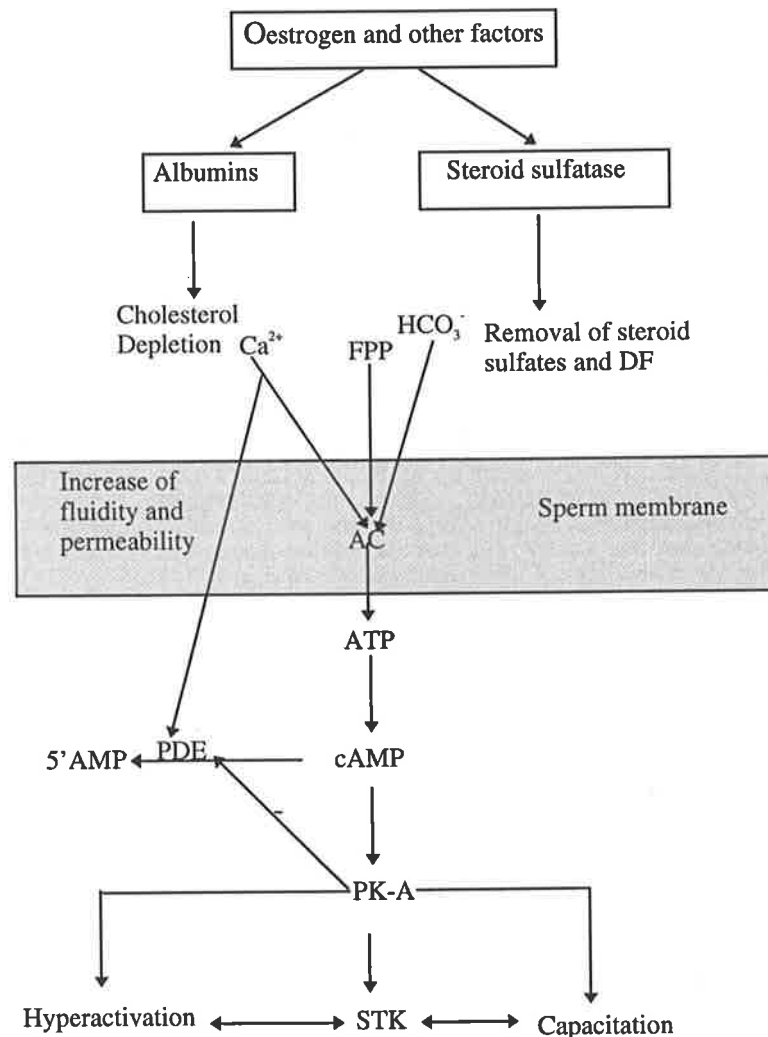
Fig 1.1 A possible mechanism of mouse sperm capacitation

(1) Oestrogen and other factors in the female reproductive tract result in an increase of both albumin and steroid sulfatases in the reproductive tract; (2) An increase of albumins leads to the loss of cholesterol from the membrane, while an increase of steroid sulfatase results in the removal of steroid sulfates and DF from the sperm plasma membrane. A total consequence of these activities leads to changes of fluidity and permeability of the plasma membrane, thus giving rise to increases in Ca^{2+} and HCO_3^- concentrations; (3) The increase in Ca^{2+} and HCO_3^- permeability and the action of FPP alter the cAMP metabolism. This could occur by the Ca^{2+} and or HCO_3^- and FPP inducing activation of adenyl cyclase (AC), resulting in an increase in intracellular cAMP. Ca^{2+} could also alter the hydrolysis of cAMP by stimulating the activity of sperm tyrosine kinase(s) (STK) and/or inactivation of phosphoprotein phosphatases, the net results being an increase in protein tyrosine phosphorylation events leading to capacitation and/or hyperactivation of motility are initiated.

(Visconti et al., 1995a,b; Adeoya-Osiguwa and Fraser, 1996; Harrison et al., 1996; Green et al., 1996).

6

During their epididymal transit, sperm absorb sialoglycoproteins (Gordon et al., 1974; Nicolson et al., 1977), sulphoglycerolipids (Ishizuka et al., 1973; Farooqui, 1978; Kornblatt, 1979), sulfates (Lalumiere et al., 1976; Legault et al., 1979a,b; Langlais et al., 1981; Bouthillier et al., 1984) and decapacitation factor (DF). These compounds are mainly localized on the plasma membrane overlying the acrosome (Langlais et al., 1981) and possess membrane-stabilizing properties. In addition, sterol sulfates inhibit in vitro sperm capacitation and fertilization in the hamster (Bleau et al., 1975) and decrease the pregnancy rate of rabbits (Burck et al., 1982).

Steroid sulfatase in the fallopian tube, endometrium and Graafian follicles of the human (Lalumiere et al., 1976) and in hamster cumulus cells (Langlais and Roberts, 1985) or the oocyte-corona-cumulus complex can stimulate sperm capacitation in mouse (Gwatkin et al., 1974), hamster (Gwatkin et al., 1972; Bavister, 1982) and human (Tarlatzis et al., 1984). Surface charge modification and a loss of sterol sulfates may result in the redistribution of attractive and repulsive forces between polar head groups of membrane lipids, the enhancement of lateral diffusion of lipids and alteration of lipid boundaries and membrane domains.

During sperm capacitation, membrane cholesterol depletion and a concomitant decrease in the cholesterol to phospholipid molar ratio (c/p) occur (Davis et al., 1979, 1980; Davis and Gergely, 1979; Davis, 1978, 1981; Langlais et al., 1981). Cholesterol has been known to bind to specific proteins in the plasma membrane (Levy, 1981). Exchange rates between the cell membranes and the extracellular compartment are mediated by serum levels of lipoproteins i.e. the high-density lipoproteins (HDL), the low-density lipoproteins (LDL) and the very-low-density lipoproteins (VLDL), apoliprotein-A-1 (ApoA-1), albumin and by lecithin:cholesterol acyltransferase (LCAT) activity (Van Deenen and Degier, 1974). The

7

concentration and distribution of lipids bound to lipoproteins in plasma are modified by gonadal hormones (Russ et al., 1955). Oestrogen increases circulating levels of HDL (Anderson et al., 1978), plasma levels of ApoA-1 (Schaefer et al., 1983) which is acceptors for cellular cholesterol (Fielding and Fielding, 1981; Oram et al., 1981) stimulates LCAT activity (Yokoyama et al., 1980) and LCAT receptors (Chao et al., 1979, 1981). Moreover, oestrogen can stimulate maximal uterine levels of albumin (Beier, 1974), cause a decrease in the c/p ratio of unfractionated plasma (Russ et al., 1955) and stimulate cholesterol efflux from cell membrane. In addition, cholesterol exchange rates increase in the presence of serum or albumin (Fogelman et al., 1977) because cholesterol acceptors are present in uterine, oviductal and follicular fluids (Davis, 1981; Langlais, et al.,1981).

In summary, sulfatase in the reproductive tract may remove steroid sulfates on sperm plasma membrane; oestrogen increases uterine levels of albumin, levels of HDL and ApoA-1 and stimulates LCAT activity, which in turn depletes the amount of the cholesterol on sperm plasma membrane. These changes may result in redistribution of phospholipid-proteins on sperm plasma membrane, change sperm plasma membrane fluidity and increase membrane permeability to ions.

There is still some dispute concerning the changes in intracellular Ca^{2+} during sperm capacitation. Early experiments suggested that Ca^{2+} is required only at the end of the capacitation (Yanagimachi and Usui, 1974). However, current evidence suggests that all capacitation processes involve a rise in intracellular Ca^{2+} concentration (White and Aitken, 1989; Fraser and McDermott, 1992; DasGupta et al., 1993; Visconti et al., 1995a). How does intracellular Ca^{2+} rise? Decapacitation factor (DF) present on the sperm plasma membrane stimulates the endogenous activity of calmodulin-regulated Ca^{2+}-ATPase which is present in the head membrane of mouse sperm (Adeoya-Osiguwa and Fraser, 1996) and

tail membranes of boar (Ashraf et al, 1982a,b), buffalo (Sidhu and Guraya, 1989), guinea pig (Gordon et al., 1983) and ram sperm (Bradley and Forrester, 1980). Ca^{2+}-ATPase actively maintains a low intracellular Ca^{2+}. Sperm transport is associated with inactivation of DF, resulting in reduced activity of Ca^{2+}-ATPase. This allows Ca^{2+} within the sperm head to rise modestly (Adeoya-Osiguwa and Fraser, 1996). The rise of intracellular Ca^{2+} induces activation of adenyl cyclase (AC), resulting in an increase in intracellular cAMP. Simultaneously, Ca^{2+} can also alter the hydrolysis of cAMP by stimulating the activity of cyclic nucleotide phosphodiesterase(s) (PED). The increase in cAMP then results in the activation of protein kinase A (PK-A) which leads to the activation of sperm tyrosine kinase(s) (STK) and/or inactivation of phosphoprotein phosphorylases. As a consequence of an increase in protein tyrosine phosphorylation, events leading to capacitation and/or hyperactivation of motility are initiated (Visconti et al., 1995a,b).

The concentration of intracellular HCO_3^- also changes during capacitation. Studies have shown that sperm capacitation (in vivo or in vitro) requires a rise of intracellular HCO_3^- (Lee and Storey, 1986; Neill and Olds-Clarke, 1987; Boatman and Robbins, 1991; Visconti et al., 1995a,b). The rise of intracellular HCO_3^- may alter cAMP metabolism, resulting in protein tyrosine phosphorylation and capacitation (Visconti et al., 1995a,b). A recent study showed that bicarbonate causes a major, rapid and reversible alteration in sperm plasma membrane lipid architecture, apparently by perturbing enzymic control processes. This novel action of bicarbonate may represent an initial permissive event in the process of capacitation (Harrison et al., 1996).

Fertilization promoting peptide (FPP), a tripeptide structurally related to thyrotrophin releasing hormone (TRH), coming from the prostate gland and/or semen of several species

including the human, rabbit and mouse (Thetford et al., 1992; Cockle, 1995), has been shown to stimulate capacitation and fertilizing ability in both mouse and human sperm (Green et al., 1996). FPP contacts sperm at ejaculation and probably remains attached to cells for some time. FPP stimulates capacitation as the sperm ascend the reproductive tract. Adenosine, present in seminal plasma and the female reproductive tract, could either augment FPP's action or replace it if FPP is lost from the cell surface. Green et al. (1996) showed that FPP and adenosine might act by modulating the adenylate cyclase/ cAMP signal transduction pathway to promote capacitation, but inhibit spontaneous acrosomal exocytosis. However, the actual mechanism of action by FPP remains unknown.

1.1.4 The acrosome and acrosome reaction

The mouse sperm acrosome is a membrane-limited organelle which is localized under the sperm plasma membrane and outside the nucleus of the mature spermatozoon. This organelle is a product of the Golgi complex and is synthesized and assembled during spermatogenesis. The acrosome includes the outer acrosomal membrane and the inner acrosomal membrane. It also consists of the anteriorly located acrosomal cap and the posteriorly located equatorial segment. The acrosomal enzymes are mainly localized in the acrosomal cap, but not in the equatorial segment. The major acrosomal enzymes are listed in Table 1.2 (Yanagimachi, 1994).

The acrosome reaction (AR) involves fusion and vesiculation of the outer acrosomal membrane with the plasma membrane, thus leading to the subsequent exposure of the acrosomal contents to the extracellular environment. This process is also considered as an exocytotic process. AR is irreversible because of changes of structures of the sperm plasma membrane and the outer acrosomal membrane. Furthermore, the AR has at least two

functions: namely rendering sperm capable of penetrating through the zona, and enabling the sperm to fuse with the ovum plasma membrane (Yanagimachi, 1994).

Table 1.2. Enzymes reported to be of acrosomal origin

Hyaluronidase	N-Acetylhexosaminidase
Acrosin	Galactosidase
Proacrosin	Glucuronidase
Acid proteinase	L-Fucosidase
Esterase	Phospholipase C
Neuraminidase	Cathepsin D
Phosphatase	Cathepsin L
Phospholipase A	Ornithine decarboxylase
N-acetyglucosaminidase	Calpain
Arylsulfatase	Metalloendoprotease
Arylamidase	Caproyl esterase
Collagenase	Peptidase

1.1.4.1 The site of the AR in vivo

It has been shown so far that the site of the AR in vivo is ovum-associated extracellular matrix and the zona pellucida. In studies where sperm are recovered from reproductive tracts of naturally mated and artificially inseminated females (rabbit, hamster and mouse), a majority of the sperm collected from the ampullary region of the oviduct and not associated with the zona pellucida appear swimming freely but have not undergone the AR (Bryan, 1974; Overstreet and Cooper, 1979; Cummins and Yanagimachi, 1982; Suarez et al., 1983). Sperm associated with the cumulus oophorus complex are either unreacted or have undergone the AR (Yanagimachi and Noda, 1970; Cummins and Yanagimachi, 1982); Only sperm associated with the zona pellucida have either undergone acrosomal changes (Shalgi et al., 1989) or have completed the AR (Cummins and Yanagimachi, 1982). In vitro studies have also shown that the zona pellucida from different species may induce the AR of homologous sperm (Cherr et al., 1986; O'Rand and Fisher, 1987; Cross et al., 1988).

11

Although the AR occurs in sperm bound to both the cumulus oophorus complex and the zona pellucida, it is not completely clear whether the AR is induced by components of the cumulus oophorus and/or the zona pellucida in the reproductive tract.

1.1.4.2 Mechanism of AR

The zona receptors (Rzp), activated by one of the zona glycoproteins (ZP3), stimulate a G protein which in turn stimulates phospholipase C (PLC) activity in the sperm plasma membrane. PLC cleaves phosphatidylinositol diphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP$_3$). IP$_3$ increases intracellular Ca^{2+} concentration by releasing Ca^{2+} from intracellular stores. DAG activates Ca^{2+}-dependent protein kinase C (PK-C) that phosphorylates proteins. A part of IP$_3$ is methylated to become IP$_4$ which then regulates the opening of voltage-dependent Ca^{2+} channels, thus allowing a massive influx of extracellular Ca^{2+}. Some of Ca^{2+} acts on membrane phospholipids directly to facilitate membrane fusion. The activated G protein also stimulates phospholipase A$_2$ (PLA) which cleaves phosphatidyl choline (PC) into lysophosphatidyl choline (LC) plus arachidonic acid (AA), both known to be highly fusogenic (namely fusion between the outer acrosomal membranes and the sperm plasma membranes). Simultaneously, phospholipase D (PLD) activated by G protein cleaves PC to choline and phosphatidic acid (PA) which is also fusogenic. One should realize that Ca^{2+} itself is fusogenic by neutralizing a negative charge of membrane phospholipids. Activated G protein also triggers another chain reaction, activating adenylate cyclase (AC) which in turn stimulates cAMP production. PK-A is cAMP-dependent and phosphorylates proteins essential for the acrosome reaction. Some of the cAMP acts on a gated Na^+ channel to allow Na^+ influx and H^+ efflux, bringing about a rise in intracellular pH (Yanagimachi, 1994).

12

The influx of Ca^{2+}, a rise in intracellular pH and the production of the fusogenic compounds decribed above are all believed to be essential for the acrosome reaction.

1.1.5 Sperm entry and penetration through the zona pellucida

Although the means by which sperm penetrate the zona is not completely known, the following mechanism is currently accepted. Acrosin, released from sperm during the AR, hydrolyzes and "softens" the zona (Urch et al., 1985a,b), allowing an acrosome reacted sperm to insert its head into the substance of the zona. In the immediate vicinity of the sperm head, the zona surface appears eroded, forming a hole or sperm track (Inoue and Wolf, 1975; Inoue et al., 1984). Subsequently, the sperm penetrates through the zona by means of its hyperactive motility (Yanagimachi, 1994).

1.1.6 Block to polyspermy

It is important that the zona pellucida (ZP) and ovum plasma membrane work synergistically to reduce the chance of polyspermy. The zona reaction refers to the combined effects of modification of ZP molecules (Fowler et al., 1986), cortical granule (CG) exocytosis (Sato, 1979) and zona hardening by means of cross-linking of tyrosine residues in the zona by the catalytic action of CG peroxidase (Gulyas and Schnell, 1980), thus preventing polyspermy. The ovum plasma membrane (oolemma) is an important site of polyspermy block and drastically reduces its ability to fuse with sperm after fertilization. Although the extracts of fertilized ova can render approximately 30% of zona-free unfertilized mouse ova incapable of fusing with sperm, the specific mechanism by which polyspermy is blocked remains unknown.

1.1.7 Activation of the mouse ovum

Following the ZP induced AR, the sperm penetrates the ZP and gains access to the perivitelline space. An activation event(s) occurs in the ovum as a result of the interaction of specific regions of the sperm plasma membrane with the ovum plasma membrane. This membrane interaction results in a transient rise in Ca^{2+} within the ovum, which will initiate all the events of ovum activation.

Activation of the mouse ovum, like the ovum of most other species, depends on an increase in the concentration of intracellular free calcium, but the mechanism by which the fertilizing sperm triggers the rise in the intracellular Ca^{2+} is not completely known. There are two hypotheses about the sperm-induced ovum activation, namely, receptor-mediated activation in the ovum and effects of soluble sperm factors on calcium release.

Several studies have shown that sperm-induced activation of mouse ova could be regulated by a heterotrimeric G protein-coupled receptor because G protein has been found in the mouse ovum. Sperm-induced ovum activation is blocked by injection of guanosine-5'-O-(2-thiodiphosphate) (GDPßs) (Connolly et al., 1982) which inactivates G protein (Williams et al., 1992; Moore et al., 1994). However, a sperm receptor in the ovum plasma membrane that might transduce a signal to one of the second messenger systems has not been demonstrated. In addition, ovum integrins and sperm disintegrins have been shown to be involved in sperm-ovum binding and fusion (Almeida et al., 1995; Evans et al., 1995). The interaction of integrins with extracellular ligands can activate several intracellular signalling events such as tyrosine phosphorylation (Lipfert et al., 1992), changes in intracellular calcium concentration (Pelletier et al., 1992), activation of phospholipase C (Cybulsky et

14

al., 1993) and changes in gene expression (Yurochko et al., 1992). However, there is no evidence yet that integrins are involved in Ca^{2+} release in ova at fertilization.

The fertilizing sperm could release factors to modulate or promote Ca^{2+} release in the ovum. Swann (1990, 1992) has shown that extracts of boar and hamster sperm initiate Ca^{2+} oscillations in the mouse ovum. Although the factors that cause Ca^{2+} release have not been identified, they are heat and trypsin sensitive, suggesting they are proteins. Moreover, sperm microinjected into mammalian ova can also activate mammalian ova (Kline, 1996).

In most animals, the centrosome introduced by the sperm at fertilization, typically organizes the radially symmetric sperm aster and after duplication, organizes the two poles of the first mitotic spindle. This requirement for the sperm centrosome ensures biparental contributions at fertilization (Schatten, 1994). However, in the mouse, the centrosome has been shown to be maternally inherited and associated equally with both parental chromosome sets at fertilization (Schatten et al., 1991). Schatten (1994) further postulated that the functional restoration of the zygotic centrosome at fertilization requires the attraction of maternal centrosomal components to the paternal reproducing elements, along with post-translational modifications.

During oocyte maturation, an endoplasmic reticulum (ER) has been found to be present in the cortex and throughout the interior of the cells. There are many large and bright clusters of ER in the cortex, just beneath the plasma membrane. These accumulations of ER are 1-2 μm in diameter and highly polarized, being localized to the cortex that contains cortical vesicles and microvilli. It has been shown that sperm preferentially fuse in the region of the ovum having surface microvilli and cortical granules in the cortex. Inositol 1,4,5-

trisphosphate (IP$_3$) receptors have been found on the ER clusters (Kline, 1996). The structural reorganization of the ER in mouse ova correlates with differences in sperm- and IP$_3$-induced Ca^{2+} release. Cytosolic Ca^{2+} levels increase the sensitivity of the IP$_3$ receptor to IP$_3$. Therefore, this evidence may suggest that the fertilizing sperm acts on IP$_3$ receptors on the ER clusters of the cortex, thus promoting Ca^{2+} release.

The transient rise in intracellular Ca^{2+} would lead to cortical granule exocytosis and the release from meiotic arrest that permits the ovum to complete the second meiotic division, pronuclear formation, development and finally formation of a zygote.

1.2 Development of Preimplantaion Embryos in the Mouse

Preimplantation mouse embryos are designated as those embryos between fertilization and intra-uterine implantation. They are found free in the oviduct or uterus.

To explore the effect of paternal heat stress on the development of preimplanttaion embryos in the mouse, it is necessary to understand the cell cycle, the uptake, metabolism and utilization of nutrients, gene regulation and their significance in the development of preimplantation embryos.

1.2.1 Biology of preimplantation mouse embryos

The biology of preimplantation embryos is not completely understood because of technical and other limitations. Therefore, in this section, only recent advances in the biology of embryos will be presented, including morphological and ultrastructural changes at the

16

various stages, the chronology of embryonic development and the polarity of intraembryonic cells.

1.2.1.1 Morphological and ultrastructural changes of preimplantation mouse embryos

Using a light microscope, preimplantation mouse embryos can be divided into two groups, namely the cleavage stages and the blastocyst. The cleavage stages involve increasing the number of cells within the embryo through mitosis, but not the formation of a blastocoele. They include the 1-cell embryo (also called the zygote), the 2-cell embryo, the 4-cell embryo, the 8-cell embryo and the morula (early or compacting). The blastocyst arises with the formation of the blastocoele and separation of the embryonic cells into trophoblast and inner cell mass. Blastocyst stages may be classified into the early blastocyst, the expanding blastocyst and the hatched blastocyst following loss of the zona pellucida (see Fig 1.2).

The ultrastructure of mouse embryos varies with the developmental stage. Microvillous distributions on the surface of cells within embryos are different at the various stages. Studies have shown that there are microvilli evenly distributed on the surface of 1-cell, 2-cell and 4-cell embryos, approximately 0.13 μm in diameter and varying from 0.1 to 4 μm in length (Calarco and Epstein, 1973). The first polar body lacks microvilli and often localizes near a smooth region on the ovum's surface, but the surface of the second polar body is covered with microvilli. At the 8-cell stage, smooth portions of the membrane are localized at and/or near the regions of blastomere contact, while microvilli localize at the regions where there are no blastomere contacts. During the morula stage, the microvilli are variable in number and in distribution. Their number often increases in the regions of cell contact within embryos. This probably strengthens the contact and aids communication between cells. At the transition from the morula to the blastocyst, the surface of the

17
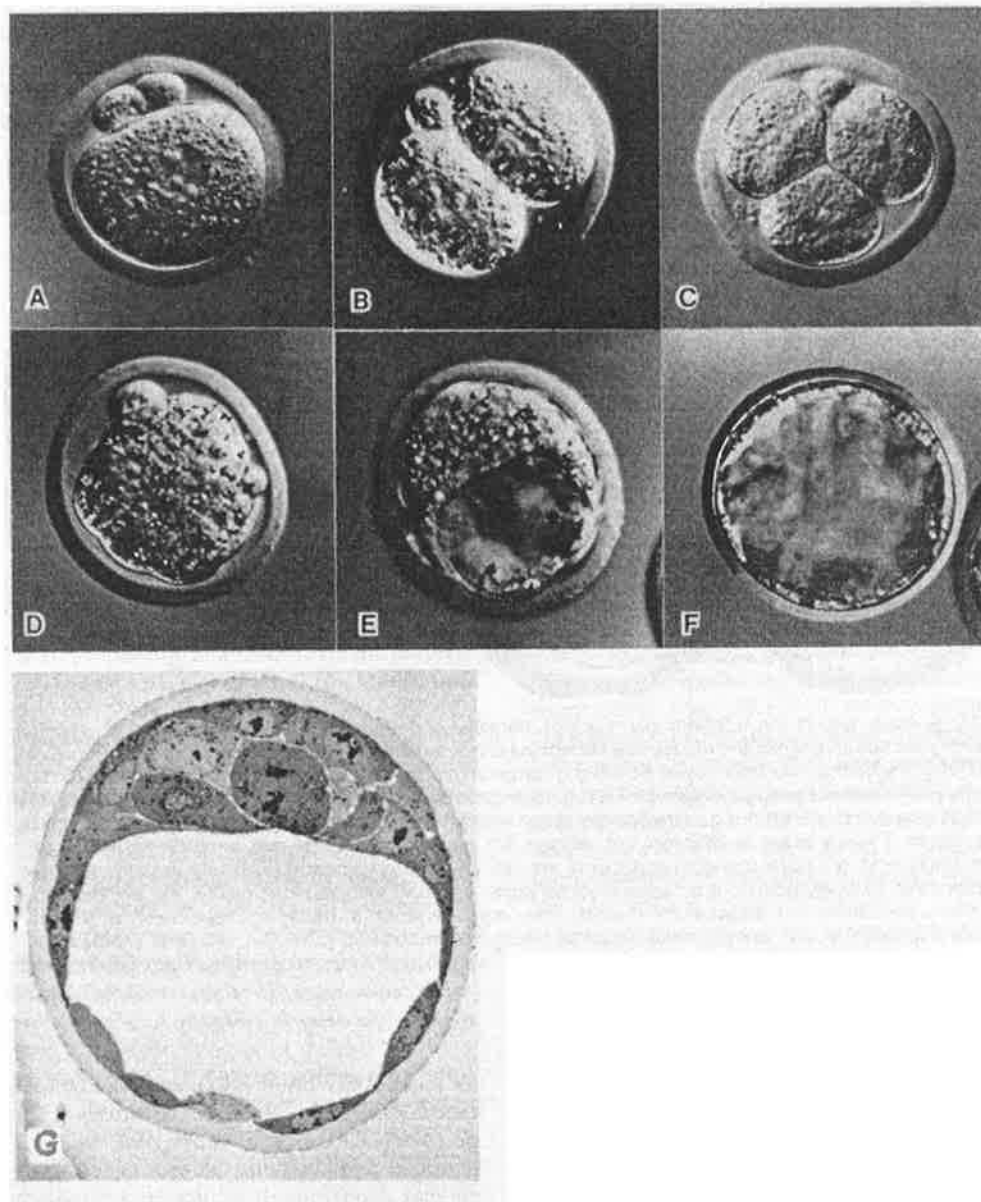
Fig 1.2. Preimplantation stages of mouse embryo development

A: Zygote, 0.5 day of gestation (d.g.); B: 2-cell stage, 1.5 d.g.; C: 4-cell stage, 2.0 d.g.; D: Compacting morula (8- to 16-cell stage), 2.5-3.0 d.g.; E: Early blastocyst stage (32 cells), 3.5 d.g.; F: Expanded blastocyst stage (~64 cells); inner cell mass is out of focal plane, X340; G: Thick-section scanning electronmicrographs of 3.5 d.g., mouse blastocyst showing large, rounded cells of inner cell mass and flattened cells of trophectoderm, X595 (selected from Pedersen and Burdsal, 1994).

18

blastocyst ranges from many long microvilli to fewer and short microvilli or be nearly smooth. Moreover, both the trophoblast cells and inner cell mass cells are significantly less microvillous than the outer surface of the embryo. Another feature involves changes in the junctions between cells at the different stages. No specialized junctions are found in the 1-cell, the 2-cell or the 4-cell embryo. However, in the 8-cell embryo, thin cell processes occasionally extend from one cell across another. During the morula stage, the cells are bound together by thin cell processes. At the blastocyst stage, the area of contact between cells increases strikingly and intercellular junctional ridges are seen, corresponding to the protruding strands. Finally, cellular morphology also changes at the different stages. Before the morula stage, the cells appear round. During the blastocyst stage, the outer cells change from a spherical to a flattened shape, with an appearance of characteristic trophoblast cells. They have larger cell processes extending from one cell across other cells and there are no gaps between cells. Intercellular boundaries are not marked by surface ridges. Compared with the trophoblast cells, cells of the inner cell mass are rounder and show gaps between cells. Microvilli occasionally are seen crossing from one cell to another at cell-cell boundaries (Calarco and Epstein, 1973; Epstein, 1975).

It is concluded from the above evidence that embryonic morphology and ultrastructure undergo a series of changes as embryo development proceeds. These could be related to the nutrient requirement of the embryo, communication between the embryo and the oviduct or uterus and differentiation of embryonic cells.

1.2.1.2 The chronology and polarity of development of preimplantation embryos

The chronology of the development of preimplantation embryos varies with the different

species. Table 1.3 shows only the chronology of mouse embryo development (Theiler, 1989).

Table1.3   The chronology of mouse embryo development (Theiler, 1989)

Stage (relative to first contact with sperm)	Time (h)
2-cell	21-23
4-cell	38-50
8-cell	50-60
16-cell	60-70
Compaction morula	60-80
Blastocyst	66-82
Hatching	96-110
Time in oviduct (relative to ovulation)	84
Development stage on entering the uterus	Morula/blastocyst

Since the speed of differentiation of blastomeres changes at each stage of development, each stage requires a different time for development. Based on the chronology of development, embryos at different stages may be recovered from the oviduct or the uterus in order to study embryo metabolism, protein patterns and gene expression at the various stages.

The polarity of preimplantation mouse embryos is associated with cell differentiation into trophoblast and inner cell mass cells. Handyside (1980) examined the distribution of binding sites for rabbit-anti-species antiserum, concanavalin A (ConA) and peanut agglutinin (PNA) on dissociated blastomeres from 2- to 16-cell mouse embryos, using direct (blastomere-fluorescein-conjugated antisera) and indirect (blastomere-antisera-fluorescein-conjugated goat anti-rabbit IgG) immunofluorescence techniques. He found that paraformaldehyde-fixed blastomeres from 2-to 8-cell precompaction embryos were

uniformly surface-labelled, but 70% of late compact 8-cell blastomeres showed quantitative polarization of surface labelling and 16-cell blastomeres were either polarization (53.3%) or uniformly surface labelled. These results suggest that the polarity mainly generates at the late 8-cell stage. During the 16-cell stage, the cells progress to be either polar or apolar. Polar cells appear relative large in size, display polar distribution of microvilli. Ligand-binding sites are less adhesive in aggregation experiments, but do tend to envelop apolar cells completely when aggregated to them and occupy an outside position in the undisturbed embryos. Apolar cells appear relatively small in size, have sparse but uniform distribution of microvilli, label uniformly over their surfaces with a variety of fluorescent ligands, are readily adhesive in cell aggregation experiments and occupy an inside position (Handyside, 1981; Reeve and Ziomek, 1981; Ziomek and Johnson, 1982; Johnson and Ziomek, 1983).

1.2.2 The cell cycle of early preimplantation embryos

During early embryonic development, cell differentiation must be temporarily and spatially regulated because in some non-mammalian species, one important component of temporal regulation has been shown to be the cell cycle (Satoh and Ikegami, 1981a,b; Newport and Kirschner, 1982a,b; Edgar et al., 1986). Moreover, studies in the mouse embryo have also shown that at least some steps in differentiation may be related to specific cell cycles or cell cycle protein kinases (Smith and McLaren, 1977; Spindle et al., 1985; Howlett, 1986; Garbutt et al., 1987; Chisholm, 1988; Moore et al., 1996). Recent studies have shown that cell receptivity to developmental signals and hence entry into a new state of differentiation and/or developmental commitment could be limited to a particular phase of the cell cycle. The passage of time during embryogenesis may be measured by the numbers of cell cycles

elapsed since a prior time-setting event. Therefore, an understanding of the kinetics of the cell cycle in blastomere cells is extremely important.

1.2.2.1 The eukaryotic mitotic cell cycle and its regulation

In order to understand the cell cycle of the blastomeres, it appears significant to firstly understand the eukaryotic mitotic cell cycle and its regulation.

The eukaryotic mitotic cell cycle consists of mitosis (M) and interphase (I) stages. M is short and produces two separate daughter cells, each containing a complete set of chromosomes. I represents the longest part of the cell cycle and includes a gap period before DNA synthesis (G1), a period of DNA synthesis (S) and another gap period (G2) after S and before M (Fig 1.3). Although G1 and G2 are gap periods of the cell cycle, their functions are different. During G1, cells undergo growth and can be affected by external factors (e.g. growth factors, cell-cell contact and cAMP) (Matsushime et al., 1994; Ando and Griffin, 1995), thus triggering proliferation or differentiation. Towards the end of G1, cells lose their ability to respond to environmental factors and commit to mitosis. During G2, cells are able to check whether DNA replication is completed before M is initiated.

Nigg (1995) reported that the different phases of the cell cycle are regulated by a family of protein kinases called cyclin-dependent kinases (cdks) within the cell. The cdks possess the kinase activity only when they combine with cyclins, proteins whose levels undergo cyclic changes throughout the cell cycle. Several different types of cyclins and cdks have been
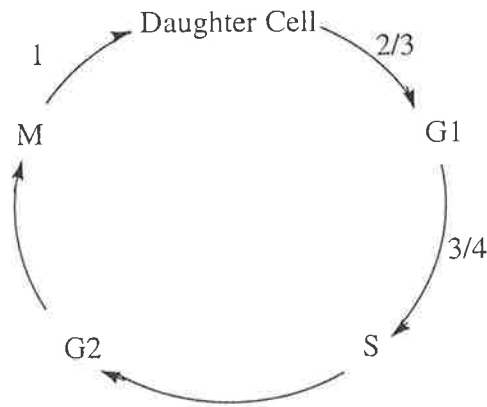
Fig1.3. The phases and regulations of the cell cycle

M--mitosis;   G1, S and G2 comprise interphase (I);   1--cyclin B-cdc2 complex; 2--cyclin D1-cdk4 complex, cyclin D2-cdk4 complex and cyclin D3- cdk6 complex;   3--cyclin E-cdk2 complex;   4--cyclin A-cdk2 complex.

found to be present within cells, which are classified as G1 or M phase cyclin-cdk complexes according to their amounts and roles in the different phases of the cell cycle although some are required for normal passage through S-phase. The different cyclin-cdk complexes regulate the different phases of the cell cycle. Cyclin B-cdc2 complex regulates the entry and exit of the cell from M-phase, while a combination of cyclin D1, D2 or D3 with cdk4 or cdk6 controls the progression through G1 and entry into S-phase (Matsushime et al., 1994; Meyerson and Harlow, 1994; Vallance et al., 1994). These conclusions have been supported by studies demonstrating that overexpression of either cyclin D1 or D2 in rat fibroblasts decrease the G1 transit time by several hours (Quelle et al., 1993) and microinjection of a monoclonal antibody to cyclin D1 results in cell cycle arrest at G1. The cyclin E-cdk2 complex has been found to be present during G1, and its kinase activity increases by late G1 and controls the early stage of DNA replication (Dulic et al., 1992; Jackson et al., 1995; Ohtsubo et al., 1995). Finally, the cyclin A-cdk2 compex and its associated kinase activity increase and peak during S phase and may control progression of DNA synthesis (Girard et al., 1991; Pagano et al., 1992; Zindy et al., 1992).

1.2.2.2 The cell cycle of preimplantation mouse embryos

Times for the first five cell cycles of mouse embryos are summarized in Table 1.4.

Table 1.4 Summary of values for phases of the first five cycles of mouse embryos

Cell Cycle	Length of phase (h)			References*
	G1	S	G2+M	
First (1-cell stage)	4.5-12[†]	4-7	1-8	1,3,6,8,9,10,11,14.
Second (2-cell stage)	0-2	4-7	12-18	2,4,5,7,9,10,14.
Third (4-cell stage)	1-1.5	7	0.5-5	5,9,11,12.
Fourth (8-cell stage)	2	7	1-3	9,12.
Fifth (16-cell stage)	2	8-9	2	13

*1. Luthardt and Donahne, 1973; 2. Luthardt and Donahne, 1975; 3. Abramczuk and Sawicki, 1975; 4. Siracusa et al., 1975; 5. Sawicki et al., 1978; 6.,Domon, 1980; 7. Bolton et al., 1984; 8. Krishna and Generoso, 1979; 9. Streffer et al., 1980; 10. Molls et al., 1983; 11. Howlett and Bolton, 1985; 12. Smith and Johnson, 1986; 13. Chisholm, 1988; 14. Moore et al., 1996.

† Includes completion of meiosis II in most studies.

The following points are relevant to Table 1.4: 1) The variation in each phase may be related to different mouse strains, different ovulation procedures and different techniques for evaluating the lengths of each phase. However, the striking point is that the length of each phase of the cell cycle is limited to a certain value; 2) The third, fourth and fifth cell cycles have a similar length in each phase of the cell cycle. Conversely, the first and second cell cycles have different lengths in G1 and G2+M, but the same length of S phase. This evidence may imply that the first two cell cycles following fertilization of mouse ova have unique characteristics when compared with the subsequent three cell cycles and play an important role in the development of the embryo.

Prior to fertilization, the mouse ovum is arrested at metaphase of the second meiotic division. Fertilization results in the completion of meiosis and the subsequent initiation of the first mitotic division. The transition from meiosis to first cell division occurs about 45-

60 min following fertilization, as evidenced by the emission of the second polar body. The formation of a pronuclear envelope that surrounds and separates the male and the female pronuclei occurs about 4-5h following polar body emission and onset of the first mitotic division. Therefore, DNA replication of the maternal and paternal chromosomes occurs in separate compartments and is initiated by mid-S phase at the first cell cycle (Worrad et al., 1994; Schultz and Worrad, 1995). The G1 to S transition in the first cell division occurs in the absence of transcription. Finally, the early cell cycles of the mouse embryo proceed in the absence of cell growth.

There are also differences between the characteristics of the first and second cell cycles in the mouse embryo. 1) Although the length of the first two cell cycles is similar (~20h), G1 in the first cell cycle is much longer than that in the second cell cycle (4.5-12 h vs 0-2 h) (see Table1.4), while G2+M in the 1-cell embryo is shorter than that in the 2-cell embryo.(1-8 h vs 12-18 h) (see Table 1.4). 2). In contrast to the first cell cycle, both sets of chromosomes replicate within a single nucleus in the 2-cell embryo. 3) The level of transcription in the 2-cell embryo is significantly higher than in the 1-cell embryo (Ram and Schultz, 1993).

Differences between the first two cell cycles could be regulated by cyclin-cdk complexes within blastomeres like somatic cells and this may also determine the fate of subsequent embryo development. Moos et al. (1995) reported changes in the levels of cdc2 and cyclin B2 proteins as well as the activity of the cyclin B-cdc2 kinase complex throughout the first cell cycle following fertilization. Subsequently, Moore et al. (1996) determined four main patterns of levels of PCR products for G1-S regulating genes during the first two cell cycles in mouse embryos. These are: 1) steady levels for cyclin A; 2) steady levels followed by a 2-3 fold increase during the G2 phase of the second mitotic cell cycle for cyclin D1, E,

cdk2 and P21; 3) a transient increase during the S and/or G2 phase of the first mitotic cell cycle for P27 (cell cycle regulatory protein), cyclin D3 and the two forms of cdk2; and 4) higher levels during the first cell cycle and then a decrease with lower levels during the second mitotic cell cycle for cyclin D2 and Rb (Retinoblastoma).

Studies have shown that many of the changes in the pattern of protein synthesis in the 1-cell mouse embryo are products of mobilization and translation of maternal mRNAs, whereas many of the changes during the second mitotic cell cycle are products of the activation of the zygotic genome (Howlett, 1986; Conover et al., 1991; Latham et al., 1991). This conclusion is supported by Moore et al. (1996) who showed that the increase in the amount of PCR products for the cdk4 gene during the first cell cycle was due to polyadenylation, whereas the increase in the amount of PCR products for cdk4, cdk2 and cyclin D1 and E in the second mitotic cell cycle was a product of activation of the embryonic genome. However, other studies have also shown a significant level of transcription at the G2 phase of the first cell cycle (Ram and Schultz, 1993; Worrad et al., 1994). This suggests that the activation of embryonic genome begins to occur at the G2 phase of first cell cycle and becomes more significant in the second cell cycle.

1.2.3 The uptake, metabolism and utilization of nutrients in preimplantation embryos

The development in vivo of embryos is controlled and regulated by many factors, such as hormones, cell paracrine and autocrine secretions and interaction and communication between mother and embryos. In contrast, when embryos are cultured in vitro, owing to unsuitable composition of medium, their development may be inferior to that in vivo. Therefore, appropriate uptake, metabolism and utilization of nutrients such as proteins, carbohydrates, amino acids, vitamins, growth factors and other substrates by preimlantation

26

embryos are important for their health and development. These nutrients have been shown to provide beneficial factors to culture milieu and improve embryo development (Bavister, 1995). In addition, the uptake, transport and the roles of carbohydrates particularly glucose and amino acids in embryo culture have also been more extensively studied, compared with other nutrients such as proteins, growth factors and vitamins. This is because glucose and amino acids are not complex and can replace other nutrients in supporting embryo development. Both glucose and some amino acids are used as energy sources for embryo development. As embryos develop, glucose is used as a precursor of macromolecules i.e., ribose moieties for DNA and RNA synthesis whose synthesis increases dramatically during the later preimplantation stages (Leese, 1991). Furthermore, glucose may act as an internal signal at the cell surface and within the cells for embryo development. For example, Chatot et al. (1994) reported that a 1-min exposure to 27 mM glucose in CZB at 42h of culture supported a high rate of development of the 1-cell mouse embryos to the blastocysts (75%). This must be due to some signalling effect because glucose can not be taken up in this short time by the mouse embryos.

Amino acids may not only be used for protein synthesis, triacylglycerols and other lipids. but also play other roles in preimplantation embryo development. Evidence from in vitro culture has shown that taurine may help them resist oxidative, osmotic and other stresses (Huxtable, 1992). Glycine protects against a rise of inorganic ions within embryos since excesses of inorganic ions in cells probably perturb the structures and activities of some enzymes. The ability of glycine to improve development of preimplantation embryos at the high ionic concentrations of oviductal fluid-like medium probably occurs by this same mechanism (Van Winkle et al., 1990).

1.2.3.1 The uptake and transport of carbohydrates in preimplantation embryos

Many studies have indicated an increase in glucose uptake between the 1- and 2-cell stages, but not between 2- and 8-cell stages, followed by a gradual increase at the morula and finally a rapid increase at the blastocyst stages (Wales and Brinster, 1968; Leese and Barton, 1984; Butler et al., 1988). Pyruvate is easily utilized by the early preimplantation stages, followed by a decrease at the early morula stage. The uptake of other carbohydrates including citrate, malate and 2-oxoglutarate increases between the 2- and 8-cell stages, with further increases in malate and oxoglutarate to the morula stage (Wales and Biggers, 1968; Kramen and Biggers, 1971). In vitro studies have also shown that glucose is inhibitory during the first 48h or at the 1- to 4-cell stages whereas at the 4- to 8-cell stages exposure to glucose for 24h, and even for only 1 min, is stimulatory for embryo development (Chatot et al., 1989, 1990, 1994). However, when KSOM is used, glucose is not inhibitory at any stages of preimplantation embryos (Summers et al., 1995) which is more consistent with the high concentrations of glucose reported in vivo in the oviduct and the uterus (Gardner and Leese, 1990; Leese, 1995).

Glucose can be transported across membranes by two different types of mechanisms, namely a sodium-coupled active carrier system that is expressed in epithelial cells (Esposito, 1984) and a sodium-independent facilitative glucose transporter system (Wheeler and Hinkle, 1985). Gardner and Leese (1988) studied glucose transporter systems in preimplantation mouse embryos by measuring glucose uptake by single mouse blastocysts at different concentrations (1, 2, 3, 4, 5mM) of glucose and found that the glucose transport system consists of a simple and a facilitated diffusion system. Subsequently, using a medium containing specific inhibitors of glucose (phloretin or cytochalasin B) or a medium containing sodium ions or no sodium ions, they found that the inhibitors significantly

inhibited glucose uptake, but the absence of sodium had no effect on glucose uptake by single mouse blastocysts. They concluded that preimplantation embryos possess a sodium-independent facilitated transporter system for glucose which strengthens transport function as embryos develop (Table 1.5).

Table 1.5 The effect of the presence and absence of phloretin on glucose uptake by different stage embryos (Gardner and Sakkas, 1993)

	Glucose uptake (pmol embryo^{-1}h^{-1})
In the absence of phloretin	
1-cell	0.05
2-cell	0.73
8-cell	0.82
Blastocyst	4.78
In the presence of phloretin	
1-cell	nd
2-cell	nd
8-cell	nd
Blastocyst	1.22

nd: not detectable (glucose uptake remained close to zero)

It has been shown that a sodium-independent, facilitated glucose transporter system is regulated by glucose transport proteins GLUT 1 to GLUT 7 (Bell et al., 1990; Kasanicki and Pilch, 1990; Mueckler, 1990; Thorens et al., 1990; Pessin and Bell, 1992; Baldwin, 1993). In preimplantation mouse embryos, by using immunohistochemistry and reverse transcription combined with the polymerase chain reaction (Hogan et al., 1991) and Western blot analysis (Aghayan et al., 1992), GLUT 1 has been found to be expressed throughout mouse preimplantation embryos and may be responsible for the low levels of glucose uptake observed from the 1-cell stage onwards. GLUT 2 begins to be expressed at the 8-cell stage and is switched on through the morula and blastocyst stage. GLUT 3 is highly expressed at blastocyst formation (Pantaleon and Kaye, 1998). Taken together, the

GLUT proteins may cooperate to be responsible for the rise in glucose uptake from the 8-cell stage to the later stage of development .

The regulation of glucose transport in preimplantation embryos is not well understood. However, recent studies have suggested that the glucose transport in the mouse blastocyst is regulated by receptors for insulin-like growth factor 1 (IGF-1). The reasons for this are as follows: 1) receptors for IGF-1 (Heyner et al., 1989; Rappolee et al., 1990) and insulin (Mattson et al., 1988; Harvey and Kaye, 1988,1991) are first expressed at the 8-cell stage in mouse embryos; 2) Insulin is almost 1,000 fold more potent in its stimulation of glucose transport than in the stimulation of protein synthesis (Harvey and Kaye, 1988), morphological development and cellular proliferation in mouse blastocysts (Harvey and Kaye, 1990); 3) In the presence of insulin or IGF-1, glucose uptake by single mouse blastocysts can be increased by from 17% to 62% (Gardner and Kaye, 1984; Willis et al., 1985; Pantaleon and Kaye, 1996); 4) Preimplantation embryos have stage specific binding of glucose which appears to be strongest in the blastocyst (Pantaleon and Kaye, 1996). Therefore, Pantaleon and Kaye (1996) proposed that IGF-1 and insulin regulate glucose transport in mouse blastocysts via IGF-1 receptors. A model for the regulation may be that IGF-1 or insulin binds to the IGF-1 receptors but not the insulin receptor, further inducing a signal of unknown nature. The signal leads to redistribution of intracellular vesicles containing glucose transport protein (GLUT 1) by means of a way of an exocytosis-like mechanism to the plasma membrane. Following fusion of GLUT 1 with the plasma membrane, GLUT 1 is then exposed to the extracellular medium and this causes an increase in glucose transport activity. However, Gardner and Leese (1988) showed that glucose can also be transported by mouse embryos in the absence of insulin, suggesting that the glucose is also likely to be transported into blastomeres by other transport mechanisms.

1.2.3.2 The uptake and transport of amino acids in preimplantation embryos

The uptake of different amino acids depends on the type of amino acid and the developmental stages of embryos. For example, there is a small amount of uptake by ova and zygotes, and the rate of uptake actually decreases somewhat between the 1- and 2-cell stages. There is then a small increase between 2- and 8-16 cell stages and a major increase between the later stage and the early blastocyst stage (Brinster, 1971; Epstein and Smith, 1973).

Transport systems of amino acids in preimplantation mouse embryos vary with the kinds of amino acids and the different stages of preimplantation embryos. The transport system for taurine is a system β (β amino acid transport system) for the selective, Na^+-dependent uptake of taurine. This system is also expressed at all stages of preimplantation development although its activity is greatest at the blastocyst stage (Van Winkle et al., 1994); the taurine content of embryos increases considerably between the 2-cell and blastocyst stages (Van Winkle and Dickinson, 1995); furthermore, mRNA encoding the mouse taurine transport protein (TAUT) is present in embryos throughout preimplantation development (Van Winkle et al., 1994). For glycine, system Gly is a Na^+-dependent transport process (Van Winkle et al., 1988). System Gly activity could depend on the developmental stage. It has a strong activity at about the 2-cell stage, then rapidly decreases to reach its lowest levels at the blastocyst stage. Glycine content in embryos also appears to change in a similar manner (Van Winkle and Campione, 1996). Additionally, the complete loss of system Gly activity at the blastocyst stage is associated with the loss of mRNA encoding the mouse glycine transport protein. The transport system of glutamine is by a Na^+-dependent and selective system and appears to rise dramatically and transiently

between the 2-cell and 4-cell stage, then decreases again (Van Winkle and Campione, 1996). From these studies, it may be concluded that transport of most amino acids is regulated by gene transcription. However, to account for a transient rise and decrease of glycine and glutamine uptake, Van Winkle and Campion (1996) proposed the possibility of a transient, pulsatile expression of transport activities which might involve activation and inactivation of extant transport proteins.

1.2.4 Zygotic gene activation in the mouse

During the growth phase, mouse oocytes synthesize and accumulate RNA (mRNA, rRNA and tRNA), proteins and organelles (e.g. mitochondria) which in turn direct the early development following fertilization. This period corresponds to the time of transition from maternal to zygotic transcription.

Zygotic gene activation (ZGA) is involved in the destruction (or loss) of most maternal transcripts and the corresponding generation of new transcripts that are unique to the developing embryo. The significance of ZGA is evident in the fact that embryos that fail to undergo the ZGA can not develop any further. Additionally, the in vitro 2-cell "developmental block" (Whitten and Biggers, 1968; Bavister, 1988) found when mouse pronuclear embryos or zygotes are cultivated in some in vitro media, is often related to an inability to undergo ZGA (Johnson and Nasr-Esfahani, 1994). However, the molecular basis for the ZGA is poorly understood in the mouse.

1.2.4.1 Time of the mouse ZGA

Most studies suggest that the ZGA occurs by the 2-cell stage in the mouse (Flach et al.,

1982; Sawicki et al., 1982; Bensaude et al., 1983; Latham et al., 1991; Manejwala et al., 1991; Schultz, 1993; Moore et al., 1996). This is supported by the following several lines of experimental evidence: 1) Expression of paternally-derived isozymes is detected at the 2-cell stage (Sawicki et al., 1982); 2) The transcription-requiring complex (TRC) is a family of structurally-related polypeptides of Mr=70,000 used to assess transcriptional activity. It is synthesized in the 2-cell embryo (Conover et al., 1991) and constitutes greater than 4% of total protein synthesis (Latham et al., 1991). In addition, it is inhibited by α-amanitin, a specific inhibitor of RNA polymerase II and III (Golbus et al., 1973; Conover et al., 1991). Thus, TRC is an accepted molecular maker for ZGA; 3) Consistent with the 2-cell embryo being transcriptionally active, analysis of cDNA libraries generated from preimplantation embryos reveals the existence of some cDNAs only detected from the 2-cell stage onwards (Rothstein et al., 1992); 4) The expression of several different reporter genes is easily detected at this time (Ueno et al., 1987; Bonnerot et al., 1991; Ram and Schultz, 1993; Wiekowski et al., 1993; Matsumoto et al., 1994); 5) Endogenous RNA polymerase activity is detected in the nuclei of 2-cell embryos, but not the pronuclei (Moore, 1975).

On the other hand, mouse ZGA is triggered in G2 of the first cell cycle (Latham et al., 1992; Ram and Schultz, 1993; Matsumoto et al., 1994; Christians et al., 1995) and also supported by experimental evidence, as presented below: 1) There is evidence for the existence of TRC in G2 of the first cell cycle. To assess the presence of functional RNA polymerase II in the recipient 1-cell embryo, a donor nucleus that could express TRC but whose endogenous RNA polymerase II was inhibited by α-amanitin, was transplanted to an enucleated recipient 1-cell embryo (Latham et al., 1992). The result showed that TRC expression can be detected and the level of synthesis is about 30% that in the mid 2-cell when the recipient embryos are radiolabeled in G2 of the first cell cycle, but little or no

TRC expression was detected when the embryos were radiolabeled in S; 2) There is also evidence for the presence of functional transcription factor in the 1-cell embryo. When a plasmid containing an Sp1-dependent luciferase reporter gene was microinjected into the pronucleus, expression of this gene could be detected and the level of luciferase expression in G2 was about 20% of that observed when the nucleus of a 2-cell embryo was injected with the reporter gene. In addition, higher levels of reporter gene expression following injection of the male pronucleus were observed, but no luciferase activity was detected following injection of the female pronucleus (Vasseur et al., 1985; Ram and Schultz, 1993). These results suggest that functional transcription factors which are present in the 1-cell embryo and the male pronucleus support a higher level of transcription.

In summary, the above evidence indicates that transcription is initiated in the 1-cell embryo and that the rate of transcription dramatically increases during the 2-cell stage. Thus, the onset of the ZGA appears to involve some activation during the 1-cell stage and a more substantial activation during the 2-cell stage. However, further investigation of this process is required.

1.2.4.2 Regulation of ZGA in the mouse

Studies have shown that changes in chromatin structure that are coupled to DNA replication, rather than changes in the activity of transcription machinery per se, may be the primary site of regulation of gene expression in the embryo (Schultz et al., 1995). Chromatin is inherently transcriptionally repressed because the nucleosomal subunit structure can prevent transcription factors from binding to their cognate sequences in DNA (Wolffe, 1991, 1994). In other situations, however, a positioned nucleosome is essential for

transcriptional initiation (Wolffe, 1994). In some cases, the role of chromatin can at least be modulated by post-translational modification of core histones.

Histones are susceptible to a wide spectrum of modification, including phosphorylation, ADP-ribosylation, ubiquitination and acetylation (Turner, 1991, 1993; Wolffe and Dimitrov, 1993). In addition, hyperacetylation of histone is highly correlated with transcriptionally permissive chromatin. Nucleosomes bearing acetylated histones can bind transcription factors in vitro, whereas nucleosomes containing hypoacetylated histones do not (Lee et al., 1993).

Chen et al. (1986) and Worrad et al. (1995), using antibodies that recognize specific isoforms of histone H_4 together with laser-scanning confocal microscopy, found the monoacetylated form of histones H_4 in a relative uniform staining pattern throughout either the pronuclei or nuclei in 1-cell and 2-cell embryos. In contrast, an enhanced localization of higher acetylated isoforms of histone H_4 at the nuclear periphery has been found and restricted to the 2-cell embryo, using those either cultured from the 1-cell to the 2-cell stage or from the oviduct. Such temporal and spatial distributions have not been observed in 4-cell embryos onwards. Moreover, RNA polymerase II also appears to manifest an enhanced peripheral localization.

The development of this regionalized domain of chromatin is under maternal control and does not require transcription since it occurs in 2-cell embryos that are cultured from the 1-cell stage in the presence of α-amanitin; it also occurs when cytokinesis is inhibited by cytochalasin D treatment. Inhibiting DNA replication by adding aphidicolin during early S phase inhibits the formation of the domain of hyperacetylated histone H_4 at the nuclear

periphery (Worrad et al., 1995). The formation of chromatin bearing nucleosomes containing these acetylated isoforms of histone H4 may be a repressive state that develops following the first mitosis (Wiekowski et al., 1991, 1993). Thus developmentally regulated histone acetylation may be critical to sustain continued gene expression that is necessary for further development. The onset of ZGA is associated with the temporally and spatially restricted localization of potentially transcriptionally permissive chromatin.

1.2.4.3 Identification of genes that manifest a transient increase in expression during the 2-cell stage

Latham et al. (1991) reported that there is a small subset of polypeptides whose relative rate of synthesis increases transiently during the 2-cell stage. This is in contrast to the vast majority of genes that are constitutively activated following ZGA (Schultz et al., 1995). A DNA sequence has been subsequently found to correspond to the protein synthesis translation initiation factor eIF-4C (Schultz et al., 1995). eIF-4C stimulates the formation of 43S preinitiation complexes and the dissociation of 80S ribosomes (Hershey, 1991). The use of two independent RT-PCR based assays indicated that there is a 3-10 fold increase in the amount of eIF-4C between the 1-cell and mid 2-cell embryos, and by the late 2-cell/early 4-cell stage. This level ultimately decreases to a level similar to that in the 1-cell embryo. Moreover, the increase in eIF-4C is inhibited when 1-cell embryos are cultured to the mid 2-cell stage in the presence of α-amanitin. This result is consistent with the increase in eIF-4C transcript levels being due to transcription.

1.2.4.4 Role of DNA replication in the onset of ZGA

Earlier results have suggested that the onset of ZGA was independent of the first round of

DNA replication (Clegg and Pikó, 1982; Poueymirou et al., 1989). The recent evidence, however, has shown that the onset of ZGA is related to the first round of DNA replication. For example, when aphidicolin is added to the 1-cell embryo already in S phase, the level of TRC expression is reduced when compared with control embryos. Therefore, inhibiting DNA replication in turn inhibits an increase in the nuclear concentration of transcription factors such as Sp1 and TBP as well as the enhanced peripheral localization of hyperacetylated histone H4 which is correlated with ZGA (Schultz et al., 1995). Furthermore, addition of aphidicolin to 1-cell embryos prior to entry into S phase substantially inhibits the increase in expression of eIF-4C that occurs during the mid 2-cell stage. Conversely, addition of aphidicolin to 1-cell embryos that were in late S phase has little effect on the increase in eIF-4C that occurs by the mid 2-cell stage (Schultz et al., 1995). These results imply that the first round of DNA replication is "permissive". Although chromatin is inherently transcriptionally repressive, DNA replication disrupts assembled nucleosomes. The fertilized ovum inherits a functional transcription machinery, e.g. RNA polymerase II associated activators and transcription factors. The clearance of nucleosomes during DNA replication would provide a window of opportunity for these proteins to gain access to their DNA-binding sites. This would account for the permissiveness of the first round of DNA replication with respect to transcription. A spectrum of genes that would be expressed during ZGA would reflect the array of enhancer and promoter elements that regulate the expression of a gene and the complement and concentration of maternally derived transcription factors and enhancers (Schultz et al., 1995).

1.3 Implantation

Implantation involves a direct interaction of the trophoblast with the luminal epithelium of the uterus when the conceptus acquires a fixed position within the uterine lumen, with the result that leads to the establishment of the placental structures and finally assures a dialogue between the maternal cells and the conceptus. This leads to a shift from a distant molecular dialogue by means of secreted factors to more intimate and local exchanges mediated by cell to cell contacts. This definition clearly shows that any factors (e.g. heat stress) influencing normal blastocyst development could disturb the normal implantation of with long term consequence. Therefore, it is neccessary and essential to briefly summarize the histological structures of the uterus and the blastocyst, preparation of the endometrium for blastocyst implantation, trophoblast-uterine adhesion and transformation of the uterus in order to understand correctly the relationship between perimplantation embryos and the uterus.

1.3.1 The histological structures of the uterus and the blastocyst

The uterus consists of several layers including the uterine epithelium, basement membrane, uterine stroma containing the uterine glands and blood vessels, myometrium and peritoneal membrane. The mouse blastocyst is composed of trophoblast, inner cell mass and endoderm. These structures are shown in figure1.4.
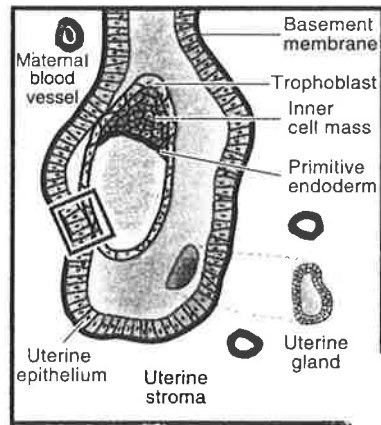
Fig1.4. The histological structures of the uterus and the blastocyst (Cross et al., 1994)

1.3.2 Preparation of the endometrium for blastocyst implantation

The oestrogen released at about the time of ovulation in the mouse initiates the first waves of epithelial proliferation and its withdrawal on the third day of pregnancy is responsible for a wave of activity in the secretory glands. Progesterone from the corpus luteum then acts on the stroma, and a little amount of oestrogen induces proliferation of the stroma on the fourth and fifth days in preparation for implantation.

Cell differentiation also occurs in response to ovarian hormones. Oestrogen results in loss of basal lipid-droplets (Fuxe and Nilssons, 1963), increases the height of the luminal epithelium and the length of microvilli, makes the nuclei occupy a more basal position and promotes an increase in the amount and prominence of the endoplasmic reticulum and Golgi complex (Nilsson, 1958, 1959). In addition, oestrogen can also increase the activity of several enzymes, such as alkaline phosphatase and nonspecific esterase (Hall, 1975; Smith and Wilson, 1971). Progesterone increases cathepsin D activity, promotes the development of extensive interdigitation of the apical microvilli on the apposed luminal surfaces (Pollard and Finn, 1972) and the formation of pinopods (Bergstrom and Nilsson, 1976). Both oestrogen and progesterone lead to the development of a generalized oedema in pre-sensitized stroma. These changes are characteristic of the first phase of uterine closure

and therefore, associated with "presensitive" endometrium. Subsequently, the apical membranes are more closely apposed and the surface becomes irregular because oestrogen promotes increases in prominence of the rough endoplasmic reticulum and a reduction in the number and the size of microvilli (Pollard and Finn, 1972). A little amount of oestrogen and progesterone dominance results in an increase in the size of uterine glandular cells and enhanced glandular secretion (Finn and Martin, 1971). Moreover, progesterone and oestrogen markedly enlarge the nucleoli and the granular component, increase the rough endoplasmic reticulum in the stroma which becomes distended. These changes lead to the second stage of uterine closure. Uterine closure is advantageous to trophoblast-uterine adhesion.

Synthesis of uterine DNA, RNA and proteins increase during implantation (Miller and Emmens, 1969). With oestrogen alone the increases of uterine proteins are greatest in the epithelium, but with progesterone alone the increases are more dramatic in stroma (Smith et al., 1970). Oestrogen reduces total lipid, primarily by influencing neutral triglycerides, whereas progesterone causes an increase. It is particularly effective in this regard if it has been preceded by oestrogen (Goswami et al., 1963). Neutral lipids provide an energy source for the developing embryo and phospholipids may be important for synthesis of such components as cell membrane and prostaglandin during decidualization.

Hormone-dependent changes in cell proliferation, in cytodifferentiation and in the different metabolic and synthetic patterns must ultimately be responsible for the development of uterine receptivity.

1.3.3 Trophoblast-uterine adhesion

At implantation, the previously nonadhesive apical surface of the trophectoderm becomes adhesive. Although the molecules that mediate binding of trophoblasts to the uterine epithelium remain to be fully determined, the materials produced by the blastocyst and the uterine epithelial cells could be related to adhesion of the trophoblast to the uterine epithelium, as described below: 1) Mouse blastocysts express several carbohydrate structures, including a selectin ligand and sialylated ligand, whereas the uterine epithelium produces lectin. Carbohydrate-lectin interactions might mediate initial blastocyst adhesion, which is then stabilized by binding of integrins to their extracellular matrix (ECM) ligands, such as laminin and fibronectin (Cross et al., 1994); 2) Mouse blastocysts synthesize proteoglycan (e.g. perlecan, the basement membrane form of heparan sulfate proteoglycan) (Carson et al., 1993), while uterine epithelial cells express heparan sulfate-binding proteins (Farach et al., 1987), that could interact with the proteoglycans; 3) Uterine epithelial cells express an H-type-1 carbohydrate around the time of implantation (White and Kimber, 1994) and the abembryonic trophectoderm acquires the ability to specifically bind H-type-1 structures at the late blastocyst stage (Lindenberg et al., 1988; Lindenberg et al., 1990). Saccharides that carry this epitope inhibit embryo attachment to endometrial monolayers in vitro. Uterine glycosaminoglycans, such as chondroitin sulfate and hyaluronic acid (HA) may also participate in adhesion because blastocysts attach and spread on HA in culture (Carson et al., 1987; Jacobs and Carson, 1991).

Integrins could be critical for trophoblast-ECM interaction. For example, intergrins recognized by an antiserum against a-V$_\text{u}$3 intergrin have been detected in the apical surface of the trophoblasts and αV integrins bind several ECM components, including perlecan. As

mouse blastocysts mature and acquire the ability to attach to ECM, $\alpha7\beta1$ integrin, a laminin receptor, is up-regulated (Sutherland et al., 1993).

1.3.4 Transformation of the uterus

The tissue-specific response of the uterus to an implanting mouse embryo is the decidual response. The initial stages share many features with the acute inflammatory responses (Welsh and Enders, 1985, 1991 and 1993). Several cell changes occur, including 1) Loss of the uterine epithelium (Starkey et al., 1988). The uterine epithelial cells exhibit all the characteristics of apoptosis, including surface blebbing, shrinkage and fragmentation of the cell, condensation of chromatin and indentation and fragmentation of nuclei. Cytoplasmic organelles remain morphologically intact, and the cytoplasm remains morphologically intact, or shows increased staining density. The epithelial cells and cell fragments are also phagocytosed by the adjacent trophoblast cells. The loss of the uterine epithelium surrounding the blastocyst is important in bringing the trophoblast into close association with the endometrial stroma during embryo implantation; 2) Poorly characterized stromal (decidual) cells undergo an epithelial transition and proliferate, producing a massively thickened uterine wall. The decidua also contains large numbers of macrophages and lymphocytes with unusual properties (Mincheva-Nilsson et al., 1992).

In addition to these changes, vascular changes also occur. At 6.5 day post coitus (p.c.), local blood vessels lose patency and blood drains into the lumen that surrounds the ectoplacental cone (El-shershaby and Hinchliffe, 1975; Welsh and Enders, 1991). At 7.5 day p.c., the integrity of the circulation is restored. Large blood sinuses form and active angiogenesis is apparent at the polar (mesometrial) end of the embryo (Welsh and Enders, 1991).

1.4 The Effects of Paternal Heat Stress on Reproduction Performance in the Animal

1.4.1 The effects of heat stress on male reproduction

In tropical and subtropical climates, high ambient temperature is an important factor influencing male reproductive performance. This conclusion has also been confirmed under experimental conditions. Therefore, the testis becomes the natural focus of attention in considering the effects of elevated temperatures on male reproduction.

The actions of heat on the testis may be seen as a dynamic process. On the one hand, heat can directly damage specific cell types within the testis. On the other hand, heat can also indirectly impair cell function within the testis, disrupting the biochemistry, molecular biology and endocrinology of the testis. Both direct and indirect effects of heat stress on the testis may finally reduce reproductive performance in animals and humans. In addition, the extent of effects of heat on the testis may depend on the magnitute of temperature, type of heat stress and species of animal.

Extensive studies have been conducted and many articles have been published so far on the effects of heat stress on male reproduction. Therefore, in this section, the effect of heat stress on male reproductive performance will be briefly summarized.

1.4.1.1 The effect of heat stress on the semen quality

1.4.1.1.1 The effect of seasonal changes on the semen quality

Animals can be divided into seasonal (sheep, goats, horse etc.) and non-seasonal breeders (cattle, pigs, mice, rats etc.) according to their reproductive behaviour and seasonal changes. This implies that seasonal changes may affect the semen quality.

In the ram, there were contradictory reports on the effect of season on reproductive performance. One study examined the effect of excessive skin folds (selected for in some breeding programs to increase wool production) on semen quality. In the summer of Australia (from late January to early March), the semen of Folds Plus Merino rams had a lower motility (5.8), proportion of live morphologically normal sperm (63.9%) and concentration of sperm (1.565 x10^9/ml) than the semen of Folds Minus rams (corresponding figures of 7.3, 80.8% and 2.081 x 10^9/ml) (Fowler and Dun, 1966). This is probably because the presence of excessive skin folds (Folds Plus) prevents the diffusion of heat within the testis. Furthermore, Sapsford (1951) found that the incidence of abnormal sperm increased during the summer months of two consecutive years, rather than during other seasons. It is suggested that these seasonal cyclic changes in ram semen were caused by environmental factors, particularly temperature. This is also supported by observations of a decrease of the semen volume, sperm concentration and total sperm per ejaculate in the ram during the summer (Dutt and Simpson, 1957). However, Fowler (1965) also demonstrated that the highest semen volumes were collected in the spring (2.0-2.4 ml) and the highest proportions of live sperm were observed during the summer months (80%) although

44

significant differences were found between months of the year for every characteristics studied.

In the bull, limited information indicates seasonal changes to not affect the semen quality (Miljovic et al., 1980; Downey et al., 1984). In contrast, other reports show that there are significant seasonal trends in semen quality, including low semen volume, sperm motility, total sperm/ejaculate, high number of abnormal sperm and testicular degeneration in the summer (Igboeli and Rakha, 1971; Fields et al., 1979; Parkinson, 1987).

In man, during the summer, the semen volume, sperm concentration and total sperm/ejaculate decreased, while the number of abnormal sperm increased (Levine et al., 1988; Levine et al., 1990). A recent study (Gyllenborg, et al., 1999) has shown that seasons affect sperm concentration and total sperm count in men. Highest sperm counts were found in the spring with a mean concentration of 77.6×10^6/ml (71.9-83.7) and lowest in summer with a mean of 57.5×10^6/ml (50.1-65.4). These results suggest that the poor quality of semen produced by both animals and man through the summer is mainly due to the detrimental effect of summer temperature on spermatogenic activity, but one can not exclude the effect of other factors (e.g. photoperiod, nutrition and other important male characteristics such as libido) during seasonal changes on reproductive performance in both animals and man.

1.4.1.1.2 The effect of artificial heat stress on the semen quality

In the bull, whole body heating did not reduce the ejaculate volume, but significantly decreased sperm motility, sperm concentration and total sperm counts (Casady et al., 1953), the number of live sperm, motility index but increased the number of abnormal sperm as

the time of exposure to the high temperatures changed (Table1.6) (Skinner and Louw, 1966).

Table1.6 Effect of exposure of bulls to 40°C on motility and percentage of
live and abnormal spermatozoa (Skinner and Louw, 1966)

Criteria		Exposure period		
		12h	24h	6 days
Motility index	MVBE*	10.4	10.1	10.6
	MVAE	9.8	8.6	8.1
Live sperm	MVBE	72.4 ± 3.1	67.7 ± 4.1	68.4 ± 4.6
% ± SE	MVAE	61.6 ± 3.7	52.5 ± 6.3	51.2 ± 4.8
Primary abnormalities	MVBE	9.9 ± 2.8	6.8 ± 2.2	7.6 ± 1.7
% ± SE	MVAE	17.2 ± 3.7	22.3 ± 5.2	22.7 ± 3.4
Secondary abnormality	MVBE	6.5 ± 1.6	6.7 ± 2.6	10.2 ± 3.7
% ± SE	MVAE	7.2 ± 2.2	10.0 ± 2.3	10.1 ± 2.9

* MVBE – mean value before exposure, semen collected at weekly intervals for 3 weeks before exposure;
MVAE – mean value after exposure, semen collected at weekly intervals for 8 weeks after exposure.

Local heating of bull testes also causes similar changes to whole body heating. Scrotal insulation for 24 or 72h reduced the number of live and normal sperm to approximately 65% and 60% respectively of the controls in the second and third weeks, returning to normal levels by 42 days after insulation and increased the number of abnormal and dead sperm (Austin et al., 1961).

Occurrence of specific types of abnormal sperm may be dependent on the time after insulation. The number of decapitated sperm increased at 6-14 days, sperm with abnormal tails at 12-23 days and those with abnormal acrosomes at 12 days after insulation for 48h (Wildeus and Entwistle, 1983). Subsequently, Vogler et al. (1993) found that predictable chronological sequences of specific abnormalities were tailess at day 12 to 15, diadem (a row of vacuoles across the nuclear ring of the sperm head) at day 18, pyriform-shaped (or pear-shaped) head and nuclear vacuoles at day 21, knobbed acrosome at day 21 and Daj-defect (as named after an infertile Danish bull with defective sperm tails) at day 30 after

insulation. In addition, epididymal sperm reserves were lower in the scrotal insulated bull (9.15×10^9) than the control bulls (17.4×10^9) (Wildeus and Entwistle, 1983), which could be due to an adverse effect of elevated testicular temperature since semen collected from bulls during day 3, 6 and 9 (sperm presumed in the epididymis or rete testis during insulation) after insulation did not differ from that collected from control bulls, but following cryopreservation, viability of sperm was significantly reduced (Vogler et al., 1991).

In rams, early studies have shown that whole body heating reduced sperm motility, and the number of live sperm, increased the number of abnormal and dead sperm, but did not affect the volume of semen (Dutt and Hamm, 1957; Moule and Waites, 1963; Smith, 1971). Moreover, the effect of whole body heating on the semen characteristics of rams varies with the temperature, time of heat stress and the time of recovery after treatment. After a single heat stress, sperm motility began to fall from 7 days after treatment, reached the lowest point at 35 days, and returned to the normal levels 52 days after treatment (Dutt and Hamm, 1957). Abnormal sperm began to increase from 9.0% in control group to 12.8% at 7 days after treatment, reached the highest point of 25.9% at 21 days, and then gradually decreased and returned to the normal levels at 42 days after treatment (Smith, 1971). Exposure of rams to 41°C for either 4h or 6h did not affect semen characteristics, but for 9 or 13.5h reduced sperm motility and increased the percentage of abnormal sperm (Smith, 1971).

Local heating of ram testes reduced the percentage of motile sperm and increased the number of dead and tailless sperm between 11 and 50 days after treatment (Waites and Setchell, 1964; Braden and Mattner, 1970; Mieusset et al., 1992). However, there are contradictory reports on the effect of local heating of testes on epididymal sperm and the

number of sperm per ejaculate. Mieusset et al. (1992) found that scrotal insulation did not affect the number of sperm per ejaculate, but adversely affected quality of epididymal sperm. However, exposure of ram testes to 40.5°C for either 1.5h or 2h did not affect sperm quality in the epididymis, but adversely affected sperm developing in the testes (Braden and Mattner, 1970). These differences could be due to differences of the time and method of heat stress and ram strains.

In the boar, an early study showed that whole body heating reduced total sperm counts (Wettemann et al., 1979), but this finding is not supported by later results (Larsson and Einarsson, 1984). Moreover, whole body heating not only reduced the number of sperm in the epididymis (both in the caput-corpus and cauda epididymidis) of boars (Wettemann et al., 1976) and sperm motility (Larsson and Einarsson, 1984; Malmgren and Larsson, 1984), but also increased the number of abnormal sperm (Larsson and Einarsson, 1984; Malmgren and Larsson, 1984). In addition, occurrences of specific types of abnormalities were related to the time after treatment. Proximal cytoplasmic droplets increased between 2 and 6 weeks, defective middle-pieces between 3 and 5 weeks, bent and coiled tails between 3 and 4 weeks, abnormal sperm head between 3 and 5 weeks and acrosome defects between 3 and 4 weeks after treatment (Larsson and Einarsson, 1984).

1.4.1.2 The effect of heat stress on the testis

1.4.1.2.1 The effect of heat stress on testicular weights

Effects of temperature on testicular weights are dependent on the magnitude of the temperature change, methods and time of heating. Mice were maintained at various ambient temperature for between 4 and 35 days or made bilaterally cryptorchid and then sacrificed

at day 4, 7, 11 and 14 after the start of treatment or at day 7, 12, 14, 21 and 30 following the operation (Meistrich et al., 1973). The testicular weights were pooled together and shown in Table 1.7 since weights of the testis were not significantly different between various points of the time following either the start of heating or the operation (Meistrich et al., 1973). Mean testicular weight decreased significantly when animals were exposed to 35°C or were made cryptorchid. Exposure to temperatures up to 30°C had no effect on the testis weight. Other studies have shown that local exposure of mouse testis to 38°C or 40°C for 60 min. did not reduce the testis weight, but exposure to 42°C dramatically decreased the testis weight to 50% of control values (Sailer et al., 1997). Exposure to 43°C for 20 min reduced the testis weight to 50% by 21 days after heating of males (Van Zelst et al., 1995). The weight loss of cryptorchid testes varies with the time after operation. Testicular weight remains unchanged for the first 6 days, followed by a 33% and a further 22% loss from days 6 to 8 and days 8 to 16 respectively (Yin et al., 1997). These losses in testicular weight are likely due to depletion of hyperthermia-sensitive cells from the testis.

Table 1.7 Effect of ambient temperature and cryptorchidism
on the testis weight of mice (Meistrich et al., 1973)

Temperature (°C)	No. of mice	Mean weight per two testes (mg)
23	21	207
26	20	199
30	20	205
35	24	133
23, cryptorchid	12	160

A reduction in testis weight resulting from heat stress has also been reported in the rat (Setchell and Waites, 1972; Fridd et al., 1975; Galil and Setchell, 1987b; Fujisava et al., 1988; Mclaren et al., 1994; Setchell et al., 1996) and ram (Lunstra and Schanbacher, 1988; Hochereau-de Reviers et al.,1993; Sanford et al., 1993).

1.4.1.2.2 Effects of heat stress on the testis cells

Paternal heat stress reduces testis weight by damaging germ cells. The extent of damage to germ cells could be related to temperature and time used during heat stress. When mice were kept at 32°C for 11 days (Meistrich et al., 1973), the testicular histology appeared abnormal. There was degeneration of the seminiferous epithelium, the absence or reduction in various generations of germ cells, with especially significant decreases in the number of both round spermatids and early pachytene spermatocytes. At 35°C, all gonads showed histological damage. After 4 days of exposure, there was an absence of pachytene spermatocytes at stages III-VIII, a reduction in the number of pachytene spermatocytes at stages IX-XII and round spermatids at step 1, with abnormal shaped spermatid nuclei at steps 11-13 (Meistrich et al., 1973). These results have been further confirmed by more recent studies (Gasinska and Hill, 1990; De Vita et al., 1990; Sailer et al., 1997). Experimental cryptorchidism in the mouse also seriously damaged primary spermatocytes and round spermatids and affected spermatogonia slightly (Meistrich et al., 1973; Yin et al., 1997). Whole body heating and local heating of the testis have been reported to increase the incidence of X-Y dissociation in primary spermatocytes in diakinesis metaphase in the mouse, which means that the primary effect was on pachytene spermatocytes (Garriott and Chrisman, 1980; Waldbieser and Chisman, 1986; Van Zelst at al., 1995).

Heat stress also results in changes in testicular-cell type ratios in the mouse. Exposure of mouse scrotum and testis to 40°C for 60 min caused a reduction in the relative percent haploid germ cells below control levels after 11 to 14 days of treatment, with a corresponding rise in the relative percentage of diploid and tetraploid germ cells. However, exposure of the scrotum and testis to 42°C for 60 min resulted in a significant decrease in

the percent haploid germ cells after day 3 through day 35, with a corresponding significant increase in the percent of diploid germ cells (Sailer et al., 1997).

In the rat, exposure of testes to 43°C for 15 min increased the number of abnormal pachytene spermatocytes, but did not affect the number of spermatogonia (Chowdhury and Steinberger, 1964, 1970). Later, Blackshaw and Hamilton (1970) found that exposure of the testis to 42°C for 30 min caused the appearance of dye chromophilia in pachytene and diplotene spermatocytes as early as 1h after heat stress. In addition, exposure of rats to an environment of 35°C for 3 months severely affected spermatogenesis in about 20% of tubular cross-sections (Sod-Morich et al, 1974). In both immature (Shikone et al., 1994) and mature (Henriksen et al., 1995) rats, experimentally-induced cryptorchidism also caused an increase of apoptosis of germ cells, and damaged spermatocytes and round spermatids in mature rats. Blanco-Rodriguez and Martinez-Garcia (1997) reported that exposure of the rat testis to 43°C for 15 min. resulted in significant increase of dying A_1 spermatogonia, pachytene spermatocytes and spermatids at stage I, dying A_2 spermatogonia, zygotene spermatocytes, pachytene spermatocytes at stage XII, and dying A_3 spermatogonia, pachytene spermatocytes and metaphase spermatocytes at stage XIV.

In rams, exposure of the testes to 40°C for 150 min resulted in significant increases in degenerating pachytene spermatocytes and dividing B-spermatogonia (Waites and Ortavant, 1968).

In pigs, an early study showed that exposure of pigs to a hot environment (34.5 ± 1°C for 8h and 31 ± 1°C for the remaining 16h during each 24h period) for 90 days reduced only the number of spermatids (Wettemann and Desjardins, 1979). However, a later study

indicated that the number of pachytene spermatocytes and early spermatids were decreased after the scrotal insulation for 100h (Malmgren and Larsson, 1989).

1.4.1.2.3 The effect of heat stress on proteins, mRNA and DNA in the testis

Paternal heat stress has been reported to reduce protein synthesis in seminiferous tubules and germ cells (Nakamura et al., 1978; Nakamura and Hall, 1980; McLaren, et al., 1994). Spermatids isolated from rat testes showed decreased incorporation of amino acids into proteins when incubated at 37°C and 40°C compared with 34°C (Nakamura et al., 1978; Nakamura and Hall, 1980). Even though adult rats were injected (s.c) with 25mg testosterone ester just before local testicular heating (43°C for 30 min.) in order to maintain normal intratesticular concentration, heat stress still reduced the synthesis of proteins in the testis, including three androgen-regulated proteins in the seminiferous tubule (McLaren et al., 1994).

Heat stress and other stress factors induce the synthesis of a small number of proteins, called heat shock proteins (hsps) in cells of all organisms, which could protect cells against the effects of adverse environmental conditions. For example, heat stress could affect protein synthesis and possibly gene expression during embryo development. Exposure of bovine 2-cell embryos to 39°C resulted in slight alteration of hsps 68, 70 and 71, but exposure of them to 42°C greatly increased the amount of hsp68, but not hsps 70 and 71 (Edwards and Hansen, 1996). However, some of these hsps have also been reported to be present in normal tissues, at normal temperatures and in different eukaryote species from insects to mammals and serve a range of vital functions in normal cells (Lindguist and Craig, 1988; Gething and Sambrook, 1992; Christians et al., 1997). For example, the hsp90

family plays an important role in cell function both under stress and non-stress conditions. It is an abundant, essential cytosolic protein, amounting to 1~2% of total soluble protein under normal conditions and becoming even more abundant with stress (Craig et al., 1994). In vivo studies have shown that the hsp90 binds to many steroid hormone receptors, forming stable, long-lived complexes, keeping them inactive until hormone binding triggers release and activation (Craig et al., 1994). Furthermore, Gruppi and Wolgemuth (1993) examined the changes of two members of the hsp90 family, designated the hsp84 and 86 in the mouse testis under normal conditions and found that the hsp86 was expressed in both germinal and somatic cells, particularly in germ cells and the hsp84 in somatic cells. In addition, specification of the hsp86 and 84 is related to mouse age. The hsp86 was lowest in the day 7 testis (only spermatogonia), increased in the day 17 testis (germ cells in all stages of meiotic prophase and spermatogonia) and highest in the adult testis (complete germ cell lineage); the hsp84 was only detected in day 7 and day 17 but not in the adult testis (Gruppi and Wolgemuth, 1993), suggesting that these two hsps may be involved in the normal process of spermatogenesis. Mouse spermatogenic cells have been shown to synthesize relatively large amounts of a 70,000 molecular weight protein (P70) that appears to be a cell-specific isoform of hsp70, the most heat-inducible protein (Allen et al., 1988a). A further study showed that relatively large amounts of P70 were present in unstressed pachytene spermatocytes and round spermatids. However, synthesis of hsp70 was not detectable in unstressed cells (32°C) but was induced in all stages of isolated germ cells following heat stress at 42.5°C for 30 min. and does not protect male germ cells from heat stress (Allen et al., 1988b). Subsequently, hsp 36 and 74 were found to be synthesized and secreted at 38°C (Lemaire and Heinlein, 1991). Heat stress also causes relocation of hsps in male germ cells. Biggiogera et al (1996) found that when the mouse testis was exposed to 42°C for 1h, hsp 90 and 27 were relocated from the cytoplasm of cells in the unstressed

53

testis to the nucleus which is actively involved in RNA synthesis and processing, thus suggesting that they may have protective functions in these processes in a tissue which is particularly sensitive to heat stress. One of main regulators of hsp gene expression is heat shock transcription factor (HSF). In the mouse, there are two HSF genes, HSF1 and HSF2. HSF1 has been shown to mediate the induction of hsp gene expression in response to elevated temperature and other environmental stresses. HSF1 activation in mouse pachytene spermatocytes occurred at 35°C and caused elevation of the hsp 72, while a preparation of somatic testis cell types exhibited HSF activation only at temperatures of 42°C and above. These results may mean that a reduced HSF1 activation temperature is a unique property of male germ cell types within the mammalian testis and demonstrate that HSF1 activated at this lower temperature threshold is fully capable of mediating a protective cellular stress response in these cell types (Sarge, 1995).

Heat stress also has differential effects on translation of normal mRNAs of the different cells in the testis. A recent study has shown that exposure to 37°C, 42.5°C and 44°C reduced incorporation of [^{35}S]-methionine into histone H1t (the testis-specific subtype of histone H1) in pachytene spermatocytes, but had minimal effects on the translation of mRNAs for transition proteins 1 and 2 in elongated spermatids. Moreover, exposure to 37°C and 44°C reduced incrementally the size of polysomes translating H1t mRNAs in pachytene spermatocytes and sulfated glycoprotein 2 mRNA in Sertoli cells, but did not affect the proportion of polysomal protamine 2 mRNA in elongated spermatids (Cataldo et al., 1997). This shows that paternal heat stress may inhibit the initiation of translation in pachytene spermatocytes and Sertoli cells, but not in elongated spermatids. However, culturing rat and mouse Sertoli cells at 41°C resulted in an increase of mRNA for clusterin by 12h and reached a maximum of 2-fold and 16-fold over that of controls by 48h after heat

stress (Clark and Griswold, 1997). The difference between these two studies could be related to heat-stress time and the temperature used. In addition, in vitro results may not really reflect in vivo situations, therefore, the effect of heat stress on expression of mRNA for clusterin in Sertoli cells in vivo merits further investigation.

Heat stress has been reported to reduce significantly DNA synthesis in spermatogonia and primary spermatocytes (see Table 1.8) (Nishimune and Komatsu, 1972) since DNA synthesis in the seminiferous tubule is attributed to these two germ cells. DNA synthesis in germ cells showed a maximal value (100) at 32.0°C and was then inhibited when temperature was elevated. Conversely, DNA synthesis in supporting cells increased with an elevation of temperature and reached the maximal level (100) at 38.5°C and then reduced again at 40.0°C.

Table 1.8 The relationship between incorporated [³H]-thymidine per cell and incubation temperature in cultured cells (Nishmune and Komatsu, 1972)

Culture cell type	Incubation temperature °C				
	32.0	35.0	37.0	38.5	40.0
Testicular floating cells (germ cells)	100	-	-	9	-
Testicular supporting cells (Sertoli cells)	8	14	43	100	64

The values represent incorporated [3H]-thymidine per cell. The maximum value in each row is taken as 100%.

DNA synthesis requires DNA polymerase α, β,γ and topoisomerase I. Fujisawa et al. (1988) examined the activities of these four enzymes in rat testis after inducing cryptorchidism and found that the activities of DNA polymerase α and topoisomerase I were unaffected at 7 days after operation, while DNA polymerase β was reduced by 43% at 5 days and by 47% at 7 days. The reduction in the activity of DNA polymerase γ was much greater and reached the lowest point of 78% at 7 days after surgery. Several studies have shown that DNA polymerase α plays a major role in chromosomal DNA replication, while

DNA polymerase β is probably responsible for DNA repair and the recombination process associated with meiosis (Weissbach, 1977; Grippo et al., 1978). DNA polymerase γ may be involved in DNA replication in mitochondria (Weissbach, 1977). In addition, heat stress (cryptorchidism) seriously depressed the recombination activity in rat spermatocytes (Hotta et al., 1988). These results indicate that the spermatocyte is more sensitive to heat stress than spermatogonia and heat stress may disturb differentiation and maturation of spermatocytes.

1.4.1.2.4 Effects of heat stress on sperm chromatin structure

Sperm chromatin structure assayed (SCSA) by studying acridine orange (AO) staining with subsequent flow cytometry (FCM) measurement (Evenson, 1989, 1990; Evenson and Jost, 1994), has been used to monitor the susceptibility of sperm chromatin DNA to abnormal conditions, such as acid-induced denaturation in situ (Darzynkiewicz et al., 1976; Evenson et al., 1980, 1985; Evenson and Jost, 1994) and heat stress (Sailer et al., 1997). AO has been reported to be intercalated into double-stranded DNA (dsDNA) (normal chromatin structure) where it fluoresces green. When bound to single-stranded RNA or single-stranded DNA (ssDNA) (abnormal chromatin structure), it fluoresces red (Darzynkiewicz, 1979). Furthermore, several studies have shown that the SCSA is a sensitive measure of sperm chromatin structure as related to fertility in the bull (Ballachey et al., 1987), the boar (Evenson et al., 1994) and man (Evenson et al., 1999; Marcello et al., 2000). A recent study has also indicated that heat stress in the mouse can increase the number of abnormal chromatin structures, as shown from the SCSA data (Sailer et al., 1997). The effect appears to depend on the degree of exposure to temperature and time of sperm recovery. In bulls, scrotal insulation for 48h detrimentally affected the chromatin structure of subsequently

ejaculated sperm from both epididymis and testes although the effect of heat stress on the chromatin of testicular sperm was more severe than that of epididymal sperm (Karabinus et al., 1997).

1.4.1.3 Effect of heat stress on endocrine function

The testis is not only an organ of gametogenesis, but also an organ of endocrine secretion and is highly sensitive to external hormones. Therefore, it is possible that heat stress may affect normal endocrine function in the testis, with indirect influences on spermatogenesis.

Many studies have shown that local heating of the testis did not change peripheral testosterone values in rats (Main et al., 1978; Kerr et al., 1979; Damber et al., 1980; Main and Setchell, 1980; Jegou et al., 1984; Galil and Setchell, 1987a). However, either exposure of male rats to 33-35°C for 3 weeks (Bedrak et al., 1980) or experimentally induced cryptorchidism (Clegg, 1961; Eik-Nes, 1968; Amatayakul et al., 1971; Hall and Gomes, 1975; Bergh et al., 1984a) reduced the peripheral levels of serum testosterone, the number of LH-binding sites per Leydig cell and increased the rate of testosterone catabolism by the liver and kidney.

Androgens are mainly produced from the interstitial tissue and in particular the Leydig cells. The effect of heat stress on steroid biosynthesis in the testis may be a gradual process. In 20-day-old rats made unilaterally cryptorchid at birth, androgen-binding protein (ABP) content of abdominal epididymides was reduced, but intratesticular concentrations of both testosterone and oestradiol did not change (Bergh et al., 1984a). Cryptorchidism of adult rats made unilaterally cryptorchid at birth resulted in decreases of the cross-sectional area of

Leydig cells, the number of the endoplasmic reticulum and an increase of the number of lipid droplets in the cytoplasm (Bergh et al., 1984a). These changes of Leydig cell morphology reduced the conversion mediated by 17α-hydroxylase and 17β-ketosteroid reductase and increased the conversion mediated by 20α-dehydrogenase, thus disturbing steroid biosynthesis (Bergh et al., 1984a). The reason for the disturbed steroid biosynthesis may be also related indirectly to altered paracrine influences from the damaged tubules produced by the effect of heat on the testis. In the scrotal testes of adult rats, the size of peritubular Leydig cells varies with the stage of spermatogenic cycle. The size of peritubular Leydig cells especially surrounding stages VII-VIII (88.7 ± 7.1 um^2) has been reported to be larger than perivascular Leydig cells (69.3 ± 5.9 um^2). Leydig cells surrounding stages IX-XIV were found to be similar in size to perivascular cells (Bergh, 1983; Bergh and Damber, 1984). Conversely, in the abdominal testes, no spermatogenic cycle and no stage-dependent variation in the size of peritubular Leydig cells were present with increased numbers of degenerating germ cells in stages XIV-III of the spermatogenic cycle and increased amounts of lipid droplets of the Sertoli cells in stages XIV-VIII (Bergh, 1983; Bergh and Damber, 1984). Jansz and Pomerantz (1986) examined Leydig cell responses to disrupted gametogenesis in vitro using tissue slices and collagenase dispersed Leydig cells from testes of unilaterally or bilaterally cryptorchid rats. Four weeks after surgery, androgen secretion per mg of tissue or per Leydig cell in response to maximal LH stimulation was greater in damaged testes than in sham-operated testes. It was concluded that disruption of spermatogenesis led to Leydig cells that were hyperresponsive to LH stimulation in vitro.

Sertoli cells have been reported to produce lactate for the energy-requiring processes in testicular germ cells and plasminogen activator (t-PA) which is controlled by follicle stimulating hormone (FSH) and preleptotene spermatocytes. However, tubules from

cryptorchid rat testes secreted less lactate than the scrotal tubules and the basal secretion of t-PA was similar but the FSH stimulation increased the content of t-PA secretion (Bergh et al., 1987), indicating a more specific dysfunction of the Sertoli cells in the cryptorchid testes (Bergh et al., 1984b). Alterations in testosterone levels could be related to an increased concentration of oestradiol (Munabi et al., 1984), altered paracrine influence by the damaged seminiferous testis, changes in the microcirculation of the testis, and a decrease in the available number of LH, FSH receptors (Steinberger, 1989). Therefore, these results indicate that cryptorchidism may damage germ cells and Sertoli and Leydig cells, further disturbing steriod biosynthesis in the testis. On the other hand, the changes of steroid biosynthesis may also promote the extent of damage of germ cells.

Heat stress not only influences production and secretion of local hormones in the testis, but also changes hormone patterns produced in the hypothalamus and the pituitary by feedback mechanisms of hormones produced by the testis. Several studies have indicated that heat stress significantly increased the values of serum LH and FSH (Collin et al., 1978; Main et al., 1978; Galil and Setchell, 1987a,b). However, Bedrak et al. (1980) reported that heat stress reduced concentrations of LH and Gonadotropin-releasing hormone (GnRH), but increased levels of FSH in peripheral blood. These changes of serum LH and FSH could be related to secretion of inhibin and/or the changed number of LH and FSH receptors in the testis. Experimentally induced cryptorchidism and local heating of the testis resulted in a decrease in inhibin production (Seethalakshmi and Steinberger, 1983; Au et al., 1987). Inhibin secretion in Sertoli cells may be directly suppressed by elevated temperature since inhibin secretion declined to near zero at 38°C, but remained unchanged at 32°C when normal rat Sertoli cells were incubated at these two temperatures for 21 days (Steinberger, 1980). Moreover, its secretion may also be affected by the types of germ cells changed by

heat stress because both in vivo and in vitro results suggested a dual stimulatory and inhibitory effect of higher temperature to inhibin production, an initial rise due to the direct effect of elevated temperature on the Sertoli cells followed by a decline due to the disruption of the seminiferous epithelium (Gonzales et al., 1989). Therefore, a decrease of inhibin in Sertoli cells may lead to an increase of FSH since inhibin plays a regulatory role of feedback on FSH secretion in the pituitary (Lincoln et al., 1990; Plymate et al., 1992). In addition, an alteration of blood circulation and a decrease in the number of LH and FSH receptors in the testis could increase peripheral concentrations of LH and FSH (Galil and Setchell, 1987b; Steinberger, 1989).

In bulls, compared with the levels during scrotal insulation, testosterone levels declined during scrotal insulation for 4 days, then increased by 7 days after scrotal insulation, followed by a decline until 28 days after scrotal insulation (Prabhakar et al., 1990), reached minimum value 42 days after scrotal insulation before returning to be normal 56 days after scrotal insulation (Sidibe et al., 1992). There were no distinct changes in the LH levels during all experimental periods (Prabhakar et al., 1990) or the level of plasma LH did not change during scrotal insulation, then gradually increased after scrotal insulation, and reached a maximum level 28 days after scrotal insulation, then decreased and reached the lowest point 105 days after scrotal insulation (Sidibe et al., 1992). These studies suggest that the feedback mechanism of testosterone regulation of LH seemed disturbed since LH and testosterone peaks were correlated inversely and seemed not to be the only component involved in the testicular regulation.

In rams, artificially induced cryptorchidism and shortened scrotum caused an increase in peripheral LH concentration (Hillard and Bindon, 1975; Schanbacher and Ford, 1977; Schanbacher, 1980; Lunstra and Schanbacher, 1988; Sanford et al.,1993) and peripheral FSH concentration (Sanford et al., 1993). However, testosterone levels in peripheral blood

were unchanged (Schanbacher and Ford, 1977; Schanbacher, 1980; Tierney and Hallford, 1985; Lunstra and Schanbacher, 1988; Sanford et al., 1993). In addition, although testicular concentrations of testosterone and oestradiol-17β were increased, the total content of testosterone within the testis was increased only in pre-pubertal testis (Barenton et al., 1982) and levels of testosterone in spermatic venous blood were normal (Lunstra and Schanbecher, 1988).

In boars, whole body heating and local heating of the testis can change endocrine function within the testis. Exposure of mature boars to 35°C slightly increased testosterone levels during the first day of heating, and thereafter continuously decreased levels for another 4 days of heating. However, the testosterone values were then increased during the next 5 days after heat exposure (Einarsson and Larsson, 1980; Larsson et al., 1983), then began to reduce and returned to control levels 20 days after exposure (Larsson et al., 1983). Lowered testosterone concentrations during heat stress and its increase during the following 5 days after the heat exposure period may be related to an increase in cortisol levels during heat stress and its decrease during the first 5 days following exposure (Larsson et al., 1983) since corticoids have been found to suppress testosterone secretion in men (Doerr and Pirke, 1976), bulls (Welsh et al., 1979) and rats (Bambino and Hsueh, 1981). During scrotal insulation of mature boars, plasma testosterone concentrations continuously decreased from 14.5 to 7.5 nmol/l and there were also falls in oestradiol and oestrone sulphate levels. Plasma testosterone values showed different patterns after the removal of scrotal insulation. One group of boars showed an increase in testosterone during the first day after removal of the device and then a decrease for the following 6 days. Another group of boars showed a rise in testosterone 4 days after the removal of the device which continued for 3 days (Malmgren, 1990). These differences may possibly be due to variation of individual boars.

Moreover, the reduction in testosterone levels may reflect the lowered testosterone secretion in heat-stressed testes which would mean impairment of Leydig cell function. The decrease in testosterone values during and after heat stress could account for the reduction in oestradiol-17β and oestrone sulphate levels (Malmgren, 1990) since oestrogens are synthesized from androgen in the testis. However, in peripubertal boars, scrotal insulation did not reduce testosterone levels and there were no changes in the values of oestradiol-17β and oestrone sulphate. After removal of the scrotal insulation device, levels of these three hormones remained similar to those in control groups (Malmgren, 1990).

1.4.1.4 Mechanism of heat damage

The mechanism of heat damage to the testis has not been well understood, but it could be related to the following possibilities.

Heat stress is likely to cause a decrease in oxygen tensions in the testicular tissue which could be responsible for damage to germ cells. An early study (Waites and Setchell, 1964) showed that local heating of ram testes increased metabolic rate in the testicular tissues. and significantly reduced the oxygen content of the blood in the spermatic veins when the temperature of the testis had risen from normal values to about 37°C. This did not change further when the temperature rose to 40°C despite a maintenance or slight increase in blood flow. Therefore, a conflict between oxygen supply and oxygen consumption may change metabolic processes within germ cells and cause cellular damage.

Heat stress also reduced fluid secretion in the testis (Setchell et al., 1971). If the germinal cells are dependent for nutrients on fluid secreted around them by the Sertoli cells, any

reduction in the production of this fluid could lead to damage of the germinal cells (Setchell, 1978).

Heat stress might directly affect the germ cells in the testis resulting in increased rates of apoptosis. Bilateral and unilateral experimental cryptorchidism or local heating to the testis can induce germ cells to undergo apoptotic cell death in the rat (Allan et al., 1987; Shikone et al., 1994; Henriksen et al., 1995) and mouse (Yin et al., 1997). The extent of germ cell apoptosis has been shown to be related to the time after surgery and the types of germ cells. In the rat, apoptotic DNA fragments significantly increased by 2.8 ± 0.3-fold and 4.2 ± 0.3-fold at days 4 and 7 after cryptorchid operation, respectively, compared with control group and the affected cells were mainly primary spermatocytes (Shikone et al., 1994). In the mouse, the percentage of apoptotic germ cells within the cryptorchid testis was 3.6% and 4.9% on days 7 and 10, respectively, while that in scrotal germ cells was 0.2%. The affected germ cells were in turn primary spermatocytes, round spermatids and occasional spermatogonia (Yin et al., 1997).

Germ cell loss induced by heat stress could also be accelerated by the p53-dependent pathway. A tumor-suppressing protein called p53, accumulates and becomes activated in response to DNA damage, thus inducing apoptosis. The testis in some mouse strains has been found to have high concentrations of p53 (Rogel et al., 1985; Almon et al., 1993) with the highest expression in primary spermatocytes (Schwartz et al., 1993). Experimental cryptorchidism in p53 +/+ mice has been shown to result in apoptosis of germ cells on day 6-7 after operation, while this result was delayed by 3 days in p53 -/- mice (Yin et al., 1998).

Heat stress may also alter contact and signal communication between germ cells and between germ cells and Sertoli cells, thus leading to damage of germ cells in the testis. It has been known that normal spermatogenesis in the testis of mammalian animals is a dynamic and highly ordered process, which consists of different stages of the spermatogenic cycle. Each stage is composed of spermatogonia, spermatocytes, spermatids and the Sertoli cells (Skinner, 1991; Jegou, 1993; Kierszenbaum, 1994, Griswold, 1995). This means that cell-cell contact devices between germ cells and between germ cells and Sertoli cells are present. Sertoli cells provide physical and biological support to these developing germ cells, while germ cells are endocrine-sensitive cells depending on the action of hormones on the Sertoli cells (Bremner et al., 1994, Vornberger et al., 1994). The Sertoli cells secrete a number of important proteins including several soluble or membrane-bound growth factors (Jegou, et al., 1993; Kierszenbaum, 1994; Griswold, 1995; Lee, et al., 1997) and their corresponding receptors of the Fas-Fas ligand system (Packer, et al., 1995; Lee, et al., 1997), integrins (Ruoslahti and Pierschbacher, 1987; Salanova et al., 1995) and cadherins (Geiger and Ayalon, 1992; Munro and Blaschuk, 1996). Most of these proteins are likely to be involved in Sertoli-germ cell communication and some of them may be extracellular regulatory signals responsible for stage-specific control of cell survival and death. Therefore, it is suggested that heat stress may disturb synthesis of these important proteins and the endocrine function in both the Sertoli cells and germ cells, thus disturbing Sertoli-germ cell normal signal communication, finally leading to apoptosis of germ cells.

Many studies have shown that epididymal spermatozoa are much less susceptible to heat than their testicular counterparts, but a few studies have shown that elevated testicular temperature reduced epididymal sperm reserves and damaged epididymal sperm (Wildeus and Entwistle, 1983, Mieusset, et al., 1992; Karabinus et al., 1997). This may be because heat stress at least changes in part the ionic and protein composition of cauda epididymal

fluid by virtue of an effect on the cauda epithelium (Bedford, 1989). These results have suggested that heat stress may disturb further maturation of spermatozoa in the epididymis.

1.4.1.5 The effect of heat stress on fertility

The effect of heat stress on the fertility of male animals varies with individual animals and the time and methods used during heating. Young (1927) found that after the testes were heated, two guinea pigs remained normally fertile, fourteen appeared temporarily sterile between 7 to 44 days and three became permanently sterile.

In the male rat, heat stress generally reduced fertility, but the effects varied between studies (see Table 1.9). In addition, treatment with hormones also influences the sterile period after heat stress. Elfving (1950) found that treatment with FSH, thyroid hormone or a low dose of testosterone delayed the onset of the sterile period in a small number of animals, but hCG or a large dose of testosterone had no effect.

Table 1.9   The effect of heat stress on the fertility of male rats

Method of heating	Sterility after heating	Authors
Infra-red Hot-air	16-82 days 34-85 days	Cunningham and Osborn (1929)
Testes were placed in a water bath at 43°C for 20 min.	Three rats were permanently sterile; twelve rats were temporarily sterile, starting between 10 and 20 days and ending between 50 and 140 days.	Elfving (1950)
Testes were placed in a water bath at 43°C for 30 min.	Sterility of rats began between 7 and 14 days and ended between 49 and 56 days.	Setchell et al. (1988)

65

In the mouse, the time of exposure of the testes to a hot environment directly affects the fertility of male animals. Male mice remained fertile after exposure of their testes to 42°C for 20 min, but if the time was extended to 30 min at the same temperature, sterility occurred between 10 and 32 days with one group and 38 days with the other group (Setchell et al., 1998). Furthermore, exposure of mice to an environment of 35°C and 65% humidity for 24h, appeared to have no effect on fertility, but exposure for 48h, 96h and 120h, reduced fertility to 36%, 27% and 18%, respectively (Garriott and Chrisman, 1981).

The effect of heat stress on fertility may also depend on the adaptability of male mice to acclimatize. Exposure of adult male mice to an environment of either 32.7°C or 36.1°C for 2 weeks resulted in sterility of 43% and 100% of the animals, respectively. However, when mice were placed in the same environment for the period from weaning to maturity, only 17% were sterile at 32.7°C and 33% at 36.1°C (Pennycuik, 1967).

1.4.2 The effect of paternal heat stress on reproductive performance in the normal female

1.4.2.1 The effect of paternal heat stress on fertilization

Burfening et al. (1970) placed male mice into an environment of 32°C and 65% relative humidity for 24h followed by mating with normal females from day1 to day 30 after their exposure. Fertilization rates (the ratio of cleaved ova to total ova recovered 48h after a successful mating) fell from 82% on day 1 to 69% on day 5, 54% on day10, 10% on day15 to 3% on day 20. This decline was followed by a recovery to 85% on day 25 and 100% on day 30 after heating (control males had fertilization rates of between 78 and 91% over the same time). Artificial insemination undertaken with epididymal sperm from mice whose

testes had been heated to 42°C for 30 min 28 days earlier resulted in fertilization rates that were reduced from 65% in controls to 45% in heated groups (Jannes et al., 1998).

In rats whose testes had been heated to 43°C for 30 min, the fertilization rates of ova collected from the oviducts of normal females, the morning after mating were reduced from 76% to 17%, 16 to 20 days after heat treatment (Setchell et al., 1988).

In rams, time and temperature used during heat stress affect the fertilization rates achieved. Howarth (1969) reported that fertilization rates were slightly reduced at 1 week and reduced to zero 2 and 3 weeks after rams were exposed to an environment of 32°C and 65% relative humidity for 4 days. Subsequently, when the testes were heated to 40.5°C for 2h or 1.5h and 39.5°C for 4h, the first procedure resulted in low fecundity between days 14 and 34, and zero between days 34 and 47. These results have been further confirmed by Rathore (1970) who found that when each male was exposed to 40.5° for 8h on one, two, three or four occasions on successive days and was then mated to eight superovulated ewes between 10 and 27 days after heating, the fertilization rate in controls was 93% of the ova recovered 60-70h after mating, with corresponding figures of 66, 42, 23 and 6% in the heated groups. However, the later two treatments did not affect fecundity (Braden and Mattner, 1970).

The results from in vitro fertilization using sperm from scrotal-insulated rams indicate that fertilization rate decreased from 73 to 7% with 16h per day insulation for 18 days. There were also significant reductions with 8h per day insulation for 35 days during both the insulation period and the following 14 days (Ekpe et al., 1992, 1993; Setchell, 1994).

When boars were exposed to 34.5°C for 8h and 31°C for 16h per day for 6 weeks, both natural mating and artificial insemination reduced fertilization rates (59 and 29%,

respectively), compared with corresponding figures of 85% and 41% in controls (Wettemann et al., 1976, 1979).

1.4.2.2 The effect of paternal heat stress on embryo development

Young (1927) found that there was an increase in the percentage of stillborn and aborted pups sired by heated male guinea pigs when mating was allowed to continue until 48 days after heating, but strangely he did not consider that this was a direct effect of heating on survival rate. He also subjected sperm to temperatures in vitro ranging from 38°C to 46°C for 30 min, prior to insemination and the results indicated an increase in early embryonic mortality with increasing temperature (Young, 1929). Howarth et al. (1965) found a significant decrease in the rate of embryo survival prior to and following implantation when sperm were capacitated in the uterus of the female rabbit maintained at an ambient temperature of 32°C compared with controls kept at 21°C. Subsequently, Burfening and Ulberg (1968) incubated ejaculated sperm at either 38°C or 40°C for 3h and found a decrease in the rate of embryo survival prior to (control, 62.5%, 38°C, 64.3%; 40°C, 42.2%) and following (control, 94.3%; 38°C, 91%; 40°C, 80%) implantation (while having no effect on fertilization rate: control, 98.2%; 38°C, 98.2%; 40°C, 95.9%).

In sheep, paternal heat stress can reduce the proportion of preimplantation embryos and increase the number of degenerated embryos. Several recent studies have demonstrated that development of embryos (2 or 4-cell, 8-cell, morula or blastocyst) produced by in vitro fertilization was slower when using semen from scrotal-insulated rams than from control rams. There was also an increased degeneration at the blastocyst stage during 11 days of insulation (Ekpe et al., 1992, 1993; Setchell, 1994). Paternal heat stress can also increase the rate of embryonic mortality after implantation (Mieusset et al., 1992). These authors

68

reported that following insemination using semen from rams after 4, 15 or 21 days of scrotal insulation, pregnancy rates were not affected at 17 days after insemination, but the rate of embryonic mortality between 17 days and 65 days after insemination was higher at days 4, 15 and 21 in the heated rams (78.7, 78.6 and 93%) than in the control rams (55.9 and 65.7%). This observation is supported by an early study (Dutt and Simpson, 1957) in which the embryonic death rate fell by 27% when rams were kept in air-conditioned chambers during the summer months.

In the mouse, Bellve (1972) exposed ICR albino male mice to an ambient temperature of 34.5°C and 65% relative humidity for 24h. When normal female mice were mated from day 3 to day 8 after heat stress of the male, the embryos produced in vivo 54h after an observed plug showed an increase of 4-cell (control 1.68 vs heat-sired 3.70 per mouse) and a decrease of 8-cell embryos (control 8.75 vs heat-sired 6.48 per mouse). Subsequently, embryos were collected from oviducts and the uterus of superovulated mice at 64, 72, 88 and 96h after hCG injection and it was found that paternal heat stress allowed normal embryonic development during early cleavage stages but caused an accumulation of embryos at the morula stage at 88h after hCG injection (control 14.01 vs heat-sired 22.12 per mouse), thus reducing the number of embryos developing to blastocysts at 96h after hCG injection (control 20.73 vs heat-sired 17.74 per mouse) (Bellve, 1973). These results were further extended by Bellve (1976). When the late 2-cell heat-sired and control-sired embryos were cultured for 66, 72, 88, 96 and 120h, respectively after hCG injection, the results demonstrated that exposure of the male to 34.5°C resulted in a marked accumulation of morula at the 120h (control 2% vs heat-sired 33.7%) and a significant decrease of the number of blastocysts (control 78.3% vs heat-sired 39.3%).

Paternal heat stress not only reduced the development of preimplantation embryos, but also influenced the development after implantation. It has been previously reported that paternal heat stress significantly increased embryonic mortality by day 10.5 of gestation (Burfening et al., 1970; Bellve, 1972, 1973). When the testes were heated by immersion in a water bath at 42°C for 20 min and mating was allowed to continue until 38 days after heating, it was found that 10.5 or 10.7-day-old embryos sired by the heated males between 4 and 35 days after heating were significantly smaller than those sired by control males (control 17. 95 $\pm$ 0.54 mg vs heat-sired 12.95 $\pm$ 0.72 mg). However, embryos produced before 4 days or after 35 days post-heating were not significantly different from controls (control 17.95 $\pm$ 0.54 mg vs heat-sired 20.89 $\pm$ 0.65 mg). The authors further examined the effects of paternal heat stress on weights of embryos of different ages(from 7.5-day-old to 18.5-day-old) and found that paternal heat stress reduced the weight of other embryos except for those collected on 7.5 and 14.5 days of gestation. Trophoblasts and yolk sacs were also smaller in 10.5-day-old embryos sired by the heated males than in those sired by the control males (trophoblast: control 36.8 $\pm$ 0.79 mg vs heat-sired 31.6 $\pm$ 0.92 mg; yolk sacs: control 4.26 $\pm$ 0.15 mg vs heat-sired 3.35 $\pm$ 0.26 mg). There were no significant differences in 15.5 and 18.5-day-old trophoblasts and yolk sacs. Litters sired between 32 and 37 days after 30 min of heating were smaller than those sired by control males (control 9.25 $\pm$ 0.20 mg vs heat - sired 3.75 $\pm$ 1.25 mg) (Setchell et al., 1998). In contrast, there were no significant differences in litter size following heating of males for 20 min (Setchell et al., 1998). These findings of smaller fetuses with normal litter sizes from heat-exposed sires have been confirmed by Jannes et al. (1998).

In rats, paternal heat stress has been reported to affect the development of pre- and post-implantation embryos, thus leading to low pregnancy rates. When normal female rats were mated with males, 0-6, 7-10, 11-15, 16-20 and 21-25 days after exposure of the testes to

43°C for 30 min, pregnancy rates were reduced significantly from 87.5% in controls to 36.3% at 16-20 days and 25.0% at 21-25 days, but did not change at the other days after heat treatment. The number of degenerating embryos significantly increased to 53.3% and 20.5% respectively at 2 and 15 days of pregnancy when females were mated with males 14 days after heat stress (Setchell et al., 1988). In contrast, exposure of rats to 35°C for 3 months reduced mating and conception rates, but once conceived, embryo development appeared normal (Sod-Moriah et al., 1974).

In pigs, the effect of paternal heat stress on embryo survival is related to the procedure for mating. When natural mating was used, heat-stressed boars did not show reduced embryo survival rates (control 82 ± 2% vs heat- sired 79 ± 4%), while following artificial insemination, heat-stressed boars did show reduced rates of embryo survival (control 74 ± 4% vs heat-sired 48 ± 5%) (Wettemann et al., 1976; Wettemann and Bazer, 1985).

Both whole body heating and local heating of the testis in male mice prior to mating caused an apparent retardation in embryonic development at the late morula and early blastocyst stage (Bellve, 1972, 1973, 1976) with embryonic mortality occurring at or shortly after implantation (Bellve, 1973, 1976; Setchell et al., 1998). This indicates that the factor(s) responsible for the delayed embryonic death are mediated by the fertilizing sperm. The effect of male hyperthermia on in vitro [^{3}H] uridine incorporation of preimplantation embryos has demonstrated that during the early cleavage stages (2-cell, 3-cell and 4-cell), the patterns of [^{3}H] uridine incorporation appear to be entirely normal. However, at the 8-cell and in particular morula and blastocyst stage, 40% of embryos failed to incorporate [^{3}H] uridine or exhibited reduced levels of incorporation (Bellve, 1972 and 1973), which could make a contribution to later embryonic mortality.

Another possibility is that heating the testes reduces the amount of a sperm-supplied factor essential for development of the early embryos, analogous to SPE 11 in Caenorhabditis elegans. This novel, very hydrophic protein is formed during spermatogenesis, but is not essential for that process, and in its absence, the embryo is incorrectly or only partly activated by the sperm (Hill et al., 1989; Browning and Strome, 1996). However, the mammalian equivalent of the protein has yet to be identified.

1.4.3 Conclusion

Natural heat stress including summer heat and cryptorchidism, and artificial heat stress such as whole body heating, local heating of testes and experimentally induced cryptorchidism have been reported to disturb spermatogenesis, endocrine and biochemical functions of testicular tissues, thus leading to temporary and permanent sterility in the animal and human. However, an exact mechanism for the effect of elevated temperature on spermatogenesis and testicular function has not been elucidated and the complexity of contact and communication devices between germ cells and between germ cells and Sertoli cells during and following heating are not well understood.

Some studies have shown that summer heat, occupational hazards or clothing fashions may contribute to infertile or subfertile problems in humans (Levine et al., 1990; Mieusset and Bujan, 1995; Parazzini et al., 1995; Tiemessen et al., 1996; Thonneau et al., 1997). This has led some to suggest that heating the testes may be a possible contraceptive technique (Kandeel and Swerdloff, 1988; Setchell, 1994) provided the technique can meet convenience, comfort, reliability and reversibility.

Environmental pollution has also been aknowledged to contribute to global warming and climatic changes which may be a further contributing factor in the reported decline in sperm counts in humans (Carlsen et al., 1992; Auger et al., 1995; van Waeleghem et al., 1996).

1.5 Aims of the Research Project

Paternal heat stress has been shown to affect the development of preimplantation, implantation, post-implantation embryos in both animals and humans (Setchell, 1998; Section 1.4 in this review). However, the relationship between the development of preimplantation embryos and the extent of damage of the germ cells during heating has not been well identified and molecular mechanisms for the effect of paternal heat stress on the development of preimplantation embryos have not yet been investigated. The present project was undertaken to examine some of these issues. Aims of the study were to establish an in vitro culture system for studying embryo development, investigate the effect of paternal heat stress on development in vitro and in vivo and to examine profiles of proteins produced by preimplantation embryos in the mouse, in an attempt to elucidate mechanisms of embryo development influenced by paternal heat stress.

Chapter Two

Materials and Methods

2.1 Reagents

Sodium bicarbonate ($NaHCO_3$) (S-5761, Lot 96H0540), Sodium chloride (NaCl) (S-5886, Lot 96H06075), Calcium chloride ($CaCl_2.2H_2O$) (C-7902, Lot 96H0955), Potassium chloride (KCl) (P-5405, Lot 85H07855), Potassium phosphate monobasic (KH_2PO_4) (P-5655, Lot 115H07485), Ethylenediamine-tetraacetic acid (EDTA) (E-6635, Lot 65H000825), D-(+)-Glucose (G-7021, Lot 76H0043), Magnesium sulfate ($MgSO_4$) (M-2643, Lot 85H04205), HEPES sodium salt (H-0763, Lot 76H5731), Bovine serum albumin (BSA) (A-9418, Lot 47H04311), Hyaluronidase type IV-S (750-1,500 U/mg) (H-4272, Lot 16H4677) were all obtained from the Sigma Chemical Co, St Louis U.S.A, having been tested as suitable for cell culture or embryo culture.

Pregnant mare serum gonadotrophin (PMSG) (Folligon, 5000 I.U, #26532) was purchased from the Intervet Pty. Ltd., Castlehill, NSW, Australia. Chorionic gonadotrophin (hCG) was obtained from either the Sigma Chemical Co, St Louis U.S.A (CG-2, Lot18F0277) or Intervet Pty. Ltd. (27524).

2.2 Animals

The use of animals was approved by the University of Adelaide Animal Ethics Committee (Ethics No. W/030/95, W/009/97 and W/19/99). C57 male and CBA female adult mice were purchased from the Adelaide University Central Animal House for use as breeders. F1 male and female mice (C57 male X CBA female) were bred in the animal house, the Department of Animal Science, the University of Adelaide throughout this study. All mice were housed under standard laboratory conditions and maintained with 14h of light (06:00-20:00) and 10h of darkness daily. A commercial pelleted diet (manufactured by Ridley Agriproducts Pty. Ltd., South Australia, Australia) and water were provided ad libitum.

2.3 Superovulation and Mating

F1 C57/CBA female mice (4 to 10 wk old) were superovulated by an intraperitoneal (i.p.) injection of 5 to 10 IU PMSG between 16:00 and 17:00h, followed 47-48h later by an i.p. injection of 5 to 10 IU hCG. After hCG injection, each female mouse was immediately paired for mating with a proven fertile male of the same strain. The presence of a copulatory plug the following morning was assumed to indicate a successful mating.

2.4 Embryo Collection

2.4.1 Hepes-HTF medium

Hepes-HTF medium (Quinn et al., 1985) was used as a flushing medium. Stock solutions for Hepes-HTF medium were prepared as indicated below:

Stock A: (lasts 3 months)

NaCl	5.931g
KCl	0.350g
KH_2PO_4	0.050g
$MgSO_4.7H_2O$	0.050g
Glucose	0.500g
Penicillin	0.060g
H_2O^*	96.3ml
Na Lactate	3.70 ml

Stock A was made up in either a glass or a plastic bottle.

*H_2O is ultra-pure water (also called Milli-Q Water) produced by Ultra-pure Water System (Millipore, Selby-Biolab, Australia). This H_2O is used through all the experiments.

Stock B: (lasts 2 weeks)

NaHCO$_3$	0.210g
Phenol Red	2/3 grains
H$_2$O	10ml

Stock B was made up in a 14ml (17x100mm) sterile Falcon tube (#80200752, Becton Dickinson, USA).

Stock C: (lasts 2 weeks)

Sodium pyruvate	0.51g
H$_2$O	10ml

Stock C was made up in a 14ml (17x100mm) sterile Falcon tube (#80200752, Becton Dickinson, USA).

Stock D: (lasts 3 months)

CaCl$_2$.2H$_2$O	0.262g
H$_2$O	10ml

Stock D was made up in a 14ml (17x100mm) sterile Falcon tube (#80200752, Becton Dickinson, USA).

Stock E: (lasts 3 months)

Hepes	3.254g
Phenol Red	2/3 grains
H$_2$O	50ml

Stock E was adjusted to pH 7.5 with 1N HCl before making up to a final volume of 50ml and stored in a 50ml tissue culture Falcon flask (#3014, Becton Dickinson, England).

All stocks were stored at 4°C.

Final preparation of the working strength medium is as shown in Table 2.1.

Table 2.1 Final preparation of Hepes-HTF medium from stock solutions

Volume (ml)	10	50	100
Stock A (ml)	1.000	5.000	10.000
Stock B (ml)	0.160	0.800	1.600
Stock C (ml)	0.071	0.356	0.710
Stock D (ml)	0.115	0.575	1.150
Stock E (ml)	0.840	4.210	8.400
H_2O	7.810	39.050	78.100

Osmolarity of this medium was measured by a Vapor Pressure Osmometer (#5520, Wescor, Surgical & Medical Supplies Pty. Ltd., USA) to ensure that it was within the range of 265 and 285 mmol/kg (mOsM). If under, drops of stock A were added into medium. Conversely, if over, drops of water were added to the medium until the appropriate osmolarity was reached.

BSA (5mg/ml) was added to the bottom of a 14ml (17x100mm) sterile Falcon tube (#80200752, Becton Dickinson, USA), followed by the medium. The medium was mixed well (not shaken) to dissolve the BSA and finally sterilized by passing it through a 0.20 µm sterile filter (#37070, Minisart, Germany).

The medium was equilibrated in an atmosphere of 5% CO_2 in air overnight at 37°C before use.

2.4.2 Embryo collection

Twenty-five to twenty-eight hours after injection of hCG, mice were euthanasized by cervical dislocation and the oviducts were recovered using a pair of sterile scissors and

forceps. The oviducts were placed into a sterile Falcon tissue culture dish (35X10mm) (#3001, Becton Dickinson, England) containing 1ml of Hepes-HTF and putative mouse zygotes were subsequently released by piercing the swollen portion of the ampulla with a 27-gauge sterile needle. Alternatively, a 27-gauge sterile needle was inserted in the ostium of the oviduct and approximately 0.1 to 0.5 ml of flushing medium injected to release embryos. The embryos were then washed three times with Hepes-HTF medium.

2.5 Culture of Preimplantation Embryos

2.5.1 Culture media

Media used included HTF, CZB and KSOM medium which were prepared as described by Quinn et al. (1985), Chatot et al. (1989) and Lawitts and Biggers (1993), respectively. The formula of HTF is based on the composition of human fallopian tubal fluid (HTF) (Quinn et al., 1985). For culture, Hepes was replaced with 25 mM $NaHCO_3$ and thus this medium was identified as HCO_3HTF. CZB was developed by Chatot, Ziomek and Bavister et al. (1989) by modifying a BMOC-2 medium. The lactate/pyruvate ratio was increased to 116, 1mM-glutamine and 0.1 mM-EDTA were added, but glucose was removed because it could inhibit the further development of 2-cell mouse embryos in outbred strains. However, because C57/CBA F1 mice used in this study do not show a 2-cell developmental block, 5.6 mM glucose was added to CZB. Lawitts and Biggers (1992) compared three different components of each medium at three different levels, in a 3^3 factorial design. Thus 27 media were produced and compared in a single trial in each experiment in order to determine the effect of each medium on mouse zygotes developing to the blastocysts. In three experiments, three factors were examined, each at three concentrations, simultaneously. A new simplex optimized medium (SOM) which is the best for embryo development was

finally produced. Following this, a modified version of SOM called KSOM has been produced by increasing the concentration of sodium (95mM rather than 85mM) and potassium chloride (2.5mM vs 0.25mM) (Lawitts and Biggers, 1993).

The composition of these three media are shown in Table 2.2.

Table 2.2. The composition (mM) of media used for embryo culture

	HCO_3HTF	CZB	KSOM
NaCl	101.60	82.70	95.00
KCl	4.69	4.68	2.50
KH_2PO_4	0.37	1.17	0.35
$MgSO_4$	0.20	1.18	0.20
Na lactate	21.40	30.10	10.00
Na pyruvate	0.33	0.26	0.20
Glucose	2.78	5.60	0.20
$NaHCO_3$	25.00	25.00	25.00
$CaCl_2$	2.04	1.71	1.71
Glutamine		1.00	1.00
EDTA		0.10	0.01
Penicillin	100U/ml	100U/ml	100U/ml
Streptomycin	50ug/ml	50ug/ml	50ug/ml
BSA	5.0mg/ml	5.0mg/ml	4.0mg/ml

Media were prepared as presented by Lawitts and Biggers (1993). Most components were stored as 0.1M solutions although NaCl and $NaHCO_3$ were stored as 1.0M solutions. Other materials were stored as follows: BSA as 100mg/ml, $CaCl_2$ as 0.171M, EDTA as 1.0mM, penicillin-G and streptomycin as 10,000U/ml and 5.0mg/ml, respectively. Most of the above components were stored at 4°C in 50 ml conical centrifuge tubes. Glutamine and the antibiotic mixture were stored at - 20°C in 5 ml polypropylene tubes. Solutions of

$NaHCO_3$, sodium pyruvate and sodium lactate were prepared weekly. BSA solution was prepared fresh every time media was made.

The day before the start of each trial, 10 ml of HCO_3HTF, CZB or KSOM solutions were prepared. Five ml of H_2O was added to a 14 ml (17x100mm) sterile Falcon culture tube (#80200752, Becton Dickinsin, USA). All components were then added to the tube. H_2O was added to each tube to 9.0 to 9.5 ml. $CaCl_2$ was then added to the tube and each tube was inverted twice to mix the components. pH and osmolarity of each medium were measured with a Digital pH meter (TPS Pty, Ltd, Brisbane, Australia) and a Vapor Pressure Osmometer (#5520, Wescor, USA), respectively. Finally, H_2O was added to make 10.0 ml of each medium. The medium was drawn into a disposable sterile syringe and pushed through a 0.2 μm sterile filter (#37070, Minisart, Germany) into a second 14 ml sterile Falcon culture tube (#80200752, Becton Dickinson, USA). BSA was then added to each medium and then the medium was drawn into a disposable sterile syringe and pushed through a 0.2 μm sterile filter (#370370, Minisart, Germany) into two 6 ml (12x75mm) sterile Falcon tubes (#70600353, Becton Dickinson, USA) for later use.

2.5.2 Culture in vitro of putative zygotes

Putative zygotes were cultured in sterile 4-well tissue culture dishes (#176740, Nunclon™, Demark) in 50 μl drops of medium under paraffin oil at 37°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The developmental progress was recorded at 24h intervals for 120h.

2.5.3 Inner cell mass and trophectoderm cell numbers in the blastocyst

Phosphate-buffered saline (PBS) solution (Hogan etal., 1986) and Acidic Tyrode's solution

(Hogan et al., 1986) for removing the zonae pellucidae of blastocysts were prepared as shown in Table 2.3 and 2.4.

Table 2.3 PBS solution pH 7.2

Composition	g/100ml
NaCl	0.800
KCl	0.020
Na_2HPO_4 or $Na2HPO_4.12H_2O$	0.115 or 0.289
KH_2PO4	0.020
H_2O	to 100.00ml

Osmolarity of the PBS solution was measured to ensure that it was maintained within the range of 280 and 285 mOsM. PBS was subsequently sterilized by passing through a 0.2μm sterile filter, prior to storage at 4°C.

Table 2.4 Acidic Tyrode's Solution for removing zonae pellucida

Composition	g/100ml
NaCl	0.800
KCl	0.020
$CaCl_2.2H_2O$	0.024
$MgCl_2.6H_2O$	0.010
Glucose	0.100
Polyvinylpyrrolidone (PVP)	0.400
H_2O	to 100.00 ml

The above solution was adjusted to pH 2.5 with 1 M HCl. The PVP was added to increase the viscosity of the solution and reduce embryo stickiness. The solution was then sterilized by passing through a 0.2μm sterile filter (#37070, Minisart, Germany) and stored in aliquots at -20°C.

Differential fluorescent labelling of trophectoderm (TE) and inner cell mass (ICM) cells in the mouse blastocyst was determined using a modified method of Handyside and Hunter (1984). Blastocysts were transferred from Hepes-HTF medium to acid Tyrode's solution, under constant observation for 30-60 sec, until the zonae pellucidae were completely dissolved. They were then washed 3 times in a Hepes-HTF medium and transferred into a CZB medium containing 10% rabbit anti-mouse whole serum (Sigma, M5774, Australia), and then incubated at 37°C for 15 min. The embryo was washed 3 times in Hepes-HTF and transferred to a CZB medium containing 10% guinea pig serum complement (Sigma, 51639, Australia) and incubated at 37°C for 5-6 min. They were washed 3 times in Hepes-HTF and stained in CZB medium containing 20 μg/ml bisbenzimide (DA) (Sigma, B2883, Australia) with 10 μg/ml propidium iodide (PI) (Sigma, P4170, Australia) at 37°C. After 30-40 min, the blastocyst was fixed in 1% paraformaldehyde in PBS for 1 min and washed 3 times in the Hepes-HTF. Then, the embryos (3-6 embryos/slide) were transferred to a glass slide (size: 76.2x25.4mm; thickness: 0.8-1.0mm) (37101-A, Livingstone International Pty.Ltd, Australia) in a droplet of less than 5 μl, covered with a microscope cover glass (size: 22X22mm) and squashed gently with the blunt end of a pen. The area under the cover glass was filled with the Hepes-HTF. Nail varnish (Supershine, Sally, Hansen, North Ryde, Australia) was used to seal the cover glass. Finally, the differentially labelled nuclei were examined quickly using an Olympus BX60 fluorescent microscope within a rage of 330 to 385 nm of UV exciting light and of 410 to 600 nm of emission in blue. The total magnification in eyepiece and photograph was x400 and x250, respectively. PI-labelled nuclei (dead cells or TE cells) appeared red while DAP-labelled nuclei (viable cells/ICM cells) appeared blue because of different emission spectra of DA and PI.

2.6 Heat Stress of Male Mice

Mature F1 C57/CBA male mice (3 to 5 months old) to be heated were placed in a chamber set at 36°C with 62-66% relative humidity for 24h. Control male mice were placed in the chamber set at 23°C. Both control and heated mice were allowed free access to diet and water. Details of the chamber are presented in Fig 2.1 and Fig 2.2.
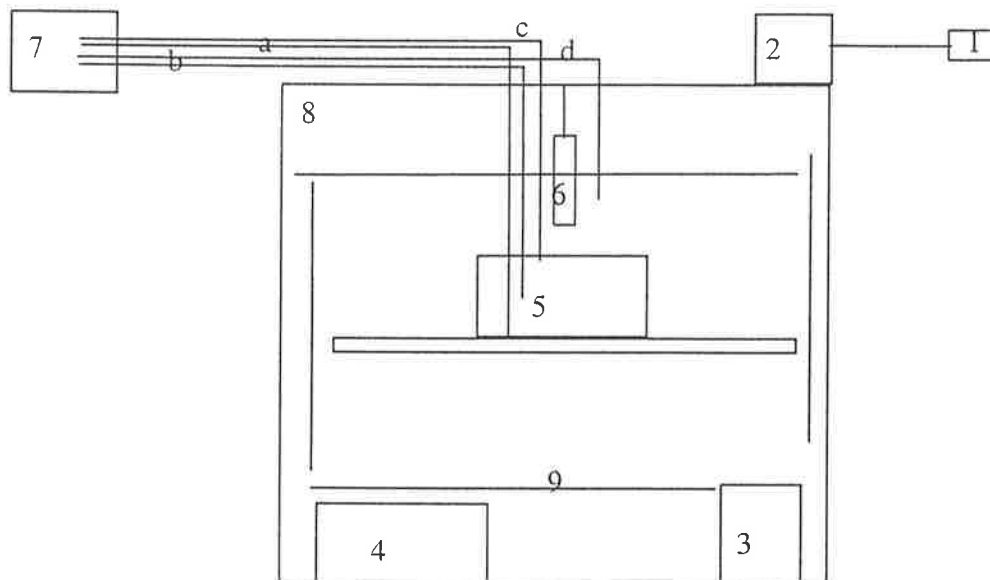
Fig 2.1 Diagrammatic outline of a heating chamber

Note: 1—Power; 2 – CAREL "IR32" universal Programming controller (R & H Wholesale (SA) Pty. Ltd., Australia); 3 – EWT heater (Model 01S, Australia); 4 – A container of water for regulating relative humidity within the chamber; 5 – A cage for male mice; 6 – Wet and dry bulb thermometer for measuring relative humidity; 7 – Tele-thermometer, a, b, c or d are thermometer's probes which can measure the temperatures from the bottom, middle or top of the cage or above the cage; 8 – Heating chamber was made of steel and perspex with doors from four sides of the chamber able to be opened in order to place the mice, or add water or diet; 9 – arrows show the flow of heat transmission during heating.

The temperatures in the bottom, middle and top of the mouse cage and above the mouse cage were measured by a, b c or d probes on the tele-thermometer and the relative humidity
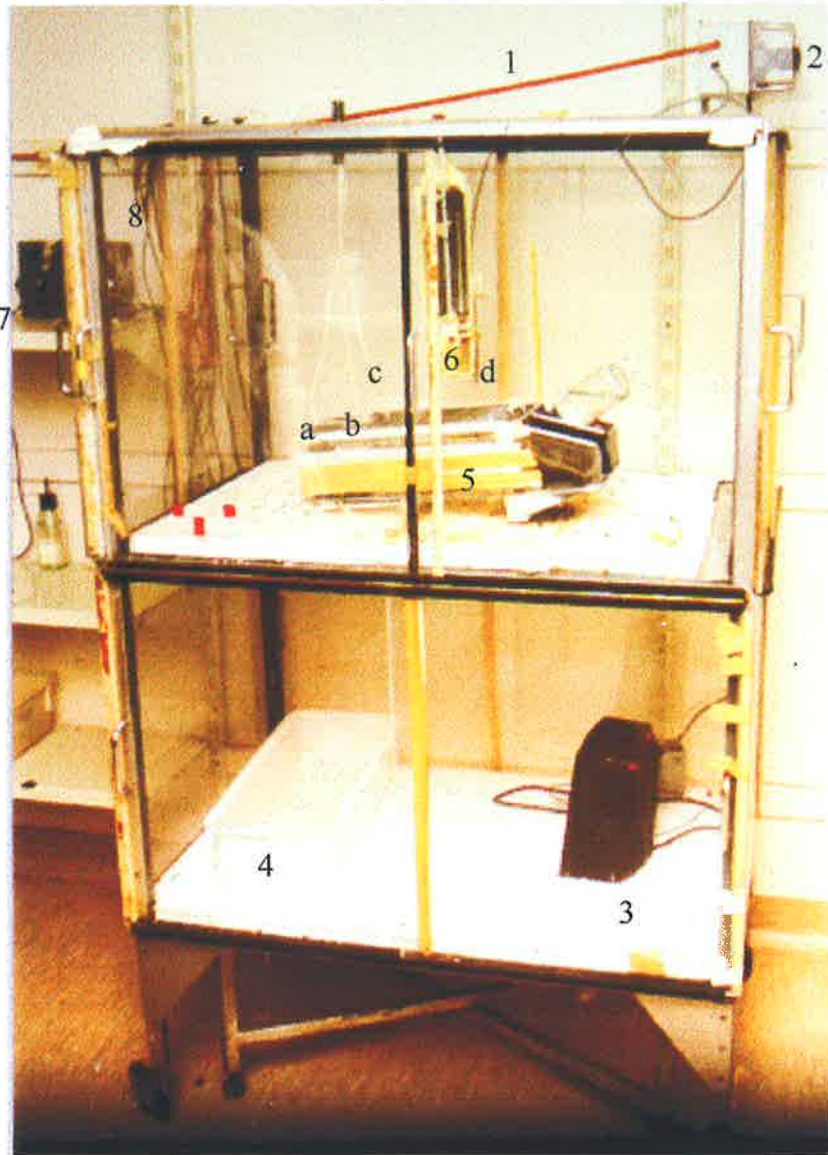
Fig 2.2 Photograph of heating chamber

1, 2, 3, 4, 5, 6, 7 (a, b, c or d) or 8 as per Fig 2.1.

within the chamber was calculated by the air temperature value (dry bulb) (t°C) and the wet bulb value (t°C) read from the wet and dry bulb thermometer.

In order to maintain constant temperatures and the relative humidity within the chamber, the chamber was pre-heated for 12h before mice were put into the cage. The mean value of the temperatures from the bottom , middle and top of the cage was used to determine the actual temperature of heating. The results of a validation experiment are shown in Table 2.5.

Table 2.5 Temperatures and relative humidity (RH) in the cage and above cage

	Temperatures of the cage (°C)				Dry	Wet	D-W	RH
	Above	Bottom	Middle	Top	(D)	(W)		(%)
Before*	35.3	36.2	36.0	36.0	35.5	29.5	6	64
When	34.8	35.3	35.0	34.8	35.5	27.0	8.5	51
After (h) 1	35.3	36.1	36.0	36.0	35.0	30.5	4.5	73**
2	35.3	36.1	36.0	36.0	35.0	30.0	5	70
3	35.3	36.1	36.0	36.0	35.0	30.0	5	70
4	35.3	36.1	36.0	36.0	35.0	30.0	5	70
5	35.3	36.1	36.0	36.0	35.0	29.5	5.5	67
6	35.3	36.2	36.0	36.0	35.0	29.0	6	64
7	35.3	36.2	36.0	36.0	35.0	28.5	6.5	62
8	35.3	36.2	36.0	36.0	35.0	28.5	6.5	62
12	35.3	36.2	36.1	36.1	35.0	28.5	6.5	62
16	35.3	36.2	36.1	36.0	35.0	28.5	6.5	62
20	35.3	36.2	36.1	36.1	35.0	28.5	6.5	62
24	35.3	36.2	36.1	36.1	35.0	28.5	6.5	62
M±SD	35.3±0.0	36.2±0.1	36.0±0.1	36.0±0.1				65.5±4.2

*   Before, when and after the mice were placed into the cage; ** Due to opening of the chamber for mouse placement and loss of humidity at this time, additional water was routinely added to the chamber after mice were placed into the cage. This resulted in a transient increase in humidity initially.

These results showed clearly that this heating system is completely suitable for the subsequent investigations of the effect of paternal heat stress on the development of preimplantation mouse embryos with the system maintaining constant temperatures and relative humidity.

2.7 Synchronization of Oestrus in the Female Mouse

Oestrous synchronization of female mice was used to increase the number of oestrous female mice available at any one time in order to investigate the effect of paternal heat stress on development in vivo of mouse embryos.

The procedures employed were adapted from Jemiolo et al. (1986). Mature female mice (2 to 3 months old) were housed in a male-free room for 14 to 28 days in order to suppress the oestrous cycle of the females. After that, male mice were placed into the room. A pheromone produced by the male causes the release of gonadotrophins from the pituitaries of female mice, stimulating the growth of smaller follicles, shortening, and synchronizing their oestrous cycles. It has been reported that a peak in the incidence of oestrus occurs on the 3rd night after exposure to the male pheromone (19% to 41%) (Whitten et al., 1968; Gangrade and Dominic, 1984; Jemiolo et al., 1986).

In order to ensure olfactory and visual contact, but not physical contact between the males and females and allow male wastes, in particular urine to enter the female cage, a small cage containing 3 to 5 male mice was put on the top of a large cage containing the females (the distance between the two cages was 1 cm).

On the 3rd day after exposure to the males, the oestrous stages in the females were determined according to the size of the vaginal opening, the degree of vaginal swelling, the color and moistness of the tissues, and the presence or absence of obvious cellular debris within the vagina (Table 2.5). Oestrous female mice were selected and mated with fertile male mice.

Table 2.5 Appearance of the vagina at various stages of the oestrous cycle
(Champlin et al., 1973)

Oestrous stage	Appearance
Dioestrus	Vagina has a small opening and the tissues are bluish-purple in color and very moist.
Prooestrus	Vagina is gaping and the tissues are reddish-pink and moist. Numerous longitudinal folds or striations are visible on both the dorsal and ventral lips
Oestrus	Vaginal signs are similar to proestrus, but the tissues are lighter pink and less moist, and the striations are more pronounced.
Metoestrus 1	Vaginal tissues are pale and dry. Dorsal lip is not as edematous as in estrus.
Metoestrus	Vaginal signs are similar to metestrus 1, but the lip is less edematous and has receded. Cellular debris may line the inner walls or partially fill the vagina.

2.8 Protein Patterns of Preimplantation Embryos Determined by One-Dimensional SDS Polyacrylamide Gel Electrophoresis (PAGE)

2.8.1 Stock solutions for SDS electrophoresis

2.8.1.1 30% Acrylamide/Bis

29.2 g acrylamide (Bio-Rad, 116533A, Australia) plus 0.8g N'N'-Bis-methylene-acrylamide (Bio-Rad, 43386A, Australia) were made up to 100 ml with H_2O, and filtered using a 0.45μm sterile filter (Medos Company Pty Ltd, Australia). Alternatively, a

commercial pre-formulated 30% acrylamide/Bis solution (37.5:1) (Bio - Rad, 161-0158, Australia) was used and stored at 4°C in the dark for a maximum of 30 days.

2.8.1.2 1.5 M Tris-HCl, pH 8.8

18.5 g Tris-base (Sigma, T8404, Australia) was made up to 50 ml with H_2O and adjusted to pH 8.8 with 1N HCl, then made to 100 ml with H_2O and stored at 4°C.

2.8.1.3 0.5 M Tris-HCl, pH 6.8

6 g Tris-base (Sigma, T8404, Australia) was made up to 60 ml with H_2O and adjusted to pH 6.8 with 1M HCl, made to 100 ml with H_2O and stored at 4°C.

2.8.1.4 10% SDS

10 g sodium dodecylsulfate (SDS) (Sigma, C-3771, Australia) was dissolved in H_2O with gentle stirring, brought to 100 ml with H_2O and stored at room temperature.

2.8.1.5 Sample buffer (SDS-reducing buffer) (Laemmli, 1970)

H_2O	4.0 ml
0.5 M Tris-HCl	1.0 ml
Glycerol (Sigma, G8773)	0.8 ml
10% SDS	1.6 ml
2-β Mercaptoethanol (Sigma, M-7154)	0.4 ml
0.05% (w/v) Bromophenol blue Sigma, B8062)	0.2 ml
	8.0ml

2.8.1.6 5X Electrode (running) buffer, pH 8.3

Tris-base (Sigma, T8404)	45g
Glycine (Sigma, G8898)	216g
SDS (Sigma, C3771)	15g

These chemicals were made up to 3 litres with H_2O and stored at 4°C. The solution was warmed to 37°C before use if precipitation occurred.

200 ml 5X concentrate was diluted with 800 ml H_2O to produce enough working strength buffer for one electrophoresis run.

2.8.2 Buffers and solutions for one-dimensional gel electrophoresis

Stacking gel (4% Acrylymide/Bis) and slab gel were prepared as shown in Table 2.6 and 2.7.

Table 2.6 Compositions of stacking gel

Compositions	Volumes (ml)
30% Acrylymide/Bis	1.400
Tris-HCl pH 6.8	2.500
10% SDS	0.100
H_2O	6.000
10% Ammonium persulfate (APS)(fresh) (Bio-Rad, 115953B)	0.050
TEMED (Bio-Rad, 51185C)	0.008

Table 2.7   Composition of slab gel (gradient gel)

Constituent	Volume (ml)
8% Light solutions (5 ml)	
30% Acrylamide/Bis	1.3300
Tris-HCl pH 8.8	1.1700
10% SDS	0.0500
H_2O	2.4500
10% APS (fresh)	0.0170
TEMED	0.0017
15% Heavy solutions (5 ml)	
30% Acrylamide/Bis	2.5000
Tris-HCl pH 8.8	1.2500
10% SDS	0.0500
Sucrose	0.8000g
H_2O	0.7700
10% APS	0.0170
TEMED	0.0017

2.8.3 Preparation and running of embryo samples and gradient gels

Superovulation and mating of female mice, and collection and culture in vitro of mouse zygotes were conducted as presented in 2.3, 2.4 and 2.5. Embryos developing to 2-cell embryos and morulae in CZB medium were collected and washed three times with BSA-, glutamine-, pyruvate- and lactate-free CZB medium. Embryos in a droplet of less than 5μl CZB medium were subsequently placed into the bottom of a 1.5ml microcentrifuge tube (Treff AG, Switzerland) and then 10μl sample buffer as described in 2.8.1.5 was added to this tube. Finally, the tube containing embryos was boiled for 2 min. in 100°C water and stored at -80°C prior to one-dimensional gel electrophoresis and silver staining.

Two-cell embryos, 4-cell embryos, morulae or blastocysts of putative mouse zygotes were collected, 18h, 39h, 63h or 87h respectively after culture in vitro in CZB medium. They were then cultured for 3h in 50µl CZB medium containing 200µCi/ml of [^{35}S]-methionine (1000Ci/mmol, Amersham Pharmacia Biotech UK Ltd), as described by Van Blerkom and Brockway (1975). Embryos were then washed three times in BSA-, glutamine-, pyruvate- and lactate-free CZB medium and placed into the bottom of a 1.5ml microcentrifuge tube in a droplet of less than 5µL CZB medium (Treff AG, Switzerland), resuspended in 10µl sample buffer as presented in 2.8.1.5 and finally boiled for 2 min. in 100°C water and stored at -80°C for one-dimensional gel electrophoresis and autoradiography.

A Bio-Rad Mini-Protein® II dual slab cell kit (Bio-Rad Laboratories, 3300 Regatta Boulevard, Richmond CA94804) was used in this study. The slab gels were of a continuous 8% to 15% (acrylamide concentration) gradient with an exponential function in order to fractionate a much wider size range of proteins on a single gel (because there are thousands of proteins in mouse embryos, reported by Latham et al. (1991). The gel was poured from a standard two-chamber gradient maker with 1.6 ml of a 15% acrylamide solution in the front chamber and 3.4 ml of a 8% acrylamide solution in the rear chamber. A small stirring bar was placed in the front chamber and the two chambers were isolated from each other by a plastic stopper.

The filled gradient maker was placed on a magnetic stirring device (#209-1, Industrial Equipment & Control Pty. Ltd., Australia), the two chambers were connected and the contents of the front chamber was gently stirred and the clamp was relaxed. During pouring gel, the volume of the front chamber remained constant and was continuously diluted by the

incoming solution from the rear chamber. This procedure generates an exponentially decreasing gradient of acrylamide.

After the gel had been allowed to polymerize for 40-45 min, a 4% stacking gel was layered over the separating gel for about 1h to polymerize. Ten sample wells were formed using a Teflon casting comb (10 wellx0.75mm) (#165-2920, Bio-Rad). 5μl protein markers were loaded into well 1 with samples loaded into subsequent wells. Gels were electrophoresed initially for 20 min at room temperature at a constant current of 20 mA per gel, followed by 2h at a constant current of 10 mA per gel.

2.9 Protein Patterns of Preimplantation Embryos Determined by Two-Dimensional Gel Eletrophoresis

To examine specific protein changes in preimplantation embryos, a 2-dimensional electrophoresis system was employed, as modified by O'Farrell (1975).

2.9.1 Isoelectric focusing (IEF) sample buffer

Urea	5.7g
H_2O	7.30 ml
10% NP - 40 (2%)	2.00ml
Ampholyte - pH 5 - 7	0.16ml
Ampholyte - pH 3 - 10	0.04ml
β - mercaptoethanol (5%)	0.50 ml
	10.00ml

2.9.2 IEF gels

	Urea	5.50g
	10% NP - 40	2.00ml
	30% Acrylamide/Bis	1.33ml
	H_2O	2.13ml
(Dissolved urea, then add)	Ampholyte-pH 5-7	0.30ml
	Ampholyte-pH 7-9	0.15ml
	Ampholyte-pH 3-10	0.15ml
(Mix, degas, then add)	10% APS	0.02ml
	TEMED	0.01ml

2.9.3 Transfer buffer or equilibration buffer

The transfer buffer employed was adapted from a laboratory manual provided by Hoefer Scientific Instruments (1994) and its composition was as follows.

0.5 M Tris-HCl pH 6.8 (0.125M)	12.500ml
10% SDS (2%)	10.000ml
Glycerol (10%)	5.000ml
DDT (4.9mM)	0.038g
Bromophenol blue (0.05%)	0.025g
H_2O	22.500ml
	50.000ml

2.9.4 Slab gels (gradient gel)

Table 2.8 Composition of slab gels for 2D-PAGE

Constituent	Light solutions (7.5%, 70ml)	Heavy solutions (15%, 30ml)
30% Acrylamide/Bis	17.5ml	15.0ml
1.5M Tris-HCl pH8.8	17.5ml	7.5ml
10% SDS	0.7ml	0.3ml
Sucrose		4.5g
H_2O	34.0ml	4.59ml
10% APS	231µl	99µl
TEMED	23.1µl	9.9µl

2.9.5 Preparation and running of samples and the first dimensional gel

Two-cell embryos and morulae of putative mouse zygotes were collected, 18h and 63h respectively after culture in vitro in CZB medium and cultured for 3h in 50µl CZB medium containing 500µCi/ml of [^{35}S]-methionine (1000Ci/mmol, Amersham Pharmacia Biotech UK Ltd) as described by Howe and Solter (1979). Embryos were then washed three times with BSA-, glutamine-, pyruvate- and lactate-free CZB medium and placed into the bottom of a 1.5ml microcentrifuge tube. The embryos were lyophilized using a speed vac concentrator (Model SVC100H, Savant, Germany), resuspended in 20µl sample buffer and stored at -80°C prior to two-dimensional gel electrophoresis.

Tube gels were used as the first dimension to separate the proteins by isoelectric points (pI). The tube gels were prepared for IEF as described in 2.9.2. After the solution was degassed

for 15 min, the APS and TEMED were added and the gels were cast in 160 mm x 1.5 mm capillary tubes to a height of marked 130 mm using a 5ml syringe connected to a gel tube loading needle (180x0.75mm) (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA94547). They were then overlaid with deionised water and left for 1h before the water was replaced with freshly prepared 20 mM sodium hydroxide (NaOH) for a further 1h. Once polymerised sufficiently, the overlay solution was replaced with fresh degassed 20 mM NaOH and the gels loaded into the electrophoresis apparatus (Protein® II xi cell, Bio-Rad, Australia) for pre-focussing at 200 V for 15 min, then 300 V for 30 min. and 400 V for 30 min. The anolyte and catholyte used for this procedure were fresh 6 mM phosphoric acid and 20 mM NaOH respectively.

Protein samples were loaded directly onto the gel, then overlaid with 10 to 20 μl half-strength sample buffer (diluted 1:2 with H_2O) in order to protect samples from exposure to the basic upper buffer. The remainder of the tube was filled with 20mM NaOH to eliminate any bubbles. The gels were then focussed at 400 V for approximately 17h and then a further 1h at 800 V (Cullen et al., 1980b). The sample gels were extruded using a 10ml syringe connected to a tube gel extrusion needle (9x0.4mm) (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA94547), then injected at the basic end with a small amount of Indian Black Ink (Superior Ink, Australia) to mark this end of the tube gel and finally placed in a test tube containing 0.5ml of transfer buffer and incubated at room temperature, for 3 min before freezing for later use, or 10-15 min if being used immediately.

The pH gradient in the tube gels was determined with the use of spare gels electrophoresed without sample. The gel was cut into 8-10 even lengths, each being placed in 1 ml 0.025 M KCl solution for at least 1h before the pH was read (Hames and Rickwood, 1983).

2.9.6 Preparation and running of the second dimensional gels

The second dimension gradient gels were prepared as detailed in Table 2.8 and cast to a size of 150 mm x 160 mm x 1.5 mm. The gel was poured from a standard two-chamber gradient maker (Gradient Mixer GM-1, Sweden) with 13.5 ml of a 15% acrylamide solution in the front chamber and 31.5 ml of a 7.5% acrylamide solution in the rear chamber. The other methods were the same as those described in 2.8.3. A two-well (one is 4x1.5mm for standard protein markers and another one is 142x1.5mm for IEF gel) Bio-Rad Telfon comb was put into the acrylamide solutions in order to form one small and one large sample wells. Water-saturated iso-butanol (1:4) was used to overlay the gels while polymerising for approximately 45 min. before being rinsed with deionised water and overlaid with 0.375 M Tris-HCl (pH 8.8). The gels were left overnight at 4°C to ensure complete polymerisation. Before use, each gel was washed with deionised water and a very thin layer of hot (50°C) 0.5-1% agarose pipetted on the top of the slab gel to be loaded. The IEF gels were then carefully transferred to the top of a very thin layer of agarose and then overlaid with a thin layer of hot (50°C) 0.5-1% agarose to seal the gel in place. Ten µl of standard protein markers were loaded into the well for protein markers.

Electrophoresis was carried out using the electrode buffer described in 2.8.1.6 at 19 mA/gel for approximately 5h.

2.10 Staining of Acrylamide Gels

2.10.1 Silver staining

In order to determine the protein profiles of preimplantation embryos, silver staining was used to stain gels after one-dimensional gel electrophoresis. Procedures employed have been detailed in Bio-Rad, Silver stain plus kit (#61578, Australia). Briefly, the protocol was essentially as follows. Gels were placed in 400ml fixative enhancer solution and fixed with gentle agitation for 20 min. Fixative enhancer solution was then decanted from the staining vessel and the gels were rinsed in 400 ml H_2O for 10 min. with gentle agitation. After 10 min., the water was replaced with fresh rinse water for a further 10 min. The gels were then placed in 100ml staining solution (35ml H_2O, 5ml silver complex solution, 5ml reduction moderator solution and 5ml image developer regent, and finally 50ml development accelerator solution) for 10-20 min. until the desired intensity of staining was reached. The staining vessel was gently agitated throughout the procedure. Finally, the gels were placed in 400ml 5% acetic acid solution for a minimum of 15 min in order to stop reaction between silver ions and proteins in the gel, and then rinsed in H_2O for a further 5 min. The gels were then stored in zip-lock bags with a small amount of H_2O or photographed.

2.10.2 Coomassie blue staining, autoradiography and phosphoimaging

In order to examine protein synthetic patterns of preimplantation embryos, Coomassie Blue staining was used for gel staining after one- or two-dimensional gel electrophoresis. Gels were stained for 25-30 min. in 0.1% Coomassie Blue R-250 (Sigma, B-0149) in fixative (40% MeOH, 10% HOAc), and then destained twice with a solution containing 40% MeOH and 10% HOAc to remove background for 15-20 min.

After destaining, gels were dried (Gel dryer, Model 583, Bio-Rad) for about 2h at 80°C for one-dimensional gels or for approximately 3h at 62°C for two-dimensional gels.

Following one-dimensional gel electrophoresis, the dried gel containing radioactive samples was exposed under Fuji medical X-ray films (18x24cm) (Fuji Photo Film Co. Ltd, Japan) in hyper-cassettes™ (Amersham Life Science, Bio-Rad) for approximately 14 days at -80°C. The cassettes were then thawed and the films developed in a dark room, using standard procedures with a developer (Phenisol X-ray developer, Ilford, Australia), deionised water wash and a fixative (Hypam rapid fixer, Hypam hardener, Ilford, Australia). The film was left in each solution for 2-3 min. under safelights (Kodak, Australia) before being rinsed and left hanging to dry.

A Phosphoimaging system was used also to examine protein spots on gels after two-dimensional gel electrophoresis. The Phosphoimager and Densitometer scanners were both part of a Molecular Dynamics system and were complemented with the IBM compatible software ImageQuant[R]. The protocol employed was as follows. The process of phosphoimaging involved the exposure of eletrophoretic gels to a phosphoimaging screen inside a BAS Fujix Molecular Dynamics cassette for 7 to 30 days before scanning with a Phosphoimager. This screen was scanned by the phosphoimager under the conditions of Orientation=R and 100 microns for 9 min./gel. After that, the ImageQuant[R] file was opened for determining the desired intensities of protein spots by regulating the range of greyscale under 1000 times sensitivities. The image of the proteins recorded were then copied as a TIFF file using Photoshop. The desired intensities of protein spots were adjusted by brightness/contrast levels and copied and pasted to Powerpoint. A desired photo was made and printed using the Powerpoint software package.

2.11 Estimation of Molecular Weights of Embryonic Proteins

Molecular weights of specific proteins in sample runs were estimated by comparison to prestained SDS-page standards-Bio-Rad-broad range run under the same conditions (#161-0318, Australia), including myosin (206,000), β-galactosidase (117,000), bovine serum albumin (79,000), ovalbumin (48,300), carbonic anhydrase (34,700), soybean trypsin inhibitor (29,300), lysozyme (21,300) and aprotinin (7,600).

Chapter Three

Determination of An

Optimised In-Vitro Culture

System

3.1 Introduction

Optimising the in vitro culture system is an essential pre-requisite if one is to produce preimplantation embryos of high quality that are free of extraneous influences.

Based on the rationale that in vitro culture conditions need to be as similar as possible to those found in the oviducts, Quinn et al. (1985) developed a culture medium that was biochemically very similar to that of human tubal fluid (HTF). In order to overcome the phenomenon of the in vitro "2-cell block" of mouse embryos from outbred stains, Chatot et al. (1989) formulated a CZB medium based on the composition of BMOC2 medium (Ebert and Brinster, 1983) that contained a high lactate/pyruvate ratio with glutamine, but without glucose. Subsequently, a modified version of a simplex optimized medium (SOM) (Lawitts and Biggers, 1991a,b) with a high K^+ concentration (KSOM) was established (Lawitts and Biggers, 1993). A high proportion of mouse zygotes from different strains have been reported to develop to blastocysts in HTF, CZB and KSOM (Quinn et al., 1985; Chatot et al., 1989; Erbach et al., 1994). However, the best medium for culture of mouse zygotes from the strain of mice used in the present study has not been determined.

Paraffin oil (Harvey and Kaye, 1992; Lonergan et al., 1996; Walker et al., 1996), mineral oil (Nagao et al., 1995; Harayuchi et al., 1996; Partridge and Leese, 1996; Zhang et al., 1996) and silicone oil (Lawitts and Biggers, 1992; Wilkinson et al., 1996) have been used extensively to protect culture medium from evaporation and contamination when early embryos are cultured. However, it was not clear which type of oil is the best for culture in vitro under various conditions. In addition, it was not known whether the volume of oil used affects the development of preimplantation embryos.

It has been reported that removing embryos from the incubator several times a day impede embryo development (Lawitts and Biggers, 1993). However, repeated observation is required in order to score temporal development of preimplantation embryos when zygotes are cultured in vitro.

Therefore, the experiments presented in this Chapter aimed to study effects of three media, volume and type of oil, and frequency of observation on the development in vitro of mouse zygotes.

3.2 Materials and Methods

3.2.1 Superovulation, embryo collection and culture in the mouse

F1 C57/CBA female mice (4 to 6 wk old) were superovulated and mated as described in 2.3.

At 25 to 28h after the injection of hCG, mice were euphonised by cervical dislocation and embryos were flushed from the excised oviduct as described in 2.4.2 into 0.5 ml of a Hepes-HTF medium containing 300 units/ml of hyaluronidase type IV-S (Sigma, H-4272, Lot 16H4677) to remove cumulus cells. The embryos then washed 3 times in 1.5 to 2 ml of Hepes-HTF medium before culture.

Zygotes with polar bodies were selected and cultured in 4-well tissue culture dishes (Medos Co PM. Ltd., Nunclon 4, 176740) in 50 µl of medium covered with paraffin oil (BDH, Prod 294375J) or mineral oil at 37°C in an atmosphere of $5\% CO_2$, $5\% O_2$ and $90\% N_2$ as

described in 2.5.2. The developmental progress was checked and recorded at either 96h and 120h or every 24h for 120h.

3.2.2 Experimental designs

3.2.2.1 The effect of medium (CZB, KSOM and HCO$_3$HTF) and the volume of paraffin oil on the development in vitro of zygotes

This experiment was designed to compare the effect of three different media on the development in vitro of zygotes in the mouse. The formulations of CZB, KSOM and HCO$_3$HTF medium were as described by Chatot et al. (1989), Lawitts and Biggers, (1993) and Quinn et al. (1985) (see in 2.5.1). Fifteen to twenty zygotes in each well were covered with either 1 ml or 0.5 ml paraffin oil. There were three replicates in this experiment.

The mammalian embryo first differentiates into two distinct cell types at the blastocyst stage. One is the inner cell mass (ICM) responsible for the embryo proper and the other is trophectoderm (TE) which contributes to blastocoel fluid accumulation and implantation, and formation of the placenta and fetal membranes after implantation (Handyside and Johnson, 1978; Hogan et al., 1994). It is suggested from these observations that the number of TE and ICM at the blastocyst stage could affect the ability of embryonic development beyond the blastocyst stage (Hogan et al., 1994). Therefore, differential fluorescent labelling of TE and ICM in the blastocyst was also investigated in this experiment to determine the effect of CZB and KSOM on the number of ICM and TE in the blastocyst. No embryos developed to blastocysts in HCO$_3$HTF.

102

At 120h of culture in CZB and KSOM media blastocysts covered with 1 ml of paraffin oil, respectively, were stained to differentiate ICM and TE cells, using a modification of a method as described previously (Handyside and Hunter, 1984, see Section 2.5.3.3). The number of ICM and TE cells in blastocysts covered with 0.5 ml of paraffin oil was not examined because the total number of blastocysts (blastocysts + hatched blastocysts) under 0.5 ml of paraffin oil was lower than that under 1 ml of paraffin oil (see Table 3.1).

3.2.2.2 The effect of paraffin oil and mineral oil on the development in vitro of mouse zygotes

To compare the effect of paraffin and mineral oil on the development in vitro of mouse zygotes, 15 to 20 zygotes in 50 μl of CZB medium within each well of the 4-well tissue culture dish were covered with either 1 ml of paraffin or mineral oil and then cultured using methods as described in 3.2.1. The number of blastocysts were examined unsung an Olympus microscope (BO61, Japan), respectively at 96h and 120h after culture in vitro. The total magnification was X100. There were two replicates in this experiment.

3.2.2.3 The effect of observation times on the development in vitro of mouse zygotes

To determine the effect of observation times on the development in vitro of mouse embryos, 20 zygotes in 50 μl of CZB medium within each well of the 4-well tissue culture dish were covered with 1 ml of paraffin oil and cultured using methods as described in 3.2.1. The developmental progress was checked using an Olympus microscope (BO61, Japan) at either 96h and 120h or every 24h. There were four replicates in this experiment.

3.2.3 Data analysis

Comparisons and differences between groups were made using the Chi-squared test and analysis of variance. A significant difference was assumed to exist when the probability of it being due to chance done was less than 5%.

3.3 Results

3.3.1 The effect of CZB, HCO$_3$HTF and KSOM and the volume of paraffin oil on the development in vitro of mouse zygotes

The results obtained with these three media and the different volumes of paraffin oil on the development in vitro of mouse embryos are shown in Table 3.1, 3.2 and 3.3.

During 96h of culture in vitro, and using 1 ml of paraffin oil, 91.7% and 65.8% zygotes developed respectively to blastocysts in CZB and KSOM, but no embryos developed to blastocysts in HCO$_3$HTF (0.0%) (Table 3.1). These differences in the development of blastocysts between the three media were highly significant and the proportion of embryos developing to the blastocyst stage was greater in CZB than KSOM (P<0.001) and greater in both CZB and KSOM than in HCO$_3$HTF (P<0.001) (Table 3.2).

During 120h of culture in vitro, significantly more zygotes developed to blastocysts (P<0.001) and hatched blastocysts (P<0.01) in CZB and KSOM than HCO$_3$HTF (Table .3.1 and 3.2). A greater proportion of embryos developed to the blastocyst stage in either KSOM or CZB than in HCO$_3$HTF (P<0.001), with CZB being better than KSOM (P<0.02) (Table

3.2), although there were no significant differences in the development to hatched

blastocysts between KSOM and CZB (Table 3.1).

Table 3.1 The effect of three media and the volume
of paraffin oil on the in vitro development of zygotes

Culture Medium	KSOM		CZB		HCO$_3$HTF		Time of culture (h)
Volume of paraffin oil (ml)	0.5	1	0.5	1	0.5	1	
No. zygotes	52	111	65	108	72	67	
1-C*	1(1.9)						
2-C	12(23.1)		2(3.1)		16(22.2)	13(19.4)	
3-C			3(4.6)		7(9.7)	3(4.5)	
4-C			1(1.5)		2(4.8)	1(1.5)	
M	6(11.5)	16(28.8)	6(9.2)	1(0.9)	20(27.8)	1(1.5)	96
B	29(55.8)	73(65.8)	42(64.6)	99(91.7)	3(4.2)	0(0.0)	
Ab		3(2.7)	3(4.6)		15(20.8)	2(3.0)	
D	4(7.7)	19(17.1)	8(12.3)	7(6.5)	9(12.5)	47(70.1)	
2-C	9(17.3)		1(1.5)		6(8.3)	6(9.0)	
3-C	1(1.9)		1(1.5)		6(8.3)	5(7.5)	
4-C						1(1.5)	
M		2(1.8)			2(2.8)	0(0.0)	
B	18(34.6)	65(58.6)	23(35.4)	80(74.1)	15(20.8)	0(0.0)	120
HB	11(21.2)	19(17.1)	20(30.8)	19(17.6)	0(0.0)	0(0.0)	
Ab	7(13.5)	2(17.1)	3(4.6)		3(4.2)		
D	6(11.5)	23(20.7)	17(26.2)	9(8.3)	40(55.6)	55(82.0)	

* C - Cell, M - Morulae, B - Blastocysts, HB - Hatched blastocysts, Ab - Abnormal, D - Dead

Values given in brackets are the number of embryos at the given stage as percentages of embryos collected.

During 96h of culture in vitro and using 0.5 ml of paraffin oil which just covered the media, the proportion of embryos developing to blastocysts in CZB and KSOM was much higher than in HCO$_3$HTF (Table 3.1). More embryos developed to blastocysts in either KSOM or in CZB than in HCO$_3$HTF ($P<0.001$) (Table 3.2).

During 120h of culture in vitro, the proportion of embryos developing to blastocysts was significantly higher in CZB and KSOM than in HCO$_3$HTF, and higher in CZB than in KSOM, but this difference was not significant. About 20% of embryos cultured in KSOM and CZB developed to hatched blastocysts, but no zygotes developed to hatched blastocysts in HCO$_3$HTF (Table 3.1). Further analysis showed that there were highly significant

differences ($P < 0.001$) in the proportion of embryos developing to blastocysts plus hatched

blastocysts in either KSOM or CZB than in HCO_3HTF ($P<0.001$) (Table 3.2).

Table 3.2 Analyses of results on the effect of three media
on in vitro development of zygotes

Time of culture (h)	Paraffin oil (ml)	Stage of Embryos*	X^2	P	Between
96	1	B	147.2408	P<0.001	KSOM, CZB, HCO_3HTF
			21.7868	P<0.001	KSOM and CZB
			74.6974	P<0.001	KSOM and HCO_3HTF
			141.4200	P<0.001	CZB and HCO_3HTF
96	0.5	B	60.7911	P<0.001	KSOM, CZB, HCO_3HTF
			0.9475	NS	KSOM and CZB
			49.9916	P<0.001	KSOM and HCO_3HTF
			56.5901	P<0.001	CZB and HCO_3HTF
120	1	B	95.2836	P<0.001	KSOM, CZB, HCO_3HTF
		B	5.8901	P<0.02	KSOM and CZB
		B	61.8026	P<0.001	KSOM and HCO_3HTF
		B	91.4230	P<0.001	CZB and HCO_3HTF
		HB	13.4936	P<0.01	KSOM, CZB, HCO_3HTF
		HB	0.0086	NS	KSOM and CZB
		B+HB	161.7960	P<0.001	KSOM, CZB, HCO_3HTF
		B+HB	10.1930	P<0.01	KSOM and CZB
120	0.5	B	4.2788	NS	KSOM, CZB, HCO_3HTF
		HB	24.6680	P<0.001	KSOM, CZB, HCO_3HTF
		HB	1.3714	NS	KSOM and CZB
		B+HB	30.9620	P<0.001	KSOM, CZB, HCO_3HTF
		B+HB	1.3163	NS	KSOM and CZB
		B+HB	16.0675	P<0.001	KSOM and HCO_3HTF
		B+HB	28.7409	P<0.001	CZB and HCO_3HTF

* B, HB, as Table 3.1.

Table 3.3 Analyses of results on the effect of volume of paraffin oil

on in vitro development of preimplantation embryos

Culture media	Time of culture (h)	Stage of embryos*	X^2	P	Between
KSOM		B	1.5111	NS	1 ml and 0.5 ml
CZB	96	B	19.6965	P<0.001	1 ml and 0.5 ml
HCO₃HTF		B	2.8532	NS	1 ml and 0.5 ml
KSOM		B	7.6063	P<0.01	1 ml and 0.5 ml
		HB	8.1229	NS	1 ml and 0.5 ml
		B + HB	0.3842	P<0.05	1 ml and 0.5 ml
CZB	120	B	6.5986	P<0.001	1 ml and 0.5 ml
		HB	25.2135	P<0.05	1 ml and 0.5 ml
		B + HB	4.0348	P<0.01	1 ml and 0.5 ml
HCO₃HTF		B	15.6468	P<0.001	1 ml and 0.5 ml

* B, HB, as Table 3.1.

When using CZB and 96h of culture in vitro, the proportion of embryos developing to blastocysts was significantly higher when covered with 1 ml of paraffin oil than in this medium covered with 0.5 ml of paraffin oil, but with KSOM, this difference was not significant (Table 3.1 and 3.3).

During 120h of culture in vitro, more zygotes developed to blastocysts in KSOM or CZB covered with 1 ml of paraffin oil than media covered with 0.5 ml of paraffin oil (Table 3.1 and 3.3).

The proportion of embryos developing to blastocysts plus hatched blastocysts was significantly higher in KSOM or CZB covered with 1 ml of paraffin oil than in medium covered with 0.5 ml of paraffin oil. However, the proportion of embryos developing to hatched blastocysts in CZB was higher if 0.5 ml oil was used (Table 3.1 and 3.3).

The effect of KSOM and CZB on the cell number in blastocysts was also determined (Table 3.4) in this experiment. The results given in Table 3.4 indicate that the number of ICM and TE+ICM cells in embryos cultured in CZB medium was higher than that in KSOM medium, while the ratio of ICM/TE in CZB medium was not significantly different from in KSOM.

Table 3.4 The effect of KSOM and CZB on the cell number of blastocysts

	No. Blastocysts	TE*	ICM*	Ratio (ICM/TE)	Total No. TE + ICM
CZB	16	40.25±17.91	24.97±8.10	0.72±0.32	65.31±21.25
KSOM	33	30.92±16.32	17.82±10.27	0.85±0.83	49.09±17.08
Anova: Single-Factor		F=3.0097 NS	F=5.7998 P<0.05	F=0.3657 NS	F=8.2763 P<0.01

* TE – trophectoderm; ICM – inner cell mass; Values given are mean ± SEM.

These results show clearly that CZB appears to be the most suitable medium for embryo culture under the conditions adopted for these investigations.

3.3.2 The effects of types of oil on development in vitro of mouse zygotes

To examine the influence of oil (paraffin vs mineral) on embryo development, embryos were cultured in CZB medium and culture droplets covered either with 1 ml of paraffin or 1 ml of mineral oil for the duration of culture. The results are shown in Table 3.5.

During 96h and 120h of culture in vitro, the proportion of embryos developing to blastocysts was significantly higher using paraffin oil than using mineral oil (P<0.001).

108

Based on these results, it was decided that paraffin oil would be used in the subsequent cultures.

Table 3.5 The effects of type of oil on in vitro development
of mouse zygote in CZB medium

	Paraffin oil	Mineral oil	X^2	P	Time of culture(h)
No. zygotes	47	42			
2-C*		6(14.3)			
3-C		3(7.1)			
4-C		1(2.4)			
8-C		2(4.8)			96
M	5(10.6)	16(38.1)			
B	**37(78.7)**	**7(16.7)**	**34.1703**	**P<0.001**	
D	5(10.6)	7(16.7)			
B	**41(87.2)**	**14(33.3)**	**27.8005**	**P<0.001**	120
D	6(12.7)	28(66.7)			

* C, M, B and D are the same as in Table 3.1.
  Values given in brackets are the number of embryos at the given stage as a percentage of the total number of embryos collected

3.3.3 The effects of the number of observation on development in vitro of mouse zygotes

The effect of observation times on embryo development are shown in Table 3.6.

After 96h and 120h of culture in vitro, the proportion of embryos developing to blastocysts was similar whether the embryos were observed only at these times or at 24, 48 and 72h as well.

These results suggest that repeated observations did not influence the proportion of embryos developing to blastocysts, allowing for routine monitoring and scoring of embryo development in subsequent experiments.

Table 3.6 The effect of observing frequency on in vitro development of zygotes

Time of culture(h)	No. zygotes	214	152	X^2	P
24	2-C*		142(93.4)		
48	4-C		10(6.5)		
	8-C		3(2.0)		
	M		134(88.2)		
72	M		99(65.1)		
	EB		45(29.6)		
96	M	11 (5.1)	3(8.6)		
	B	172 (80.4)	119(78.3)	0.2370	NS
120	M	2 (0.9)	2(1.3)		
	B	181 (84.6)	130(85.5)	0.0624	NS

* C, M and B are the same meanings as those in Table 3.1; EB is referred as early blastocysts
  Values given in brackets are the number of embryos at the given stage as a percentage of the total number of embryos collected.

3.4 Discussion

The proportion of embryos developing to blastocysts (96h) and blastocysts plus hatched blastocysts during 120h in culture (Table 3.1) was similar to those reported by Chatot et al. (1989) using CZB, but lower than those reported of Erbach et al. (1994) using KSOM and of Quinn et al. (1985) using HCO_3HTF. These differences could be related to different mouse strains and local culture conditions. However, under the present conditions, when all 3 media were compared directly, CZB appeared to be the most satisfactory medium. Therefore, all subsequent studies were undertaken using this medium.

It is known that the main differences among these media are in the concentrations of NaCl and glucose and the addition of glutamine and ethylenediaminetetraacetic acid (EDTA). High concentrations (125 mM) of NaCl in culture media have been reported to decrease

110

rates of protein synthesis (Anbari and Schultz, 1993), change the patterns of protein synthesis and reduce the rates of mRNA synthesis and its stability, and the relative amount of mRNAs for IGF-I and IGF-II and their cognate receptors (Ho et al., 1994). These changes of protein and mRNA synthesis subsequently inhibit the development of mouse zygotes to blastocysts (Lawitts and Biggers, 1991a,b; Biggers et al., 1993) since the development and differentiation of preimplantation embryos require protein synthesis. In contrast, media containing a low sodium concentration (85 mM) may overcome these problems and support embryo development (Anbari and Schultz, 1993; Biggers et al., 1993; Ho et al., 1994). The CZB medium found be the most appropriate in the present study contains only 82 mM of NaCl.

In the mouse, zygotes can utilize only pyruvate (or oxaloacetate) as energy sources, but lactate can support development from the 2-cell stage onwards and together with glutamine helps support development in vitro. Many studies have indicated an increase in glucose uptake between the 1-cell and 2-cell stage, but not between 2- and 8-cell stage, and that glucose can be used as the sole substrate from the 8-cell stage onwards (Whitten, 1957; Brinster, 1963, 1965a,b; Biggers, et al., 1967; Whitten and Biggers, 1968; Whittingham, 1969, 1971; Biggers and Stern, 1973; Biggers, 1987; Leese, 1991; Bavister, 1995). In this experiment, glucose concentrations in CZB, KSOM and HCO_3HTF were 5.6, 0.2 and 2.78 mM, respectively (Table 2.2 in 2.5.2) and the high concentrations of glucose in CZB might better support the development of mouse zygotes onward because there were a higher proportion of 2-cell embryos in KSOM and HCO_3HTF than CZB, during 96h and 120h of culture in vitro.

Glutamine may improve the development of mouse zygotes from outbred strains (Chatot et al., 1989). Similar beneficial effects of glutamine on embryonic development have been

reported in other species, including the hamster (Bavister et al., 1983; Carney and Bavister, 1987), rabbit (Brinster, 1970), pig (Petters et al., 1990) and sheep (Szell, 1995). The role of glutamine in embryonic development may be to act as a source of amino groups for transamination and/or as a source of energy, and a regulator of the detrimental effects of excessively high NaCl concentrations (Lawitts and Biggers, 1992; Biggers et al., 1993). The combination of 1 mM of glutamine with 105 mM of NaCl appears to be optimal (Biggers et al., 1993). In the present study, glutamine concentrations in CZB, KSOM and HCO_3HTF were respectively 1 mM, 1 mM and 0.0 mM (Table 2.2 in 2.5.2) and our results showed clearly that CZB and KSOM were much better than HCO_3HTF.

Previous studies have indicated that ethylenediamine-tetraacetic acid (EDTA) may exert its beneficial effect on embryo development by chelating toxic metals, or by facilitating the transport of other factors important for embryo development (Loutradis et al., 1987; Chatot et al., 1989; Fissore et al., 1989; Uranga and Arechaga, 1997), or by increasing protein synthesis (Poueymiou et al., 1989), through its action on the cell surface of the embryo (Abramczuk et al., 1977). Furthermore, EDTA may be more important for embryo in vitro development beyond the 2-cell stage than either protein or amino acids (Fissore et al., 1989) and improves the development of postimplantation embryos (Mehta and Kiessling, 1990). The EDTA content used varies with different studies reported. Some studies have shown that the optimum EDTA concentration is 10 μM (Abramczuk et al., 1977; Erbach et al., 1994), while other studies have indicated that the optimum EDTA concentration is 100 uM (Chatot et al., 1989; Poueymiou et al., 1989; Mehta and Kiessling, 1990). This value is supported by our results because CZB containing 100 μM of EDTA was better at supporting embryo development in vitro than KSOM containing 10 μM of EDTA (Table 2.2 in 2.5).

The results presented here on the number of TE, ICM and TE+ICM are not in agreement with those previously reported by Erbach et al. (1994) who showed that during 96h of culture in vitro of mouse zygotes, the number of TE, ICM and TE+ICM in the blastocysts was higher in KSOM than in CZB. This difference may be due to the differences between mouse strains, between culture conditions and the time at which the blastocysts were sampled. However, the rate of success for transfer of embryos cultured for 4 days in KSOM and CZB indicated that the former is not better than the latter (30% vs 50%) (Erbach et al., 1994), and this is supported by the present study which suggests that the number of ICM, and the total number of ICM plus TE cells are higher in embryos cultured in CZB than in KSOM.

The proportion of embryos developing to blastocysts in the presence of mineral oil was significantly lower than in the presence of paraffin oil. Mineral oil (Sigma, M-3516) is known to cause irritant damage to some tissues (e.g. Eyes, Sigma Company) and therefore may disturb developmental in vitro capacity of preimplantation embryos. On the other hand, the paraffin oil used (BDH, 294375J) contains the lowest toxin and impurity level according to the manufacturer's specifications and these features appear to be an advantage in the development in vitro of preimplantation embryos.

During repeated observations, embryos are exposed to light and transient temperature fluctuation as they are moved repeatedly from the incubator to the atmosphere and returned. This could result in an interference with the optimal in vitro development of mouse embryos. It has been shown that increasing the length of exposure of mouse zygotes to room temperature significantly reduced the rate of development to blastocysts since temperature reductions may slow the metabolic processes of zygotes and disrupt their

113

enzyme systems and mitotic spindle formation. However, 2-cell embryos were less affected by temperature fluctuation (Scott et al., 1993). Whilst handling conditions were optimized in the present studies to minimize temperature fluctuation by reduced number of observation, the results in the present study have shown that repeated observations did not subsequently reduce development, compared with those observed only once at the end of the developmental period.

Chapter Four

The Effect of Paternal Heat Stress on Development In Vitro of Preimplantation Embryos in the Mouse

4.1 Introduction

Bellve (1973) first studied the development in vitro of late 2-cell mouse embryos sired by males from day3 to day8 after heat stress and showed that paternal heat stress can cause some developmental retardation at the 8-cell stage and a marked accumulation of morulae at 120h. Heat-stressed rams have also been shown to have a reduced in vitro fertilisation rate (Ekpe et al., 1992), and a slower in vitro development of preimplantation embryos with an increased degeneration at the blastocyst stage (Ekpe et al., 1992, 1993; Setchell, 1994). The present experiment aimed to study the effect of paternal heat stress between 3 days and 42 days previously on the development in vitro of preimplantation embryos in the mouse, and determine which stage of preimplantation embryos are most seriously affected by the heat-stressed male.

4.2 Materials and Methods

4.2.1 The effect of heat stress on body temperature and testicular temperature in the male mouse

Body temperature and testicular temperature under heat-stressed conditions were first determined in order to understand the actual insult of heat stress on the mouse body and testes. Sixty C57/CBA F1 male mice (13 to 22 weeks old) were used in this experiment. Control (23°C) and heated (36°C, 62% relative humidity) mice were anaesthetised using fluothane (ICI Australia Operations Pty. Ltd., AUSTR 11216, Australia) at 0, 6, 12, 18 and 24h during heat stress and core body temperature (intra-peritoneal) and testicular

temperature were measured using a "Digi-sense" thermometer (Model 91100-200, Extech Equipment Pty. Ltd., USA) collected with a type T needle probe in 0.75 length (Model E08505-93, Extech. Equipment Pty. Ltd., USA). This thermometer has a sensitivity of ± 0.1°C at 36-40°C. Six control and six heated mice were used at each time point.

4.2.2 Heat stress of the male mouse

To investigate the effect of paternal heat stress on the development of preimplantation mouse embryos, six mature and fertile C57/CBA F1 male mice (13 to 22 weeks old) were placed in a psychrometric chamber set at 36 ± 0.1°C and 62 ± 0.4% relative humidity for 24h. Two control male mice were similarly maintained at 23°C for 24h with other conditions being the same as those in the experimental group. There were two replicates in this experiment.

4.2.3 Superovulation and mating of female mice

One-hundred and twelve normal C57/CBA F1 female mice (4 to 10 weeks old) were superovulated as method presented in 2.3. Briefly, female mice were superovulated by the i.p. injection of 5 to 10 IU PMSG (Folligon, 26532, Australia) at approximately 16:00h, followed 47 to 48h later by an i.p. injection of 5 to 10 IU of hCG (CG-2, Lot 18F0277, Australia). Eight female mice were used each time, six mice in the experimental group and two in the control group.

Following the injection of hCG, each superovulated female mouse was caged overnight with either a control or a heated male from one of experimental groups on Day 3 (D3), Day 7 (D7), Day 14 (D14), Day 21 (D21), Day 28 (D28), Day 35 (D35) or Day 42 (D42) after heating of the male. The presence of a vaginal plug the following morning was taken as an indication of a successful mating.

4.2.4 Collection and culture in vitro of control-sired and heat-sired putative zygotes in the mouse

Materials and methods employed for these studies have been described in detail in sections 2.4.2 and 2.5.2. Briefly, 25 to 28h after the injection of hCG, the females were euthanized by cervical dislocation and control-sired and heat-sired embryos were collected from the oviducts by piercing the swollen portion of the ampulla with a needle or by inserting a 27-gauge needle into the ostium of the oviduct and injecting approximately 0.5 ml of Hepes HTF. Cumulus cells were dispersed by a brief treatment with a Hepes HTF containing 300 μg/ml of hyaluronidase type IV-S (Sigma, H-4272, Lot 16H4677). Embryos with two pronuclei or one-cell /zygotes were selected and washed in a Hepes HTF for 3 times.

Control-sired and heat-sired embryos were cultured in 4-well tissue culture dishes (Falcon, 3001, Australia) in 50 ul of a CZB medium (Chatot et al., 1989) under paraffin oil (BDH, 294375J, Australia) at 37°C in an atmosphere of 5%CO_2, 5%O_2 and 90%N_2. The developmental progress was checked and recorded every 24h, for 120h.

Control-sired (n = 2 sires) and heat-sired (n = 6 sires) embryos were collected at D3, 7, 14,

21, 28, 35 and 42 after paternal heat stress.

4.2.5 Morphological evaluation of preimplantation embryos in the mouse

The development of preimplantation mouse embryos was examined using an Olympus CK2 inverted microscope (Olympus Optical Co., Japan). Embryos can be classified into 4 categories according to their morphology after culture in vitro (Wright and Ellington, 1995). They are defined as:

Normal embryos (Fig 4.1, 1-10): Putative zygotes have developed to a specific stage after a defined time of culture in vitro, i.e. to 2-C (after 24h), 4-C to morulae (48h), 8-C to early blastocysts (72h), morulae and early blastocysts to blastocysts (96h), and blastocysts (120h). Developed embryos appear symmetrical with cells of uniform size, colour and texture;

Non-developing embryos (Fig 4.1, 11): Embryos did not develop during culture in vitro;

Abnormal embryos (Fig 4.1, 12-14): Embryonic morphology is not spherical, but shows extruded blastomeres, irregular shape, colour and texture;

Dead/dying embryos (Fig 4.1, 15-16): Cells within embryos have dissolved during culture in vitro.

The appropriate development stage of preimplantation embryos at a given time were first defined in order to analyse the effect of paternal heat stress on the development in vitro of mouse embryos. Embryos were examined at 5 times namely 24, 48, 72, 96 and 120h of

118
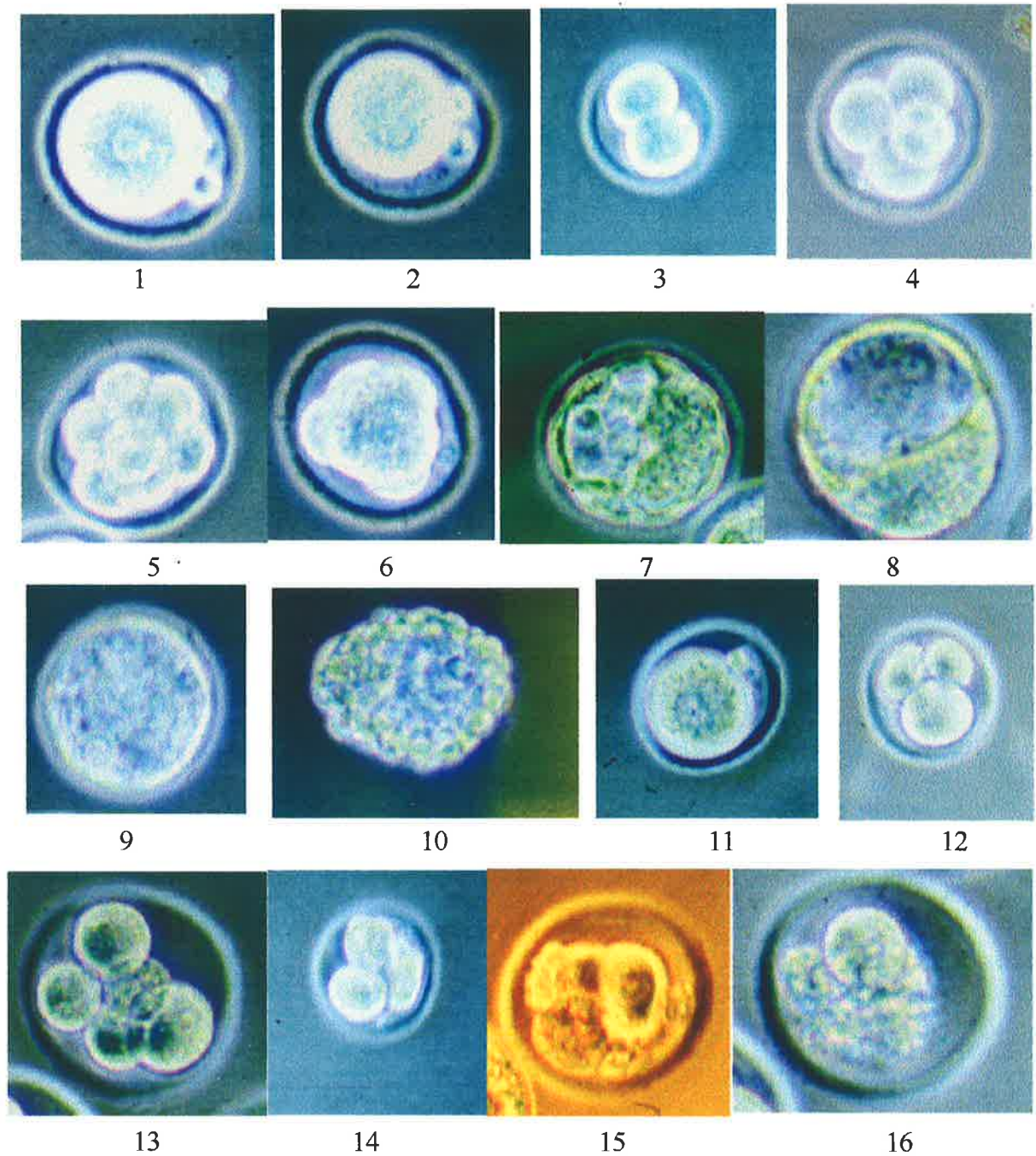
Fig 4.1 Development in vitro of mouse embryos

1 and 2. Zygotes collected respectively from either a control mouse oviduct or a experimental mouse oviduct 25-28h after hCG injection; 3. 2-cell embryo (24h of culture in vitro); 4. 4-cell embryo (48h of culture in vitro); 5. 8-cell embryo (48h or 72h of culture in vitro); 6-7. Morula and early blastocyst (72h of culture in vitro); 8-9. Blastocyst and expanded blastocyst (96h of culture in vitro); 9-10. Expanded and hatched blastocysts (120h of culture in vitro); 11. Not developed embryo; 12-14. Abnormal embryos; 15-16. Dead/dying embryos. X250.

culture to determine the development stage. At each of these five times, the number of fertilised ova to have reached the appropriate development stage out of the total number of fertilised ova 'Risk Set' was determined. The "appropriate development stage was taken to be that stage normally reached by healthy control embryos in that time frame as summarised below Table 4.1.

Table 4.1 The appropriate development stage
for each time of observation

Time (h)	Development stage
24	2-cell
48	4-cell to morula
72	8-cell to early blastocyst
96	Morula to blastocyst
120	Blastocyst

Survival, as an outcome, was scored when an embryo was deemed to have reached the appropriate development stage. Consequently, the event of interest, namely the failure to reach the appropriate development stage was also recorded. Failure also included fertilised ova that were abnormal, dying/dead or not developed.

4.2.6 Statistics

Comparisons and differences of body temperature and testicular temperature between groups and within groups were tested for significance using analysis of variance. A significant difference was assumed to exist when the probability of it being due to chance was less than 5%.

Analysis of developmental competence of embryos was found to be complicated by variation over time in the rate of development of normal, control-sired embryos and the lack of independence of the data. In particular, for each replicate, at each day after heating, in each heat treatment group, the same embryos were examined over the development times between 24 and 120h (repeated measures). Most statistical methods require independence of observations. To address this, the assistance of a professional biometrical consultant was sought for statistical analysis of these data. The assistance of Ms. H. Oakey of Biometrics SA is acknowledged again at this point.

Survival analysis (Cox and Oakes, 1984) provides one way to overcome the lack of independence of these data and was used to investigate the effect on embryonic in vitro development of heat treatment and days after heating on the "response time until failure" [where failure is deemed to be not reaching the appropriate development stage at the appropriate time (24, 48, 72, 96 or 120h) and includes embryos that are abnormal, dead or not developed].

At the last development time (120h), any embryos that have reached the correct development stage are treated as 'right' censored. Censoring implies that we only know that this embryo has reached the correct stage at this particular time, we do not know what happens to the development of the embryo after this time i.e. failure has not yet occurred at the last time that we examined the embryo.

In summary, our response is time until failure. This response can take on the value 24, 48, 72, 96 or 120h, or greater (but unknown). It was of interest to determine the effect of the

explanatory variables heat treatment and days after heating on the response.

Preliminary investigation of the time to failure (hours) was performed by graphing Kaplan Meier (KM) estimated Survival Curves (Cox and Oakes, 1984) for each control and each heat treatment group at each day after heating.

A Log-Rank test (Cox and Oakes, 1984) was then used to determine whether the plotted Kaplan-Meier (KM) survival curves were different. This test is approximately Chi-squared with 1 degree of freedom. The null hypothesis was that there was no overall difference between survival curves for the control and heat-treated groups. The graphs and testing were performed using Splus 2000 (Splus 2000 Professional Edition for Windows Network, Release 1, 1988-1999 MathSoft, Inc., Math. Soft International, Knightway House, Park St. Bagshot, Survey, GU19 5AQ, UK).

A Cox Proportional Hazard (PH) Model (Cox and Oakes, 1984), which is a semi-parametric model was used to determine whether the hazard of failure differed with the two explanatory factors of interest-heat treatment and days after heating.

For data in the present study, the Cox PH model fitted was:

$$h(t)=h_0(t)e^{(A)* (B)}$$

Note: (A)*(B) = A+B+A.B, where A and B represented the main factors for heat treatment and days after heating, respectively, and A.B was an interaction term between the two explanatory factors.

Where:

The hazard function h (t) was interpreted as the instantaneous potential per unit time for failure of a fertilised ovum given that this fertilised ovum had survived up to a specific development stage.

h_0 (t) is the unspecified baseline hazard, which was taken the form of a non-negative function and t was time until failure.

The two explanatory factors included in the model were:

Heat treatment which had two levels, corresponding to the control and heat-treated group and *days after heating* which had 7 levels, corresponding to the following times 3, 7, 14, 21, 28, 35 and 42 days. An interaction between these two variables was also investigated.

Both these factors were examined as categorical variables, and were fitted such that one level was a baseline to which the other level(s) were compared (known as corner point parameterisation). Thus instantaneous relative risks or 'hazard ratios' of the baseline level to the other levels were determined. For example, the hazard ratio for the control as compared to the heat-treated group at a particular day after heating was determined if appropriate.

Replicate had two levels corresponding to the two replicates. It was included in the model to allow for variation between replicates and was fitted as a categorical explanatory variable. However, as opposed to corner-point constraints, we imposed a zero sum constraints on this effect, thus $\sum$ replicate = 0 and the mean was zero. As a result of this the

other explanatory variables (heat treatment and days after heating) were estimated at the 'mean' replicate level, providing an estimate of the hazard ratios at the mean replicate level. Therefore, the contribution by the replicate to the hazard ratio was taken as zero.

The importance of the explanatory variables was determined using a likelihood ratio test (-2*Change in log likelihood), (Cox and Oakes, 1984). This test is approximately Chi-squared. A significant difference was assumed to exist when the probability of it being due to chance was less than 5%. The importance of model terms was tested in a hierarchical manner, so that the interaction term between days after heating and heat treatment was tested before main effects. If the interaction term was significant it means the two variables did not act independently of each other and therefore it was not appropriate to test for the main effects of these variables.

The Cox PH model was fitted using the SAS procedure PROC PHREG (SAS for Windows Version 6.12, 1989-1996, SAS Institute Inc., Cary, NC, USA), with the "exact" method for dealing with ties.

4.3 Results

4.3.1 The effect of heat stress on body temperature and testicular temperature in the mouse

As can be seen in Fig. 4.2, exposure of male mice to the elevated environmental temperature of 36°C for 24h slightly increased body mean temperature ($P<0.005$), but significantly increased testicular mean temperature ($P<0.001$).
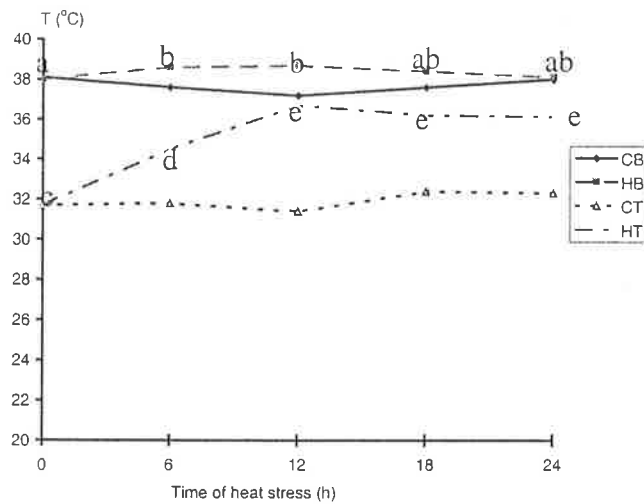
Fig. 4.2. Changes of body and testicular temperature in the mouse during heat stress

Note: CB – Mean body temperature in control group; HB – Mean body temperature in heated groups;
CT – Mean testicular temperature in control groups; HT – Mean testicular temperature in heated groups.
N= 6 mice per time point.
Values with a and b differ significantly (P<0.005) in mean body temperatures between HB groups and CB groups at each time point and between HB groups over time;
Values with c, d, and e differ significantly in mean testicular temperatures (P<0.001) between CT groups and HT groups and between HT groups over time.

4.3.2 The effect of paternal heat stress on the development in vitro of preimplantation embryos in the mouse

A summary of the raw results of the effect of paternal heat stress on the development in vitro of preimplantation mouse embryos is presented in Table 4.2. KM survival curves for each treatment group, each day after heating, and the log-rank tests for embryonic survival are presented in Figures 4.3 and Table 4.3, respectively.

Table 4.2. The effect of paternal heat stress on in vitro development
of preimplantation embryos in the mouse

Embryo stage	C**	D3	D7	D14	D21	D28	D35	D42	Time of culture (h)
	28 males	12 males	18 males	12 males	12 males	12 males	12 males	12 males	
Zygotes	257	128	136	114	108	138	185	106	
2-C*	247 (96.1)	111 (86.7)	99 (72.8)	14 (12.3)	21 (19.4)	39 (28.3)	68 (36.8)	97 (91.5)	
3-C to 4-C	0 (0.0)	4 (3.1)	4 (2.9)	9 (7.9)	3 (2.8)	33 (23.9)	33 (17.8)	1 (0.9)	24
Nd+Ab+D	10 (3.9)	13 (10.2)	33 (24.3)	91 (79.8)	84 (77.8)	66 (47.8)	84 (45.4)	8 (7.5)	
2-C to 3-C	1 (0.3)	8 (6.2)	17 (12.5)	48 (42.1)	33 (30.6)	34 (24.6)	60 (32.4)	1 (0.9)	
4-C to M	249 (96.9)	111 (86.7)	97 (71.3)	37 (32.5)	9 (8.3)	53 (38.4)	55 (29.7)	95 (89.6)	48
Nd+Ab+D	7 (2.7)	9 (7.0)	22 (16.2)	29 (25.4)	66 (61.1)	51 (37.0)	64 (34.6)	8 (7.5)	
2-C to 6-C	0 (0.0)	2 (1.6)	12 (8.8)	21 (18.4)	22 (20.4)	16 (11.6)	17 (9.2)	0 (0.0)	
8-C to EB	249 (96.9)	118 (92.2)	95 (69.9)	23 (20.2)	19 (17.6)	60 (43.5)	75 (40.5)	98 (92.5)	72
Nd+Ab+D	8 (3.1)	8 (6.3)	29 (21.3)	70 (61.4)	67 (62.0)	61 (44.2)	93 (50.2)	8 (7.5)	
2-C to 4-C	0 (0.0)	0 (0.0)	1 (0.7)	8 (5.9)	4 (3.7)	0 (0.0)	8 (4.3)	0 (0.0)	
M to B	246 (95.7)	112 (87.5)	71 (52.2)	21 (18.4)	12 (11.1)	42 (30.4)	55 (29.7)	98 (92.5)	96
Nd+Ab+D	11 (4.3)	16 (12.5)	64 (47.1)	85 (74.6)	92 (85.2)	91 (65.9)	122 (65.9)	8 (7.5)	
B	237 (92.2)	100 (78.1)	64 (47.1)	5 (4.4)	1 (0.9)	24 (17.4)	36 (19.5)	98 (92.5)	120
Nd+Ab+D	19 (7.4)	28 (21.9)	70 (51.5)	106 (93.0)	107 (99.1)	112 (81.2)	148 (80.0)	7 (6.6)	

*C – Cell, M – Morulae, EB – Early blastocysts, B – Blastocysts, Nd – Not developed embryos, Ab – Abnormal embryos, D – Dying/dead embryos;

**Pooled data from the control groups across days after heating; The values given in brackets were the number of embryos developing to given stages as a percentage of the total number of putative zygotes.

During 24-120h of culture in vitro, the proportion of embryos developing to 2-C, 4-C to M, 8-C to EB, M to B and B showed no detectable difference between control and heat-treated groups at day3 and day 42 after heating of the male (Fig 4.3, A and G, respectively, Table 4.3), but were significantly reduced in other heat-treated groups compared with control

Figure 4.3 KM Survival curves for embryos sired by control and heat treated males
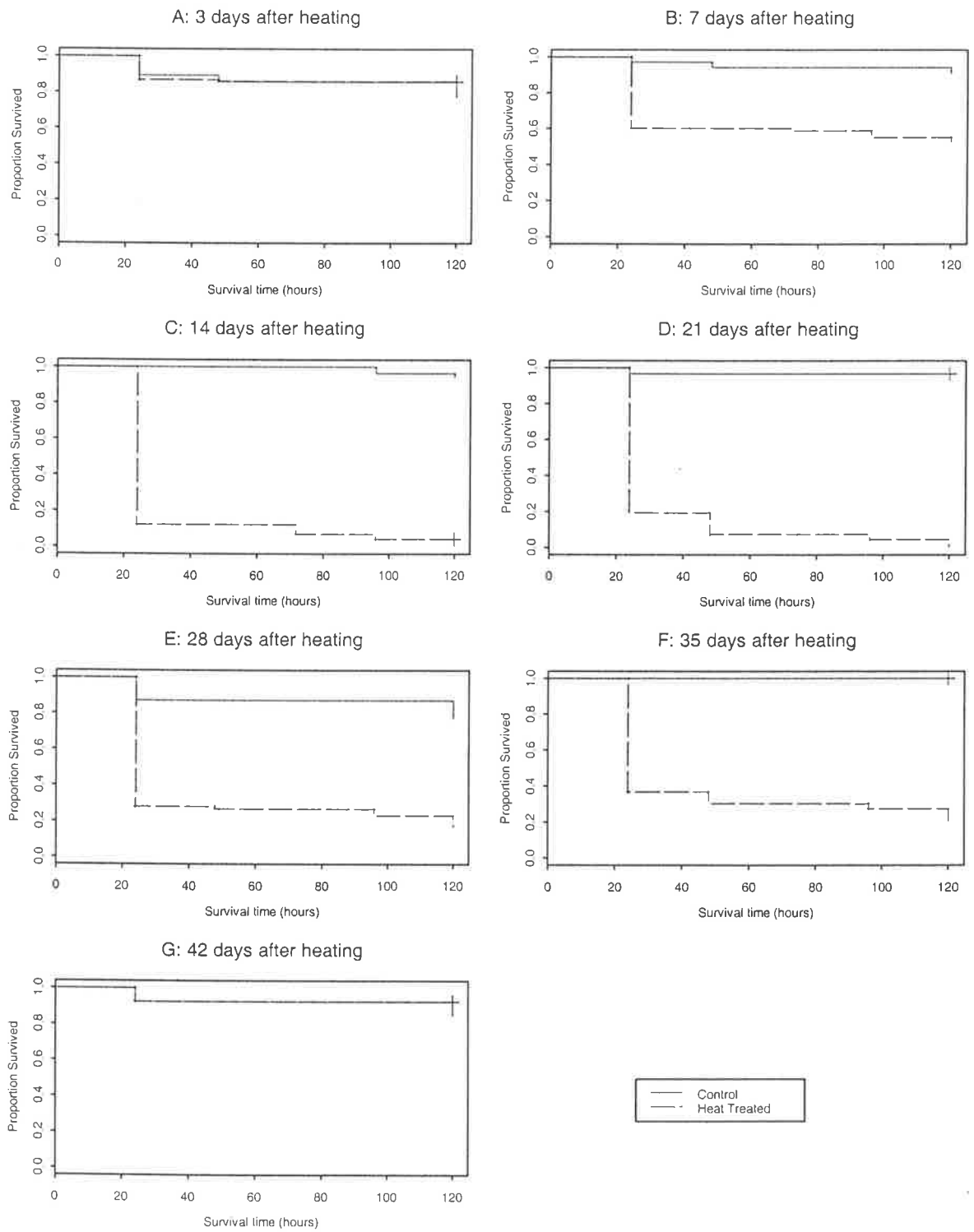
groups (Table 4.2, Fig 4.3, E-F, Table 4.3). The proportion of zygotes undergoing further development was significantly (P<0.0001) decreased by day 7 (Fig 4.3, B, Table 4.3) after heating, and had not recovered to control levels by day 35 after heating of the male (Fig 4.3, F, Table 4.3). Maximum impairment to development occurred at day 14 and day 21 after heating of the male (Fig 4.3, C and D, respectively, Table 4.3).

Whilst all nominated stages of embryo development were affected by paternal heat stress, the number of embryos at the 2-C stage were severely reduced in all heat-treated groups except day 3 and day 42 after heating (Table 4.2). Furthermore, at days 14, 21, 28 and 35 after heating of the male, development 4-C to M stage and M to B stage also demonstrated substantial impairment (Table 4.2).

Paternal heat stress increased the number of 3-C to 5-C embryos after 24h of culture in vitro. However, the proportion of 2-C to 3-C at 48h, 2-C to 6-C at 72h and 2-C to 4-C at 96h were higher at days 7, 14, 21, 28 and 35 after heating than that in control groups and day 42 after heating of the male (Table 4.2).

Table 4.3 Log-rank tests comparing the control and heat-treated group survival curves separately at each day after heating using a Chi-square test with 1 degree of freedom

Day after heating	Chi-square	p-value	
Day3	0.8	0.357	NS
Day7	17.1	0.00003	**
Day14	154	0	**
Day21	173	0	**
Day28	39.7	0	**
Day35	35.8	0	**
Day42	0.9	0.342	NS

NS indicates no significant difference between the control and heat-treated group at the 5% level; **indicates a highly significant difference between the control and heat-treated group significant at least the 0.01% level.

Modelling showed that the interaction between heat treatment and days after heating was highly significant when tested using the likelihood ratio test (X2 = 42.8, df = 6, P<0.001). This indicates that the hazard ratio of the control and heat-treated groups are different depending on the day after heating.

Using different days after heating as the baseline level when model fitting, we can determine the hazard ratios of the control to the heat-treated groups for each day after heating. These are shown in Table 4.4.

Table 4.4 Hazard ratios comparing the control and heat-treated groups
for each day after heating

Day after heating	Parameter Estimate ◆	Standard error	Hazard Ratio ◆ ◆	95% CI for hazard ratio	
Day3	0.467	0.534	1.595	(0.549, 4.637)	NS
Day7	2.081	0.599	8.016	(2.420, 26.554)	*
Day14	4.154	0.589	63.705	(19.580, 206.917)	*
Day21	4.550	0.716	94.673	(22.600, 396.594)	*
Day28	2.155	0.394	8.630	(3.926, 18.968)	*
Day35					
Day42	-0.633	0.791	0.531	(0.109, 2.583)	NS

NS indicates hazard ratio not significantly different from 1 i.e. No difference between the control and heat-treated group;

* indicates hazard ratio significantly different from 1 i.e. Control and heat-treated group have significantly different hazards at the 5% level;

◆ parameter estimate obtained from Cox PH model by fitting day x and control as the baseline level;

◆ ◆ the hazard ratio was given by the exponential of the parameter estimate.

Note: the results for day35 were not shown because there was 100% censorship in the control group at day35, meaning none of the fertilised ova in the control group had failed. The Cox PH model was unable to cope with this degree of censorship and the model fit for this parameter was unreliable.

Paternal heat stress at day 3 before conception did not increase the hazard ratio compared with that in control groups (Table 4.4). However, by day 7, the heat-treated groups had a hazard of failure 8.0 times that of the control group (Table 4.4). The hazard ratio rose and peaked over day 14 and day 21, such that the hazard of a failure occurring in the heat-

treated groups was 63.7 and 94.7 times the hazard of a failure in control group on day 14 and day 21 respectively (Table 4.4). At 28 days after heating, the hazard of failure in the treatment group had returned to be only 8.6 times that of the control group, and by day 42 the hazard of failure was not significantly different to the controls (Table 4.4).

4.4 Discussion

It is clear from the results of the present study that paternal heat stress affects the development of mouse preimplantation embryos and the extent of the effect may be related to the time of mating after heat stress of the male. The present results support a previous report in the mouse by Bellve (1973) who found paternal heat stress to reduce the number of blastocysts at 120h of culture in vitro when heat-stressed males were mated 3 to 8 days later. However, in contrast to his results (Bellve, 1973), we did not find a marked accumulation of morulae after 120h of culture. One possible reason for these differences is that mouse strains were different with mice in our experiment being C57/CBA F1 mice, while Bellve used ICR albino mice. The heat-stressed conditions used in the two experiments were also different, with Bellve's experiment being conducted with an environmental temperature and relative humidity of $34.5 \pm 0.2°C$ and $65 \pm 5\%$, while in our experiment, the values were $36.1 \pm 0.1°C$ and $62 \pm 0.4\%$, respectively. Exposure of male mice to higher temperature in our experiment resulted in higher temperatures in the testis, which is likely to have contributed additional damage to spermatogenesis and endocrine functions, finally influencing male fertility. The mating times after paternal heat stress were also different between the two studies. In Bellve's experiment, the male was kept with females only from days 3 to 8 inclusive, while in our experiment, the female was mated with the male after days 3 and 7, but also at later times after paternal heat stress.

Furthermore, the in vitro culture system and embryo stages used for investigation were different. We used 50 µl of CZB medium to culture 1-cell embryos in an incubator maintained at 37°C and in an atmosphere of $5\%CO_2/5\%O_2/90\%N_2$, while Bellve used 200 µl of BMOC-2 medium to culture late 2-cell embryos in the incubator kept at 37.5°C and in an atmosphere of 5% CO_2 in air. Nevertheless, the results of both experiments confirm that paternal heat stress has an effect on the development of preimplantation embryos in the mouse.

Heat stress is well documented in affecting spermatogenesis in the mouse and other species. The extent of the influence seems to be related to the temperature to which the individual is exposed during heat stress and to the sensitivity of the different types of germ cells in the testis. It has been previously demonstrated that late spermatocytes are the cell type most susceptible to heat exposure, followed by spermatids, spermatogonia and finally sperm in the mouse (De Vita et al., 1987, 1990; Sailer et al., 1997). According to the timetable of spermatogenesis, sperm involved in fertilization at day 3 and day 7 are those which would have been already in the epididymis at the time of heating (Meistrich et al., 1975; Dadoune and Alfonsi, 1984). That paternal heat stress 7 days earlier reduced the development of preimplantation embryos suggests that heat stress can damage sperm from the epididymis.

Paternal heat stress 14 and 21 days prior to mating had a greater effect on embryo development, than at days 3, 7 or 42. Sperm at D14 and D21 arise from round spermatids (D14) or diplotene and pachytene spermatocytes (D21), respectively (Oakberg, 1957) which are known to be seriously damaged by heat stress (De Vita et al., 1987, 1990; Sailer

et al., 1997). The susceptibility of these cells to heat stress might also be expected to influence the development of preimplantation embryos which they produce.

Similarly, paternal heat stress at 28 and 35 days prior to mating also reduced significantly the development of embryos. These results suggest that heat stress might also damage preleptotene spermatocytes and/or spermatogonia (Oakberg, 1957).

Paternal heat stress 42 days before mating did not affect the development of embryos to the different stages studied, compared with those in control group. This indicates that the new cycle of spermatogenesis which begins from Type A spermatogonia within this time frame is not affected by the heat exposure, and suggests that Type A spermatogonia appear not to be affected directly by the heating.

It is of interest that paternal heat stress reduced significantly the proportions of embryos developing to the 2-cell stage in particular, and therefore also 4-cell to morula stages, and the blastocyst stage in this experiment. Several studies have shown that endogenous transcription takes place at the late 1-cell stage of mouse embryos and that transcription is first initiated in the paternal pronucleus (Ram and Schultz, 1993; Matsumoto et al., 1994; Bouniol et al., 1995; Christians et al., 1995; Nothias et al., 1996). The results of our experiments have shown that heat stress of the male reduced significantly the proportions of embryos developing from 1-cell stage to 2-cell stage. This may suggest that sperm from heat-stressed male mice could be damaged such that the paternal pronucleus produced after fertilisation with these sperm is unable to initiate appropriate transcription at the late 1-cell stage. It is also possible that even though these sperm have fertilized an ovum, the sperm

asters can not be appropriately formed (Schatten, 1994; Navara et al., 1995; Sutovsky and Schatten, 2000), thus influencing the development of embryos after fertilization.

The two-cell stage of the mouse embryo is a key period which determines the later developmental fate of embryos since zygotic gene activation (ZGA) occurs at this stage. It is possible that, when normal ova have been fertilised by sperm from heat-stressed male mice, they can not destroy the maternal transcripts and produce the corresponding new transcripts that are unique to the developing embryos, thus leading possibly to a failure of ZGA. The low proportions of embryos developing to 4-cell to morula stages are in agreement with the failure of the ZGA which occurs only at the 2-cell stage (Flach et al., 1982; Bensaude et al., 1983; Latham et al., 1991; Manejwala et al., 1991; Schultz, 1993; Moore et al., 1996). Differentiation of cells within the morulae is responsible for the transition from morula to blastocyst. In the mouse, the first process of differentiation begins at the 8-cell stage with compaction of the embryo and development of cell polarity (Ducibella et al., 1975; Ziomek and Johnson, 1980). Moreover, these morphological changes require zygotic transcription (De Sousa et al., 1993; Fleming et al., 1993). If paternal heat stress damages or reduces zygotic transcription, the differentiation events of cells within morulae will possibly be disturbed, reducing the proportion of embryos developing from morulae to blastocysts.

Paternal heat stress not only reduced the proportion of preimplantation embryos at the different stages, but also changed the developmental capacity of some preimplantation embryos. The present study has shown that during 24h of culture, paternal heat stress increased the number of embryos developing to 3-cell to 5-cell stages, especially at D28 and D35 after heating of the male. This suggests that heat stress could increase the

functions of some sperm processes, such as sperm motility, sperm metabolism or fertility, which could accelerate sperm entry and penetration through the zona pellucida, activation of the ovum and fertilisation, and thus promote indirectly greater development and differentiation of the early preimplantation embryos. However, whether the 3-cell, 4-cell and 5-cell embryos produced in this way continue developing normally or not is not clear, although it seems unlikely.

After 48h-120h of culture, respectively, the number of 2-cell, 3-cell and 4-cell embryos in experimental groups, especially at D14, D21, D28 and D35 were much higher than in control groups. One possibility for this is that paternal heat stress could result in the failure of zygotic gene activation at the 2-C stage in the mouse, reducing development past the 4-cell stage; another reason could be related to the cell cycle of preimplantation embryos since the first and second cell cycles have longer G1 and G2+M stages, compared with the later cell cycles (Streffer et al., 1980; Molls et al., 1983; Howlett and Bolton, 1985; Smith and Johnson, 1986; Chrisholm, 1988; Moore et al., 1996). If paternal heat stress disturbs the normal process of the first and second cell cycle of preimplantation embryos in the mouse, it may disturb cell differentiation and subsequent development. In addition, it might mean that although 2-cell embryos have differentiated into 3-cell or 4-cell embryos, these may not be able to further differentiate if DNA replication has not been completed.

Paternal heat stress also increased the number of non-developing, abnormal and dead/dying embryos in the mouse. The extent of this increase is also related to the different types of germ cells damaged by heat stress. The present results suggest that once an ovum is fertilised by a sperm from a heat stressed male, these fertilised ova are likely to express

abnormal metabolism, abnormal protein synthesis and/or abnormal gene expression, thus leading to an increase of non-developing, abnormal and dead/dying embryos.

Chapter Five

The Effect of Paternal Heat Stress on Development In Vivo of Preimplantation Embryos in the Mouse

5.1 Introduction

Paternal heat stress has been reported to reduce fertilization rates (Burfening et al., 1970; Jannes et al., 1998) and the development in vivo of preimplantation embryos (Bellve, 1972) in the mouse. Setchell et al. (1998) further reported that local heating of the testis between day 7 and day 35 prior to mating reduced the size and weight of litters in the mouse. Studies in this thesis have also indicated that in vitro developmental rates of heat-sired preimplantation embryos begin to decrease when sired at day 3 after heat-stress, were lowest when embryos were sired on day 21 after heat-stress, and then gradually returned to control levels when sired by day 42 (Chapter 4). Although an optimised in vitro culture system has been established to minimise the effect of in vitro culture conditions on embryo development (Chapter 3), it remains highly likely that the in vitro environment is inferior to in vivo environment. Furthermore, given the significant effect of paternal heat stress on development in vitro, it was of interest to determine whether embryo development was improved when allowed to occur in vivo. Therefore, this study have investigated the effect of paternal heat stress on the development in vivo of preimplantation embryos conceived at 7, 21 and 35 days after heating of the male.

5.2 Materials and Methods

5.2.1 Heat stress of the male mouse

Materials and methods employed are described in detail in Section 2.6. Briefly, twelve normal fertile C57/CBA F1 male mice (13 to 15 weeks old) were maintained in a psychrometric chamber set at $36 \pm 0.3°C$ and $65.8 \pm 5.6 \%$ relative humidity and heated for

24h, while another twelve comparable, fertile F1 male mice were kept at 23°C as a control group. There were three replicates in this experiment.

5.2.2 Oestrous synchronization and mating of female mice

Normal C57/CBA F1 female mice (9 to 13 weeks old) were synchronized to be oestrous as the described in Section 2.7.

Each control male and each heat-stressed male was caged with 2 oestrous females in 7, 21 and 35 days after heating of the respective male. The existence of a vaginal copulation plug the following morning, was taken as an indication of a successful mating.

5.2.3 Collection of preimplantation mouse embryos

Successfully mated mice were selected and euthanasized by cervical dislocation. The mating time was defined as midnight (0:00h) and female mice were allocated randomly for the collection of preimplantation embryos. One-cell, 2-cell, morula and blastocyst stage embryos were collected 14-16h, 34-39h, 61-65h and 85-90h after mating, respectively. The numbers of corpora lutea were recorded. The number of preimplantation embryos recovered at the different stages were also scored.

5.2.4 Morphological evaluation of preimplantation mouse embryos

Embryos were examined using an Olympus CK2 inverted microscope (Olympus Optical Co., Japan) at a magnification of X200. The quality of individual embryos was determined

by a method modified from Wright and Ellington (1995), scoring Normal, Abnormal or Unfertilized ovum. The definitions applied were:

Normal: The embryo was spherical and symmetrical with cells of uniform size, colour and texture, and was at an appropriate stage of development, i.e. embryos recovered at 14-16h, 34-39h, 61h-65h and 85-90h after mating were zygotes (judged by the presence of the second polar body or pronuclei), 2-cell, 8-cell to morulae or morulae to blastocysts, respectively.

Abnormal: Embryos showed extruded blastomeres, a few cells of variable size, and contained numerous large vesicles.

Unfertilized ovum: An ovulated ovum which did not contain two polar bodies or pronuclei.

Embryos were photographed using professional colour reversal film (Kodak Ektachrome 64T Tungsten, Q-LAB Service, Australia). Slides were then scanned into a PC computer, for subsequent reporting.

5.2.5 Differential fluorescent labelling of trophectoderm and inner cell mass in the blastocyst

To examine blastocyst quality and the development of cellular polarisation, blastocysts were stained for differential fluorescent labelling of trophectoderm (TE) and inner cell mass (ICM) cells as described in Section 2.5.3 and the relative proportions of each cell type scored between control and heat-sired embryos.

5.2.6 Data analysis

Comparisons of differences between groups were made using the Chi-squared test for embryo development and analysis of variance for the number of TE and ICM cells in the blastocyst. A significant difference was assumed to exist when the probability of it being due to chance was less than 5%.

5.3 Results

5.3.1 The effect of paternal heat stress 7 days earlier on development in vivo of preimplantation mouse embryos

Paternal heat stress 7 days prior to mating had no significant effect on the proportion of embryos developing to the zygote and 2-cell stages at 14-16h and 34-39h respectively after mating, but significantly decreased the percentage of 8-cell embryos to morulae recovered at 61-65h after mating and the number of hatched blastocysts at 85-90h after mating (Table 5.1). However, the number of zygotes at 61-65h and morulae at 85-90h after mating were significantly higher in the heat-sired group (H7) (Table 5.1) than controls (C7) (Table 5.1).

Morphological changes in control-sired and heat-sired embryos collected from female mice at the different times after mating are shown from Fig 5.1 to 5.4. Morphology of the zygotes in the control group (Fig 5.1, A) were not obviously different to those in the heated group (Fig 5.1, B). However, as embryos developed further, paternal heat stress 7 days

Table 5.1 The effect of paternal heat stress 7 days prior to mating

on the in vivo development of embryos

Time of embryos recovered after mating	14-16h		34-39h		61-65h		85-90h	
Groups	C7	H7	C7	H7	C7	H7	C7	H7
No. female mice	4	4	4	4	4	4	7	11
Total No. corpora lutea	43	42	35	37	31	32	58	91
Recovered embryos[1]	41(95.3)	42(100.0)	34(97.1)	33(89.2)	31(100.0)	31(96.9)	54(93.1)	82(90.1)
Zygotes[2]	38(92.7)	42(100.0)	2(5.9)	3(9.1)	1(3.2)	9(29.0)**		
2-C[3]			32(94.1)	29(87.9)	0(0.0)	2(6.5)		
4-C					9(29.0)	5(16.1)		
8-C to M					21(67.7)	11(35.5)*		
M							0(0.0)	14(17.1)**
B							22(40.7)	43(52.4)
EB							12(22.2)	25(30.5)
HB							20(37.0)	0(0.0)***
Total No. B							54(100.0)	68(82.9)**
Abnormal	3(7.3)	0(0.0)	0(0.0)	1(3.0)	0(0.0)	3(9.7)		
Unfertilized					0(0.0)	1(3.2)		

1 The values given in brackets are the total number of embryos collected as a percentage of the total number of corpora lutea.

2 The values given in brackets are the number of embryos at the given stage as a percentage of the total number of embryos collected.

3. C - Cell, M - Morula, B - Blastocyst, EB - Expanded blastocyst, HB - Hatched blastocyst, Abnormal - Abnormal embryo, Unfertilized - Unfertilized ovum.

Values with *, ** and *** superscripts differ significantly (P<0.05 - 0.001) between the heat-sired group (H7) and controls (C7) for a given group.

previously appeared to have reduced developmental capacity. Although most heat-sired zygotes had developed to the 2-cell stage by 34h after mating, some remained undivided (Fig 5.2, B). In contrast, all control-sired zygotes had developed to the 2-cell stage (Fig 5.2, A). By 64h after mating, all control-sired embryos had developed to the morula stage (Fig 5.3, A), but some heat-sired embryos remained as zygotes (Fig 5.3, B and C), 2-cell and 3-cell embryos (Fig 5.3, C). Some did develop to the morula stage (Fig 5.3, B and C). By 89h after mating, control-sired embryos had developed to either the expanded blastocyst stage or the hatched blastocyst stage (Fig 5.4, A). In contrast, although there were no abnormal embryos seen in heat-sired groups, the number of morulae and blastocysts was increased, and no embryos had developed to the hatched blastocyst stage (Fig 5.4, B, C and D).
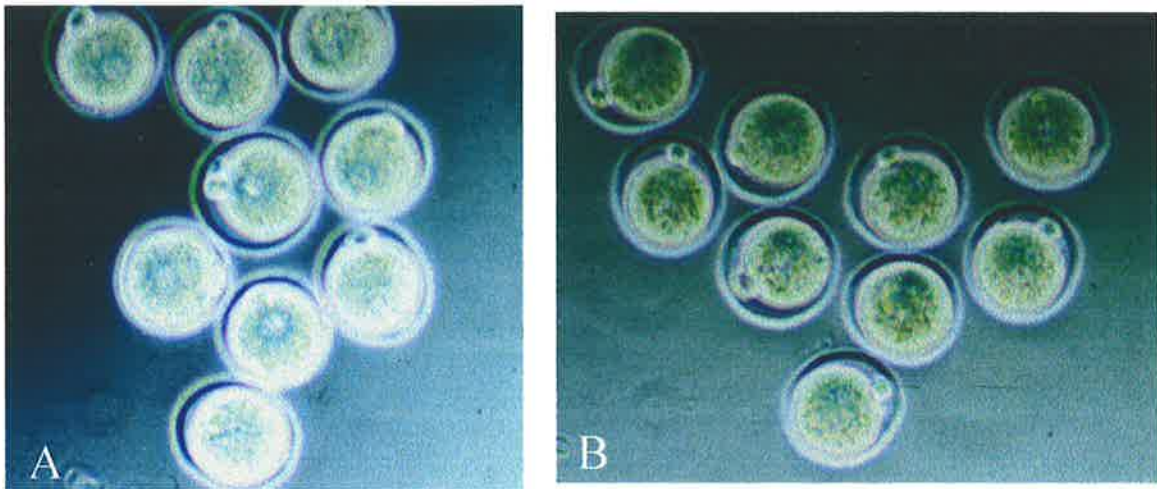
Fig 5.1 Embryos collected 14h after mating

A. Zygotes from a female mouse after mating with a control male; B. Zygotes from a female mouse after mating with a male heat-stressed 7 days previously. X120
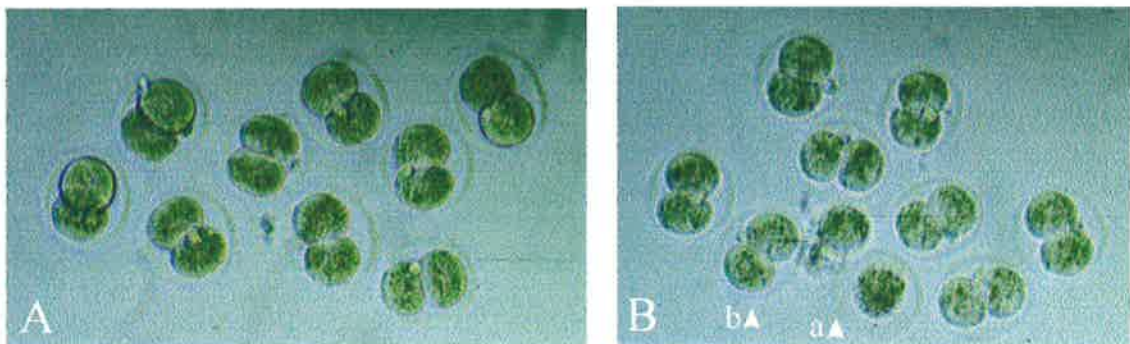
Fig 5.2 Embryos collected 34h after mating

A. Embryos (2-cell) from one female mouse after mating with a control male; B. Embryos from one female mouse after mating with a male heat-stressed 7 days previously, a ▶ zygotes , b▶ 2-cell embryos. X100

141
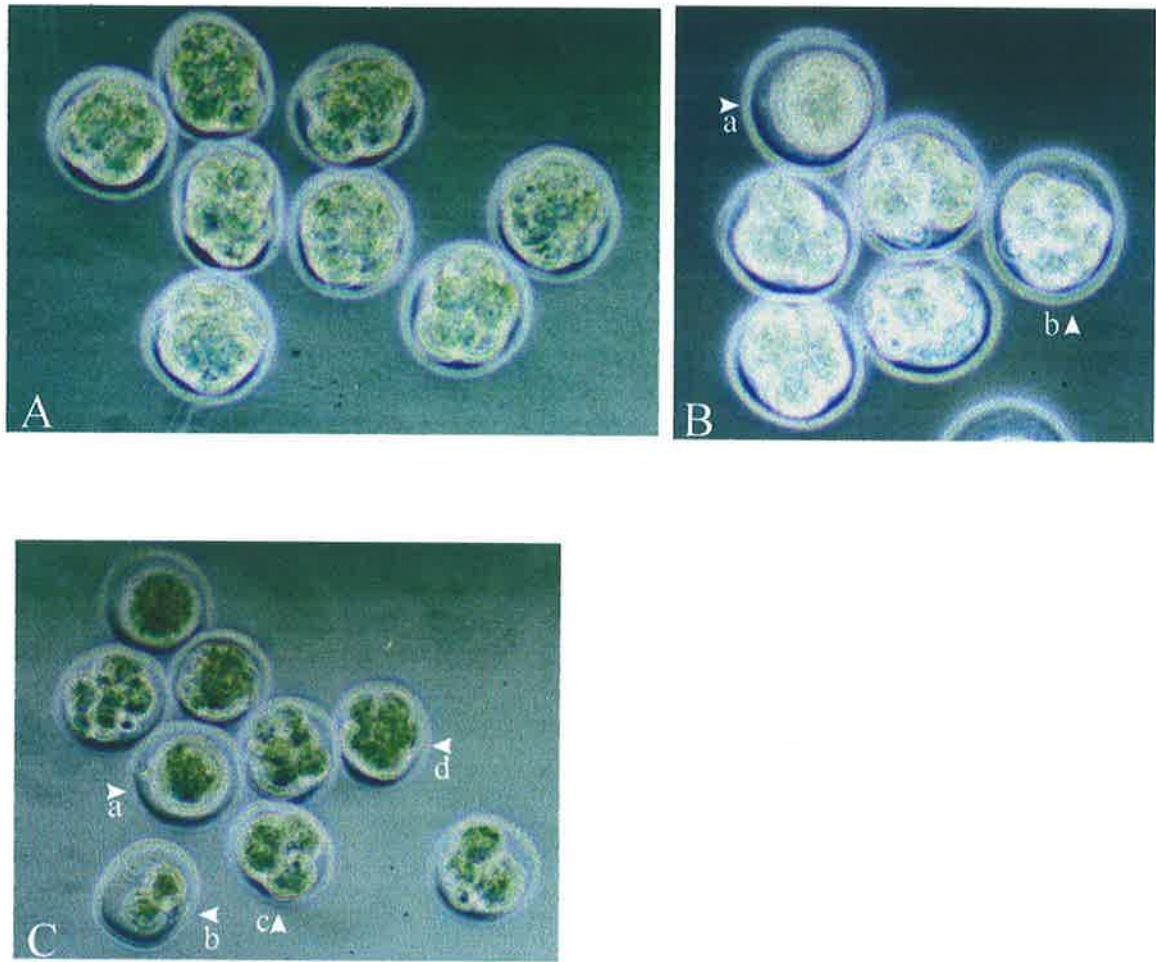
Fig 5.3 Embryos collected 64h after mating

A. Embryos (morulae) from a female mouse after mating with a control male; B. Embryos from a female mouse after mating with a male heat-stressed 7 days previously, a▶ zygote , b▶ morulae; C. Embryos from one female mouse after mating with a male heat-stressed 7 days previously, a▶ zygote, b▶ 2-cell embryo, c▶ 3-cell embryo, d▶ morulae. X150

Fig 5.4 Embryos collected 89h after mating

A. Embryos from a female mouse after mating with a control male, a➤ expanded
blastocysts, b➤ hatched blastocysts; B. Embryos from a female mouse after
mating with a male heat-stressed 7 days previously, a➤ morulae, b➤ blastocyst,
c➤ expanded blastocysts; C. Embryos from one female mouse after mating with a
male heat-stressed 7 days previously, a➤ morulae, b➤ blastocysts, c➤ expanded
blastocysts; D. Embryos from one female after mating with a male heat-stressed 7
days previously, a➤ morulae, b➤ blastocysts, c➤ expanded blastocysts.
X130

5.3.2 The effect of paternal heat stress 21 days earlier on the development in vivo of preimplantation mouse embryos

Paternal heat stress 21 days prior to mating significantly (P<0.01-0.001) reduced the proportion of zygotes developing to 2-cell embryos, 8-cell to morulae and prevented any embryos becoming blastocysts up to 90h after mating. The number of zygotes and 2-cell embryos that did not further develop, and the number of abnormal embryos (Table 5.2) were increased in this time frame, compared with control groups (Table 5.2).

Table 5.2 The effect of paternal heat stress 21 days prior to mating
on in vivo development of embryos

Time of embryos recovered after mating	14-16h		34-39h		61-65h		85-90h	
Groups	C21	H21	C21	H21	C21	H21	C21	H21
No. female mice	4	5	5	6	5	6	6	7
Total No. corpora lutea	43	49	46	52	40	50	46	63
Recovered embryos[1]	43(100.0)	46(93.9)	45(97.8)	50(96.2)	39(97.5)	41(82.0)	44(95.7)	49(77.8)
Zygotes[2]	43(100.0)	44(95.7)	1(2.2)	20(40.0)***	0(0.0)	21(51.2)***	0(0.0)	11(22.4)***
2-C[3]			42(93.3)	27(54.0)***	0(0.0)	3(7.3)	0(0.0)	8(16.3)**
3-C							0(0.0)	1(2.0)
4-C					10(25.6)	0(0.0)***		
8-C to M					29(74.4)	1(2.4)***		
M							12(27.3)	1(2.0)***
B							16(36.4)	0(0.0)
EB							15(31.4)	0(0.0)
HB							1(2.2)	0(0.0)
Total No. B							32(72.4)	0(0.0)***
Abnormal	0(0.0)	2(4.3)	1(2.2)	3(6.0)	0(0.0)	16(39.0)***	0(0.0)	28(57.1)***
Unfertilized			1(2.2)	0(0.0)				

1, 2, 3, are as defined in Table 5.1.

Values with ** and *** superscripts differ significantly (P<0.01 - 0.001) between the heat-sired group (H21) and controls (C21) for a given group.

As can be seen in Fig 5.5 (B and C), paternal heat stress 21 days earlier reduced the number of embryos developing to the 2-cell stage, compared with that in the control group (Fig 5.5,

A). This decrease may be related to individual males after heating (Fig 5.5, B and C). In Fig 5.5 (B), 2 zygotes developed to the 2-cell stage and 7 did not develop. Conversely, in Fig 5.5 (C), 7 zygotes developed to the 2-cell stage and 2 did not. This suggests that the extent of testes insulted by heat stress could be related to individuals although differences in time of fertilization could be responsible. However, as embryos developed further, paternal heat stress 21 days prior to mating increased significantly the proportion of abnormal embryos and the number of zygotes and 2-cell embryos that did not develop beyond the 2-cell stage, indicating that time of fertilization is not a confounding factor. In fact, no embryos developed to the blastocyst stage at 64h (Fig 5.6, B, C, D, E)or at 88h after mating (Fig 5.7, B, C and D). it was of interest to note the morphology of abnormal embryos recovered at 64h and 88h after mating. Most abnormal embryos showed extruded and loosed blastomeres, cells of variable size, cellular fragments and large vesicles. In Fig 5.6 (B), three abnormal embryos appeared pear-shaped, indicating that paternal heat stress may inhibit cell differentiation and/or influence the nature of cell contact. In contrast, control-sired embryos had developed to morulae (Fig 5.6, A) and blastocysts (Fig 5.7, A) with spherical and symmetrical with blastomeres of uniform size, colour and texture at 64h and 88h after mating respectively.
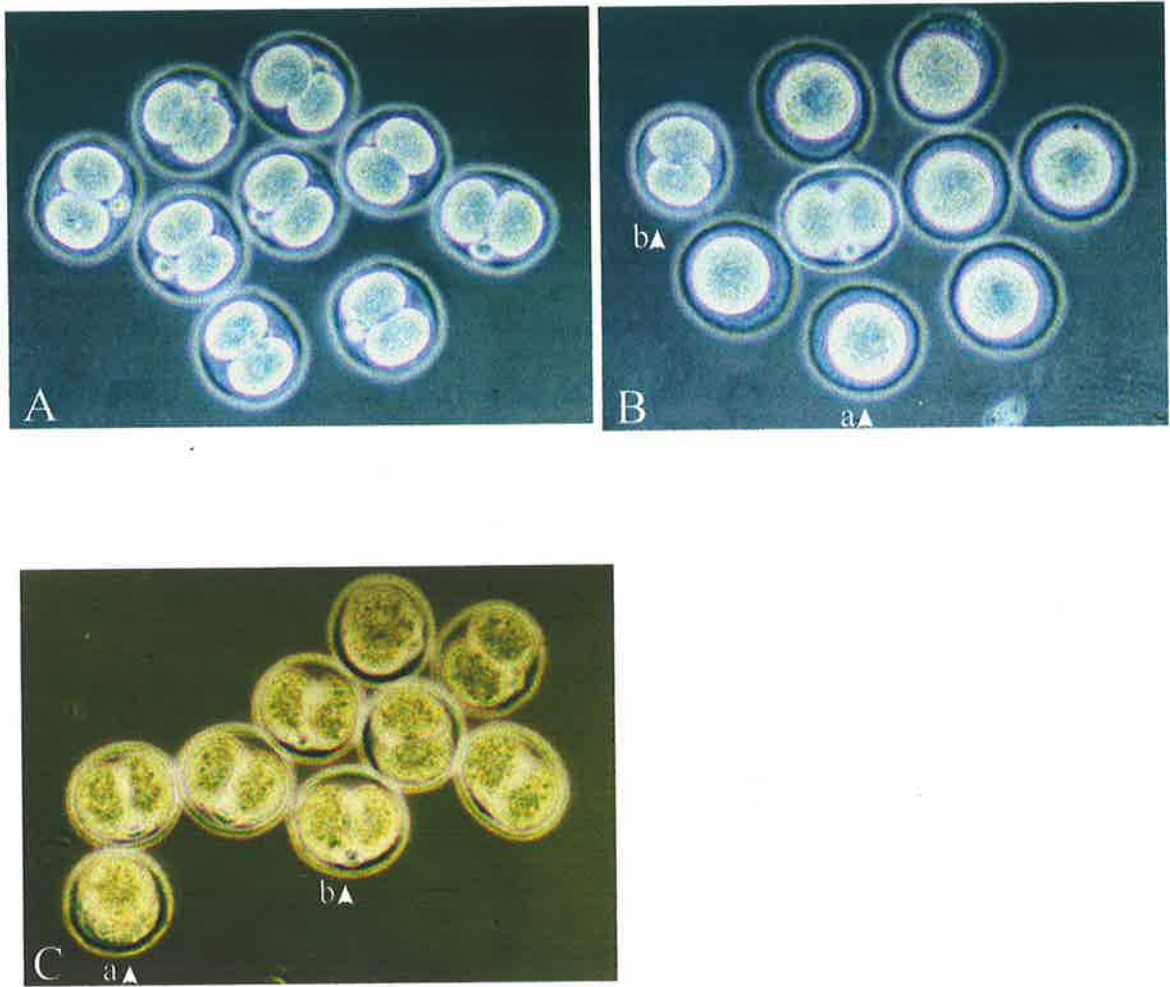
Fig 5.5 Embryos collected 35h after mating

A. Embryos (2-cell) from a female mouse after mating with a control male; B.
Embryos from a female mouse after mating with a male heat-stressed 21 days
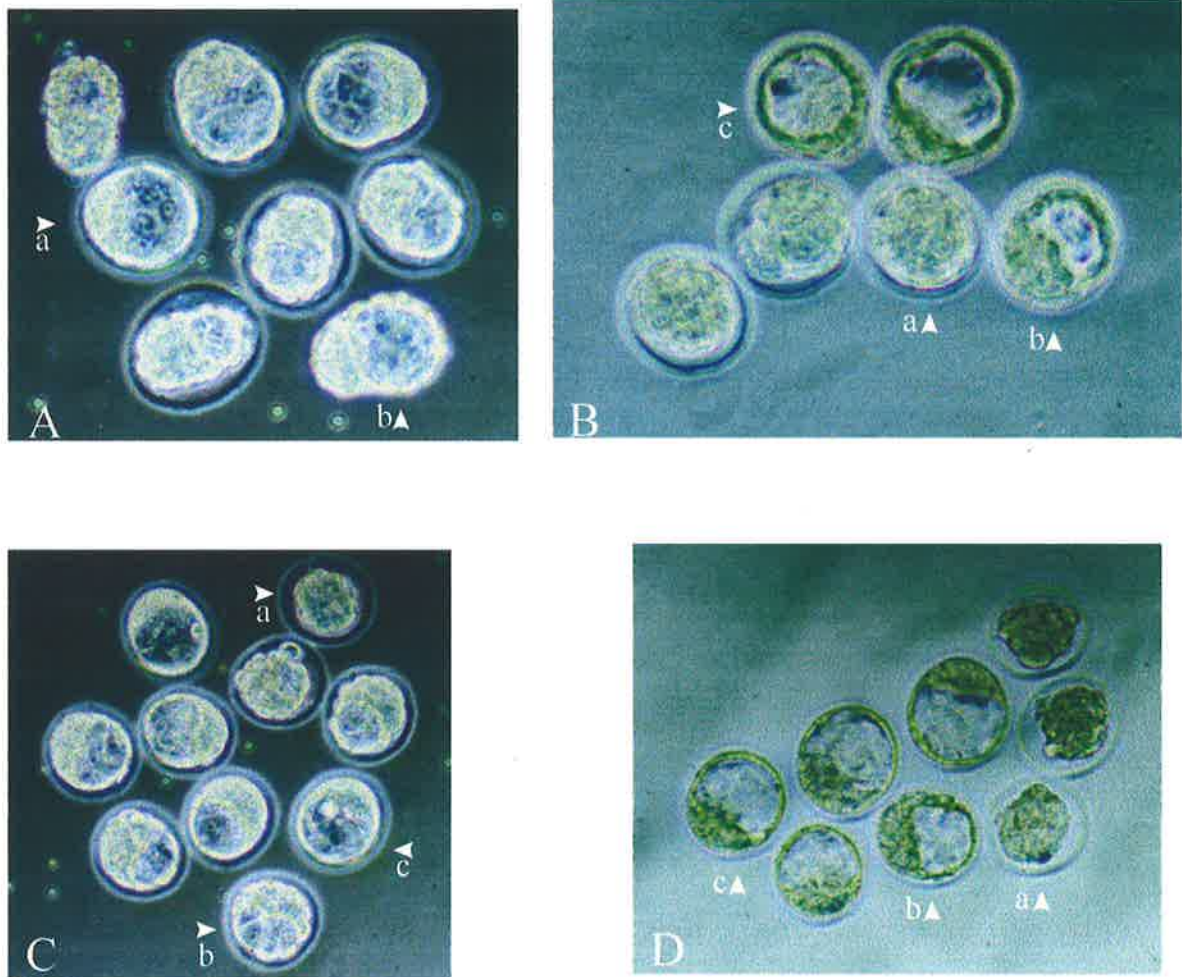previously, a► zygotes,   b► 2-cell embryos; C. Embryos from a female
mouse after mating with a male heat-stressed 21 days previously,   a► zygotes,
b► 2-cell embryos. X120
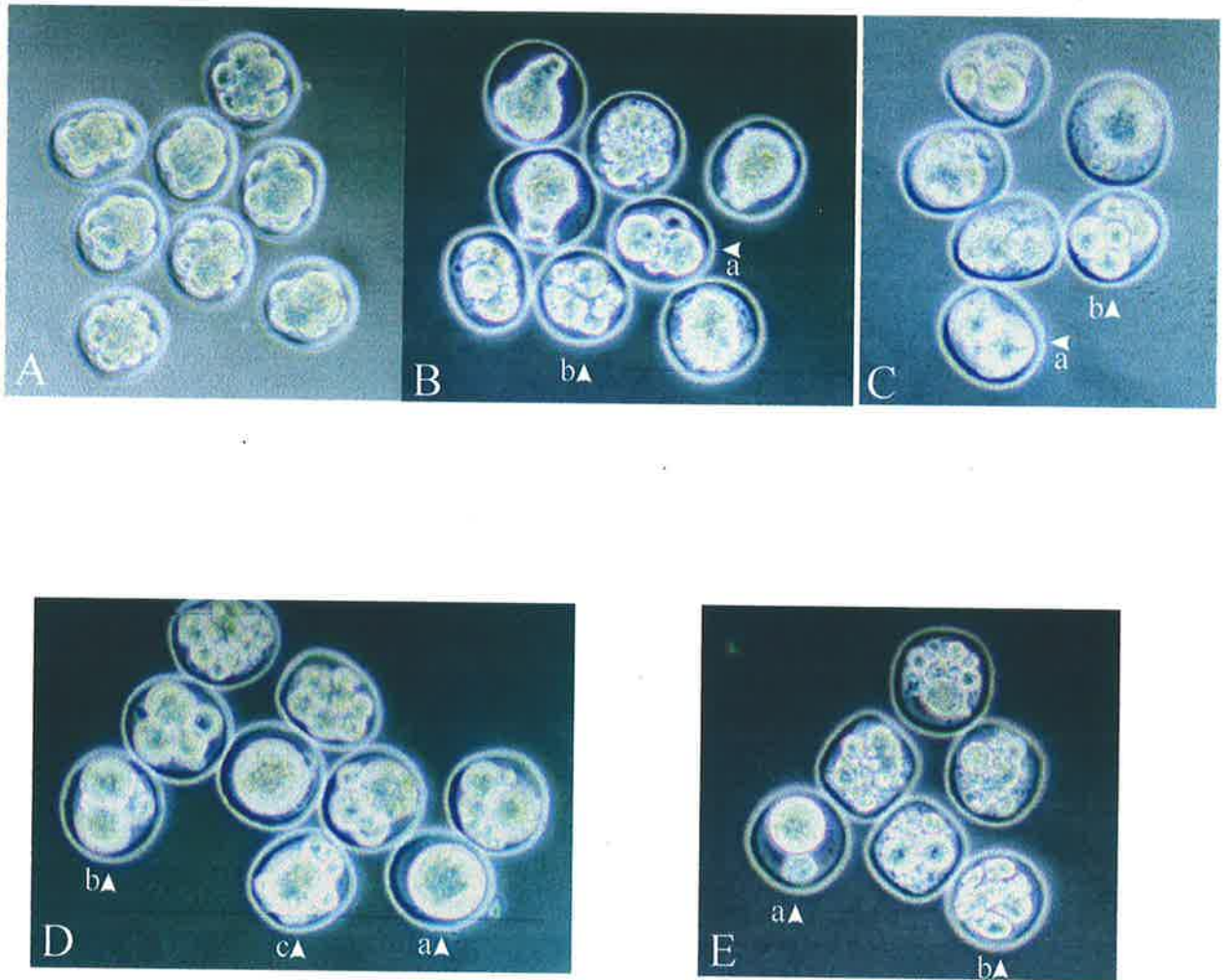
Fig 5.6 Embryos collected 64h after mating

A. Embryos (morulae) from a female mouse after mating with a control male mouse; B. Embryos from a female mouse after mating with a male heat-stressed 21 days previously, a▸ 2-cell embryo, b▸ abnormal embryos; C. Embryos from a female mouse after mating with a male heat-stressed 21 days previously, a▸ 2-cell embryo, b▸ abnormal embryos; D. Embryos from one female mouse after mating with a male heat-stressed 21 days previously, a▸ zygotes, b▸ 2-cell embryo, c▸ abnormal embryos; E. Embryos from a female mouse after mating with a male heat-stressed 21 days previously, a▸ zygote, b▸ abnormal embryos. X100

147

Fig 5.7 Embryos collected 88h after mating

A. Embryos from a female mouse after mating with a control male, a►
expanding/expanded blastocysts, b► hatched blastocyst; B. Embryos from a
female mouse after mating with a male heat-stressed 21 days previously,
a► zygote, b► abnormal embryos; C. Embryos from one female mouse
after mating with a male heat-stressed 21 days previously, a► zygotes,
b► 2-cell embryo, c► abnormal embryos; D. Embryos from one female
mouse after mating with a male heat-stressed 21 days previously, a► zygote,
b► 2-cell embryos, c► 3-cell embryo, d► abnormal embryos. X160

148

5.3.3 The effect of paternal heat stress 35 days earlier on the development in vivo of preimplantation mouse embryos

Paternal heat stress 35 days prior to mating significantly (P<0.01-0.001) increased the number of zygotes that did not develop further and reduced the proportion of embryos developing to the 2-cell stage at 34 to 39h after mating (Table 5.3), compared with the control group. Furthermore, Paternal heat stress 35 days before mating also reduced significantly (P<0.01) the proportion of embryos developing to the expanded blastocyst stage (Table 5.3).

Table 5.3 The effect of paternal heat stress 35 days prior to mating
on in vivo development of embryos

| Time of embryos recovered after mating	14-16h		34-39h		61-65h		85-90h	
Groups	C35	H35	C35	H35	C35	H35	C35	H35
No. female mice	4	4	4	4	4	4	6	5
Total No. corpora lutea	44	43	33	44	30	29	57	43
Recovered embryos[1]	44(100.0)	41(95.3)	33(100.0)	42(95.5)	25(83.3)	25(86.2)	57(100.0)	42(97.0)
Zygotes[2]	38(86.4)	41(100.0)	0(0.0)	12(28.6)***				
2-C[3]			32(97.0)	28(66.7)***				
3-C			0(0.0)	1(2.4)				
4-C			0(0.0)	1(2.4)	2(8.0)	1(4.0)		
5-C					1(4.0)	0(0.0)		
8-C to M					20(80.0)	24(96.0)		
M							6(10.5)	3(7.1)
B							20(35.1)	28(66.7)**
EB							30(52.6)	11(26.2)**
HB							1(2.0)	0(0.0)
Total No. B							51(89.5)	39(92.9)
Abnormal	6(13.6)	0(0.0)	1(3.0)	0(0.0)	1(4.0)	0(0.0)		
Unfertilized					1(4.0)	0(0.0)		

1, 2, 3, are as defined in Table 5.1.

Values with ** and *** superscripts differ significantly (P<0.01 - 0.001) between the heat-sired group (H35) and controls (C35) for a given group.

Morphological changes in control-sired and heat-sired embryos at 35h and 87h after mating are presented in Fig 5.8 and 5.9. Paternal heat stress 35 days earlier increased the number of

zygotes at 35h after mating (Fig 5.8, B). In contrast, all control-sired embryos had developed to the 2-cell stage (Fig 5.8, A) at that time. However, by 88h after mating, there were no obviously morphological differences seen between control- (Fig 5.9, A) and heat-sired (Fig 5.9, B) blastocysts.

5.3.4 The effect of paternal heat stress 7 and 35 days earlier on the number of TE and ICM cells in the blastocyst

There were significantly (P<0.001) fewer total cells in blastocysts sired by heat-stressed males on day 7 after heating. Reductions occurred equally in both the TE and the ICM, with no change in the ICM/TE ratio (Table 5.4). However, these differences were no longer apparent in embryo sired 35 days after heating (Table 5.4). No blastocysts were available in the H21 group for analysis due to developmental retardation (refer Table 5.2).

Table 5.4 The effect of paternal heat stress on the number of
TE and ICM cells in blastocysts

Sire	No. blastocysts	ICM	TE	Total No. TE + ICM	Ratio (ICM/TE)
Control(C7)	20	18.2±2.92	25.2±6.17	43.8±7.08	0.75±0.19
Heated (H7)	20	14.8±5.30*	19.0±6.27**	33.7±10.58***	0.82±0.33
Control(C35)	21	17.1±4.81	26.3±8.35	43.5±12.65	0.66±0.12
Heated (H35)	17	16.4±4.37	22.9±5.37	39.4±8.68	0.73±0.17

Values are mean ± standard error (M±SEs).

Values with *, ** and *** superscripts differ significantly (P< 0.02, 0.005 and 0.001) between the heat-sired group (H7) and controls (C7) for the number of ICM and TE cells.
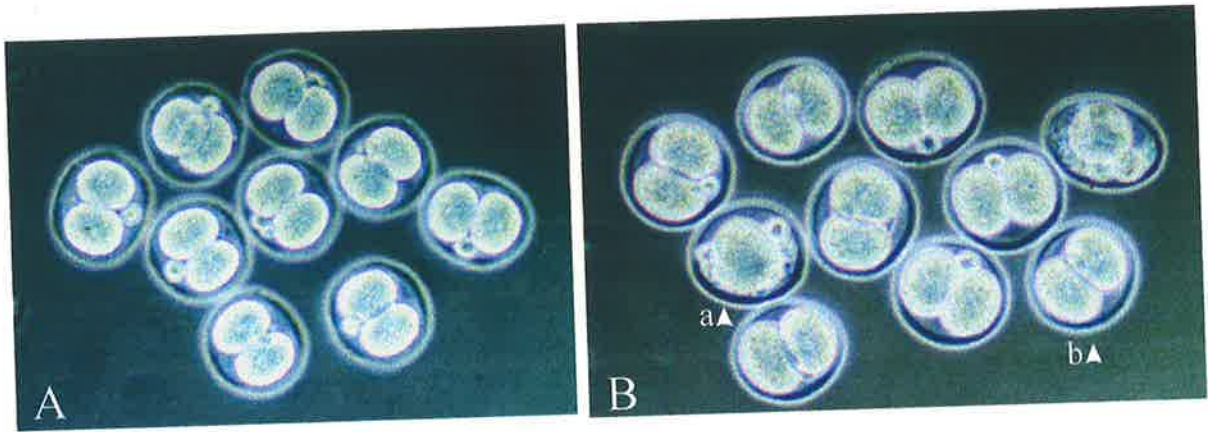
Fig 5.8 Embryos collected 35h after mating

A. Embryos (2-cell) from a female mouse after mating with a control male;
B. Embryos from a female mouse after mating with a male heat-stressed 35
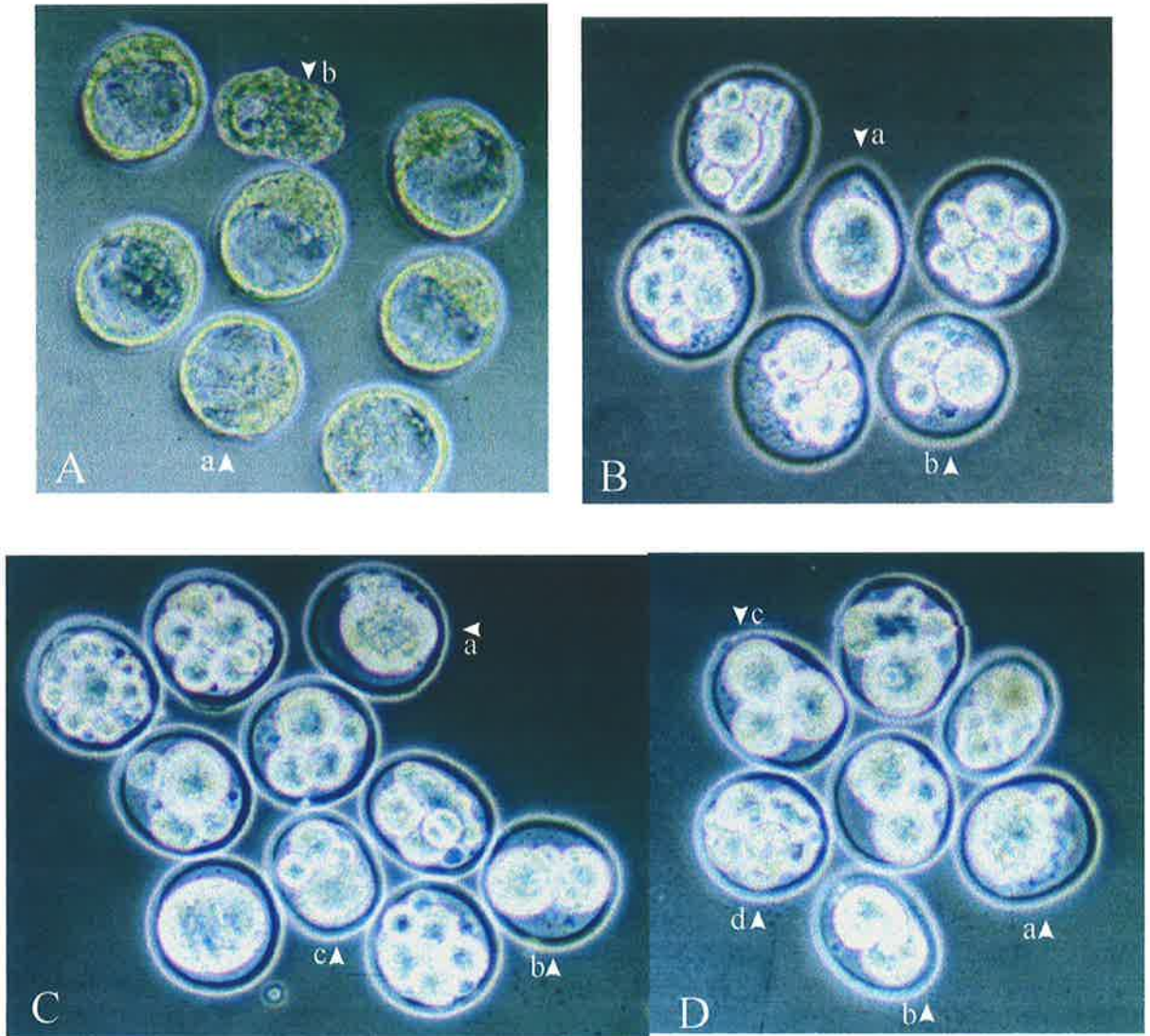days previuosly, a➤ zygotes, b➤ 2-cell embryos. X120
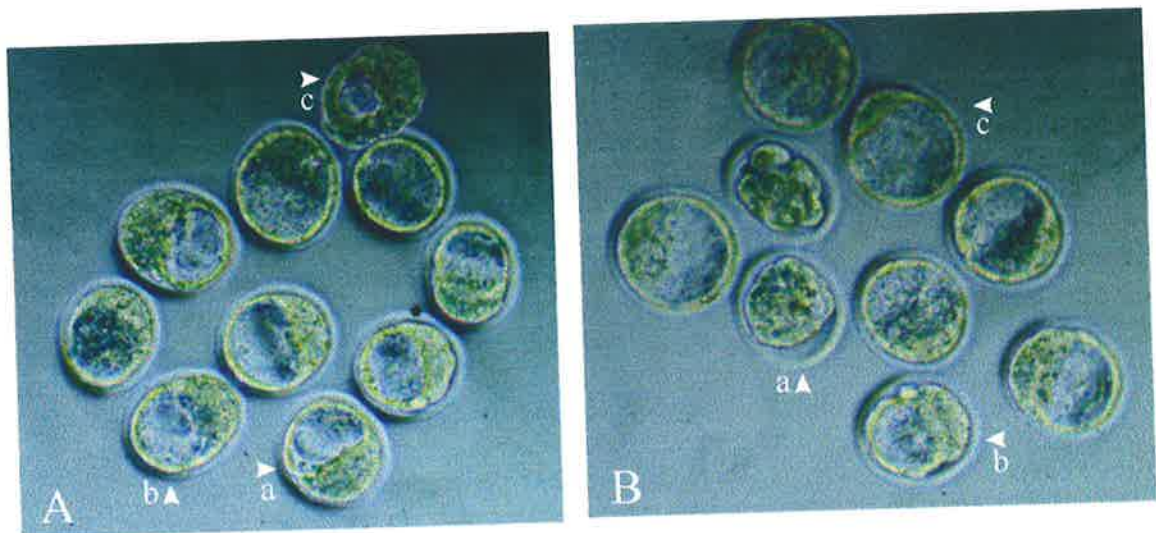
Fig 5.9 Embryos collected 88h after mating

A. Embryos from a female mouse after mating with a control male, a➤ blastocysts,
b➤ expanded blastocysts, c➤ hatched blastocyst (1); B. Embryos from a female
mouse after mating with a male heat-stressed 35 days previously, a➤ morulae,
b➤ blastocysts, c➤ expanded blastocysts. X120

151

5.4 Discussion

The results of this study in the mouse confirm previous reports for the rabbit (Howarth et al., 1965; Burfening and Ulberg, 1968) and mouse (Bellve, 1972, 1973; Chapter 4) which have shown that when sperm are subjected to an elevated temperature in the uterus or in vitro or when males are exposed to high ambient temperatures, there are reductions in the in vivo and in vitro developmental rates of preimplantation embryos. The present finding that the number of 8-cell embryos plus morulae found 61-65h after mating is significantly lower in experimental groups at 7 and 21 days after heat stress, with a higher proportion 1 or 2-cell embryos differs from the findings of previous studies which suggested that paternal heat stress significantly increased the number of 4-cell embryos 54h after an observed vaginal plug (Bellve,1972). This discrepancy could be attributed to the differences of mouse strains used, heat-stress conditions and the time of collecting embryos. However, the results are also completely consistent with the results of the in vitro studies presented in Chapter 4. In addition, the decrease in the number of blastocysts sired 7 days after heating of the male is in accord with earlier results (Bellve, 1973). Therefore, a common feature in this experiment and previous studies (Bellve, 1972, 1973; Setchell et al., 1988) is that the heat-stressed male can reduce the developmental rate of preimplantation embryos and increase the number of retarded embryos.

Sperm ejaculated on day 7 after heating would have been in the epididymis at the time of heating (Oakberg, 1957; Dadoune and Alfonsi, 1984). An earlier study showed that scrotal insulation for 48h in the bull did not damage sperm of the epididymis (Vogler et al., 1991). However, recent studies with scrotal insulation for 48h in the bull (Karabinus et al., 1997) or exposure of mouse testes to 40 or 42°C for 60 min. (Sailer et al., 1997) have indicated

disturbed maturation of the sperm at the epididymis, with heat stress reported to damage the chromatin structure of sperm in the epididymis of the mouse and the bull. The results of the present study have shown that when embryos arise from epididymal sperm of heat-stressed males, the developmental rates during preimplantation are reduced and this is in agreement with an increased mortality of embryos produced with immature, epididymal sperm collected from rams during scrotal insulation (Mieusset et al., 1992).

The mammalian embryo first differentiates into two distinct cell types at the blastocyst stage. One is ICM cells responsible for embryo proper and the other is TE cells which contribute to blastocoel fluid accumulation, implantation and formation of the placenta (Handyside and Johnson, 1978; Cross et al., 1994). Recently, homozygous integrin β-subunit null mouse embryos, produced by gene targeting in embryonic stem cells, have been reported to develop normally to the blastocyst stage and initiate implantation, but die thereafter (Fassler and Meyer, 1995). Analysis of the β-null blastocyst shows a delayed growth of the ICM (Stephen et al., 1995). This suggests that appropriate differentiation of ICM and TE cells in the blastocyst may play an important role in subsequent healthy embryonic development. In the present study, paternal heat stress 7 days prior to mating reduced the number of TE and ICM cells in the blastocyst although the ratio of ICM/TE was not different. This suggests that paternal heat stress may reduce the rate of cell mitosis and affect the cell cycle stages in the blastocyst stage. A reduction of TE and ICM in the blastocyst stage could further decrease its autocrine capacity which could be extremely important in any communication between the conceptus and the uterus. This could affect directly the subsequent implantation and establishment of pregnancy because paternal heat stress has been reported to reduce implantation rates (Bellve, 1972, 1973) and fetal weight, litter size, and weights of trophoblast and yolk sac in the mouse (Setchell et al., 1998 and

153

Jannes et al., 1998), and pregnancy rates in sheep (Mieusset et al., 1992), rat (Setchell et al., 1988), pig (Wettemann and Bazer, 1985) and humans (Thonneau et al., 1997).

Twenty-one days after heating, there were significant reductions in the development rate of 2-cell embryos and an increase in the number of abnormal embryos. Additionally, no embryos developed into blastocysts when sired 21 days after heating. These results strongly support the results of in vitro studies in the mouse presented in Chapter 4. Furthermore, an increase in the number of abnormal embryos in this study is similar to results published previously for the rat at 15 days after heating (Setchell et al., 1988), and with the reduced implantation rate reported in the mouse at 21 days after heating (Burfening et al., 1970). A model proposed by Haig and Westoby (1989) predicts that paternally imprinted genes will promote growth and maternal genes will inhibit growth. A paternally imprinted gene encoding U2afbp-rs, an RNA processing protein is transiently transcribed at the 2-cell stage in the mouse (Latham et al., 1995). It is possible that heat stress could impair and/or disturb one or more paternally imprinted genes during spermatogenesis, thus inhibiting subsequent embryo development. Furthermore, a higher proportion of 2-cell embryos 85-90h after mating may arise because endogenous markers of zygotic gene activation including a transcription requiring complex (TRC) (Poueymiron and Schultz, 1989; Latham et al., 1991 and Schultz, 1993), HSP70.1 (Bensuade et al., 1983; Thompson et al., 1995 and Christians et al., 1997) and a translation initiation factor (eIF-4C) (Davis et al., 1996) might either not be transcribed transiently and/or be abnormally transcribed during the 2-cell stage. These suggestions merit further investigation.

Sperm ejaculated 21 days after heating would have been pachytene and diplotene spermatocytes at the time of heating (Oakberg, 1957; Dadoune and Alfonsi, 1984) and

154

these cells have previously been reported to be seriously damaged by heat exposure (De Vita et al., 1987, 1990; Sailer et al., 1997). Apparently this damage can extend to an effect on the development of preimplantation embryos because the present study has clearly indicated that paternal heat stress significantly reduced the number of 2-cell embryos and morulae, and increased the proportion of abnormal embryos and zygotes.

Paternal heat stress 21 and 35 days prior to mating also increased the number of embryos remaining as the zygotes at 34-39h, 61-65h and 85-90h after mating, compared with control groups. This may be because paternal heat stress could damage nuclear remodelling and pronuclear formation (Worrad and Schultz, 1997; Latham, 1999), inhibit the first round of DNA replication (Forlani et al., 1998) and/or impair the regulatory balance between cell death genes and cell survival genes (Jurisicova et al., 1998a). Furthermore, paternal heat stress 35 days earlier reduced the number of 2-cell embryos at 34-39h after mating, which is consistent with our own results of in vitro studies (Chapter 4) and the number of expanded blastocysts 85 to 90h after mating. However, the number of the total blastocysts in the present study are higher than those in the in vitro study (Chapter 4). This difference may be attributed to different environments of embryo development and it would not be unreasonable to suggest that development may be better supported in vivo than in vitro. These results also suggest that spermatogonia in the testis can be damaged by heat insult because sperm ejaculated 35 days after heating would have been spermatogonia at the time of heating (Oakberg, 1957; Dadoune and Alfonsi, 1984).

In summary, the present results have further demonstrated that paternal heat stress not only reduces the development in vivo of preimplantation embryos, significantly reducing the number of 2-cell embryos, 8-cell to morula and blastocysts, but also decreases the number of both ICM and TE cells in the blastocyst. These results also suggest that heat stress has

damaged germ cells in both the epididymis and the testis, and significantly damaged spermatocytes, and possibly also spermatogonia.

Chapter Six

Protein Profiles of Control-sired and Heat-sired Preimplantation Embryos

6.1 Introduction

The development of preimplantation embryos involves specific alterations in gene transcription, mRNA content and protein synthesis. Both one- and two-dimensional gel electrophoretic analyses have revealed concomitant stage-specific changes and extensive remodelling in protein synthesis as well as alterations in post-translational modification in preimplantation embryos in the mouse (Epstein and Smith, 1974; Van Blerkom and Brockway, 1975; Levinson et al., 1978; Howe and Solter, 1979; Cullen et al., 1980a,b; Johnson and Calarco, 1980; Howlett and Bolton, 1985; Howlett, 1986; Conover et al., 1991; Latham et al., 1991; Latham et al., 1992; Latham et al., 1994; Shi et al., 1994; Sasaki et al., 1999) and in the rabbit (Manes and Daniel, 1969; Van Blerkom and Manes, 1974; Van Blerkom and McGaushey, 1978; Van Blerkom, 1979). Early development in embryos of most species, if not all, has been shown to be controlled, largely or exclusively by the maternal genome (Denny and Tyler, 1964; Brachet et al., 1968; Woodland et al., 1979; Braude et al., 1979; Rosenthal et al., 1980; Woodland and Ballantine, 1980; Wells et al., 1981; Van Blerkom, 1981; Pratt et al., 1983; Bolton et al., 1984; Howlett and Bolton, 1985; Howlett, 1986), but the duration of this period of exclusive maternal control varies in the different species (Davidson, 1976; De Sousa et al., 1998). In the mouse embryo, although the period of maternal control appears to extend from ovulation through cleavage to the early 2-cell stage (Johnson, 1981; Pratt et al., 1983), several groups of proteins such as 30kD, 35kD and 46kD appear to change significantly between the unfertilized ovum and the 1-cell embryo (Van Blerkom and Brockway, 1975; Braude et al., 1979; Cullen et al., 1980a,b; Bolton et al., 1984; Howlett and Bolton, 1985). This evidence represents a fertilization-accelerated and/or fertilization-dependent polypeptide synthetic change (Pratt et al., 1983, Howlett, 1986). In addition, transcriptional activity from the embryonic

genome is first detected at the early to mid 2-cell stage (Young et al., 1978; Levey et al., 1978; Flach et al., 1982; Bensaude et al., 1983). These reports suggest paternally important roles in regulating and controlling the development of preimplantation embryos involving gene transcription, mRNA content and protein synthesis.

Both in vivo and in vitro studies (Chapter 4 and 5) have clearly shown that paternal heat stress significantly reduced the proportion of developing preimplantation mouse embryos. This effect is detected between 7 and 35 days after heating of the male, with the greatest effect occurring 21 days after heating of the male. In an attempt to explore the influence of paternal heat stress on embryo development via a change in protein synthetic potential, the present study examined the protein synthetic patterns of control-sired and heat-sired embryos at 7, 14 and 21 days after heating of the male.

6.2 General Materials and Methods

6.2.1 Heat stress of the male mouse

Materials and methods used are largely described in Section 2.6. Briefly, mature and proven fertile C57/CBA F1 male mice (13 to 17 weeks old) were maintained in a psychrometric chamber set at $36 \pm 0.3°C$ and $62 \pm 2.7\%$ relative humidity for 24h, while control male mice were similarly kept at 23°C for 24h. Other conditions were the same in both the control and the experimental groups.

6.2.2 Superovulation and mating of female mice

Normal fertile C57/CBA F1 female mice were superovulated as described in section 2.3. After the injection of hCG, each superovulated mouse was caged overnight with either a control male or one which had been subjected to heat stress either 7, 14 or 21 days earlier. The presence of a vaginal plug the following morning was taken to indicate a successful mating.

6.2.3 Collection and culture in vitro of control-sired and heat-sired putative zygotes in the mouse

Materials and methods employed were adapted from Section 3.1. In brief, 25 to 28h after hCG injection, the females were euthanasized by cervical dislocation and embryos were collected from the oviducts by piercing the swollen portion of the ampulla with a 27-gauge needle. Control-sired and heat-sired putative zygotes were selected, washed three times using Hepes HTF medium and finally cultured in 50 μl of CZB medium under paraffin oil (BDH, Australia) at 37°C in an atmosphere of 5% CO_2/5%O_2/90%N_2 for later use (see Section 2.5).

6.3 Specific Methods and Results

6.3.1 The effect of paternal heat stress on protein patterns of preimplantation embryos in the mouse: determination using one-dimensional gel electrophoresis and silver staining

To investigate the effect of paternal heat stress on protein profiles of preimplantation mouse embryos, 2-cell embryos and morulae were used. Zygotic gene activation in the mouse embryo definitely occurs by the 2-cell stage (Flach et al., 1982; Manejwala et al., 1991; Moore et al., 1996) with concomitant changes in the profile of synthesised proteins (Latham et al., 1991). Furthermore, a transition from the morula to the blastocyst leads to cellular polarity and differentiation, thus probably resulting in a change of protein profiles in preimplantation embryos (Cullen et al., 1980b). Our own previous studies have shown that during the transition from the zygote to the 2-cell stage and from the morula to the blastocyst, paternal heat stress significantly reduced the proportion of the in vivo and in vitro development of 2-cell embryos and blastocysts (Chapter 4 and 5). In addition, the protein patterns of heat-sired abnormal embryos were also determined because paternal heat stress, 7, 14 or 21 days after heating of the male significantly increased the number of abnormal embryos (Chapter 4 and 5), suggesting that paternal heat stress may disturb normal expression of genes, further affecting protein synthesis.

6.3.1.1 Materials and methods

140 control- and 200-300 heat-sired zygotes, sired 7, 14 or 21 days after heating of the male, were collected and cultured. Sixty two-cell embryos and 30 morulae and abnormal

embryos (selection criteria are as described in Section 5.2.4) were collected, 20 and 64h respectively after culture of zygotes. Embryos were washed three times with BSA-, glutamine-, pyruvate- and lactate-free CZB medium, placed in 10 μl sample buffer (Laemmli, 1970), boiled for 2 min and finally stored at -80°C prior to one-dimensional (1-D) gel electrophoresis.

Materials and methods employed in 1-D gel electrophoresis and silver staining for detecting proteins were as described in Sections 2.8 and 2.10.1, respectively.

There were two replicates in this experiment.

6.3.1.2 Results

The results, as shown in Fig. 6.1 and Fig 6.2, did not suggest detectable differences in protein patterns between control-sired (Fig 6.1, C7, C14 or C21) and heat-sired 2-cell embryos (Fig 6.1, H7, H14 or H21), or between control-sired (Fig 6.2, C7 and C21), heat-sired morulae (Fig 6.2, H7 and H21) and heat-sired abnormal embryos (Fig 6.2, H21-A).
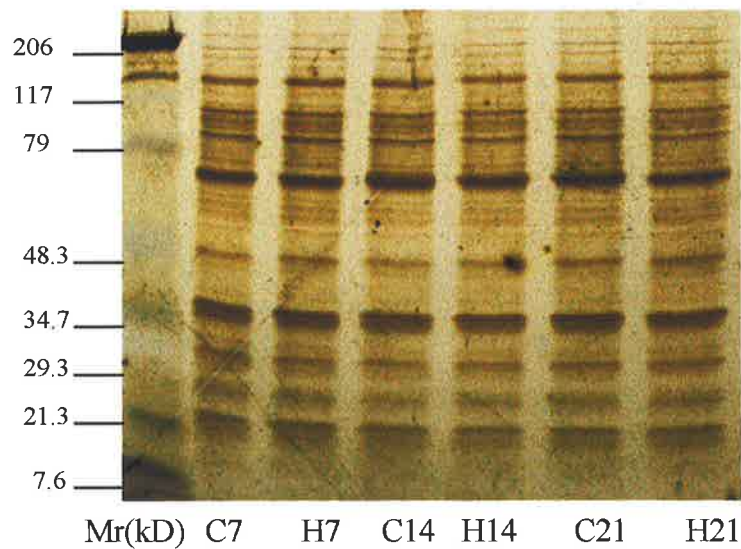
Fig 6.1 Protein profiles of 2-cell embryos

Mr, relative molecular mass markers in the range of 7.6- 206kD; C7, C14 and C21 are protein patterns of 60 control-sired 2-cell embryos while H7, H14 and H21 are protein profiles of 60 heat-sired 2-cell embryos, sired 7, 14 or 21 days after heating of the male.
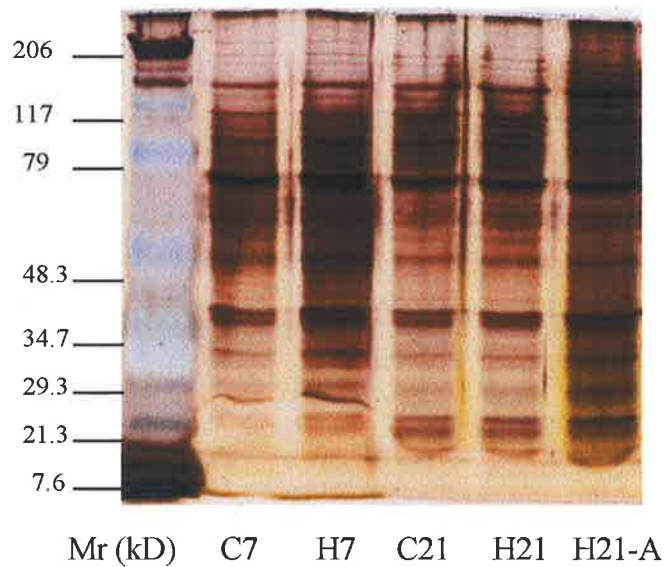
Fig 6.2 Protein patterns of morulae

Mr, relative molecular mass markers as for Fig 6.1; C7 and C21 are protein patterns of 30 control-sired morulae while H7 and H21 are protein patterns of 30 heat-sired morulae, sired 7 or 21 days after heating of the male; H21-A is protein profile of 30 heat-sired abnormal embryos sired 21 days after heating of the male.

162

6.3.2 The effect of paternal heat stress on protein synthetic profiles of preimplantation embryos in the mouse: determination using 1-D gel electrophoresis and autoradiography

Since that there were no obvious differences seen in the protein profiles between control-sired and heat-sired 2-cell embryos and morulae, sired 7, 14 or 21 days after heating of the male when protein patterns of mouse embryos were determined by 1-D gel electrophoresis and silver staining, and given the relative insensitivity of this approach, it seemed important to further examine the effect of paternal heat stress on protein synthetic profiles of preimplantation mouse embryos after the embryos were labelled using [^{35}S]-methionine.

6.3.2.1 Materials and methods

Labelling of embryonic proteins by [^{35}S]-methionine was as described in section 2.8.3. Briefly, 50 two-cell embryos, 45 4- to 8-cell embryos, 30-32 morulae or 10-16 blastocysts of control-sired and heat-sired putative zygotes, sired 7, 14 or 21 days after heating of the male were collected, 18h, 39h, 63h or 87h respectively after culture in vitro in 50μl CZB medium. They were then cultured for 3h in 50 μl CZB medium containing 200 μCi/ml of [^{35}S]-methionine (1000Ci/mmol, Amershan Pharmacia Biotech UK Ltd), as described by Van Blerkom and Brockway (1975). Embryos were then washed three times with BSA-, glutamine-, pyruvate- and lactate- free CZB medium and placed in 10 μl sample buffer (Laemmli, 1970), boiled for 2 min. and stored at -80°C prior to 1-D gel electrophoresis.

Materials and methods used in 1-D gel electrophoresis and Commassie blue staining or autoradiography for detecting proteins were as described in Sections 2.8 and 2.10.2 respectively. There were two replicates in this experiment.

6.3.2.2 Results

Preliminary investigations demonstrated that use of 50 embryos and 14 days exposure of the gel to X-ray film were optimal for determination of protein profiles although protein bands would be detected when between 30-60 2-cell embryos and between 7-14 days exposure were used.

Paternal heat stress 7 and 14 days prior to mating did not have any detectable effects on the protein patterns of 2-cell embryos (Fig 6.3, H7 and H14), 4-cell to 8-cell embryos (Fig 6.4, H7-4-8), morulae (Fig 6.4, H7-M, Fig 6.5, H14-M and Fig 6.6, H21-M) or blastocysts (Fig 6.4, H7-B; Fig 6.5, H14-B), compared with those of the control groups (Fig 6.3, C7 and C14; Fig 6.4, C7-4-8, C7-M or C7-B; Fig 6.5, C14-M and C14-B; Fig 6.6, C21-M). However, paternal heat stress 21 days earlier significantly reduced the synthesis of some groups of proteins in 2-cell embryos including molecular weights 7.6, 40.3, 43.4, 45.2, 53.3, 57.2, 71, 78, 83.8, 88.5, 117.0kD. In particular, there were very significantly decreases in the synthesis of proteins with molecular weights of 34.7, 47.1, 51.3 67, 68, 70, 75.6, 78.1 or 162kD indicated by arrows (Fig 6.3, H21). Furthermore, paternal heat stress 21 days before mating also significantly reduced the synthesis of several groups of proteins with molecular weights of 44.2, 52.5, 76, 87.4kD, indicated by arrows (Fig 6.6, H21-A) in the abnormal embryos, but did not apparently change protein patterns of heat-sired morulae (Fig 6.6, H21-M), compared with those of control groups (Fig 6.3, C21; Fig 6.6, C21-M).
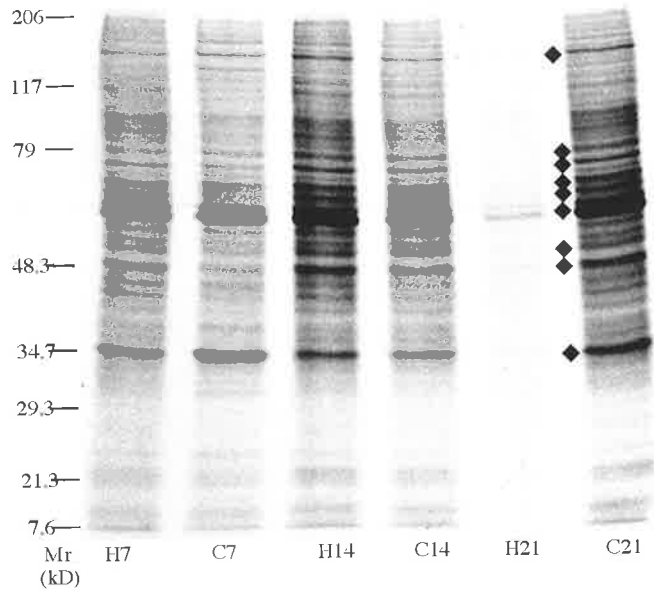
Fig 6.3 Protein patterns of the 2-cell embryos

Mr, relative molecular mass markers as for Fig 6.1; C7, C14 and C21 are protein patterns of 50 control-sired 2-cell embryos while H7, H14 and H21 are protein patterns of 50 heat-sired 2-cell embryos, sired 7, 14 or 21 days after heating of the male.

◆ Indicates significant difference of protein bands between C21 and H21.
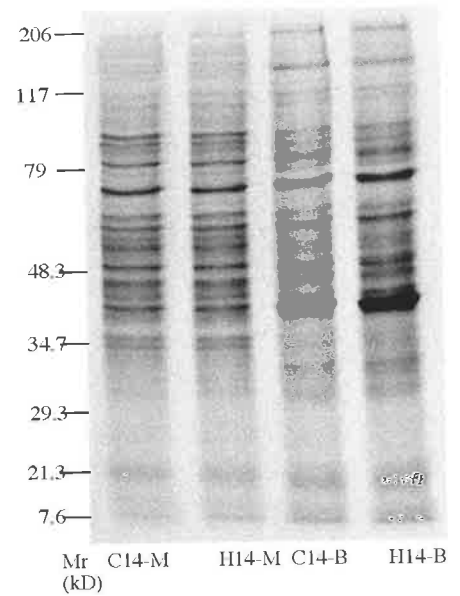
Fig 6.5 Protein patterns of morulae and blastocysts

Mr, Relative molecular mass markers as for Fig 6.1; C14-M and C14-B are protein patterns of respectively 30 and 16 control-sired morulae and blastocysts while H14-M and H14-B represent protein patterns of respectively 30 and 16 heat-sired morulae and blastocysts sired 14 days after heating of the male.
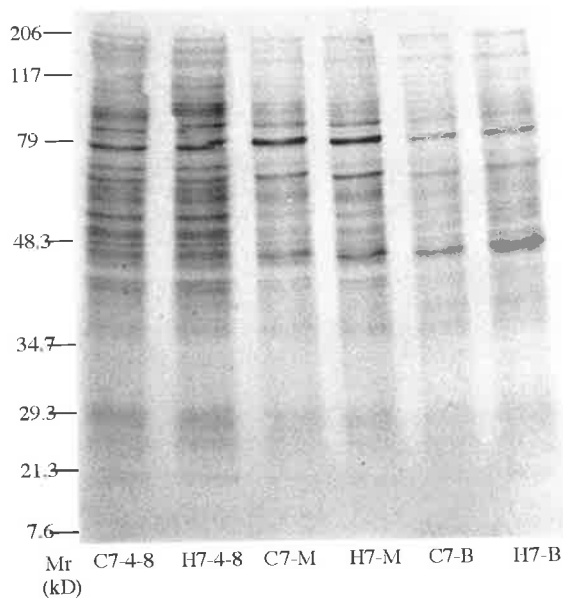
Fig 6.4 Protein patterns of preimplantation embryos

Mr, relative molecular mass markers as for Fig 6.1; C7-4-8, C7-M and C7-B are respectively protein patterns of control-sired 45 4-cell to 8-cell embryos, 32 morulae and 10 blastocysts while H7-4-8, H7-M and H7-B represent protein patterns of 48 4-cell to 8-cell heat-sired embryos, 30 morulae and 10 blastocysts, sired 7 days after heating of the male.
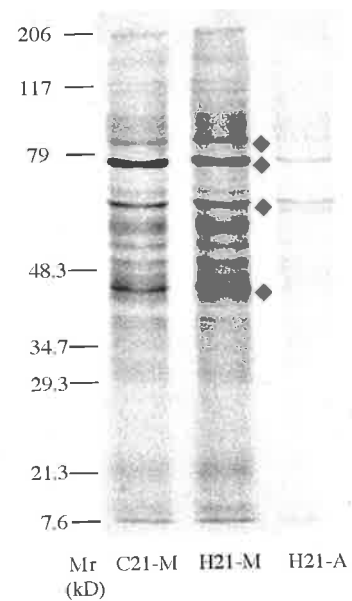
Fig 6.6 Protein patterns of morulae

Mr, relative molecular mass markers as for Fig 6.1; C21-M is protein profile of 30 control-sired morulae while H21-M and H21-A are protein profiles of respectively 30 heat-sired morulae and 32 heat-sired abnormal embryos (No. of cells > 4) sired 21 days after heating of the male.

◆ Indicates significant difference of protein bands between C21-M and H21-A.

165

6.3.3 The effect of paternal heat stress 21 days earlier on protein synthetic profiles of preimplantation embryos in the mouse: determination using 2-D gel electrophoresis and phosphoimaging

The results obtained by 1-D gel electrophoresis and autoradiography have shown that paternal heat stress 7 or 14 days earlier did not significantly change the protein synthetic patterns of preimplantation mouse embryos. However, paternal heat stress 21 days prior to mating did significantly affect the protein profiles of both 2-cell embryos and abnormal embryos, compared with those of the control groups. In order to extend these results and increase experimental sensitivity, two-dimensional (2-D) gel electrophoresis following labelling of embryonic proteins by [^{35}S]-methionine was undertaken using control- and heat-sired 2-cell embryos and morulae, sired 21 days after heating of the male.

6.3.3.1 Materials and methods

Labelling of embryonic proteins by [^{35}S]-methionine was as detailed in Section 2.9.5. In brief, 70 two-cell embryos and 40 morulae of control-sired and heat-sired putative zygotes, sired 21 days after heating of the male were collected, 18h and 63h respectively after culture in 50μl CZB medium. They were cultured for 3h in 50μl CZB medium containing 500 μCi/ml of [^{35}S]-methionine (1000 Ci/mmol, Amershan Pharmacia Biotech UK Ltd), as described by Howe and Solter (1979). Embryos were then washed three times with BSA-, glutamine-, pyruvate- and lactate- free CZB medium, collected into a 1.5 ml microcentrifuge tube (CH-9113, Treff AG, Switzerland) and lyophilised. Twenty μl of lysis buffer was then added and the tubes stored at -80°C prior to 2-D gel electrophoresis.

To determine the number of counts incorporated as a means of assessing the likely sensitivity of detection required to examine embryonic protein synthesis, randomly sampled 2-cell embryos labelled using [^{35}S]-methionine incorporation in a 3h period of culture were collected and washed three times in BSA-, glutamine-. pyruvate- and lactate-free CZB medium. Following the addition of 3ml biodegradable counting scintillant (BCS) solution (NBCS104, Amersham International Plc., USA), radioactivity incorporation was measured using a Liquid Scintillation Counter (Beckman, LS3801, USA). On average, 6000-8500 CPM per embryo were recorded for control-, heat-sired 2-cell embryos, and heat-sired abnormal embryos.

Materials and methods employed in 2-D gel electrophoresis were as described in Sections 2.9.

Determination of the pH value in IEF gels was as described in Section 2.9.5. Briefly, the pH gradient in the tube gels was detected with the use of spare gels poured and electrophoresed at the same time as the sample gels, but run without sample. Each gel was cut into 8-9 even lengths with each piece being placed in 1 ml 0.025 M KCl for at least 1h before the pH was read.

Phosphoimaging for detecting proteins was as described in Section 2.10.2. Briefly, the process of phosphoimaging involved the exposure of gels to a phosphoimaging screen for 2 weeks at room temperature. This screen was scanned by the phosphoimager under conditions of Orientation=R and 100 microns for 9min/gel. The imageQuant® file was opened in a Macintosh computer for determining the desired intensities of protein spots by regulating the range of greyscale under 1000 times sensitivities. The images of the proteins

167

recorded were copied as a TIFF file using Photoshop program and then copied to the Powerpoint program. To facilitate the comparison of protein profiles between control- and heat-sired embryos, protein spots determined by the Macintosh computer using molecular weight and isoelectric point as coordinates, were identified and certain groups of proteins collectively identified as "constellations". Each constellation was marked by drawing a box or a circle around it, but neither the box nor the circle has any specific meaning. The position of each constellation and positions of protein spots within each constellation must be emphasised to be consistent both in gels from the control-sired embryos and in gels from the heat-sired embryos in order to compare accurately pattern differences seen in different 2-D gels from control-sired and heat-sired embryos. Each constellation was given an arbitrary name and a spot number for each protein within each constellation was also allocated. In this study, each 2-D gel is therefore presented both in its original form and in a plotted form.

There were two replicates in this experiment.

6.3.3.2 Results

6.3.3.2.1 The effect of paternal heat stress 21 days earlier on protein profiles of the 2-cell embryos in the mouse

As seen in Fig 6.7 (A, C or E), paternal heat stress significantly changed protein synthetic patterns of both 2-cell embryos and abnormal embryos, thus supporting the results of the 1-D gel electrophoresis study. In order to facilitate comparisons in protein profiles, the main

protein spots identified in both the control and experimental groups and their molecular weights are summarised in Table 6.1, based on data given in Fig 6.7 (B, D or F).

Paternal heat stress 21 days prior to mating appears to have inhibited the synthesis of several groups of proteins including molecular weights 13-16.3kD in constellation A, 14.9kD in B, 39kD in H, 45-48kD in J, 53.7kD in K and 70kD in M (Table 6.1, Fig 6.7, D; or F) compared with those in the control-sired 2-cell embryos. Paternal heat stress also reduced the number of protein spots in several constellations. In constellation C, control-sired 2-cell embryos had two protein spots (spot 1 and 2) in Fig 6.7 (B) and Table 6.1, while heat-sired 2-cell embryos contained only protein spot 2 (Fig 6.7, D and Table 6.1). In constellation D, control-sired 2-cell embryos had three protein spots (spot 1, 2 or 3; in Fig 6.7, B and Table 6.1), but heat-sired 2-cell embryos and abnormal embryos contained only spot 3 (Fig 6.7, D and Table 6.1) and spots 2 and 3 respectively (Fig 6.7, F and Table 6.1). Furthermore, in constellation N, control-sired 2-cell embryos contained 3 protein spots (1, 2 or 3) of molecular weights 67-70kD, but heat-sired 2-cell embryos contained protein spot 1 of molecular weight 67kD and heat-sired abnormal embryos did not have protein spots in this constellation. However, there were two protein spots (spots 1 and 2) including molecular weights 35kD in heat-sired 2-cell embryos, but one protein spot (spot 2) respectively in control-sired 2-cell embryos and heat-sired abnormal embryos in constellation F. Furthermore, the spot in constellation I had shifted its isoelectric point from position in pH 7.4 in control-sired 2-cell embryos to position in pH 7.8 in heat-sired 2-cell embryos.

Table 6.1 The effect of paternal heat stress 21 days earlier on protein
patterns of 2-cell embryos and abnormal embryos in the mouse

Constellations	Control-sired 2-cell		Heat-sired 2-cell		Heat-sired abnormal	
	No.spots	MW (kD)	No.spots	MW (kD)	No.spots	MW (kD)
A	3	13-16.3	None	None	None	None
B	1	14.9	None	None	None	None
C	2	21.3	1	21.3	None	None
D	3	29-33.6	1	33.6	2	31.3-33.6
E	1	30	1	30	None	None
F	1	35	2	35	1	35
G	1	37.8	1	37.8	1	37.8
H	1	39	None	None	None	None
I	1	39.4	1	39.4	None	None
J	2	45-48	None	None	None	None
K	1	53.7	None	None	None	None
L	1	67	1	67	1	67
M	1	70	None	None	None	None
N	3	67-70	1	67	None	None

Fig 6.7 Protein profiles of 2-cell embryos in the mouse

Mr, relative molecular mass weights as for Fig 6.1; A and B are respectively the original and plotted protein patterns of 70 control-sired 2-cell embryos; C and D are respectively the original and plotted protein patterns of 70 heat-sired 2-cell embryos; E and F are respectively the original and plotted protein patterns of 70 heat-sired abnormal embryos.

SDS, gels in the SDS (sodium dodecylsulfate) dimension were of a 7.5-15% polyacrylamide gradient.

171

6.3.3.2.2 The effect of paternal heat stress 21 days earlier on protein profiles of morulae in the mouse

Paternal heat stress 21 days prior to mating reduced significantly the protein synthetic capacity of morulae (Fig 6.8, C compared with A). The main protein spots and their molecular weights in the control-sired and heat-sired morulae are summarised in Table 6.2, based on data presented in Fig 6.8 (B or D).

Table 6.2 The effect of paternal heat stress 21 days earlier on protein profiles of morulae

Constellation	Control-sired morulae		Heat-sired morulae	
	No. spots	MW (kD)	No. spots	MW (kD)
A	3	12.6-14.3	3	12.6-14.3
B	5	18.8-21.3	3	18.8-21.3
C	3	21.3	2	21.3
D	3	23.6-25.7	3	23.6-25.7
E	3	23.6-26.6	3	23.6-26.6
F	5	22.8-24.7	2	22.8
G	3	29.3	1	29.3
H	3	29.3-31.5	None	None
I	5	30.8-33	4	30.8-33
J	3	33.7-34.7	2	33.7-34.7
K	5	33-34.7	3	33-34.7
L	2	36.7-38.8	None	None
M	3	36.7-37.6	None	None
N	5	41.9-48.3	3	41.9-43.5
O	5	44.5-48.3	3	44.5-48.3
P	16	54.4-72.9	13	54.4-72.9
Q	10	82.5-99.4	7	82.5-90.4

Paternal heat stress 21 days earlier appears to have inhibited the synthesis of three groups of proteins including molecular weights, 29.3-31.5kD in constellation H, 36.7-38.8kD in constellation L and 36.7-37.6kD in constellation M because there were not comparable proteins detected in the heat-sired morulae (Table 6.2 and Fig 6.8, D). Furthermore, paternal heat stress 21 days prior to mating also reduced the number of protein spots in
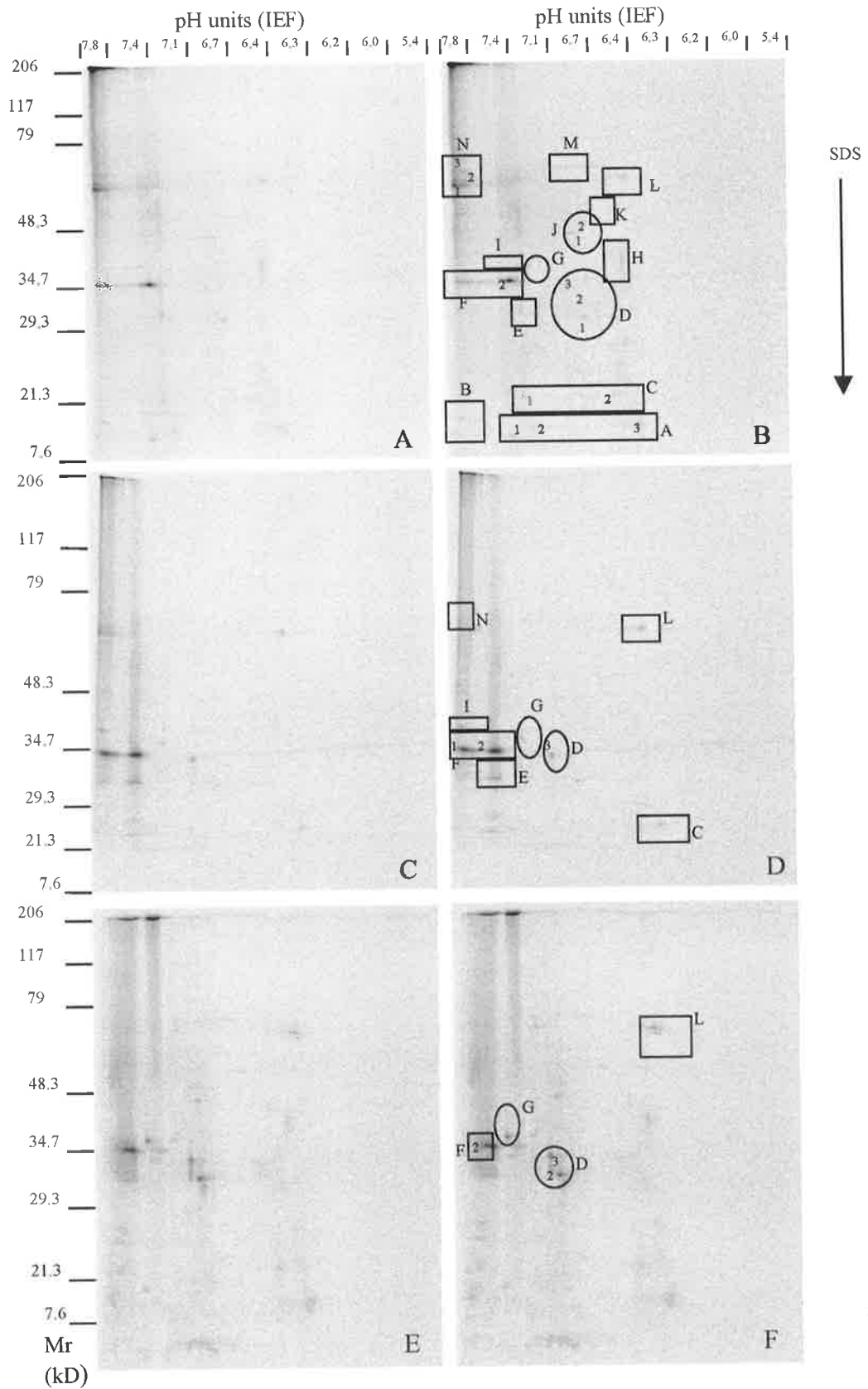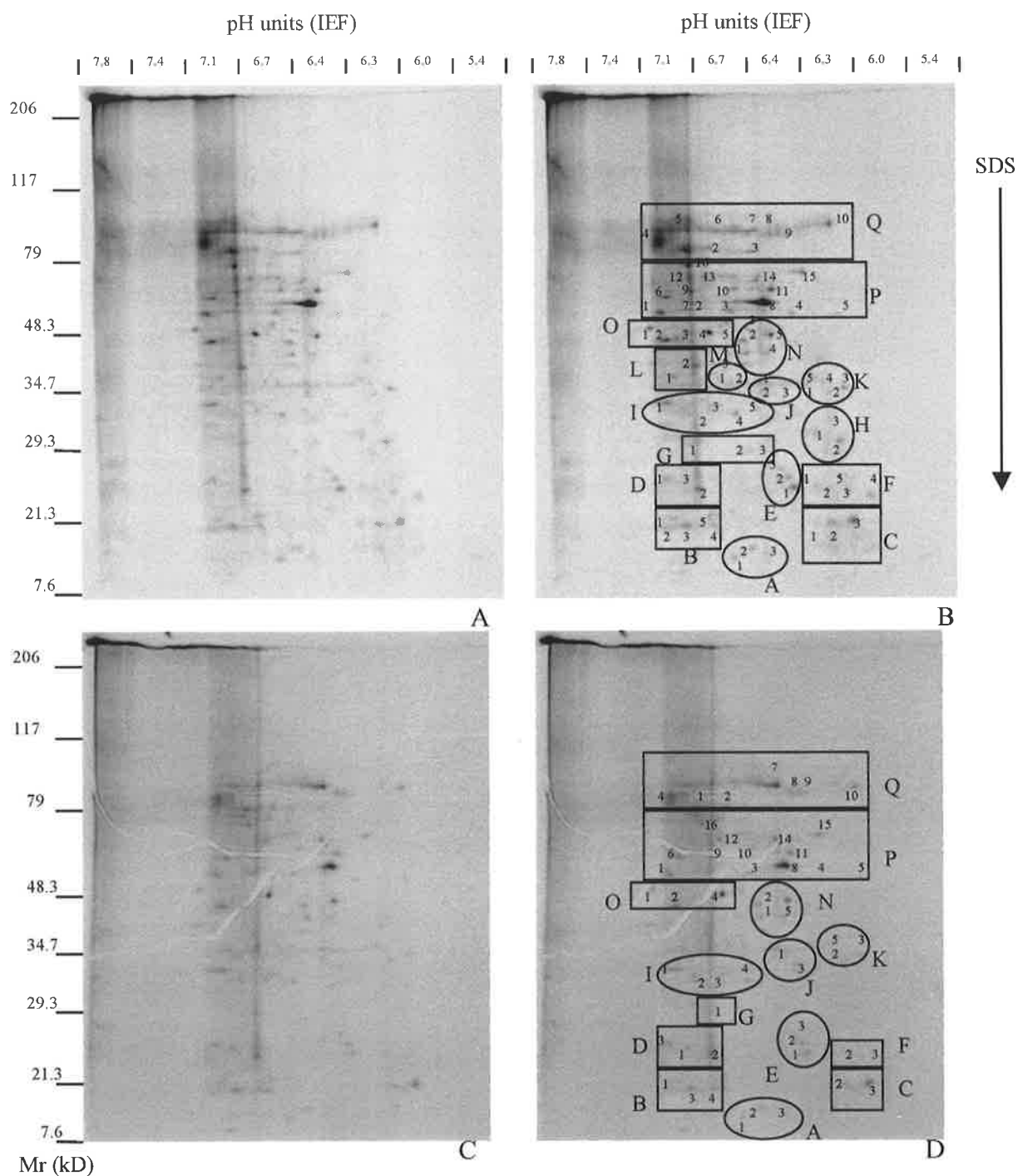
Fig 6.8 Protein profiles of morulae in the mouse

Mr, relative molecular mass markers as for Fig 6.1; A and B are respectively the original and plotted protein patterns of 40 control-sired morulae while C and D are respectively the original and plotted protein patterns of 40 heat-sired morulae.

SDS, as for Fig 6.7.

173

constellations B, C, F, G, I, J, K, N, O, P, or Q (Table 6.2, Fig 6.8, D), compared with those of control groups (Table 6.2 and Fig 6.8, B). The number of protein spots observed in constellations A, D, E did not change between the control and the heated groups, but the intensities of spots are reduced in the heated groups and spot 3 in constellation D seemed to shift from a pI of 6.7 in control-sired morulae (Fig 6.8, A or B) to a pI of 7.1 in heat-sired morulae (Fig 6.8, C or D).

6.4 Discussion

The results obtained using both 1-D and 2-D gels clearly indicate that paternal heat stress, 21 days prior to mating, significantly reduced protein synthesis by 2-cell and abnormal embryos. This result was particularly noticeable in the synthesis of proteins that are dependent on zygotic genomic activation such as 68kD and 70kD (Bensaude et al., 1983; Poueymirou and Schultz, 1987) and 67 kD (Flach et al., 1982), the transcription-requiring complex (TRC) of molecular weights 68-73 kD (Conover et al., 1991). In addition, The developing embryo requires large amounts of 30kD, 35kD and 46kD proteins at a time when a sudden burst of transcription is inconvenient or impossible, and the embryo therefore relies on a post-transcriptional control mechanism (Braude et al., 1979; Howlett, 1986), thus assuring the later development of embryos. However, as zygotic genomic activation occurs at the 2-cell stage in the mouse, this normally causes a reduction in the amount of these proteins (Pratt et al., 1983) and they are gradually replaced by new synthesised proteins. The present study showed that paternal heat stress 21 days prior to mating increased the number of spots of proteins with molecular weight 35kD and the intensity of the protein spot of molecular weight 30kD (Fig 6.8, C and D). This may mean that paternal heat stress has damaged sperm, further disturbing and/or reducing the function

174

of these sperm following ovum penetration and fertilization, limiting the functionality of the genome and impacting on subsequent embryogenesis. Furthermore, paternal heat stress 21 days prior to mating also disturbed the synthesis of several other groups of proteins such as 13-16.3, 21.3, 34.7, 45-48, 51.3, 53.7, 75.6, 78.1 or 162kD in the 2-cell embryos, compared with control embryos. These proteins have been reported to be first produced in mouse embryos at the 2-cell stage (Van Blerkom and Brockway, 1975; Cullen et al., 1980b). Although their roles in embryonic development have not been elucidated, both the in vivo and in vitro studies on paternal heating indicate that these proteins are likely to play important roles in the later development of these embryos.

During the morula stage, position, morphology and polarization of cells within the morula are changed in order to contribute to the subsequent formation of the blastocyst (Watson, 1992; Gueth-Hallonet and Maro, 1992). These changes seem to be regulated by changes in previously synthesized proteins (Gueth-Hallonet and Maro, 1992). In addition, proteins synthesized initially at the 2-cell stage are again synthesised at the morula stage (Cullen et al., 1980b) and paternal heat stress significantly influences the protein profiles of 2-cell embryos. It is possible that paternal heat stress may continue affecting the synthesis of such proteins at the morula stage. Furthermore, a set of proteins of molecular weights around 20-42kD first appear at the morula stage (Cullen et al., 1980b). The results obtained by 2-D gel electrophoresis and phosphoimaging have shown that paternal heat stress 21 days previously appears to have inhibited the synthesis of proteins in this molecular weight range (29.3-31.5, 36.7-37.6 and 36.7-38.8kD) and reduced the number of protein spots in constellations B, C, F, G, I, J, K, N, O, P, or Q. This suggests that changes in the protein profiles caused by paternal heat stress may further affect expression of new gene products, post-translational modification of proteins appearing at the earlier stages, cell lineage-specific proteins, differentiation requiring peptides and housekeeping proteins, finally

175

decreasing significantly the proportion of in vivo and in vitro development from the morula to the blastocyst in the mouse (Chapter 4 and 5).

It has been well documented that paternal heat stress increased significantly the proportion of abnormal embryos (Chapter 4 and 5). In the present study, it was of interest to note that heat-sired abnormal embryos appear to have inhibited protein synthesis including proteins of molecular weights 44.2, 52.5, 67, 70 and 87.4kD (Fig 6.6, H21-A; Fig 6.8, E and F). Whilst this could be a consequence of the abnormal development of these embryos, it remains possible that a significant decrease in protein synthetic capacity of embryos caused by paternal heat stress may at least contribute to subsequent abnormal development.

In summary, paternal heat stress significantly affects many but not all protein synthesised patterns of preimplantation embryos. At the 2-cell stage, paternal heat stress influenced mainly the later development of embryos by reducing the protein synthesis normally associated with zygotic genomic activation such as 67kD, 68kD and 70kD, altering fertilization-accelerated and/or fertilization-dependent polypeptide synthetic changes including molecular weights 30kD, 35kD and 46kD, and also affecting the synthetic profiles of other proteins. At the morula stage, paternal heat stress reduced the later development of embryos with the synthesis of specific constellations of proteins being inhibited (29.3-31.5kD in H, 36.7-38.8kD in L and 36.7-37.6 in M) and the number of protein spots being reduced in some constellations. Furthermore, paternal heat stress increased the number of abnormal embryos and this was associated with altered protein synthetic patterns. Therefore, the effect of paternal heat stress on mRNA content and gene expression of embryos merit further investigation.

Chapter Seven

General Discussion

The results of both in vitro and in vivo studies presented in this thesis show clearly that paternal heat stress retarded the development of preimplantation embryos in the mouse. These results have extended previous findings which covered the period from 3 days to 8 days after heat-stress of the males (Bellve, 1972, 1973 and 1976) and extend this to 42 days after heating of males. In the earlier studies, the fertilizing sperm would have been in the epididymis at the time of heating, while the present study extends the observations to cells present in the testis at the time of heat stress.

In mice and other animals such as rats, rabbits, sheep, pigs, cattle, even humans, most studies have to-date focused on investigating the effects of paternal heat stress on fertilization and post-implantation embryos (Setchell, 1998; reviewed in Chapter 1). In contrast, only a few investigations have examined the effects of paternal heat stress on the development of preimplantation embryos in mice (Bellve, 1972, 1973 and 1976), rats (Setchell et al., 1988) and sheep (Ekpe et al., 1992, 1993; Setchell, 1994). These studies have demonstrated that paternal heat stress reduced the developmental capacity of preimplantation embryos and increased the number of degenerated blastocysts in the sheep sired during 11 days of scrotal insulation (Ekpe et al., 1992, 1993; Setchell, 1994), and caused an accumulation of morulae at 88h and a significant decrease of blastocysts at 120h after hCG injection in the mouse (Bellve, 1972, 1973 and 1976). However, the relationship between the stage of the spermatogenetic cycle at which the cells were affected by heat stress and the subsequent development of preimplantation embryos has not been completely understood. The present studies have indicated that the extent of effect of paternal heat stress on the development of preimplantation embryos is related to the stage of development of the male germ cells at the time of heat stress.

It is known that heat stress may change endocrine function and induce intracellular damage to germ cells in the testis of mice, and other animals including humans (Setchell, 1998; reviewed in Chapter 1). Experimental cryptorchidism, whole body heating and local heating of the testis in the mouse have been shown to seriously damage primary spermatocytes, followed by round spermatids and spermatogonia (Meistrich et al., 1973; Van Zelst et al., 1995; Sailer et al., 1997; Yin et al., 1997). Subsequent development of heat-sired mouse preimplantation embryos is directly affected by the extent of germ cell damage caused by heat stress. In the other words, the more germ cells in the testis that are damaged by heat stress, the lower the proportions of subsequent embryo development in the mouse.

The finding that paternal heat stress 14 and 21 days before mating significantly reduced the proportion of preimplantation embryos developing normally is particularly interesting because the sperm involved in the fertilization of these embryos would have come from spermatids (day14) and spermatocytes (day 21) at the time of heating. These results suggest that heat stress might impair the suitable allocation of chromosomes within each spermatocyte and/or damage the chromatin and acrosome structures in spermatids.

The spermatocyte is a key stage in the spermatogenetic cycle because it is characterized by meiosis and the formation of pairs of chromosomes and doubling of the chromosome number (Setchell, 1978). Several studies have demonstrated that heat stress increases the incidence of X-Y dissociation in the primary spermatocytes in diakinesis of metaphase in the mouse (Garriott and Chrisman, 1980; Waldbieser and Chisman, 1986; Van Zelst et al., 1995) and X-Y chromosome dissociation may be related to sterility in the mouse (Beechey, 1973) and humans (Chandley, 1973; Chandley et al., 1976; Burgoyne, 1979). Therefore, heat stress may disturb the normal process of meiosis, further affecting the appropriate allocation of chromosomes within each spermatocyte. Spermatids are the cells resulting

178

from the second meiotic division. These cells are finally transformed into the very complex spermatozoa, undergoing condensation of the nucleus, formation of the acrosome, virtual elimination of the cytoplasm, development of a tail and the arrangement of its mitochondria into a helix to produce the midpiece (Setchell, 1978). In addition, chromatin progressively aggregates from a fine dusty state in the early spermatid stage into the chromatin granules which then produce the highly condensed nucleus of the late spermatid (Setchell, 1978). Sperm chromatin structures have been shown to be related to fertility in the bull (Ballachey et al., 1987), boar (Evenson et al., 1994) and man (Evenson et al., 1999; Marcello et al., 2000). Furthermore, heat stress in both mouse (Sailer et al., 1997) and bull (Karabinus et al., 1997) can damage the chromatin structure of sperm cells. The effect of heat stress on the chromatin of testicular sperm was more severe than that of epididymal sperm (Karabinus et al., 1997; Sailer et al., 1997).

The preimplantation period represents a time where fundamental developmental decisions are made that culminate in the formation of the blastocyst. Preimplantation development is dependent mainly on three key events, namely zygote genomic activation, compaction and the cavitation. The present results have also shown that paternal heat stress not only reduced significantly the proportion of 2-cell embryos, morulae and blastocysts (although it affects the development of preimplantation embryos at all stages), but also changed the protein synthetic profiles of 2-cell embryos and morulae.

Certain maternal and paternal autosomal genes are differentially expressed in mammals, a phenomenon termed genomic imprinting (Bartolomei and Tilghman, 1997). Imprinting of genes must be established during gametogenesis since this is the only time when maternal and paternal genes can be subject to differing influences (Bartolomei and Tilghman, 1997; Morison and Reeve, 1998;). Furthermore, imprinting must be capable of being erased and

reset during the production of the germ cells such that the appropriate sex-specific imprint is transmitted to the progeny(Bartolomei and Tilghman, 1997; Tilghman, 1999). Haig and Westoby (1989) early proposed a model of parent-offspring conflict and further referred to this as a parental "tug-of-war" in which the weapons are imprinted genes (Moore and Haig, 1991; Haig, 1992). This model predicts that paternally expressed genes will promote growth and maternal genes will inhibit fetal growth. One example of this model is that insulin-like growth factor II (IGF2), a paternally expressed gene that promotes growth, and H19, and IGF2 receptor (IGF2r), two maternally expressed genes that inhibit IGF2 by transcriptional or biochemical ways (Bartolomei and Tilghman, 1997). This balance between IGF2 and IGF2r or H19 may at least maintain partly healthy development of embryos. IGF2 has been reported to stimulate the growth of both pre-implantation (Harvey and Kaye, 1992) and post-implantation embryos (Baker et al., 1993). Furthermore, another paternally imprinted gene encoding U2afbp-rs, an RNA processing protein, is transiently transcribed at the 2-cell stage (Latham et al., 1995). This imprinted gene may promote early embryo growth because its expression in androgenetic embryos is greater than biparental zygotes, which in turn is greater than gynogenetic embryos (De Sousa et al., 1998). In the present studies, paternal heat stress increased the proportion of abnormal embryos and reduced the developmental capacity of preimplantation embryos, compared with control-sired embryos. The possibility that the effects of paternal heat stress are mediated through imprinted genes remains an option that results require further investigation.

During pronuclear formation in the mouse, sperm chromatin packing protamines are replaced by histones, resulting in the nuclear remodelling and different pronuclear transcriptional activity. This exchange has been supported by evidence that the transcription factors Sp1 and TATA-box binding protein (TBA) appear to be more concentrated in male than female pronuclei (Worrad et al., 1994; Worrad and Schultz, 1997). The capacity of

RNA polymerase I, II and III to mediate embryonic transcription at the 2-cell stage appears to develop in paternal pronuclei during the G2 phase of the first cell cycle (Ram and Schultz, 1993; Nothias et al., 1996). In addition, the frequency of mouse embryo cytoplasmic fragmentation is affected by maternal age, fertilization conditions and genetic background (Jurisicova et al., 1995, 1998a,b). Fragmentation occurring in 1-cell embryos has been shown to be related to an elevation of the temporal expression of some cell death genes (MA-3, P53, Bad and Bcl-xS) and a decrease of expression of Bcl-2 involved in cell survival (Jurisicova et al., 1998a). Furthermore, treatment of male rats with 5-aza-cytidine (5-AZAC) interferes with proper DNA methylation during spermatogenesis and causes DNA damage. After mating to normal females, embryos sired by 5-AZAC treated males appeared fragmented 24h after fertilization (Doerksen and Trasler, 1996). In the present studies, paternal heat stress reduced significantly the proportions of 1-cell embryos developing to the 2-cell stage. This may imply that paternal heat stress could damage nuclear remodelling, reduce the production of RNA polymerases and disturb the regulatory balance between cell death genes and cell survival genes, thus causing fragmentation of the 1-cell embryo.

Transcription stops in the mouse oocyte during metaphase II (Wassarman and Letourneau, 1976) and transcriptional permissiveness is re-established in the late 1-cell embryo prior to the activation of zygotic genes at the 2-cell stage (Matsumoto et al., 1994; Aoki et al., 1997; Forlani et al., 1998). However, a transcriptionally repressive environment is produced between the end of oocyte maturation and the 2-cell stage. This appears to involve the absence of an active transcriptional apparatus, suppression of long-range activation due to absence of a putative coactivator activity (Majumder et al., 1997) and a non-permissive state of the genome (Forlani et al., 1998). A transcriptionally repressive environment in the 1-cell mouse embryo may prevent the premature expression of genes whose effects are only

required at a subsequent stage (Majumder et al., 1997; Forlani et al., 1998). One example of this is that if genes involved in differentiation of the trophoblast are expressed at the 1-cell stage, this would be lethal to the embryo (Forlani et al., 1998). Moreover, gene activation at the beginning of development is controlled by a zygotic clock (Wiekowski et al., 1991) which may prevent the premature expression of paternal genes although the chromatin of paternal genes has an open configuration, allowing the formation of active transcriptional complexes without the need of enhancer elements (Majumder et al., 1993). The first round of DNA replication has been shown to mediate the resumption of transcriptional activity through activating of a transcriptional apparatus, long-range activation and establishing a permissive genomic state and control of ZGA (Howlett, 1986; Davis et al., 1996; Latham, 1999). Endogenous markers of ZGA, including a transcription requiring complex (TRC) (Poueymirou and Schultz, 1989; Latham et al., 1991; Schultz, 1993), HSP70.1 (Bensuade et al., 1983; Thompson et al., 1995; Christians et al., 1997) and a translation initiation factor (eIF-4C) (Davis et al., 1996) are transiently transcribed during the 2-cell stage in the mouse. The present studies have indicated that paternal heat stress reduced significantly synthesis of 70KD proteins at the 2-cell embryo. This suggests that paternal heat stress may disturb the transient accumulation of mRNA and protein for these proteins.

Blastocyst formation, essential for implantation, is initiated during compaction. E-cadherin mediates cell-to-cell adhesion and drives trophectoderm cell differentiation. In E-cadherin null embryos, cell polarization is not prevented, but rather the orientation of cell polarity is delayed and randomized, thus preventing the formation of an ordered trophectoderm cell layer (Larue et al., 1994; Riethamacher et al., 1995) and normal blastocysts (Riethamacher et al., 1995). The formation of tight junctions is also important in blastocyst formation because it plays a role in the regulation of paracellular transport (the movement of water and solutes between epithelial cells) and maintenance of epithelial cell polarity (Biggers et

al., 1988; Stevenson et al., 1988; Watson, 1992; Citi, 1993). One of the proteins involved in the formation of the tight junction, ZO-1 was localized at contact sites between outer blastomeres following compaction (Rajasekaran et al., 1996). Furthermore, ZO-1α isoform proteins first appear in compacting mouse morulae as perinuclear foci, followed by membrane accumulation between the outer blastomeres (Fleming et al., 1989). By the late morula stage, Na/K-ATPase localized to the basolateral domains of the outer trophectoderm may play a very important role in the onset of fluid accumulation and the formation of blastocyst cavitation through regulating cell volume and ion concentration (Watson and Kidder, 1988; Watson et al., 1990; Watson 1992; MacPhee et al., 1994). Watson et al. (1999) proposed a hypothesis that the blastocyst cavity forms as a result of a trans-trophectoderm ion gradients established, in part, by a polarized trophectoderm Na/K-ATPase, which facilitates the osmotic movement of water into the extracellular space to form the fluid-filled cavity of the blastocyst. The tight junction permeability seal regulates blastocyst formation by restricting the leakage of fluid via paracellular routes, thereby ensuring the expansion of the cavity as fluid accumulates. In addition, DNA replication may also control the compaction of the 8-cell embryos (Smith and Johnson, 1985) and the timing of blastocoele formation (Smith and McLaren, 1977; Mayor and Izguierdo, 1994). In the present study, paternal heat stress inhibited the synthesis of many proteins in the morulae (Chapter 6), thus probably affecting normal cell-to-cell adhesion, cell differentiation and DNA replication in the morulae, and finally influencing the formation of blastocysts (Chapter 4 and 5).

In summary, the present studies and previous studies have clearly indicated that paternal heat stress may influence the development of embryos from preimplantation, through to implantation and postimplantation. The present investigation has contributed new

information about the heat-susceptible stages of male germ cells in the testis, and possible events in preimplantation embryo development that are affected by paternal heat-stress. This study has highlighted a number of issues that require further investigation, and future studies should concentrate on the effect of paternal heat stress on chromatin structure, DNA replication, the zygotic gene activation and paternally expressed genomic imprinting of preimplantation embryos in the mouse. It is suggested that further investigations of the mechanisms by which paternal heat stress affects embryonic development will be beneficial as a model for examining the importance of paternal contributions to reproductive biology and embryo development, and may provide important information relevant to improving the success of in vitro fertilization, embryo culture, transgenic and cloning techniques.

Chapter Eight

Bibliography

Abramczuk, J. and Sawicki, W. (1975). Pronuclear synthesis of DNA in fertilized and parthenogenetically activated mouse eggs. Exp. Cell Res., **92**, 361-369.

Abramczuk, J., Solter, D. and Koprowski, H. (1977). The beneficial effect of EDTA on development of mouse one-cell embryos in chemically defined medium. Dev. Biol., **61**, 378-383.

Adams, C.E. and Chang, M.C. (1962). Capacitation of rabbit spermatozoa in the fallopian tube and in the uterus. J. Exp. Zool., **151**, 159-165.

Adeoya-Osiguwa, S.A. and Fraser, L.R. (1996). Evidence for Ca^{2+}-dependent ATPase activity stimulated by decapacitation factor and calmodulin in mouse sperm. Mol. Reprod. Dev., **44**, 111-120.

Aghayan, M., Rao, L.V., Smith, R.M., Jarett, L., Charron, M.J., Thorens, B. and Heyner, S. (1992). Developmental expression and cellular localisation of glucose transporter molecules during mouse preimplantation development. Development, **115**, 305-312.

Allan, D.J., Harmon, B.V., Kerr, J.F.R. (1987). Cell death in spermatogenesis. In "Perspective on Mammalian Cell Death". Oxford Univ. Press, London, 229-258.

Allen, R.L., O'Brien, D.A. and Eddy, E.M. (1988a). A novel hsp70-like protein (P70) is present in mouse spermatogenic cells. Mol. Cellular Biol., **8**, 828-832.

Allen, R.L., O'Brien, D.A., Jones, C.C., Rockett, D.L. and Eddy, E.M. (1988b). Expression of heat shock proteins by isolated mouse spermatogenic cells. Mol. Cellular Biol., **8**, 3260-3266.

Almeida, E.A.C., Huovila, A-P.J., Sutherland, A.E., Stephens, L.E., Calarco, P.G., Shaw, L.M., Mercurio, A.M., Sonnenberg, A., Primakoff, P., Myles, D.G. and White, J.M. (1995). Mouse egg integrin α6β1 functions as a sperm receptor. Cell, **81**, 1095-1104.

Almon, E., Goldfinger, N., Kapon, A., Schwartz, D., Levine, A.J. and Rotter, V. (1993). Testicular tissue-specific expression of the p53 suppressor gene. Dev. Biol., **156**, 107-116.

Amatayakul, K., Ryan, R., Uozumi, T. and Albert, A. (1971). Reinvestigation of testicular-anterior pituitary relationships in the rat. I. Effect of castration and cryptorchidism. Endocrinol., **88**, 872-880.

Anbari, K. and Schultz, R.M. (1993). Effect of sodium and betaine in culture media on development and relative rates of protein synthesis in preimplantation mouse embryos in vitro. Mol. Reprod. Dev., **35**, 24-28.

Anderson, D.W., Nichol, A.V., Pan, S.S. and Lindgren, F.T. (1978). High density lipoprotein distribution, resolution and determination of three major components in a normal population sample. Atherosclerosis, **29**, 161-179.

Anderson, G.B. (1977). Fertilization, early development and embryo transfer. In "Reproduction in Domestic Animals". Eds. H.H. Cole and P.T. Cupps. Academic Press, New York, 285-314.

Ando, K. and Griffin, J.D. (1995). Cdk4 integrates growth and inhibitory signals during G1 phase of hematopoietic cells. Oncogene, **10**, 751-755.

Aoki, F., Worrad, D.M. and Schultz, R.M. (1997). Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. Dev. Biol., **181**, 296-307.

Ashraf, M., Peterson, R.N. and Russel, L.D. (1982a). Activity and location of cation-dependent ATPase on the plasma membrane of boar spermatozoa. Biochem. Biophys. Res. Commun., **107**, 1273-1278.

Ashraf, M., Peterson, R.N. and Russel, L.D. (1982b). Evidence for Ca^{2+}/Na^{+} antiport in boar sperm plasma membrane vesicles. Biol. Reprod., **26**, 37A.

Au, C.L., Robertson, D.M. and de Kretser, D.M. (1987). Changes in testicular inhibin after a single episode of heating of rat testes. Endocrinol., **120**, 973-977.

Auger, J., Kunstmann, J.M., Czyglik, F. and Jouannet, P. (1995). Decline in semen quality among fertile men in Paris during the past 20 years. New Engl. J. Med., **332**, 281-285.

Austin, C.R. (1969). Sperm capacitation-biological significance in various species. In "Advances in Biosciences, 4, Schering Symposium on Mechanisms Involved in Conception". Pergamon Press, Berlin, 5-11.

Austin, C.R. and Braden, A.W.H. (1956). Early reaction of the rodent egg to spermatozoa penetration. J. Exp. Biol., **33**, 358-365.

Austin, J.W., Hupp, E.W. and Murphree, R.L. (1961). Effect of scrotal insulation on semen of Hereford bulls. J. Animal Sci., **20**, 307-310.

Baker, J., Liu, J-P, Robertson, E.J. and Efstratiadis, A. (1993). Role of insulin-like growth factors in embryonic and post-natal growth. Cell, **75**, 73-82.

Baldwin, S.A. (1993). Mammalian glucose transporters: members of an ubiquitous family of active and passive transport proteins. Biochim. Biophys. Acta, **1154**, 17-49.

Ballachey, B.E., Hohenboken, W.D. and Evenson, D.P. (1987). Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. Biol. Reprod., **36**, 915-925.

Bambino, T.H. and Hsueh, A.J.W. (1981). Direct inhibition of glucocorticoids upon testicular luteinizing hormone receptor and steroidogenesis in vivo and in vitro. Endocrinol., **108**, 2142-2148.

Barenton, B., Blanc, M.R., Caraty, A., Hochereau-de Reviers, M.T. and Perreau, C. (1982). Effect of cryptorchidism in the ram: changes in the concentration of testosterone and

estradiol and receptors for LH and FSH in the testis and its histology. Mol. Cellular Endocrinol., **28**, 13-25.

Bartolomei, M.S. and Tilghman, S.M. (1997). Genomic Imprinting in mammals. Annu. Rev. Genet., **31**, 493-525.

Bavister, B.D. (1982). Evidence for a role of post-ovulatory cumulus components in supporting fertilizing ability of hamster spermatozoa. J. Androl., **3**, 365-372.

Bavister, B.D. (1988). Role of oviductal secretions in embryonic growth in vivo and in vitro. Theriogenology, **29**, 143-154.

Bavister, B.D. (1995). Culture of preimplantation embryos: facts and artefacts. Hum. Reprod., **1**, 91-148.

Bavister, B.D., Edwards, R.G. and Steptoe, P.C. (1969). Identification of the mid-piece and tail of the spermatozoon during fertilization of human eggs in vitro. J. Reprod. Fertil., **20**, 159-160.

Bavister, B.D., Leibfried, M.L. and Lieberman, G. (1983). Development of preimplantation embryos of the golden hamster in a defined culture medium. Biol. Reprod., **28**, 235-247.

Bedford, J.M. (1970). Sperm capacitation and fertilization in mammals. Biol. Reprod. (suppl), **2**, 128-158.

Bedford, J.M. (1989). Effect of elevated temperature on the epididymis and testis: experimental studies. In "Temperature and Environmental Effects on the Testis". Ed. A. Zorgniotti. Plenum, New York, 19-32.

Bedford, J.M. and Shalkovsky, S. (1967). Species specificity of sperm capacitation in the rabbit. J. Reprod. Fertil., **13**, 361-364.

Bedrak. E., Chap, Z. and Fried, K. (1980). Factors for consideration in the interpretation of the adverse effects of elevated environmental temperatures on reproduction in the

male rat. Int. J. Androl., **24**, 117-128.

Beechey, C.V. (1973). X-Y chromosome dissociation and sterility in the mouse. Cytogenet. Cell Genet., **12**, 60-67.

Behboodi, E., Anderson, G.B., BonDurant, R.H., Cagill, S.L., Kreuscher, B.R., Medrano, J.F. and Murray, J.D. (1995). Birth of large calves that developed from in vitro-derived embryos. Theriogenology, **44**, 227-232.

Beier, H.M. (1974). Oviductal and uterine fluids. J. Reprod. Fertil., **37**, 221-237.

Bell, G.I., Kayano, T., Buse, J.B., Burnant, C.F., Takeda, J., Lin, D., Fukomoto, H. and Seino, S. (1990). Molecular biology of mammalian glucose transporters. Diabetes Care, **13**, 198-208.

Bellve, A.R. (1972). Viability and survival of mouse embryos following parental exposure to high temperature. J. Reprod. Fertil., **30**, 71-81.

Bellve, A.R. (1973). Development of mouse embryos with abnormalities induced by parental heat stress. J. Reprod. Fertil., **35**, 393-403.

Bellve, A.R. (1976). Incorporation of [^{3}H] uridine by mouse embryos with abnormalities induced by parental hyperthermia. Biol. Reprod., **15**, 632-646.

Bensaude, O., Babinet, C., Morange, M. and Jacob, F. (1983). Heat shock proteins, first major products of zygotic gene activity. Nature, **305**, 331-333.

Bergh, A. (1983). Paracrine regulation of Leydig cells by the seminiferous tubules. Int. J. Androl., **6**, 57-65.

Bergh, A., Ason-Berg, A., Damber, J.E., Hammar, M. and Selstam, G. (1984a). Steroid biosynthesis and Leydig cell morphology in adult unilaterally cryptorchid rats. Acta Endocrinol., **107**, 556-562.

Bergh, A. and Damber, J.E. (1984). Local regulation of Leydig cells by the seminiferous tubules: effect of short-term cryptorchidism. Int. J. Androl., **7**, 409-418.

Bergh, A., Damber, J.E., Jacobsson, H. and Nilsson, T.K. (1987). Production of lactate and tissue plasminogen activator in vitro by seminiferous tubules obtained from adult unilaterally cryptorchid rats. Arch. Androl., **19**, 177-182.

Bergh, A., Damber, J.E. and Ritzen, M. (1984b). Early signs of Sertoli and Leydig cell dysfunction in the abdominal testes of immature unilaterally cryptorchid rats. Int. J. Androl., **7**, 398-408.

Bergstrom, S. and Nilsson, O. (1976). Blastocyst attachment and early invasion during oestradiol-induced implantation in the mouse. Anat. Embryol., **149**, 149-154.

Biggers, J.D. (1987). Pioneering mammalian embryo culture. In "The Mammalian Preimplantation Embryo", Ed. B.D. Bavister, Plenum Press, New York, 1-22.

Biggers, J.D., Bell, J.E. and Benos, D.J. (1988). Mammalian blastocyst: transport function in a developing epithelium. Am. J. Physiol., **255**, C419-432.

Biggers, J.D., Lawitts, J.A. and Lechene, C.P. (1993). The protective action of betaine on the deleterious effects of NaCl on preimplantation mouse embryos in vitro. Mol. Reprod. Dev., **34**, 380-390.

Biggers, J.D. and Stern, S. (1973). Metabolism of the preimplantation mammalian embryo. Adv. Reprod. Physiol., **6**, 1-59.

Biggers, J.D., Whittingham, D.G. and Donahue, R.P. (1967). Pattern of energy metabolism in the mouse oocyte and zygote. Proc. Natl. Acad. Sci. USA, **58**, 560-567.

Biggiogera, M., Tangury, R.M., Marin, R., Wu, Y., Martin, T.E. and Fakan, S. (1996). Localization of heat shock proteins in mouse male germ cells: an immunoelectron microscopical study. Exp. Cell Res., **229**, 77-85.

Blackshaw, A.W. and Hamilton, D. (1970). The effect of heat on hydrolytic enzymes and spermatogenesis in the rat testis. J. Reprod. Fertil., **22**, 569-571.

Blanco-Rodriguez, J. and Martinez-Garcia, C. (1997). In vivo analysis of germ cell apoptosis reveals the existence of stage-specific 'social' control of germ cell death in the seminiferous epithelium. Int. J. Androl., **20**, 373-379.

Bleau, G., Vandenheuvel, W.J.A., Andersen, O.F. and Gwatkin, R.B.L. (1975). Desmosteryl sulfate of hamster spermatozoa, a potent inhibitor of capacitation in vitro. J. Reprod. Fertil., **43**, 175-178.

Boatman, D.E. and Robbins, R.S. (1991). Bicarbonate: carbon-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. Biol. Reprod., **44**, 806-813.

Bolton, V.N., Oades, P.J. and Johnson, M.H. (1984). The relationship between cleavage, DNA replication, and gene expression in the mouse 2-cell embryo. J. Embryol. Exp. Morph., **79**, 139-163.

Bonnerot, C., Vernet, M., Grimber, G., Briand, P. and Nicolas, J.F. (1991). Transcriptional selectivity in early mouse embryos: a qualitative study. Nucl. Acid. Res., **25**, 7251-7557.

Bouniol, C., Nguyen, E. and Debey, P. (1995). Endogenous transcription occurs at the 1-cell stage in the mouse embryo. Exp. Cell Res., **218**, 57-62.

Bouthillier, M., Bleau, G., Chapdelaine, A. and Roberts, K.D. (1984). Distribution of steroid sulfotransferase in the male hamster reproductive tract. Biol. Reprod., **31**, 936-941.

Brachet, J., Ficq, A. and Tencer, R. (1968). Amino acid incorporation into proteins of nucleate and enucleate fragments of sea urchin eggs: effect of parthenogenetic activation. Exp. Cell Res., **32**, 168-170.

Braden, A.W.H. and Mattner, P.E. (1970). The effects of scrotal heating in the ram on semen characteristics, fecundity and embryonic mortality. Australian J. Agric. Res., **21**, 509-518.

Bradley, M.P. and Forrester, I.J. (1980). A $(Ca^{2+}-Mg^{2+})$ ATPase and active Ca^{2+} transport in the plasma membranes isolated from ram sperm flagella. Cell Calcium, **1**, 381-390.

Braude, P.R., Pelham, H.R.B., Flach, G. and Lobatto, R. (1979). Post transcriptional control in the early mouse embryo. Nature, **282**, 102-105.

Bremner, W.J., Millar, M.R., Sharpe, R.M. and Saunders, P.T. (1994). Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. Endocrinol., **135**, 1227-1234.

Brinster, R.L. (1963). A method for in vitro cultivation of mouse ova from two-cell to blastocyst. Exp. Cell Res., **32**, 205-207.

Brinster, R.L. (1965a). Studies on the development of mouse embryos in vitro. II. The effect of energy source. J. Exp. Zool., **158**, 59-68.

Brinster, R.L. (1965b). Studies on the development of mouse embryos in vitro. IV. Interaction of energy source. J. Reprod. Fertil., **10**, 227-240.

Brinster, R.L. (1970). Culture of two-cell rabbit embryos to morulae. J. Reprod. Fertil., **21**, 17-22.

Brinster, R.L. (1971). Uptake and incorporation of amino acids by the preimplantation mouse embryo. J. Reprod. Fertil., **27**, 329-338.

Browning, H. and Strome, S. (1996). A sperm-supplied factor required for embryogenesis in C. elegans. Development, **122**, 391-404.

Bryan, J.H.D. (1974). Capacitation in the mouse: the response of murine acrosomes to the

environment of the female genital tract. Biol. Reprod., **10**, 414-421.

Burck, P.J., Thakkar, L. and Zimmerman, R.E. (1982). Antifertility action of a sterol sulphate in the rabbit. J. Reprod. Fertil., **66**, 109-112.

Burfening, P.J., Elliott, D.S., Eisen, E.J. and Ulberg, L.C. (1970). Survival of embryos resulting from spermatozoa produced by mice exposed to elevated ambient temperature. J. Anim. Sci., **30**, 578-582.

Burfening, P.J. and Ulberg, L.C. (1968). Embryonic survival subsequent to culture of rabbit spermatozoa at 38°C and 40°C. J. Reprod. Fertil., **15**, 87-92.

Burgoyne, P.S. (1979). Evidence for an association between univalent Y chromosomes and spermatocyte loss in XYY mice and men. Cytogenet. Cell Genet., **23**, 84-89.

Butler, J.E., Lechene, C. and Biggers, J.D. (1988). Noninvasive measurement of glucose uptake by two populations of murine embryos. Biol. Reprod., **39**, 779-786.

Calarco, P.C. and Epstein, C.T. (1973). Cell surface changes during preimplantation development in the mouse. Dev. Biol., **32**, 208-213.

Carlsen, E., Giwercman, A., Keiding, N. and Skakkebaek, N.E. (1992). Evidence for decreasing quality of semen during past 50 years. Brit. Med. J., **305**, 609-612.

Carney, E.W. and Bavister, B.D. (1987). Stimulatory and inhibitory effects of amino acids on the development of hamster eight-cell embryos in vitro. J. IVF Embryo Transfer, **4**, 162-167.

Carson, D.D., Dutt, A. and Tang, J.P. (1987). Glycoconjugate synthesis during early pregnancy: hyaluronate synthesis and function. Dev. Biol. **120**, 228-235.

Carson, D.D., Tang, J.P., Julian, J. (1993). Heparan sulfate proteoglycan (Perlecan) expression by mouse embryos during acquisition of attachment competence. Dev. Biol., **155**, 97-106.

Casady, R.B., Myers, R.M. and Legates, J.E. (1953). The effect of exposure to high ambient temperature on spermatogenesis in the dairy bull. J. Dairy Sci., **36**, 14-23.

Cataldo, L., Mastrangelo, M.A. and Kleene, K.C. (1997). Differential effects of heat shock on translation of normal mRNAs in primary spermatocytes, elongated spermatids and Sertoli cells in seminiferous tubule culture. Exp. Cell Res., **231**, 206-213.

Champlin, A.K., Dorr, D.L. and Gates, A.H. (1973). Determining the stage of the estrous cycle in the mouse by the appearance of the vagina. Biol. Reprod., **8**, 491-494.

Chandley, A.C. (1973). Univalent sex chromosomes and meiotic arrest in man. Heredity, **30**, 262.

Chandley, A.C., Maclean, N., Edmond, P., Fletcher, J. and Watson, G.S. (1976). Cytogenetics and infertility in man. II. Testicular histology and meiosis. Ann Hum. Genet., **40**, 165-176.

Chang, M.C. (1955). Development of fertilizing capacity of rabbit spermatozoa in the uterus. Nature, **175**, 1036-1037.

Chang, M.C. and Sheaffer, D. (1957). Number of spermatozoa ejaculated at copulation, transported into the female and present in the male tract of the golden hamster. J. Hered., **48**, 107-109.

Chang, M.C. and Yanagimachi, R. (1963). Fertilization of ferret ova by deposition of epididymal sperm into the ovarian capsule with special reference to the fertilizable life of ova and the capacitation of sperm. J. Exp. Zool., **154**, 175-187.

Chao, Y-S, Jones, A.L., Hradek, G.T., Windler, E.E.T. and Havel, R.T. (1981). Autoradiographic localization of the sites of uptake, cellular transport and catabolism of low density lipoproteins in the liver of normal and estrogen-treated rats. Proc. Natl. Acad. Sci. USA, **78**, 579-601.

Chao, Y-S, Windler, E.E.T, Chen, G.C. and Havel, R.T. (1979). Hepatic catabolism of rat and human lipoproteins in rats treated with 17α-ethynylestradiol. J. Biol. Chem., **254**, 11360-11366.

Chatot, C.L., Lewis, J.L., Torres, I. and Ziomek, C.A. (1990). Development of 1-cell embryos from different strains of mice in CZB medium. Biol. Reprod., **42**, 432-440.

Chatot, C.L., Lewis, W.J., Torres, I. and Ziomek, C.A. (1994). One-minute exposure of 4-cell mouse embryos to glucose overcomes morula block in CZB medium. Mol. Reprod. Dev., **37**, 407-412.

Chatot, C.L., Ziomek, C.A., Bavister, B.D., Lewis, J.L. and Torres, I. (1989). An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J. Reprod. Fertil., **86**, 679-688.

Chen, H.Y., Trumbauer, M.E., Ebert, K.M., Palmiter, R.D. and Brinster, R.L. (1986). Developmental changes in the response of mouse eggs to injected genes. In "Molecular and Developmental Biology". Ed. L. Bogorad. Alan R Liss, Inc, New York, 149-159.

Cherr, G.N., Lambert, H., Meizel, S. and Katz, D.F. (1986). In vitro studies of the golden hamster sperm acrosome reaction: completion on the zona pellucida and induction by homologous soluble zonae pellucida. Dev. Biol., **114**, 119-131.

Chisholm, J.C. (1988). Analysis of the fifth cell cycle of mouse development. J. Reprod. Fertil., **84**, 29-36.

Chowdhury, A.K. and Steinberger, E. (1970). Early changes in the germinal epithelium of rat testes following exposure to heat. J. Reprod. Fertil., **22**, 205-212.

Chowdhury, A.K. and Steinberger, E. (1964). A quantitative study of the effect of heat on the germinal epithelium of the rat testis. American J. Anatomy, **115**, 509-524.

Christians, E., Campion, E., Thompson, E.M. and Renard, J.P. (1995). Expression of the HSP 70.1 gene, a landmark of early zygotic gene activity in the mouse embryo, is restricted to the first burst of transcription. Development, **121**, 113-122.

Christians, E., Michel, E., Adenot, P., Mezger, V., Rallu, M., Morange, M. and Renard, J.P. (1997). Evidence for the involvement of mouse heat shock factor 1 in the atypical expression of the HSP70.1 heat shock gene during mouse zygotic genome activation. Mol. Cell Biol., **17**, 778-788.

Christians, E., Michel, E. and Renard, J.P. (1997). Developmental control of heat shock and chaperone gene expression. Cell Mol. Life Sci., **53**, 168-178.

Citi, S. (1993). The molecular organization of tight junctions. J. Cell Biol., **122**, 485-489.

Clark, A.M. and Grisword, M.D. (1997). Expression of clusterin/sulfated glycoprotein-2 under conditions of heat stress in rat Sertoli cells and a mouse Sertoli cell line. J. Androl., **18**, 257-263.

Clegg, E.J. (1961). Further studies on artificial cryptorchidism: quantitative changes in the interstitial cells of the rat testis. J. Endocrinol., **21**, 433-441.

Clegg, K. B. and Piko, L. (1982). RNA synthesis and cytoplasmic polyadenylation in the one-cell mouse embryo. Nature, **295**, 342-345.

Cockle, S.M. (1995). Fertilization promoting peptide: a novel peptide, structurally similar to TRH, with patent physiological activity. J. Endocrinol., **146**, 3-8.

Collins, P.M., Collins, W.P., McNeilly, A.S. and Tsang, W.N. (1978). Plasma FSH, LH and testosterone levels in the male rat during degeneration of the germinal epithelium caused by severe heat treatment or ligation of the vasa efferentia. J. Reprod. Fertil., **54**, 285-291.

Connolly, B.A., Romaniuk, P.J. and Eckstein, F. (1982). Synthesis and characterization of diastereomers of guanosine-5'-O- (1-thiotriphosphate) and guanosine-5'-O- (2-thiotriphosphate). Biochem., **21**, 1983-1989.

Conover, J.C., Temeles, G.L., Zimmermann, J.W., Burke, B. and Schultz, R.M. (1991). Stage-specific expression of a family of proteins that are major products of zygotic gene activation in the mouse embryo. Dev. Biol., **144**, 392-404.

Cox, D.R. and Oakes, D. (1984). Analysis of Survival Data. Chapman and Hall, London and New York.

Craig, E.A., Weissman, J.S. and Horwich, A.L. (1994). Heat shock proteins and molecular chaperones: mediators of protein conformation and turnover in the cell. Cell, **78**, 365-372.

Cross, N.L., Morales, P., Overstreet, J.W. and Hanson, F.W. (1988). Induction of acrosome reactions by human zona pellucida. Biol. Reprod., **38**, 235-244.

Cross, J.C., Werb, Z. and Fisher, S.J. (1994). Implantation and the placenta: key pieces of the development puzzle. Science, **266**, 1508-1518.

Cullen, B., Emigholz, K. and Monahan, J. (1980a). The transient appearance of specific proteins in 1-cell mouse embryos. Dev. Biol., **76**, 215-221.

Cullen, B., Emigholz, K. and Monahan, J.(1980b). Protein patterns of early mouse embryos during development. Differentiation, **17**, 151-160.

Cummins, J.M. and Yanagimachi, R. (1982). Sperm-egg ratios and the site of the acrosome reaction during in vivo fertilization in the hamster. Gamete Res., **5**, 239-256.

Cunningham, B. and Osborn, G. (1929). Infra-red sterility: preliminary report. Endocrinol., **13**, 93-96.

Cybulsky, A.V., Carbonetto, S., Cyr, M-D, McTavish, A.J. and Huang, Q. (1993). Extracellular matrix-stimulated phospholipase activation is mediated by β_1-integrin. Am. J. Physiol., **264**, 323-332.

Dadoune, J.P. and Alfonsi, M.F. (1984). Autoradiographic investigation of sperm transit through the male mouse genital tract after tritiated thymidine incorporation. Reprod. Nutr. Dev., **24**, 927-935.

Damber, J-E., Bergh, A. and Janson, P.O. (1980). Leydig cell function and morphology in the rat testis after exposure to heat. Andrologia, **12**, 12-19.

Darzynkiewicz, Z. (1979). Acridine orange as a molecular probe in studies of nucleic acids in situ. In "Flow Cytometry and Sorting". Eds. M. Melamed, P. Mullaney and M. Mendelson. John Wiley and Sons, New York, 285-316.

Darzynkiewicz, Z., Traganos, F., Sharpless, T., Melamed, M.R. (1976). Lymphocyte stimulation: a rapid multiparameter analysis. Proc. Natl. Acad. Sci. USA, **73**, 2881-2884.

DasGupta, S., Mills, C.C. and Fraser, L.R. (1993). Ca^{2+}-related changes in the capacitation state of human spermatozoa assessed by a chlortetracycline fluorescence assay. J. Reprod. Fertil., **99**, 135-143.

Davidson, E.H. (1976). Gene Activity in Early Development. 2nd edn, New York/London, Academic Press.

Davis, W.J., DeSousa, P.A. and Schultz, R.M. (1996). Transient expression of translation initiation factor eIF-4C during the 2-cell stage of the preimplantation mouse embryo: identification by mRNA differential display and the role of DNA replication in zygotic gene activation. Dev. Biol., **174**, 190-201.

Davis, B.K.(1978). Inhibition of fertilizing capacity in mammalian spermatozoa by natural and synthetic vesicles. In "ACCS monograph No.5, Symposium on the Pharmacological Effects of Lipids", 145-158.

Davis, B.K. (1981). Timing of fertilization in mammals: sperm cholesterol/phospholipid ratio as a determinant of the capacitation internal. Proc. Natl. Acad Sci. USA, **78**, 7560-7564.

Davis, B.K., Byrne, R. and Bedigian, K. (1980). Studies on the mechanism of capacitation: albumin-mediated changes in plasma membrane lipids during in vitro incubation of rat sperm cells. Proc. Natl. Acad Sci. USA, **77**, 1546-1550.

Davis, B.K., Byrne, R. and Hungund, B. (1979). Studies on the mechanism of capacitation. II. Evidence for lipid transfer between plasma membrane of rat sperm and serum albumin during capacitation in vitro. Biochem. Biophys. Acad., **558**, 257-266.

Davis, B.K. and Gergely, A.F. (1979). Studies on the mechanism of capacitation: changes in plasma membrane proteins if rat spermatozoa during incubation in vitro. Biochem. Biophys. Res. Commun., **88**, 613-618.

Denny, P.C. and Tyler, A. (1964). Activation of protein biosynthesis in non-nucleate fragments of sea urchin eggs. Biochem. Biophys. Res. Comm., **14**, 245-249.

De Sousa, P.A., Caveney, A., Westhusin, M.E. and Watson, A.J. (1998). Temporal patterns of embryonic gene expression and their dependence on oogenetic factors. Theriogenology, **49**, 115-128.

De Sousa, P.A., Valdimarsson, G., Nicholson, B. and Kidder, G.M. (1993). Connexin trafficking and the control of gap junction assembly in mouse preimplantation embryos. Development, **117**, 1355-1367.

De Vita, R., Calugi, A., Chiarantano, C., Forte, D., Mauro, F. and Uccelli, R. (1990). Effects on mouse spermatogenesis monitored by flow cytometry. Int. J. Hyperthermia, **6**, 543-551.

De Vita, R., Calugi, A., Forte, D., Chiarantano, C., Uccell, R., Vizzone, A. and Mauro, F. (1987). Cellular effects of heat on mouse spermatogenesis. In "Clinical Cytometry and Histometry". Eds. G. Burger, J.S. Ploem and K. Goerttler. Academic Press, London, 300-302.

Doerksen, T. and Trasler, J. (1996). Developmental exposure of male germ cells to 5-azacytidine results in abnormal preimplantation embryo development in rats. Biol. Reprod., **55**, 1155-1162.

Doerr, P. and Pirke, K.M. (1976). Cortisol-induced suppression of plasma testosterone in normal adult males. J. Clin. Endocrinol. Metab., **43**, 622-629.

Domon, M. (1980). Cell cycle-dependent radiosensitivity in two-cell mouse embryos in culture. Radiat. Res., **81**, 236-248.

Downey, B.R., Pierce, A.R.J and Sandford, L.M. (1984). Seasonal differences in pituitary and testicular function in bulls. Proc. 10th Int. Congr. Anim. Reprod. and AI, Urbana, USA, **2**, 146.

Ducibella, T., Albertini, D.F., Anderson, E. and Biggers, J.D. (1975). The preimplantation mammalian embryo: characterization of intercellular junctions and their appearance during development. Dev. Biol., **45**, 231-250.

Dulic, V., Lees, E. and Reed, S.I. (1992). Association of human cyclin E with a periodic G1-S phase protein kinase. Science, **257**, 1958-1961.

Dutt, R.H. and Hamm, P.T. (1957). Effect of exposure to high environmental temperature and shearing on semen production of rams in winter. J. Anim. Sci., **16**, 328-334.

Dutt, R.H. and Simpson, E.C. (1957). Environmental temperature and fertility of Southdown rams early in the breeding season. J. Anim. Sci., **16**, 136-143.

Ebert, K.M. and Brinster, R.L. (1983). Rabbit α-globin messenger RNA translation by the mouse ovum. J. Embryol. Exp. Morph., **74**, 159-168.

Edgar, B.A., Kiehle, C.P. and Schubiger, G. (1986). Cell cycle control by the nucleo-cytoplasmic ratio in early Drosophila development. Cell, **44**, 365-372.

Edwards, J.L. and Hansen, P.J. (1996). Elevated temperature increases heat shock protein 70 synthesis in bovine two-cell embryos and compromises function of maturing oocytes. Biol. Reprod., **55**, 340-346.

Edwards, R.G., Bavister, B.D. and Steptoe, P.C. (1969). Early stages of fertilization in vitro of human oocytes matured in vitro. Nature, **221**, 632-635.

Eik-Nes, K.B. (1968). Effects of the temperature changes on secretion of testosterone by the canine testis in vivo. Proc. 6th Int. Congr. Anim. Reprod. and AI, Paris, **1**, 269.

Einarsson, S. and Larsson, K. (1980). Endocrine effects of elevated ambient temperature in boars. Proc. 9th .Int. Congr. Anim. Reprod. and AI, Madrid, **4**, 736-740.

Ekpe, G., Seamark, R.F., Sowerbutts, S.F. and Setchell, P.B. (1992). Effect of intermittent scrotal insulation on fertilizing ability of ram spermatozoa and the development of the embryo to blastocysts in vitro. Proc. Aust. Soc. Reprod. Biol., **24**, 51.

Ekpe, G., Zupp, J.L., Seamark, S.F. and Setchell, B.P. (1993). Fertilising ability of spermatozoa from rams subjected to intermittent scrotal insulation and development of the resultant embryos in vitro. Proc. Aust. Soc. Reprod. Biol., **25**, 88.

Elfving, G. (1950). Effects of the local application of heat on the physiology of testis. An Experimental Study in Rats Doctoral Thesis, Helsinki.

El-Shershaby, A.M. and Hinchliffe, J.R. (1975). Epithelial autolysis during implantation of the mouse blastocyst: an ultrastructural study. J. Embryol. Exp. Morph., **33**, 1067-1080.

Epstein, C.J. (1975). Gene expression and macromolecular synthesis during preimplantation embryonic development. Biol. Reprod., **12**, 82-105.

Epstein, C.J. and Smith, S.A. (1973). Amino acid uptake and protein synthesis in preimplantation mammalian embryos. Dev. Biol., **33**, 171-184.

Epstein, C.J. and Smith, S.A. (1974). Electrophoretic analysis of proteins synthesized by preimplantation mouse embryos. Dev. Biol., **40**, 233-244.

Erbach, G.T., Lawitts, J.A., Papaioannou, V.E. and Biggers, J.D. (1994). Differential growth of the mouse preimplantation embryo in chemically defined media. Biol. Reprod., **50**, 1027-1033.

Esposito, G. (1984). Intestinal permeability of water-soluble nonelectrolytes: sugars, amino acids, peptides. In "Pharmacology of Intestinal Permeability", Ed. T. Z. Czaky. Springer-Verlag, New York, 567-611.

Evans, J.P., Schultz, R.M. and Kopf, G.S. (1995). Identification and localization of integrin subunits in oocytes and eggs of the mouse. Mol. Reprod. Dev., **40**, 211-220.

Evenson, D.P. (1989). Flow cytometry evaluation of male germ cells. In "Flow Cytometry Advanced Research and Clinical Applications. Vol I". Ed. A. Yen. CRC Press, Boca Raton, Florida, 217-246.

Evenson, D.P. (1990). Flow cytometry assays of male fertility. In "Methods in Cell Biology. Vol 33, Flow Cytometry". Eds. Z. Darzynkiewicz, H. Crissman. Academic Press, New York, 401-410.

Evenson, D.P., Darzynkiewicz, Z. and Melamed, M.R. (1980). Relation of mammalian sperm chromatin heterogeneity to fertility. Science, **210**, 1131-1133.

Evenson, D.P., Higgins, P.H., Grueneburg, D. and Ballachey, B.E. (1985). Flow cytometric analysis of mouse spermatogenic function following exposure to ethylnitrosourea. Cytometry, 238-253.

Evenson, D.P. and Jost, L.K. (1994). Sperm chromatin structure: DNA denaturability. In "Flow Cytometry. 2nd ed. A Volume of Methods in Cell Biology. Vol 42". Eds. Z. Darzynkiewicz, J.P. Robinson and H.A. Crissman. Academic Press, Orlando, Florida, 159-176.

Evenson, D.P., Jost, L.K., Marshall, D., Zinaman, M.J., Clegg, E. and Purvis, K. (1999). Utility of the sperm chromatin structure assay (SCSA) as a diagnosis and prognostic tool in the human fertility clinic. Hum. Reprod., **14**, 1039-1049.

Evenson, D.P., Thompson, L. and Jost, L. (1994). Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. Theriogenology, **41**, 637-651.

Farach, M.C., Tang, J.P., Decker, G.L. and Carson, D.D. (1987). Heparin/heparan sulfate is involved in attachment and spreading of mouse embryos in vitro. Dev. Biol., **123**, 401-410.

Farooqui, A.A. (1978). Metabolism and role of sulpholipids in mammals. Int. J. Biochem., **9**, 709-716.

Fassler, R. and Meyer, M. (1995). Consequences of lack of beta 1 integrin gene expression in mice. Genes Dev., **9**, 1896-1908.

Fields, M.J., Burns, W.C. and Warwick, A.C. (1979). Age, season and breed effects on testicular volume and semen traits in young beef bulls. J. Anim. Sci., **48**, 1299.

Fielding, C.J. and Fielding, P.E. (1981). Evidence for a lipoprotein carrier in human plasma catalyzing sterol efflux from cultured fibroblasts and its relationship to lecithin: cholesteryl acyltransferase. Proc. Natl. Acad Sci. USA, **78**, 3911-3914.

Finn, C.A. and Martin, L. (1971). Endocrine control of the proliferation and secretion of uterine glands in the mouse. Acta Endocrinol. (suppl), **155**, 139.

Fissore, R., Jackson, K.V. and Kiessling, A.A. (1989). Mouse zygote development in culture medium without protein in the presence of ethylenediaminetetraacetic acid. Biol. Reprod., **41**, 835-841.

Flach, G., Johnson, M.H., Braude, P.R., Taylor, R.A.S. and Bolton, V.N. (1982). The transition from maternal to embryonic control in the 2-cell mouse embryo. EMBO J., **1**, 681-686.

Fleming, T.P., McConnel, J., Johnson, M.H. and Stevenson, B.R. (1989). Development of tight junctions de novo in the mouse early embryo: control of assembly of the tight junction-specific protein, ZO-1. J. Cell Biol., **108**, 1407-1418.

Fleming, T.P., Qamar, J., Collins, J. and Hay, M. (1993). Biogenesis of structural intercellular junctions during cleavage in the mouse embryo. J. Cell Sci., (suppl.), **17**, 119-125.

Fogelman, A.M., Seager, J., Edwards, P.A. and Popjak, G. (1977). Mechanism of induction of 3-hydroxy-3-methylglutaryl coenzyme: a reduction in human leukocytes. J. Biol. Chem., **252**, 644-651.

Forlani, S., Bonnerot, C., Capgras, S. and Nicolas, J.F. (1998). Relief of a repressed gene expression state in the mouse 1-cell embryo requires DNA replication. Development, **125**, 3153-3166.

Fowler, D.G. (1965). Semen quality of Merino rams. Aust. J. Exp. Agric. Anim. Husb., **5**, 243-246.

Fowler, D.G. and Dun, R.B. (1966). Skin folds and Merino breeding 4. The susceptibility of rams selected for a high degree of skin wrinkle to heat induced infertility. Aust. J. Exp. Agric. Anim. Husb., **6**, 121-127.

Fowler, R.E., Kaufman, M.H. and Grainge, C. (1986). The secretions of the cumulus-oocyte complex in relation to fertilization and early mouse embryonic development: a histochemical study. Histochem. J., **18**, 541-550.

Fraser, L.R. and Maudlin, I. (1979). Analysis of aneuploidy in first-cleavage mouse embryos fertilized in vitro and in vivo. Environ. hlth. Perspect., **31**, 141-149.

Fraser, L.R. and McDermott, C.A. (1992). Ca^{2+}-related changes in the mouse sperm capacitation state: a possible role for Ca^{2+}-ATPase. J. Reprod. Fetrtil., **96**, 363-377.

Fridd, C.W., Murphy, J., Linke, C.A. and Bonfoglio, T.A. (1975). Response of rat testis to localized induced hyperthermia. Urology, **5**, 76-82.

Fujisawa, M., Matsumoto, O., Kamidono, S., Hirose, F., Kojima, K. and Yoshida, S. (1988). Changes of enzymes involved in DNA synthesis in the testes of cryptorchid rats. J. Reprod. Fertil., **84**, 123-130.

Fuxe, K. and Nilssons, O. (1963). The effect of oestrogen on the histology of the uterine epithelium of the mouse. Exp. Cell. Res., **32**, 109-117.

Galil, K.A.A. and Setchell, B.P. (1987a). Effects of local heating of the testes on the concentration of testosterone in jugular and testicular venous blood of rats and on testosterone production in vitro. Int. J. Androl., **11**, 67-72.

Galil, K.A.A. and Setchell, B.P. (1987b). Effects of local heating of the testis on testicular blood flow and testosterone secretion in the rat. Int. J. Androl., **11**, 73-85.

Gangrade, B.K. and Dominic, C.L. (1984). Oestrus induction in unisexually grouped mice by multiple short-term exposures to males. Biol. Reprod., **31**, 89-96.

Garbutt, C.L., Chisholm, J.C. and Johnson, M.H. (1987). The establishment of the embryonic-abembryonic axis in the mouse embryo. Development, **100**, 125-134.

Gardner, H.G. and Kaye, P.C. (1984). Effects of insulin on preimplantation mouse embryos. Proc. 16th. Ann. Congr. Aust. Soc. Reprod. Biol., 107.

Gardner, D.K. and Leese, H.J. (1988). The role of glucose and pyruvate transport in regulating nutrient utilization by preimplantation mouse embryos. Development, **104**, 423-429.

Gardner, D.K. and Leese, H.J. (1990). Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. J. Reprod. Fertil., **88**, 361-368.

Gardner, D.K. and Sakkas, D. (1993). Mouse embryo cleavage, metabolism and viability: role of medium composition. Hum. Reprod., **8**, 288-295.

Garriott, M.L. and Chrisman, C.L. (1980). Hyperthermia induced dissociation of the X-Y bivalent in mice. Environ. Mutagen., **2**, 465-471.

Garriott, M.L. and Chisman, C.L. (1981). Investigation of hyperthermia-induced dominant lethal mutations in male mice. J. Heredity, **72**, 338-342.

Gasinska, A. and Hill, S. (1990). The effect of hyperthermia on the mouse testis. Neoplasma, **37**, 357-366.

Geiger, B. and Ayalon, O. (1992). Cadherins. Annu. Rev. Cell Biol., **8**, 81-116.

Gething, M.J. and Sambrook, J. (1992). Protein folding in the cell. Nature, **355**, 33-45.

Girard, F., Strausfeld, U., Fernandez, A. and Lamb, N.J.C. (1991). Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. Cell, **67**, 1169-1179.

Golbus, M.S., Calarco, P.G. and Epstein C.J. (1973). The effects of inhibitors of RNA synthesis (a-amanitin and actinomycin D) on preimplantation mouse embryogenesis. J. Exp. Zool., **186**, 207-216.

Gonzales, G.F., Risbridger, G.P., and de Krester, D.M. (1989). In vivo and in vitro production of inhibin by cryptorchid testes from adult rats. Endocrinol., **124**, 1661-1668.

Gordon, M., Dandekar, P.V. and Bartoszewicz, W. (1974). Ultrastructural localization of surface receptor for concanavalin A on rabbit spermatozoa. J. Reprod. Fertil., **36**, 211-214.

Gordon, M., Morris, E.G. and Young, R.J. (1983). The localization of Ca^{2+}-ATPase and Ca^{2+} binding proteins in the flagellum of guinea pig sperm. Gamete Res., **8**, 49-55.

Goswami, A., Kar, A.B. and Chowdhury, S.R. (1963). Uterine lipid metabolism in mice during the oestrous cycle: effect of ovariectomy and replacement therapy. J. Reprod. Fertil., **6**, 287-295.

Green, C.M., Cockle, S.M., Watson, P.F. and Fraser, C.R. (1996). A possible mechanism of action for fertilization promoting peptide, a TRH-related tripeptide that promotes capacitation and fertilizing ability in mammalian spermatozoa. Mol. Reprod. Dev., **45**, 244-252.

Grippo, P., Geremia, R., Locorotondo, G. and Monsei, V. (1978). DNA-dependent DNA polymerase species in male germ cells of the mouse. Cell Differentiation, **7**, 237-248.

Griswold, M.D. (1995). Interactions between germ cells and Sertoli cells in the testis. Biol. Reprod., **52**, 211-216.

Gruppi, C.M. and Wolgemuth, D.J. (1993). HSP86 and HSP84 exhibit cellular specificity of expression and co-precipitate with an HSP70 family member in the murine testis. Dev. Genet., **14**, 119-126.

Gueth-Hallonet, C. and Maro, B. (1992). Cell polarity and cell diversification during early mouse embryogenesis. Reviews, **8**, 274-279.

Gulyas, B.J. and Schnell, E.D. (1980). Ovoperoxidase activity in ionophore treated mouse eggs. I. Electron microscopic localization. Gamete Res., **3**, 267-277.

Gwatkin, R.B.L., Andersen, O.F. and Hutchinson, C.F. (1972). Capacitation of hamster spermatozoa in vitro: the role of cumulus components. J. Reprod.Fertil., **30**, 389-394.

Gwatkin, R.B.L., Andersen, O.F. and Williams, D.T. (1974). Capacitation of mouse spermatozoa in vitro: involvement of epididymal secretions and cumulus oophorus. J. Reprod. Fertil., **41**, 253-256.

Gyllenborg, J., Skakkebaek, N.E., Nielsen, N.C., Keiding, N. and Giwercman, A. (1999). Secular and seasonal changes in semen quality among young Danish men: a statistical analysis of semen samples from 1927 donor candidates during 1977-1995. Int. J. Androl., **22**, 28-36.

Haig, D. (1992). Genomic imprinting and the theory of parent-offspring conflict. Semin. Dev. Biol., **3**, 153-160.

Haig, D. and Westoby, M. (1989). Parent-specific gene expression and the triploid endosperm. American Naturalist, **134**, 147-155.

Hall, K. (1975). Lipids in the mouse uterus during pregnancy. J. Endocrinol., **65**, 233-243.

Hall, R.W. and Gomes, W.R. (1975). The effect of artificial cryptorchidism on serum oestrogen and testosterone levels in the adult male rats. Acta Endocrinol., **80**, 583-591.

Hames, B.D. and Rickwood, D. (1983). Gel electrophoresis of proteins: a practical approach. Publ. IRL Press, Oxford, 162-163.

Hamner, C.E. and Sojka, N.J. (1967). Capacitation of rabbit spermatozoa, species and organ specificity. Proc. Soc. Exp. Biol. Med., **124**, 689-691.

Handyside, A.H. (1980). Distribution of antibody- and lectin-binding sites on dissociated blastomeres from mouse morulae: evidence of polarization at compaction. J. Embryol. Exp. Morph., **60**, 99-116.

Handyside, A.H. (1981). Immunofluorescence techniques for determining the numbers of inner and outer blastomeres in mouse morulae. J. Reprod. Immunol., **2**, 339-350.

Handyside, A.H. and Hunter, S. (1984). A rapid procedure for visualising the inner cell mass and trophectoderm nuclei of mouse blastocysts in situ using polynucleotide-specific fluorochromes. J. Exp. Zool., **231**, 429-434.

Handyside, A.H. and Johnson, M.H. (1978). Temporal and spatial pattern of the synthesis of tissue-specific polypeptides in the preimplantation mouse embryos. J. Embryol. Exp. Morph., **87**, 27-45.

Harayuchi, S., Naito, K., Azuma, S. Sato, E., Nagahama, Y., Yamashita, M. and Toyota, Y. (1996). Effects of phosphate on in vitro 2-cell block of AKR/N mouse embryos based on changes in cdc2 kinase activity and phosphorylation states. Biol. Reprod., **55**, 598-603.

Harper, M.J.K. (1994). Gamete and zygote transport. In "The Physiology of Reproduction". Eds. E. Knobil and J. D. Neill. Raven Press, New York, 1, II, 123-187.

Harrison, R.A.P., Ashworth, P.J.C. and Miller, N.G.A. (1996). Bicarbonate/CO_2 an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. Mol. Reprod. Dev., **45**, 378-391.

Harvey, M.B. and Kaye, P.L. (1988). Insulin stimulates protein synthesis in compacted mouse embryos. Endocrinol., **122**, 1182-1184.

Harvey, M.B. and Kaye, P.L. (1990). Insulin increases the cell number of the inner cell mass and stimulates morphological development of mouse blastocysts in vitro. Development, **110**, 963-967.

Harvey, M.B. and Kaye, P.L. (1991). Visualisation of insulin receptors on mouse preimplantation embryos. Reprod. Fertil. Dev., <u>3</u>, 9-15.

Harvey, M.B. and Kaye, P.L. (1992). Insulin-like growth factor-1 stimulates growth of mouse preimplantation embryos in vitro. Mol. Reprod. Dev., <u>31</u>, 195-199.

Hasler, J.F., Henderson, W.B., Hurtgen, P.J., Jin, Z.Q., McCauley, A.D., Mower, S.A., Neely, B., Shuey, L.S., Stokes, J.E. and Trimmer, S.A. (1995). Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology, <u>43</u>, 141-152.

Henriksen, K., Hakovirta, H. and Parvinen, M. (1995). In-situ quantification of stage-specific apoptosis in the rat seminiferous epithelium: effects of short-term experimental cryptorchidism. Int. J. Androl., <u>18</u>, 256-262.

Hershey, J.W.B. (1991). Translational control in mammalian cells. Ann Rev. Biochem., <u>60</u>, 717-755.

Heyner, S., Mattson, B.A., Smith, R.M. and Rosenblum, I.Y. (1989). Insulin and insulin-like growth factors in mammalian development. In "Growth Factors in Mammalian Development". Eds. I.Y. Rosenblum, S. Heyner. CRC Press, Boca Raton, 19-32.

Hill, D.P., Shakes, D.C., Ward, S. and Strome, S. (1989). A sperm-supplied product essential for initiation of normal embryogenesis in Caenorhabditis elegans is encoded by the paternal-effect embryonic-lethal gene spe-11. Dev. Biol., <u>136</u>, 154-166.

Hillard, M.A. and Bindon, B.M. (1975). Plasma LH patterns in cryptorchid rams and wethers. J. Reprod. Fertil., <u>43</u>, 379-380.

Ho, Y., Doherty, A.S. and Schultz, R.M. (1994). Mouse preimplantation embryo development in vitro: effect of sodium concentration in culture media on RNA synthesis and accumulation and gene expression. Mol. Reprod. Dev., <u>38</u>, 131-141.

Hochereau-de Reviers, M.T., Locatelli, A., Perreau, C., Pisselet, C. and Setchell, B.P. (1993). Effects of a single brief period of moderate heating of the testis on seminiferous tubules in hypophysectomized rams treated with pituitary extract. J. Reprod. Fertil., **97**, 381-387.

Hoefer Scientific Instruments (1994). Two-dimensional gel electrophoresis. In"Protein Electrophoresis: Application Quide." Published and Printed in USA, 65-76.

Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994). Manipulation the mouse embryo. Cold Spring Harbor Laboratory, New York, 20-113.

Hogan, B., Costantini, F. and Lacy, E. (1986). Chemicals and supplies. In"Manipulating the Mouse Embryo: A Laboratory Manual." Cold Spring Harbor, Cold Spring Laboratory, New York, 271-277.

Hogan, A., Heyner, S., Charron, M.J., Copeland, N.J., Gilbert, D.J., Jenkins, N.A., Thorens, B. and Schultz, G.A. (1991). Glucose transporter gene expression in early mouse embryos. Development, **113**, 365-374.

Hotta, Y., Fujisawa, M., Tabata, S., Stern, H. and Yoshida, S. (1988). The effect of temperature on recombination activity in testes of rodents. Exp. Cell Res., **178**, 163-168.

Howarth, B. (1969). Fertility in the ram following exposure to elevated ambient temperature and humidity. J. Reprod. Fertil., **19**, 179-183.

Howarth, B., Alliston, C.W. and Ulberg, L.C. (1965). Importance of uterine environment on rabbit sperm prior to fertilization. J. Anim. Sci., **24**, 1027-1032.

Howe, C.C. and Solter, D. (1979). Cytoplasmic and nuclear protein synthesis in preimplantation mouse embryos. J. Embryol. Exp. Morph., **52**, 209-225.

Howlett, S.K. (1986). A set of proteins showing cell cycle development modification in the early mouse embryo. Cell, **70**, 387-396.

Howlett, S.K. and Bolton, V.N. (1985). Sequence and regulation of morphological and molecular events during the first cell cycle of mouse embryogenesis. J. Embryol. Exp. Morph., **87**, 175-206.

Hunter, R.H.F. (1988). The fallopian tubes: their role in fertility and infertility. Springer-Verlag, Berlin.

Hunter, R.H.F. (1996). Ovarian control of very low sperm/egg ratios at the commencement of mammalian fertilization to avoid polyspermy. Mol. Reprod. Dev., **44**, 417-422.

Hunter, R.H.F. and Dziuk, P.J. (1968). Sperm penetration of pig eggs in relation to timing of ovulation and insemination. J. Reprod. Fertil., **15**, 199-208.

Huxtable, R.J. (1992). Physiological actions of taurine. Physiol. Rev., **72**, 101-163.

Igboeli, G. and Rakha, A.M. (1971). Seasonal changes in the ejaculate characteristics of Angoni (short horn zebu) bulls. J. Anim. Sci., **33**, 651-654.

Inoue, M., Kobayashi, Y., Kaneko, M. and Fujii, A. (1984). Sperm-zona interaction in the mouse-special reference to the time of acrosome reaction. J. Mamm. Ova Res.(Japan), **1**, 77-80.

Inoue, M. and Wolf, D.P. (1975). Sperm binding characteristics of the murine zona pellucida. Biol. Reprod., **13**, 340-346.

Ishizuka, I., Suzuki, M. and Yamakawa, T. (1973). Isolation and characterization of a novel sulphoglycerolipid "seminolipid" from boar testis and spermatozoa. J. Biochem. (Tokyo), **73**, 77-87.

Iwamatsu, T. and Chang, M.C. (1969). In vitro fertilization of mouse eggs in the presence of bovine follicular fluid. Nature, **224**, 919-920.

Jackson, P.K., Chevalier, S., Philippe, M. and Kirschner, M.W. (1995). Early events in DNA replication require cyclin E and are blocked by p21. J. Cell Biol., **130**, 755-769.

Jacobs, A.L. and Carson, D.D. (1991). Proteoglycan synthesis and metabolism by mouse uterine stroma cultured in vitro. J. Biol. Chem., **266**, 15464-15473.

Jannes, P., Spiessens, C., Van der Auwera, I., D'Hooghe, T., Verhoeven, G. and Vanderschueren, D. (1998). Male subfertility induced by acute scrotal heating affects embryo quality in normal female mice. Hum. Reprod., **13**, 372-375.

Jansz, G.F. and Pomerantz, D.K. (1986). A comparison of Leydig cell function after unilateral and bilateral cryptorchidism and efferent-duct-ligation. Biol. Reprod., **34**, 316-321.

Jegou, B. (1993). The Sertoli-germ cell communication network in mammals. Int. Rev. Cytol., **147**, 25-96.

Jegou, B., Laws, A.O. and de Kretser, D.M. (1984). Changes in testicular function induced by short-term exposure of the rat testis to heat: further evidence for interaction of germ cells. Sertoli cells and Leydig cells. Int. J. Androl., **7**, 244-257.

Jegou, B., Pineau, C., Velez de la Calle, J.F., Touzalin, A.M., Bardin, C.W. and Cheng, C.Y. (1993). Germ cell control of testin production is inverse to that of other Sertoli cell products. Endocrinol., **132**, 2557-2562.

Jemiolo, B., Harvey, S. and Novotny, M. (1986). Promotion of the Whitten effect in female mice by synthetic analogs of male urinary constituents. Proc. Natl. Acad. Sci. USA, **83**, 4576-4579.

Johnson, L.V. and Calarco, P.G. (1980). Stage specific embryonic antigens detected by an antiserum against mouse blastocysts. Dev. Biol., **79**, 224-231.

Johnson, M.H. (1981). The molecular and cellular basis of preimplantation mouse development. Biol. Rev., **56**, 463-498.

Johnson, M.H. and Nasr-Esfahani, M.H. (1994). Radical solutions and cultural problems: could free oxygen radicals be responsible for the impaired development of mammalian embryos in vitro? BioEssays, <u>16</u>, 31-38.

Johnson, M.H. and Ziomek, C.A. (1983). Cell interactions influence the fate of mouse blastocysts undergoing the transition from the 16- to the 32-cell stage. Dev. Biol., <u>95</u>, 211-218.

Jurisicova, A., Latham, K.E., Casper, R.F. and Varmuza, S. (1998a). Expression and regulation of genes associated with cell death during murine preimplantation embryo development. Mol. Reprod. Dev., <u>51</u>, 243-253.

Jurisicova, A., Rogers, I., Fraciani, A., Casper, R.F. and Varmuza, S. (1998b). Effect of maternal age and conditions of fertilization on programmed cell death during murine preimplantation embryo development. Mol. Hum. Reprod., <u>4</u>, 139-145.

Jurisicova, A., Varmuza, S. and Casper, R.F. (1995). Involvement of programmed cell death in preimplantation embryo demise. Hum. Reprod. Update, <u>1</u>, 558-566.

Kandeel, F.R. and Swerdloff, R.S. (1988). Role of temperature in regulation of spermatogenesis and the use of heating as a method for contraception. Fertil. Steril., <u>49</u>, 1-23.

Karabinus, D.S., Vogler, C.J., Saacke, R.G. and Evenson, D.P. (1997). Chromatin structural changes in sperm after scrotal insulation of Holstein bulls. J. Androl., <u>18</u>, 549-555.

Kasanicki, M.A. and Pilch, P.F. (1990). Regulation of glucose-transporter function. Diabetes Care, <u>13</u>, 219-227.

Kerr, B.J., Rich, K.A. and de Kretser, D.M.(1979). Alterations of the fine structure and androgen secretion of the interstitial cells in the experimentally cryptorchid rat testis. Biol. Reprod., <u>20</u>, 409-422.

Kierszenbaum, A.L. (1994). Mammalian spermatogenesis in vivo and in vitro: a partnership of spermatogenic and somatic cell lineages. Endocrinol. Rev., **15**, 116-134.

Kitayama, T. (1965). Study on testicular temperature in man. Acta Urologica Japonica, **11**, 435-437.

Kline, D. (1996). Activation of the mouse egg. Theriogenology, **45**, 81-95.

Kornblatt, M.J. (1979). Synthesis and turnover of sulphogalactoglycerolipid, a membrane lipid, during spermatogenesis. Can. J. Biochem., **57**, 255-258.

Kramen, M.A. and Biggers, J.D. (1971). Uptake of tricarboxylic acid cycle intermediates by preimplantation mouse embryos in vitro. Proc. Natl. Acad. Sci. USA, **68**, 2656-2659.

Krishna, M. and Generoso, W.M. (1979). Timing of sperm penetration, pronuclear formation, pronuclear DNA synthesis, and first cleavage in naturally ovulated mouse eggs. J. Exp. Zool., **202**, 245-253.

Kruip, Th.A.M, den Dass, J.H.G. (1997). In vitro produced and cloned embryos: effect on pregnancy, parturition and offspring. Theriogenology, **47**, 43-52.

Krzanowska, H. (1974). The passage of abnormal spermatozoa through the uterotubal junction of the mouse. J. Reprod. Fertil., **38**, 81-90.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**, 680-685.

Lalumiere, C., Bleau, G., Chapdelaine, A. and Roberts, K.D. (1976). Cholesteryl sulfate and sterol sulfatase in the human reproductive tract. Steroids, **27**, 247-260.

Langlais, J. and Roberts, K.D. (1985). A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa. Gamete Res., **12**, 183-224.

Langlais, J., Zollinger, M., Plante, L., Chapedelaine, A., Bleau, G. and Roberts, K.D. (1981). Location of cholesterol sulfate in human spermatozoa in support of a hypothesis of the mechanism of capacitation. Proc. Natl. Acad. Sci. USA, **78**, 7266-7270.

Larsson, K. and Einarsson, S. (1984). Seminal changes in boars after heat stress. Acta Vet. Scand., **25**, 57-66.

Larsson, K., Einarsson, S., Lundstrom, K. and Hakkarainen, J. (1983). Endocrine effects of heat stress in boars. Acta. Vet. Scand., **24**, 305-314.

Larue, L., Ohsugi, M., Hirchenhain, J. and Kemler, R. (1994). E-cadherin null mutant embryos fail to form a trophectoderm epithelium. Proc. Natl. Acad. Sci. USA, **91**, 8263-8267.

Latham, K.E. (1999). Mechanisms and control of embryonic genome activation in mammalian embryos. Inter. Rev. Cytology, **193**, 71-124.

Latham, K.E., Garrels, J.I., Chang, C. and Solter, D. (1991). Quantitative analysis of protein synthesis in mouse embryos. I. Extensive reprogramming at the one- and two-cell stages. Development, **112**, 921-932.

Latham, K.E., Garrels, J.I. and Solter, D. (1994). Alteration in protein synthesis following transplantation of mouse 8-cell stage nuclei to enucleated 1-cell embryos. Dev. Biol., **163**, 341-350.

Latham, K.E., Rambhatla, L., Hayashizaki, Y. and Chapman, V.M. (1995). Stage-specific induction and regulation by genomic imprinting of the mouse U2afbp-rs gene during preimplantation development. Dev. Biol., **168**, 670-676.

Latham, K.E., Solter, D. and Schultz, R.M. (1992). Acquisition of a transcriptionally permissive state during the one-cell stage of mouse embryogenesis. Dev. Biol., **149**, 457-462.

Lawitts, J.A. and Biggers, J.D. (1991a). Optimization of mouse embryo culture media using simplex methods. J. Reprod. Fertil., **91**, 543-556.

Lawitts, J.A. and Biggers, J.D. (1991b). Overcome the 2-cell block by modifying components in mouse embryo culture medium. Biol. Reprod., **45**, 245-251.

Lawitts, J.A. and Biggers, J.D. (1992). Joint effects of sodium chloride, glutamine and glucose in mouse preimplantation embryo culture media. Mol. Reprod. Dev., **31**, 189-194.

Lawitts, J.A. and Biggers, J.D. (1993). Culture of preimplantation embryos. In "Methods in Enzymology". Eds. P.M. Wassarman and M.L. Depamphilis, V125 (Guide to Techniques in Mouse Development), Academic Press, San Diego, CA, 153-164.

Lee, D.Y., Hayes, J.J., Pruss, D. and Wolffe, A.P. (1993). A possible role for histone acetylation in transcription factor access to nucleosomal DNA. Cell, **72**, 73-84.

Lee, J., Richburg, J.H., Younkin, S.C. and Boekelheide, K. (1997). The Fas system is a key regulator of germ cell apoptosis in the testis. Endocrinol., **138**, 2081-2088.

Lee, M.A. and Storey, B.T. (1986). Evidence for plasma membrane impermeability to small ions in acrosome-intact mouse spermatozoa bound to mouse zona pellucida, using an aminoacridine fluorescent probe: time course of the zona-induced acrosome reaction monitored by both chlortetracycline and pH probe fluorescence. Biol. Reprod., **33**, 235-246.

Leese, H.J. (1991). Metabolism of the preimplantation mammalian embryo. Oxf. Rev. Reprod. Biol., **13**, 35-72.

Leese, H.J. (1995). Metabolic control during preimplantation mammalian development. Hum. Reprod., **1**, 63-72.

Leese, H.J. and Barton, A.M. (1984). Pyruvate and glucose uptake by mouse ova and preimplantation embryos. J. Reprod. Fertil., **72**, 9-13.

Legault, Y., Bleau, G., Chapdelaine, A. and Roberts, K.D. (1979a). The binding of sterol sulfates to hamster spermatozoa. Steroids, **27**, 247-260.

Legault, Y., Bouthillier, M., Bleau, G., Chapdelaine, A. and Roberts, K.D. (1979b). The sterol and sterol sulfate content of the male hamster reproductive tract. Biol. Reprod., **20**, 1213-1219.

Lemaire, L. and Heinlein, U.A.O. (1991). Detection of secreted and temporarily inducible heat shock responsive proteins in mouse testicular tissue. Life Sci., **48**, 365-372.

Levey, I.L., Stull, G.B. and Brinster, R.L. (1978). Poly (A) and synthesis of polyadenylated RNA in the preimplantation mouse embryo. Dev. Biol., **64**, 140-148.

Levine, R.J., Bordson, B.L., Mathew, R.M., Brown, M.H., Stanley, J.M. and Starr, T.B. (1988). Deterioration of semen quality during summer in New Orleans. Fertil. Steril., **49**, 900-907.

Levine, R.J., Mathew, R.M., Chenault, C.B., Brown, M.H., Hurtt, M.E., Bentley, K.S., Mohr, K.L. and Working, P.K. (1990). Differences in the quality of semen in outdoor workers during summer and winter. New Engl. J. Med., 12-16.

Levinson, J., Goodfellow, P., Vadeboncoeur, M. and Mcdevitt, H. (1978). Identification of stage-specific polypeptides synthesized during murine preimplantation development. Proc. Natl. Acad. Sci. U.S.A., **75**, 3332-3336.

Levy, R.I. (1981). Cholesterol, lipoproteins, apoproteins and heart disease: present status and future prospects. Clin. Chem., **27**, 653-662.

Lincoln, G.A., Lincoln, C.E., McNeilly, A.S. (1990). Seasonal cycles in the blood plasma concentration of FSH, inhibin and testosterone, and testicular size in rams of wild, feral and domesticated breeds of sheep. J. Reprod. Fertil., **88**, 623-633.

Lindenberg, S., Kimber, S.J. and Kallin, E. (1990). Carbohydrate binding properties of mouse embryos. J. Reprod. Fertil., **89**, 431-437.

Lindenberg, S., Sundberg, K., Kimber, S.J. and Lundblad, A. (1988). The milk oligosaccharide, lacto-N-fucopentaose I inhibits attachment of mouse blastocysts on endometrial monolayers. J. Reprod. Fertil., **83**, 149-158.

Lindquist, S. and Craig, E.A. (1988). The heat-shock proteins. Annu. Rev. Genet., **22**, 631-677.

Lipfert, L., Haimovich, M., Schaller, M.D., Cobb, B.S., Parson, J.T. and Brugge, J.S. (1992). Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125^{FAK} in platelets. J. Cell Biol., **119**, 905-912.

Lonergan, P., Carolan, C., Langendonckt, A.V., Donny, I., Khatir, H. and Mermillod, P. (1996). Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development in vitro. Biol. Reprod., **54**, 1420-1429.

Loutradis, D.K., John, D. and Kiessling, A.A. (1987). Hypoxanthine cause a 2-cell block in random-bred mouse embryo. Biol. Reprod., **37**, 311-316.

Lunstra, D.D. and Schanbacher, B.D. (1988). Testicular function and Leydig cell ultrastructure in long-term bilaterally cryptorchid rams. Biol. Reprod., **38**, 211-220.

Luthardt, F.W. and Donahue, R.P. (1973). Pronuclaer DNA synthesis in mouse eggs. Exp. Cell Res., **82**, 143-151.

Luthardt, F.W. and Donahue, R.P. (1975). DNA synthesis in developing two-cell mouse embryos. Dev. Biol., **44**, 210-216.

MacPhee, D.J., Barr, K.J., De Sousa, P.A., Todd, S.D.L. and Kidder, G.M. (1994). Regulation of Na/K-ATPase α subunit gene expression during mouse preimplantation development. Dev. Biol., **162**, 259-266.

McLaren, T.T., Foster, P.M.D. and Sharpe, R.M. (1994). Identification of stage-specific changes in protein secretion by isolated seminiferous tubules from rats following exposure to short-term local testicular heating. J. Reprod. Fertil., **102**, 293-300.

Main, S.J., Davies, R.V. and Setchell, B.P. (1978). Feedback control by the testis of gonadotrophin secretion: an examination of inhibin hypothesis. J. Endocrinol., **79**, 255-270.

Main, S.J. and Setchell, B.P. (1980). Responsiveness of the pituitary gland to androgens and of the testis to gonadotrophins following damage to spermatogenesis in the rats. J. Endocrinol., **87**, 445-454.

Majumder, S., Miranda, M. and DePamphilis, M.L. (1993). Analysis of gene expression in mouse preimplantation embryos demonstrates that the primary role of enhancers is to relieve repression of promoters. EMBO J., **12**, 1131-1140.

Majumder, S., Zhao, Z., Kaneko, K. and DePamphilis, M.L. (1997). Developmental acquisition of enhancer function requires a unique coactivator activity. EMBO J., **16**, 1721-1731.

Malmgren, L. (1990). Experimentally induced testicular alterations in boars: hormonal changes in mature and peripubertal boars. Acta Vet. Scand., **31**, 97-107.

Malmgren, L. and Larsson, K. (1984). Semen quality and fertility after heat stress in boars. Acta Vet. Scand., **25**, 425-435.

Malmgren, L. and Larsson, K. (1989). Experimentally induced testicular alterations in boars: histological and ultrastructural findings. J. Vet. Med., **A36**, 3-14.

Manejwala, F.M., Logan, C.F. and Schultz, R.M. (1991). Regulation of hsp 70 mRNA levels during oocyte maturation and zygotic gene activation in the mouse. Dev. Biol., **144**, 301-308.

Manes, C. and Daniel, Jr.J.C. (1969). Quantitative and qualitative aspects of protein synthesis in the preimplantation rabbit embryo. Exp. Cell Res., **55**, 261-268.

Marcello, S., Bonde, J.P., Hjollund, H.I., Kolstad, H.A., Cordelli, E., Leter, G. and The Danish First Pregnancy Planner Study Team (2000). Sperm chromatin damage impairs human fertility. Fertil. Steril., **73**, 43-50.

Marston, J.H. and Kelly, W.A. (1968). Time relationship of spermatozoa penetration into the egg of the rhesus monkey. Nature, **217**, 1073-1074.

Matsumoto, K., Anzai, M., Nakagata, N., Takahashi, A., Takahashi, Y. and Miyata, K. (1994). Onset of paternal gene activation in early mouse embryos fertilized with transgenic mouse sperm. Mol. Reprod. Dev., **39**, 136-140.

Matsushime, H., Quelle, D.E., Shurtleff, A.S., Shibuya, M., Sherr, C.J. and Kato, J-Y (1994). D-type cyclin-dependent kinase activity in mammalian cells. Mol. Cell Biol., **14**, 2066-2076.

Mattner, P.E. (1963). Capacitation of ram spermatozoa and penetration of the ovine egg. Nature, **199**, 772-773.

Mattson, B.M., Rosenblum, I.Y., Smith, R.M. and Heyner, S. (1988). Autoradiographic evidence for insulin and insulin-like growth factor binding to early mouse embryos. Diabetes, **37**, 585-589.

Maudlin, I. and Fraser, L.R. (1978). The effect of sperm and egg genotype on the incidence of chromosomal anomalies in mouse embryos fertilized in vitro. J. Reprod. Fertil., **52**, 107-112.

Mayor, R. and Izquierdo, L. (1994). Morulae at compaction and the pattern of protein synthesis in mouse embryos. Differentiation, **55**, 175-184.

Mehta, T.S. and Kiessling, A.A. (1990). Development potential of mouse embryos conceived in vitro and cultured in ethylenediaminetetraacetic acid with or without amino acids or serum. Biol. Reprod., 43, 600-606.

Meistrich, M.L., Eng, V.W.S. and Loir, M. (1973). Temperature effects on the kinetics of spermatogenesis in the mouse. Cell Tissue Kinet., 6, 379-393.

Meistrich, M.L., Hughes, T.J. and Bruce, W.R. (1975). Alteration of epididymal sperm transport and maturation in mice by oestrogen and testosterone. Nature, 258, 145-147.

Meuckler, M. (1990). Family of glucose-transporter genes: implication for glucose homeostasis and diabetes. Diabetes, 39, 6-11.

Meyerson, M. and Harlow, E. (1994). Identification of G1 kinase activity for cdk6, a novel cyclin D partner. Mol. Cell Biol., 14, 2077-2096.

Mieusset, R. and Bujan, I. (1995). Testicular heating and its possible contributions to male infertility: a review. Int. J. Androl., 18, 169-184.

Mieusset, R., Quintana Casares, P., Sanchez Partida, L.G., Sowerbutts, S.F., Zupp, J.L. and Setchell, B.P. (1992). The effects of heating the testes and epididymides of rams by scrotal insulation on fertility and embryonic mortality in ewes inseminated with frozen semen. J. Reprod. Fertil., 94, 337-343.

Miljovic, V., Veselinovic, S., Mrvos, G., Perkucin, R., Jouanovic, V., Stojadinovic, V.and Bacic, M. (1980). Results of investigation of fertile capability of the bull sperm in the course of the year. Proc. 9th Int. Congr. Anim. Reprod. and AI, Madrid, 3, 289.

Miller, B.G. and Emmens, C.W. (1969). The effect of oestradiol and progesterone on the incorporation of tritiated uridine into the genital tract of the mouse. J. Endocrinol., 43, 427-436.

Mincheva-Nilsson, L., Hammarstrom, S. and Hammarstrom, M.L. (1992). Human decidual leukocytes from early pregnancy contain high numbers of $\gamma\delta^+$ cells and show selective down-regulation of alloreactivity. J. Immunol., **149**, 2203-2211.

Molls, M., Zamboglon, N. and Streffer, C. (1983). A comparison of the cell kinetics of preimplantation mouse embryos from two different mouse strains. Cell Tiss. Kinet., **16**, 277-283.

Moore, G.D., Ayabe, T., Kopf, G.S. and Schultz, R.M. (1996). Temporal patterns of gene expression of G1-S cyclins and cdks during the first and second mitotic cell cycles in mouse embryos. Mol. Reprod. Dev., **45**, 264-275.

Moore, G.D., Ayabe, T., Visconti, P.E., Schultz, R.M. and Kopf, G.S. (1994). Roles of heterotrimeric and monomeric G proteins in sperm-induced activation of mouse eggs. Development, **120**, 3313-3323.

Moore, G.P.M. (1975). The RNA polymerase activity of the preimplantation mouse embryo. J. Embryol. Exp. Morph., **34**, 291-298.

Moore, T. and Haig, D. (1991). Genomic imprinting in mammalian development: a parental tug-of-war. Trends Gen., **7**, 45-89.

Moos, J., Visconti, P.E., Moore, G.D., Schultz, R.M. and Kopf, G.K. (1995). Potential role of mitogen-activated protein kinase in pronuclear envelope assembly and disassembly following fertilization of mouse eggs. **53**, 692-699.

Morison, I.M. and Reeve, A.E. (1998). A catalogue of imprinted genes and parent-of-origin effects in humans and animals. Hum. Mol. Genet., **7**, 1599-1609.

Moule, G.R. and Knarp, B. (1950). Observation on intratesticular temperatures of Merino rams. Aust. J. Agric. Res., **1**, 456 - 464.

Moule, G.R. and Waites, G.M.H. (1963). Seminal degeneration in the ram and its relation to the temperature of the scrotum. J. Reprod. Fertil., **5**, 433-446.

MRC (1990). Working Party on Children conceived by in vitro fertilization. Births in Great Britain resulting from assisted conception, 1978-87. Brit. Med. J., **300**, 1229-1233.

Munabi, A.K., Cassorla, F.G., D'Agata, R., Albertson, B.D., Loriaux, D.L. and Lipsett, M.B. (1984). The effects of temperature on the activity of testicular steroidogenic enzymes. Steroids, **43**, 325.

Munro, S.B. and Blaschuk, O.W. (1996). A comprehensive survey of the cadherins expressed in the testes of fetal, immature, and adult mice utilizing the polymerase chain reaction. Biol. Reprod., **55**, 822-827.

Nagao, Y., Saeki, K., Hoshi, M., Takahashi, Y. and Kanagawa, H. (1995). Effects of water quality on in vitro fertilization and development of bovine oocytes in protein-free medium. Theriogenology, **44**, 433-444.

Nakamura, M. and Hall, P.F. (1980). The mechanism by which body temperature inhibits protein biosynthesis in spermatids of rat testes. J. Biol. Chem., **255**, 2907-2913.

Nakamura, M., Romrell, L.J. and Hall, P.F. (1978). The effects of temperature and glucose on protein biosynthesis by immature (round) spermatids from rat testes. J. Cell Biol., **79**, 1-9.

Navara, C.S., Simerly, C., Zoran, S. and Schatten, G. (1995). The sperm centrosome during fertilization in mammals: implications for fertility and reproduction. Reprod. Fertil. Dev., **7**, 747-754.

Neill, J.M. and Olds-Clarke, P. (1987). A computer-assisted assay for mouse sperm hyperactivation demonstrates that bicarbonate but not bovine serum albumin is required. Gamete Res., **18**, 121-140.

Newport, J. and Kirschner, M. (1982a). A major developmental transition in early Xenopus embryos. I. Characterization and timing of cellular changes at the midblastula stage. Cell, **30**, 675-686.

Newport, J. and Kirschner, M. (1982b). A major developmental transition in early Xenopus embryos. II. Control of onset of transcription. Cell, **30**, 687-696.

Nicolson, G.L., Yanagamachi, R. and Yanagamachi, H. (1975). Ultrastructural localisation of lectin-binding sites on the zonae pellucidae and plasma membranes of mammalian eggs. J. Cell Biol., **66**, 263-274.

Nicolson, G.L., Usui, N., Yanagimachi, R., Yanagimachi, H. and Smith, J.R. (1977). Lectin-binding sites on the plasma membrane of rabbit spermatozoa: changes in surface receptors during epididymal maturation and after ejaculation. J. Cell Biol., **74**, 950-962.

Nigg, E.A. (1995). Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. Bioessays, **17**, 471-480.

Nilsson, O. (1958). Ultrastructure of mouse uterine surface epithelium under different estrogenic influences. 1. Spayed animals and oestrus animals. J. Ultrastruct. Res., **1**, 375-396.

Nilsson, O. (1959). Ultrastructure of mouse uterine surface epithelium under different estrogenic influences. 4. Uterine secretion. J. Ultrastruct. Res., **2**, 331-341.

Nishimune, Y. and Komatsu, T. (1972). Temperature sensitivity of mouse testicular DNA synthesis in vitro. Exp. Cell Res., **75**, 514-517.

Niwa, K. (1993). Effectiveness of in vitro maturation and in vitro fertilization techniques in pigs. J. Reprod. Fertil. (suppl), **48**, 49-59.

Nothias, J-Y., Miranda, M. and Depamphilis, M.L. (1996). Uncoupling of transcription and translation during zygotic gene activation in the mouse. EMBO J., **15**, 5715-5725.

Noyes, R.W. (1953). Fertilizing capacity of spermatozoa. Western. J. Surg. Obstet. Gynecol., **61**, 342-349.

Noyes, R.W., Walton, A. and Adams, C.E. (1958). Capacitation of rabbit spermatozoa. J. Endocrinol., **17**, 374-380.

Oakberg, E.F. (1957). Duration of spermatogenesis in the mouse. Nature, **180**, 1137-1138.

O'Farrell, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem., **250**, 4007-4021.

Ohtsubo, M., Theodoras, A.M., Schumacher, J., Roberts, J.M. and Pagano, M. (1995). Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Mol. Cell Biol., **15**, 2612-2624.

Oram, J.F., Albers, J.J., Cheung, M.C. and Bierman, E.C. (1981). The effects of subfractions of high density lipoproteins on cholesterol efflux from cultured fibroblasts. J. Biol. Chem., **256**, 8348-8356.

O'Rand, M.G. and Fisher, S.J. (1987). Localization of zona pellucida binding sites on rabbit spermatozoa and induction of the acrosome reaction by solubilized zonae. Dev. Biol., **119**, 551-559.

Overstreet, J.W. and Cooper, G.W. (1979). Effect of ovulation and sperm motility on the migration of rabbit spermatozoa to the site of fertilization. J. Reprod. Fertil., **55**, 53-59.

Packer, A.I., Besmer, P. and Bachvarova, R.F. (1995). Kit ligand mediates survival of type A spermatogonia and dividing spermatocytes in postnatal mouse testes. Mol. Reprod. Dev., **42**, 303-310.

Pagano, M., Pepperkok, P., Verde, F., Ansorge, W. and Draetta, G. (1992). Cyclin A is required at two points in the human cell cycle. EMBO J., **11**, 961-971.

Pantaleon, M. and Kaye, P.L. (1996). IGF-1 and insulin regulation glucose transport in mouse blastocysts via IGF-I receptor. Mol. Reprod. Dev., **44**, 71-76.

Pantaleon, M. and Kaye, P.L. (1998). Glucose transporters in preimplantation development. Rev. Reprod., **3**, 77-81.

Parazzini, F., Marchini, M., Luchini, L., Tozzi, L., Mezzopane, R. and Fedele, L. (1995). Tight underpants and trousers and the risk of dysspermia. Int. J. Androl., **18**, 137-140.

Parkinson, T.J. (1987). Seasonal variations in semen quality of bulls: correlations with environmental temperature. Vet. Rec., **120**, 479-482.

Partridge, R.T. and Leese, H.J. (1996). Comsumption of amino acids by bovine preimplantation embryos. Reprod. Fertil. Dev., **8**, 945-950.

Pedersen, R.A. and Burdsal, C.A. (1994). Mammalian embryogenesis. In "The Physiology of Reproduction." Eds. E. Knobil and J.D. Neill, Paven Press, New York, 1, Vol II, 319-390.

Pelletier, A.J., Bodary, S.C., and Levinson, A.D. (1992). Signal transduction by the platelet integrin $a_{IIb}?3$: induction of calcium oscillations respreading of stably transfected cells. Mol. Biol. Cell, **3**, 989-998.

Pennycuik, P.R. (1967). A comparison of the effect of a range of high temperatures and of two different periods of acclimatization on the reproductive performances of male and female mice. Aust. J. Exp. Biol. Med. Sci., **45**, 527-532.

Pessin, J.E. and Bell, G.I. (1992). Mammalian facilitative glucose transporter family: structure and molecular regulation. Annu. Rev. Physiol., **54**, 911-930.

Petters, R.M., Johnson, B.H., Reed, M.L. and Archibong, A.E. (1990). Glucose, glutamine, and inorganic phosphate in early development of the pig embryo in vitro. J. Reprod. Fertil., **89**, 269-275.

Plymate, S.R., Paulsen, C.A., McLachlan, R.I. (1992). Relationship of serum inhibin levels to serum follicle stimulating hormone and sperm production in normal men and men with varicoceles. J. Clin. Endocrinol. Metab., **74**, 859-864.

Pollard, R.M. and Finn, C.A. (1972). Ultrastructure of the uterine epithelium during the hormonal induction of sensitivity and insensitivity to a decidual stimulus in the mouse. J. Endocrinol., **55**, 293-298.

Poueymiou, W.T., Conover, J.C. and Schultz, R.M. (1989). Regulation of mouse preimplantation embryo development: differential effects of CZB and Whitten's medium on random-bred and patterns of protein synthesis in 2-cell embryos. Biol. Reprod., **41**, 318-322.

Poueymiou, W.T. and Schultz, R.M. (1987). Differential effects of activators of cAMP-dependent protein kinase and protein kinase C on cleavage of one-cell mouse embryos and protein synthesis and phosphorylation in one- and two-cell embryos. Dev. Biol., **121**, 489-498.

Poueymiou, W.T. and Schultz, R.M. (1989). Regulation of mouse preimplantation development: inhibition of synthesis of proteins in the two-cell embryo that require transcription by inhibitors of cAMP-dependent protein kinase. Dev. Biol., **133**, 588-599.

Prabhakar, J., Chimbombi, M., Malmgren, L., Fredriksson, G. and Madej, A. (1990). Effects on testosterone and LH concentrations of induced testicular degeneration in bulls. Acta Vet. Scand., **31**, 505-507.

Pratt, H.P.M., Bolton, V.N. and Gudgeon, K.A. (1983). The legacy from the oocyte and its role in controlling early development of the mouse embryo. In "The Molecular Biology of Egg Maturation". CIBA Foundation Symposium 98, Pitman Publishing, London, 197-227.

Quelle, D.E., Ashmun, R.A., Shurtleff, S.A., Kato, J-Y, Bar-Sagi, D., Roussel, M.F. and Sherr, C.J. (1993). Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. Genes. Dev., 7, 1559-1571.

Quinn, P., Kerin, J.F. and Wanes, G.M. (1985). Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertil. Steril., 44, 493-498.

Rajasekaran, A.K., Hojo, M., Huima, T. and Rodriguez-Boulan, E. (1996). Catenins and zonula occludens form a complex during early stages in the assembly of tight junctions. J. Cell Biol., 132, 451-463.

Ram, P.T. and Schultz, R.M. (1993). Reporter gene expression at G2 of the 1-cell mouse embryo. Dev. Biol., 156, 552-556.

Rappolee, D.A., Sturm, K.S., Schultz, G.A., Pedersen. R.A. and Werb, Z. (1990). The expression of growth factor ligands and receptors in preimplantation mouse embryos. In "Early Embryo Development and Paracrine Relationships". Eds. S. Heyner, L. Wiley. Alan R. Liss, Inc, New York, 11-25.

Rathore, A.K. (1970). Fertility of rams heated for 1, 2, 3, and 4 days, mated to superovulated ewes. Aust. J. Agric. Res., 21, 355-358.

Reeve, W.J.D. and Ziomek, C.A. (1981). Distribution of microvilli on dissociated blastomeres from mouse embryos: evidence for surface polarization at compaction. J. Embryol. Exp. Morphol., 62, 339-350.

Riethmacher, D., Brinkmann, V. and Birchmeteier, C. (1995). A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. Proc. Natl. Acad. Sci. USA, 92, 855-859.

Robinson, D. and Rock, J. (1967). Intrascrotal hyperthermia induced by scrotal insulation: effect of spermatogenesis. Obstet. Gynecol., 29, 217-223.

Rogel, A., Popliker, M., Webb, C.G. and Oren, M. (1985). p53 cellular tumor antigen: analysis of mRNA levels in normal adult tissues, embryos, and tumors. Mol. Cell Biol., **5**, 2851-2855.

Rosenthal, E.T., Hunt, T. and Ruderman, J.V. (1980). Selective translation of mRNA controls the pattern of protein synthesis during early development of the surf clam Spisula solidissima. Cell, **20**, 487-494.

Rothstein, J.L., Johnson, D., Deloia, J.A., Skowronski, J., Solter, D. and Knowles, B. (1992). Gene expression during preimplantation mouse development. Genes. and Develop., **6**, 1190-1201.

Ruoslahti, E. and Pierschbacher, M.D. (1987). New perspectives in cell adhesion: RGD and integrins. Science, **238**, 491-497.

Russ, M.E., Eder, H.A. and Barr, D.P. (1955). Influence of gonadal hormones on protein, lipid relationships in human plasma. Am. J. Med., **19**, 4-24.

Sailer, B.L., Sarkar, L.J., Bjordahl, J.A., Jost, L.K. and Evenson, D.P. (1997). Effects of heat stress on mouse testicular cells and sperm chromatin structure. J. Androl., **18**, 294-301.

Salanova, M., Stefanini, M., Curtis, I. and Palombi, F. (1995). Integrin receptor $\alpha6\beta1$ is localized at specific sites of cell-to-cell contact in rat seminiferous epithelium. Biol. Reprod., **52**, 79-87.

Sanford, L.M., Voglmayr, J.K., Vale, W.W. and Robaire, B. (1993). Photoperiod-mediated increases in serum concentrations of inhibin, follicle-stimulating hormone, and luteinizing hormone are accentuated in adult shortened-scrotum rams without corresponding decreases in testosterone and estradiol. Biol. Reprod., **49**, 365-373.

Sapsford, C.S. (1951). Seasonal changes in spermatogenesis in rams: their relation to plane of nutrition and vitamin A status. Aust. J. Agric. Res., **2**, 331-341.

Sarge, K.D. (1995). Male germ cell-specific alteration in temperature set point of the cellular stress response. J. Biol. Chem., **270**, 18745-18748.

Sasaki, R., Nakayama, T. and Kato, T. (1999). Microelectrophoretic analysis of changes in protein expression patterns in mouse oocytes and preimplantation embryos. Biol. Reprod., **60**, 1410-1418.

Sato, K. (1979). Polyspermy-preventing mechanisms in mouse eggs fertilised in vitro (1). J. Exp. Zool., **210**, 353-359.

Satoh, N. and Ikegami, S. (1981a). A definite number of a phidicolin-sensitive cell-cyclic events are required for acetylcholinesterase development in the presumptive muscle cells of the ascidian embryos. J. Embryol. Exp. Morph., **61**, 1-13.

Satoh, N. and Ikegami, S. (1981b). On the "clock" mechanism determining the time of tissue-specific enzyme development during ascidian embryogenesis. II. Evidence for association of the clock with the cycle of DNA replication. J. Embryol. Exp. Morph., **64**, 61-71.

Sawicki, W., Abramczuk, J. and Blaton, O. (1978). DNA synthesis in the second and third cell cycles of mouse preimplantation development. Exp. Cell Res., **112**, 199-205.

Sawicki, J.A., Magnuson, T. and Epstein, C.L. (1982). Evidence for the expression of the paternal genome in the two-cell mouse embryo. Nature, **294**, 450-451.

Schaefer, E.J., Fosfer, D.M., Zech, L.A., Lindgren, F.T., Brewer, H.B. and Levy, R.I. (1983). The effects of estrogen administration on plasma lipoprotein metabolism in premenopausal females. J. Clin. Endocrinol. Metab., **57**, 262-267.

Schanbacher, B.D. (1980). Androgen response of cryptorchid and intact rams to ovine LH. J. Reprod. Fertil., **59**, 151-154.

Schanbacher, B.D. and Ford, J.J. (1977). Gonadotropin secretion in cryptorchid and castrate rams and the acute effects of exogenous steroid treatment. Endocrinol., **100**, 387-393.

Schatten, G. (1994). The centrosome and its mode of inheritance: the reduction of the centrosome during gametogenesis and its restoration during fertilization. Dev. Biol., **165**, 299-335.

Schatten, G., Simerly, C. and Schatten, H. (1991). Maternal inheritance of centrosomes in mammals? Studies on parthenogenesis and polyspermy in mice. Proc. Natl. Acad. Sci. USA, **88**, 6785-6789.

Schultz, R.M. (1993). Regulation of zygotic gene activation in the mouse. Bioessays, **8**, 531-538.

Schultz, R.M. and Worrad, D.M. (1995). Role of chromatin structure in zygotic gene activation in the mammalian embryo. Semin Cell Biol., **6**, 201-208.

Schultz, R.M., Worrad, D.M., Davis, W.Jr and De Sousa, P.A. (1995). Regulation of gene expression in the preimplantation mouse embryo. Theriogenology, **44**, 1115-1131.

Schwartz, D., Goldfinger, N. and Rotter, V. (1993). Expression of p53 protein in spermatogenesis is confined to the tetraploid pachytene primary spermatocytes. Oncogene, **8**, 1487-1494.

Scott, L.F., Sundaram, S.G. and Smith, S. (1993). The relevance and use of mouse embryo bioassays for quality control in an assisted reproductive technology program. Fertil. Steril., **60**, 559-568.

Seethalakshmi, L. and Steinberger, A. (1983). Effect of cryptorchidism and orchidopexy on inhibin secretion by rat Sertoli cells. J. Androl., **4**, 131.

Setchell, B.P. (1978). Naturally occurring and induced dysfunctions of the testis. In "The Mammalian Testis". Ed. B.P. Setchell. Elek Books, London Cornell University Press, Ithaca, 181-218 and 359-366.

Setchell, B.P. (1994). Possible physiological bases for contraceptive techniques in the male. Hum. Reprod., **9**, 1081-1087.

Setchell, B.P. (1998). The Parkes lecture: Heat and the testis. J. Reprod. Fertil., **114**, 179-194.

Setchell, B.P., D'Occhio, M.J., Hall, M.J., Laurie, M.S., Tucker, M.J. and Zupp, J.L. (1988). Is embryonic mortality increased in normal females mated to subfertile males. J. Reprod. Fertil., **82**, 567-574.

Setchell, B.P., Ekpe, G., Zupp, J.L. and Surani, M.A.H. (1998). Transient retardation in embryo growth in normal female mice made pregnant by males whose testes had been heated. Hum. Reprod., **13**, 342-347.

Setchell, B.P., Tao, L. and Zupp, J.L. (1996). The penetration of chromium-EDTA from blood plasma into various compartments of rat testes, as an indicator of function of the blood-testis barrier, following exposure of the testes to heat. J. Reprod. Fertil., **106**, 125-133.

Setchell, B.P., Voglmayr, J.K. and Hinks, N.T. (1971). The effect of local heating on the flow and composition of rete testis fluid in the conscious ram. J. Reprod. Fertil., **24**, 81-89.

Setchell, B.P. and Waites, G.M.H. (1972). The effects of local heating of the testis on the flow and composition of rete testis fluid in the rat, with some observations on the effects of age and unilateral castration. J. Reprod. Fertil., **30**, 225-233.

Shi, C.Z., Collins, H.W., Garside, W.T., Buettger, C.W., Matschinsky, F.M. and Heyner, S. (1994). Protein databases for compacted eight-cell and blastocyst-stage mouse embryos. Mol. Reprod. Dev., **37**, 34-47.

Shikone, T., Billig, H. and Hsueh, A.J.W. (1994). Experimentally induced cryptorchidism increases apoptosis in rat testis. Biol. Reprod., **51**, 865-872.

Shalgi, R., Phillips, D.M. and Jones, R. (1989). Status of the rat acrosome during sperm-zona pellucida interactions. Gamete Res., **22**, 1-13.

Short, R.V. (1972). Role of hormones in sex cycles. In "Reproduction in Mammals". Eds. C. R. Austin and R. V. Short. Cambridge Univesity Press, Vol III, Cambridge, 42-72.

Sidhu, K.S. and Guraya, S.S. (1989). Calmodulin-like protein in buffalo (Bubalus bulalis) seminal plasma and its effect on sperm Ca^{2+}, Mg^{2+}-ATPase. Int. J. Androl., **12**, 148-154.

Sidibe, M., Franco, L.A., Fredriksson, G., Madej, A. and Malmgren, L. (1992). Effects on testosterone, LH and cortisol concentrations and on testicular ultrasonographic appearance of induced testicular degeneration in bulls. Acta Vet. Scand., **33**, 191-196.

Sinclair, K.D., Broadbent, P.J. and Dolman, D.F. (1995). In vitro produced embryos as a means of achieving pregnancy and improving productivity in beef cows. Anim. Sci., **60**, 55-64.

Siracusa, G., Coletta, M. and Monesi, V. (1975). Duplication of DNA during the first cell cycle in the mouse embryo. J. Reprod. Fertil., **42**, 395-398.

Skinner, M.K. (1991). Cell-cell interactions in the testis. Endocrinol. Rev., **12**, 45-77.

Skinner, J.D. and Louw, G.N. (1966). Heat stress and spermatogenesis in Bos indicus and Bos taurus cattle. J. Appl. Physiol., **21**, 1784-1790.

Smith, J.F. (1971). The effect of temperature on characteristics of semen in rams. Aust. J. Agric. Res., **22**, 481-490.

Smith, R.K.W. and Johnson, M.H. (1985). DNA replication and compaction in the cleaving embryo of the mouse. J. Embryol. Exp. Morph., **89**, 133-148.

Smith, R.K.W. and Johnson, M.H. (1986). Analysis of the third and fourth cell cycles of mouse early development. J. Reprod. Fertil., **76**, 393-399.

Smith, R. and McLaren, A (1977). Factors affecting the time of formation of the mouse blastocoele. J. Embryol. Exp. Morph., **41**, 79-92.

Smith, J. A., Martin, L., King, R.J.B. and Vertes, M. (1970). Effects of oestradiol- 17β and progesterone on total and nuclear-protein synthesis in epithelial and stromal tissues of the mouse uterus and of progesterone on the ability of these tissues to bind oestradiol-17β. Biochem. J., **119**, 773-784.

Smith, M.S.R. and Wilson, I.B. (1971). Histochemical observations on early implantation in the mouse. J. Embryol. Exp. Morph., **25**, 165-174.

Sod-Moriah, U.A., Goldberg, G.M. and Bedrak, E. (1974). Intrascrotal temperature, testicular histology and fertility of heat-acclimatized rats. J. Reprod. Fertil., **37**, 263-268.

Spindle, A.I., Nagaro, H. and Pedersen, R.A. (1985). Inhibition of DNA replication in preimplantation mouse embryos by aphidicolin. J. Exp. Zool., **235**, 289-295.

Starkey, P.M., Sargent, I.L. and Redman, C.W. (1988). Cell populations in human early pregnancy decidua: characterization and isolation of large granular lymphocytes by flow cytometry. Immunol., **65**, 129-134.

Steinberger, A. (1980). Factors affecting in vitro secretion of inhibin by isolated Sertoli cells. In "Endocrinology". Eds. I.A. Cumming, J.W. Funder and F.A.O. Mendelsohn. The Australian Academy of Sci., Canberra, Australia, 259-262.

Steinberger, A. (1989). Effects of temperature on the biochemistry of the testis. In "Temperature and Environment Effects on the Testis". Ed. A. Zorgniotti. Plenum, New York, 33-49.

Stephens, L.E., Sutherland, A.E., Klimanskaya, I.V., Andrieux, A., Meneses, J., Pedersen, R.A. and Damsky, C.H. (1995). Deletion of beta 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. Genes Dev., 9, 1883-1895.

Stevenson, B.R., Anderson, J.M. and Bullivant, S. (1988). The epithelial tight junction: structure, function and preliminary biochemical characterization. Mol. Cell Biochem., 83, 129-145.

Strauss, F. (1956). The time and place of fertilization of the golden hamster egg. J. Embryol. Exp. Morph., 4, 42-45.

Streffer, C., Van Beuningen, D., Molls, M., Zamboglon. N. and Schultz, S. (1980). Kinetics of cell proliferation in the preimplanted mouse embryo in vivo and in vitro. Cell Tiss. Kinet., 13, 135-143.

Suarez, S.S., Katz, D.F. and Overstreet, J.W. (1983). Movement characteristics and acrosomal status of rabbit spermatozoa recovered at the site and time of fertilization. Biol. Reprod., 29, 1277-1287.

Suarez, S.S. (1987). Sperm transport and motility in the mouse oviduct: observation in situ. Biol. Reprod., 36, 203-210.

Summers, M.C., Bhatnagar, P.R., Lawitts, J.A. and Biggers, J.D. (1995). Fertilization in vitro of mouse ova from inbred and outbred strains: complete preimplantation embryo development in glucose-supplemented KSOM. Biol. Reprod., 53, 431-437.

Sutherland, A.E., Calarco, P.G. and Damsky, C.H. (1993). Developmental regulation of integrin expression at the time of implantation in the mouse embryo. Development, 119, 1175-1186.

Sutovsky, P. and Schatten, G. (2000). Paternal contribution to the mammalian zygote: fertilization after sperm-egg fusion. Inter. Rev. Cytology, **195**, 1-65.

Swann, K. (1990). A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. Development, **110**, 1295-1302.

Swann, K. (1992). Different triggers for calcium oscillations in mouse eggs involve a ryanodine-sensitive calcium store. Biochem. J., **287**, 79-84.

Szell, A.Z. (1995). The effect of glutamine on the development of sheep embryos in vitro. Theriogenology, **44**, 673-680.

Tarlatzis, B.C., Laufer, N., De Cherney, A.H., Makler, A., Haseltine, F.P., Polan, M.L. and Naftolin, F. (1984). Human sperm capacitation: evidence for a role of human oocyte-corona-complex (occc). J. In Vitro. Fertil. Embry. Trans., **1**, 143.

Theiler, K. (1989). In "The House Mouse: Altas of Embryonic Development". Ed. K. Theiler. Springer-Verlag, New York, 1-14.

Thetford, C.R., Morrell, J.M. and Cockle, S.M. (1992). TRH-related peptides in the rabbit prostate complex during development. Biochim. Biophys. Acta, **1115**, 252-258.

Thompson, E.M., Legouy, E.L., Christians, E. and Renard, J.P. (1995). Progressive maturation of chromatin structure regulates HSP70.1 gene expression in the preimplantation mouse embryo. Development, **121**, 3425-3427.

Thonneau, P., Ducot, B., Bujan, L., Mieusset, R. and Spira, A (1997). Effect of male occupational heat exposure on time to pregnancy. Int. J. Androl., **20**, 274-278.

Thorens, B., Cheng, Z.Q., Brown, D. and Lodish, H.F. (1990). Liver glucose transporter: a basilateral protein in hepatocytes and intestine and kidney cells. Am. J. Physiol., **259**, 279-285.

Tiemessen, C.H.J., Evers, J.L.H. and Bots, R.S.G.M. (1996). Tight-fitting underwear and sperm quality. Lancet., **347**, 1844-1845.

Tilghman, S.M. (1999). The sins of the fathers and mothers: genomic imprinting in mammalian development. Cell, **96**, 185-193.

Tierney, L.A. and Halford, D.M. (1985). Mating behaviour, serum testosterone and serum characteristics in vasectomized and short scrotum rams. Theriogenology, **23**, 535-545.

Turner, B.M. (1991). Histone acetylation and control of gene expression. J. Cell Sci., **99**, 13-20.

Turner, B.M. (1993). Decoding the nucleosome. Cell, **75**, 5-8.

Ueno, K., Hiramoto, Y., Hayashi, S. and Kondoh, H. (1987). Introduction and expression of recombinant β-galactosidase genes in cleavage stage mouse embryos. Dev. Growth Differ., **30**, 61-73.

Uranga, J.A. and Arechaga, J. (1997). Comparative analysis of in vitro development of outbred mouse embryos cultured in Krebs-Ringer or Tyrode-derived media. Reprod. Nutr. Dev., **37**, 41-49.

Urch, U.A., Wardrip, N.J. and Hedrick, J.L. (1985a). Limited and specific proteolysis of the zona pellucida by acrosin. J. Exp. Zool., **233**, 479-483.

Urch, U.A., Wardrip, N.J. and Hedrick, J.L. (1985b). Proteolysis of the zona pellucida by acrosin: the nature of the hydrolysis products. J. Exp. Zool., **236**, 239-243.

Vallance, S.J., Lee, H-M, Roussel, M.F., Shurtleff, S.A., Kato, J-Y, Strom, D.K. and Sherr, C.J. (1994). Monoclonal antibodies to mammalian D-type G1 cyclins. Hybridoma, **13**, 37-44.

Van Blerkom, J. (1979). Molecular differentiation of the rabbit ovum. III Fertilization-autonomous polypeptides synthesis. Dev. Biol., **72**, 188-194.

Van Blerkom, J. (1981). Structural relationship and post translational modification of stage-specific proteins synthesized during early preimplantation development in the mouse. Proc. Natl. Acad. Sci. U.S.A., **78**, 7629-7633.

Van Blerkom, J. and Brockway, G.O. (1975). Qualitative patterns of protein synthesis in the preimplantation mouse embryo. Dev. Biol., **44**, 148-157.

Van Blerkom, J. and McGaushey, R.W. (1978). Molecular differentiation of the rabbit ovum. II During the preimplantation development of in vivo and in vitro matured oocytes. Dev. Biol., **63**, 151-164.

Van Blerkom, J. and Manes, C. (1974). Development of preimplantation rabbit embryos in vivo and in vitro. II A comparison of qualitative aspects of protein synthesis. Dev. Biol., **40**, 40-51.

Van Deenen, L.L.M. and De Gier, J. (1974). Lipids of the red cell membrane. In "The Red Blood Cell". Ed. D. MacN. Surgenor. Academic Press, Vol II, New York, 147-211.

Van Waeleghem, K., de Clerq, N., Vermeulen, L., Schoonjans, F. and Comhaire, F. (1996). Deterioration of sperm quality in young healthy Belgian men. Hum. Reprod., **11**, 325-329.

Van Winkle, L.J. and Campione, A.L. (1996). Amino acid transport regulation in preimplantation mouse embryos: effects on amino acid content and pre- and peri-implantation development. Theriogenology, **45**, 69-80.

Van Winkle, L.J. and Dickinson, H.R. (1995). Differences in amino acid content of preimplantation mouse embryos that develop in vitro vs. in vivo: in vitro effects of five amino acids that are abundant in oviductal secretions. Biol. Reprod., **52**, 96-104.

Van Winkle, L.J., Haghighat, N. and Campione, A.L. (1990). Glycine protects preimplantation mouse conceptuses from a detrimental effect on development of the inorganic ions in oviductal fluid. J. Exp. Zool., **253**, 215-219.

Van Winkle, L.J., Haghighat, N., Campione, A.L. and Gorman, J.M. (1988). Glycine transport in mouse eggs and preimplantation conceptuses. Biochim. Biophys. Acta., **941**, 241-256.

Van Winkle, L.J., Patel, M., Wasserlauf, H.G., Dickinson, H.R. and Campione, A.L. (1994). Osmotic regulation of taurine transport via system β and novel processes in mouse preimplantation conceptuses. Biochim. Biophys. Acta, **1191**, 244-255.

Van Zelst, S.J., Zupp, J.L., Hayman, D.L. and Setchell, B.P. (1995). X-Y dissociation in mice and rats exposed to increased testicular or environmental temperatures. Reprod. Fertil. Dev., **7**, 1117-1121.

Vasseur, M., Condamine, H. and Duprey, P. (1985). RNAs containing B2 repeated sequences are transcribed in the early stages of mouse embryogenesis. EMBO J., **4**, 1749-1753.

Visconti, P.E., Bailey, J.L., Moore, G.D., Pan, D., Olds-Clarke, P. and Kopf, G.S. (1995a). Capacitation in mouse spermatozoa. I: Correlation between the capacitation state and protein tyrosine phosphorylation. Development, **121**, 1129-1137.

Visconti, P.E., Moore, G.D., Bailey, J.L., Leclerc, P., Connors, S.A., Pan, D., Olds-Clarke, P. and Kopf, G.S. (1995b). Capacitation of mouse spermatozoa. II: Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development, **121**, 1139-1150.

Vogler, C.J., Bame, J.H., DeJarnette, J.M., McGilliard, M.L. and Saacke, R.G. (1993). Effects of elevated testicular temperature on morphology characteristics of ejaculated spermatozoa in the bovine. Theriogenology, **40**, 1207-1219.

Vogler, C.J., Saacke, R.G., Bame, J.H., DeJarnette, J.M. and McGilliard, M.L. (1991). Effects of scrotal insulation on viability characteristics of cryopreserved bovine semen. J. Dairy Sci., **74**, 3827-3835.

Vornberger, W., Prins, G., Musto, N.A. and Suarez-Quian, C.A. (1994). Androgen receptor distribution in rat testis: new implications for androgen regulation of spermatogenesis. Endocrinol., **134**, 2307-2316.

Waites, G.M.H. and Ortavant, R. (1968). Effects precoces d'une breve elevation de la temperature testiculaire sur la spermatogenese du belier. Ann. Biol. Anim. Bioch. Biophys., **8**, 323-331.

Waites, G.M.H. and Setchell, B.P. (1964). Effect of local heating on blood flow and metabolism of the testes of the conscious ram. J. Reprod. Fertil., **8**, 339-344.

Waldbieser, G.C. and Chisman, C.L. (1986). X-Y chromosome univalency in the testes of hyperthermic mice: I. Concomitant formation of multinucleated giant cells. Gamete Res., **15**, 153-160.

Wales, R.G. and Biggers, J.D. (1968). The permeability of two- and eight-cell mouse embryos to L-malic acid. J. Reprod. Fertil., **15**, 103-111.

Wales, R.G. and Brinster, R.L. (1968). The uptake of hexoses by preimplantation mouse embryos in vitro. J. Reprod. Fertil., **15**, 415-422.

Walker, S.K., Hill, J.L., Kleemann, D.O. and Nancarrow, C.D. (1996). Development of ovine embryos in synthetic oviductal fluid containing amino acids at oviductal fluid concentrations. Biol. Reprod., **55**, 703-708.

Wassarman, P.M., and Letourneau, G.E. (1976). RNA synthesis in fully-grown mouse oocytes. Nature, **261**, 73-74.

Watson, A.J. (1992). The cell biology of blastocyst development. Mol. Reprod. Dev., **33**, 492-504.

Watson, A.J. and Kidder, G.M. (1988). Immunofluorescence assessment of the timing of appearance and cellular distribution of Na/K-ATPase during mouse embryogenesis. Dev. Biol., **126**, 80-90.

Watson, A.J., Pape, J.R., Emanuel, J.R., Levenson, R. and Kidder, G.M. (1990). Expression of Na/K-ATPase α and β subunit genes during preimplantation development of the mouse. Dev. Genet., **11**, 41-48.

Watson, A.J., Westhusin, M.E., De Sousa, P.A., Betts, D.H. and Barcroft, L.C. (1999). Gene expression regulating blastocyst formation. Theriogenology, **51**, 117-133.

Weissbach, A. (1977). Eukaryotic DNA polymerases. Rev. Biochem., **46**, 25-47.

Wells, D.E., Showman, R.M., Klein, W.H. and Raff, R.A. (1981). Delayed recruitment of maternal histone H3 mRNA in sea urchin embryos. Nature, **292**, 477-478.

Welsh, A.O. and Enders, A.C. (1985). Light and electronmicroscopic examination of mature decidual cells of the rat with emphasis on the antimesometrial decidua and its degeneration. Am. J. Anat., **172**, 1-29.

Welsh, A.O. and Enders, A.C. (1991). Chorioallantoic placenta formation in the rat: II. Angiogenesis and maternal blood circulation in the mesometrial region of the implantation chamber prior to placenta formation. Am. J. Anat., **192**, 347-365.

Welsh, A.O. and Enders, A.C. (1993). Chorioallantoic placenta formation in the rat: III Granulated cells invade the uterine luminal epithelium at the time of epithelial cell death. Biol. Reprod., **49**, 38-57.

Welsh, T.H., Randel, R.H. and Johnson, B.H. (1979). Temporal relationships among peripheral blood concentrations of corticosteroids, luteinizing hormone and testosterone in bulls. Theriogenology, **12**, 169-179.

Wettemann, R.P and Bazer, F.W. (1985). Influence of environmental temperature on prolificacy of pigs. J. Reprod. Fertil. (suppl.), **33**, 199-208.

Wettemann, R.P. and Desjardins, C. (1979). Testicular function in boars exposed to elevated ambient temperature. Biol. Reprod., **20**, 235-241.

Wettemann, R.P., Wells, M.E. and Johnson, R.K. (1979). Reproductive characteristics of boars during and after exposure to increased ambient temperature. J. Anim. Sci., **49**, 1501-1505.

Wettemann, R.P., Wells, M.E., Omtvedt, I.T., Pope, C.E. and Turman, E.J. (1976). Influence of elevated ambient temperature on reproductive performance of boars. J. Anim. Sci., **42**, 664-669.

Wheeler, T.J. and Hinkle, P.C. (1985). The glucose transporter of mammalian cells. Annu. Res. Physiol., **47**, 503-517.

White, D.R. and Aitken, R.J. (1989). Relationship between calcium, cyclic AMP, ATP, and intracellular pH and the capacity of hamster sperm to express hyperactivated motility. Gamete Res., **22**, 163-177.

White, S. and Kimber, S.J. (1994). Changes in α (1-2)-fucosyltransferase activity in the murine endometrial epithelium during the estrous cycle, early pregnancy, and after ovariectomy and hormone replacement. Biol. Reprod., **50**, 73-81.

Whitten, W.K. (1957). Culture of tubal ova. Nature, **179**, 1081-1082.

Whitten, W.K. and Biggers, J.D. (1968). Complete development in vitro of the preimplantation stages of the mouse in a simple chemically defined medium. J. Reprod. Fertil., **17**, 399-401.

Whitten, W.K., Bronson, F.H. and Greenstein, J.A. (1968). Estrus-inducing pheromone of male mice. Science, **161**, 584-585.

Whittingham, D.G. (1968). Fertilization of mouse eggs in vitro. Nature, **220**, 592-593.

Whittingham, D.G. (1969). Biochemical aspects of early gestation. In "Congenital Malformations", Excerpta Medica International Series, No. 204, The Hague, Netherlands, 147-156.

Whittingham, D.G. (1971). Culture of mouse ova. J. Reprod. Fertil.(suppl.). **14**, 7-21.

Wiekowski, M., Miranda, M. and DePamphilis, M.L. (1991). Regulation of gene expression in preimplantation mouse embryos: effects of the zygotic clock and the first mitosis on promoter and enhancer activities. Dev. Biol., **147**, 403-414.

Wiekowski, M., Miranda, M. and DePamphilis, M.L. (1993). Requirements for promoter activity in mouse oocytes and embryos distinquish paternal pronuclei from maternal and zygotic nuclei. Dev. Biol., **159**, 366-378.

Wildeus, S. and Entwistle, K.W. (1983). Spermiogram and sperm reserves in hybrid Bos indicus X Bos taurus bulls after scrotal insulation. J. Reprod. Fertil., **69**, 711-716.

Wildeus, S. and Entwistle, K.W. (1986). Effects of scrotal insulation and unilateral vasoligation on ejaculate characteristics and sperm reserves in the bull. Anim. Reprod. Sci., **10**, 11-21.

Wilkinson, R.F., Ming, R., Anderson, B., Bunch, T.S. and White, K.L. (1996). The use of neural networks in developing novel embryo culture media-formulations. Theriogenology, **45**, 41-49.

Williams, C.J., Schultz, R.M. and Kopf, G.S. (1992). Role of G proteins in mouse egg activation: stimulatory effects of acetylcholine on the ZP2 to ZP2$_f$ conversion and pronuclear formation in egg expressing a functional ml muscarinic receptor. Dev. Biol., **151**, 288-296.

Willis, M., Howell, S.C. and Taylor, K.W. (1985). The Biochemistry of the Polypeptide Hormones. John Wiley and Sons, Chichester.

Wolffe, A.P. (1991). Implications of DNA replication for eukaryotic gene expression. J. Cell Sci., **99**, 201-206.

Wolffe, A.P. (1994). The transcription of chromatin templates. Curr. Opinion Gen. Dev., **4**, 245-254.

Wolffe, A.P. and Dimitrov, S. (1993). Histone-modulated gene activity: developmental implications. Critical Rev. Eukaryotic Gene Expression, **3**, 167-191.

Woodland, H.R. and Ballantine, J.E.M. (1980). Paternal gene expression in developing hybrid embryos of Xenopus laevis and Xenopus Borealis. J. Embryol. Exp. Morph., **60**, 359-372.

Woodland, H.R., Flynn, J.M and Wyllie, A.J. (1979). Utilization of stored mRNA in Xenopus embryos and its replacement by newly synthesized transcripts: histone H1 synthesis using interspecies hybrid. Cell, **18**, 165-171.

Worrad, D.M., Ram, P.T. and Schultz, R.M. (1994). Regulation of gene expression in the mouse oocyte and preimplantation embryo: developmental changes in Sp1 and TATA box-binding protein TBP. Development, **120**, 2347-2357.

Worrad, D.M. and Schultz, R.M. (1997). Regulation of gene expression in the preimplantation mouse embryo: temporal and spatial patterns of expression of the transcription factor Sp1. Mol. Reprod. Dev., **46**, 268-277.

Worrad, D.M., Turner, B.M. and Schultz, R.M. (1995). Temporally restricted spatial localization of acetylated isoforms of histone H4 and RNA polymerase II in the 2-cell mouse embryo. Development, **121**, 2949-2959.

Wright, Jr.R.W. and Ellington, J. (1995). Morphological and physiological differences between in vivo- and in vitro-produced preimplantation embryos from livestock species. Theriogenology, **44**, 1167-1189.

Yanagimachi, R. (1994). Mammalian Fertilization. In "The Physiology of Reproduction". Eds. E. Knobil and J. D. Neill. Raven Press, New York, 1, Vol II, 189-318.

Yanagimachi, R. and Noda, Y.D. (1970). Physiological changes in the postnuclear cap region of mammalian spermatozoa: a necessary preliminary to the membrane fusion between sperm and egg cells. J. Ultrastruct. Res., **31**, 486-493.

Yanagimachi, R. and Usui, N. (1974). Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. Exp. Cell Res., **89**, 161-174.

Yin, Y., Dewolf, W.C. and Morgentaler, A. (1998). Experimental cryptorchidism induces testicular germ cell apoptosis by p53-dependent and -independent pathways in mice. Biol. Reprod., **58**, 492-496.

Yin, Y., Hawkins, K.L., Dewolf, W.C. and Morgentaler, A. (1997). Heat stress causes testicular germ cell apoptosis in adult mice. J. Androl., **18**, 159-165.

Yokoyama, S., Fukushima, D., Kupferberg, J.P., Kezdy, F.J. and Kaiser, E.T. (1980). The mechanism of activation of lecithin, cholesterol acyltransferase by apolipoprotein A-1 and an amphiphilic peptide. J. Biol. Chem., **255**, 7333-7339.

Young, R.J., Sweeney, K. and Bedford, J.M. (1978). Uridine and guanosine incorporation by the mouse one-cell embryo. J. Embryol. Exp. Morph., **44**, 133-148.

Young, W.C. (1927). The influence of high temperature on the guinea pig testis: histologic changes and effects on reproduction. J. Exp. Zool., **49**, 459-499.

Young, W.C. (1929). The influence of high temperature on the reproductive capacity of guinea pig spermatozoa as determined by artificial insemination. Physiol. Zool., **2**, 1-8.

Yurochko, A.D., Lui, D.Y., Eierman, D. and Haskill, S. (1992). Integrins as a primary signal transduction molecule regulating monocyte immediate-early gene induction. Proc. Natl. Acad. Sci. USA, **89**, 9034-9038.

Zamboni, L. (1972). Fertilization in the mouse. In "Biology of mammalian fertilization and implantation". Eds. K.S. Moghissi and E.S.E. Hafez. Thomas, Springfield, III, 213-262.

Zhang, X., Shu, M.A., Harvey, M.B. and Schultz, G.A. (1996). Regulation of urokinase plasminogen activator production in implanting mouse embryo: effects of embryo interaction with extracellular matrix. Biol. Reprod., **54**, 1052-1058.

Zindy, F., Lamas, E., Chenivesse, X., Sobczak, J., Wang, J., Fesquet, D., Henglein, B. and Brechot, C. (1992). Cyclin A is required in S phase in normal epithelial cells. Biochem Biophys. Res. Commun., **182**, 1144-1154.

Ziomek, C.A. and Johnson, M.H. (1980). Cell surface interaction induces polarization of mouse 8-cell blastomeres at compaction. Cell, **21**, 935-942.

Ziomek, C.A. and Johnson, M.H. (1982). The roles of phenotype and position in guiding the fate of 16-cell mouse blastomeres. Dev. Biol., **91**, 440-447.