

THE EFFECTS OF SHORT – TERM ENERGY RESTRICTION IN OVERWEIGHT / OBESE FEMALES ON REPRODUCTIVE OUTCOMES

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Thesis abstract

In the general population, one in five couples experiences difficulty in conceiving a child. The role of obesity on women's fecundity has become a focus of attention in recent years.

Successful treatment of infertility through Assisted Reproductive Technology (ART) is also compromised by the presence of obesity, which occurs in 30 % of women seeking treatment. A negative correlation exists between increased body mass index (BMI) and the number of collected oocytes and a lower birth rate after ART. Furthermore, a number of studies have established that weight loss improves natural conception rates in overweight women. Whether weight management can improve success rates in overweight / obese women undergoing in vitro fertilisation (IVF) has not been studied.

The purpose of this project was to explore the role of short-term weight loss on potential pregnancy outcomes in overweight / obese women undergoing IVF programme. However, to establish this relationship, we proposed to carry out two studies to assess the following:

- (I) The feasibility of very low calorie diet (VLCD) during IVF treatment with respect to duration, level of restriction and tolerability of the diet during hormonal down regulation in women (Chapter 2).
- (II) How energy restriction may affect the quality of an early embryo in diet - induced obese mice with respect to various body weight and caloric intake (Chapter 3).

In study (I), women preferred a shorter dietary intervention with greater energy restriction (456 kcal per day) to gradual energy restriction (1200 kcal / day for the first week, and afterward, 456 kcal / day) prior to oocyte transfer. Women were able to comply with the VLCD during IVF treatment and both dietary groups achieved a significant weight loss (mean 6.3 %).

In study (II), by using obese mice, the effect of rapid weight loss (mean 12 %) was observed after 5 days of energy restriction. Ovulation rate was greater in the Obese group (HFD) (55.6%) and equal in both Control (CD) and Energy Restricted (HF / ER) (44.4 %) groups. The HF / ER group showed higher fertilisation rate (80 %) than HFD and CD (55% and 45.5%, correspondingly). The blastocyst stage was reached by half of the cultured embryos in both HF / ER and HFD groups and 33 % in the CD group. The quality of embryos that completed blastocyst formation did not differ between groups. However, postfertilisation development in females fed a high fat diet was slower compared to CD and HF / ER groups.

In conclusion, this work illustrated a weight management prior conception and use of VLCD during IVF treatment in clinical study needs further investigation with regard to the dietary duration, level of energy restriction and how this combination will influence IVF treatment outcomes. Furthermore, as we were unable to determine the question of how the dietary intervention affects the quality of oocytes and the animal study illustrated a promising result, thus further studies are required.

Statement

I hereby state that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where stated.

I give consent for this thesis to be available for photocopying and loan purposes after depositing in The University of Adelaide library.

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The experiments reported in this work were performed by myself and any assistance received from others is acknowledged. To my knowledge, there are no intellectual property issues or conflicts of interest with other persons or organizations with respect to the data presented in this thesis.

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Publications

Tsagareli V, Noakes M, Norman RJ. Effect of a very-low-calorie diet on in vitro fertilization outcomes. *Fertil Steril* 2006; 86:227-9.

Minge CE, Bennett BD, **Tsagareli V**, Lane M, Owens JA, Norman RJ, Robker RL. Ovulation and Oocyte quality are adversely affected by a High fat diet. *In press*.

Abbreviations

ACTH	Adrenocorticotrophic hormone
ART	Assisted Reproductive Technology
BMI	Body mass index
CC	Clomiphene Citrate
CD	Control diet
CSIRO	Commonwealth Scientific Industrial Research Organization
CVD	Cardiovascular disease
DHEA-s	Dehydroepiandrosterone sulphate
DHT	Dihydrotestosterone
DIVA	Diet and IVF assessment
DNA	Deoxyribonucleic acid
DOB	Date of birth
ESHRE	European Society for Human Reproduction & Embryology
ET	Embryo transfer
FET	Frozen embryo transfer
FSH	Follicle stimulating hormone
GH	Growth hormone
GIFT	Gamete intra fallopian transfer
HC	High carbohydrate
hCG	Human chorionic gonadotrophin
HDL - C	High density lipoprotein cholesterol
HFD	High fat diet
HF / ER	High fat / Energy restricted
HP	High protein
ICSI	Intracytoplasmic sperm injection
IUD	Intrauterine device
IUGR	Intrauterine growth retardation
IUI	Intrauterine insemination
IVF	In vitro fertilisation
LC	Low carbohydrate
LCD	Low calorie diet
LDL - C	Low density lipoprotein cholesterol
LH	Luteinizing hormone
NHANES	National Health and Nutrition Examination Survey
NH&MRC	National Health and Medical Research Council
OC	Oral contraceptives
OPU	Oocyte pick up
PCO	Polycystic ovaries
PCOS	Polycystic ovarian syndrome
RDI	Recommended dietary intake
RM	Recurrent miscarriage
RMU	Reproductive Medicine Unit
SHBG	Serum testosterone-binding globulin
SEM	Standard Error Mean
STIs	Sexually transmitted infections
TWD	The Total Wellbeing Diet
VLCD	Very low calorie diet
WHO	World Health Organization

CHAPTER ONE

Literature review

1. Introduction

During a woman's reproductive years, ovarian function is regulated by the neuro-endocrine system. The quality of oocytes, and receptivity of the uterine endometrium for the successful implantation and maintenance of pregnancy to delivery of a live child, primarily determines a woman's fertility. A small change in any parameter of these systems may have a significant influence on a woman's reproductive function leading to infertility. The prevalence and complexities of infertility have assumed an increased importance in health care systems in recent years. A survey reported that 7 % of married couples in their reproductive years had not conceived in the first year of unprotected intercourse (National Survey of Family Growth, USA 2002). Infertility affects about 10 – 15 % of all couples of reproductive age (Mosher et al, 1991) and approximately 60 % of infertility issues are due to female factors, approximately 25 % to male factors (Spira, 1986) and the rest remains as combined or unexplained cases.

Abnormal physiological and pathological processes during the reproductive years may diminish the influence of oocyte quality and affect the uterine environment, fertilisation and embryo quality. These factors may include maternal age, lifestyle factors (smoking, alcohol and caffeine intake), nutritional status and environmental factors. Decreased natural fertility occurs with increasing age, reducing the chance of pregnancy following any type of infertility therapy (Gindoff et al 1986; Jacobs et al. 1990; Scott et al. 1993). Munne et al. 1995 found that the frequency of chromosomal abnormalities in embryos is higher with an increased maternal age (Munne et al. 1995). Moreover, it appears that lifestyle factors such as caffeine intake (Bolumar et al. 1997; Spinelli et al. 1997), smoking (Cooper et al. 1995; Winter et al. 2002), alcohol consumption (Henriksen et al. 2004), stress and excessive physical exercise (Bullen et al. 1985) have a negative effect on fertility.

Furthermore, initiation and maintenance of reproductive function is also related to BMI in women. Being underweight (Knuth et al. 1977; Frisch 1981) or overweight or obese are associated with an increased risk of anovulatory infertility (Grodstein et al. 1994; Rich-Edwards et al. 2002). Reduced fecundity in underweight and overweight women is related to altered metabolic and hormonal processes, which may affect follicle growth, embryo development and implantation (Poretsky et al. 1999; Pasquali et al. 2003) and is also associated with lower success in live birth after in vitro fertilisation (IVF) (Fedorcsak et al. 2004).

In overweight and obese infertile women, the presence of polycystic ovary syndrome (PCOS) is frequently observed, which exacerbates infertility due to chronic anovulation and the co-existence of hyperandrogenism and insulin resistance. Previous studies found that obese women with PCOS have a high frequency of infertility (50 %) (Kiddy et al. 1992) and hirsutism (59 %) (Isik et al. 1997). Furthermore, overweight / obesity is a major factor contributing to endocrine disturbance leading to insulin resistance in approximately 80 % of PCOS women (Campbell and Gerich 1990; Morales et al. 1996; Carmina and Lobo 2004) and in 95 % of obese women (Carmina and Lobo 2004). It can be concluded, that abnormal BMI, hormonal and metabolic profile are related to a woman's fertility and all or any combination of these issues may lead to compromised infertility treatment, especially in the use of Assisted Reproductive Technology (ART).

Approximately 1 % of all births in the developed world arise from the application of ART (Centres for Disease Control, 2002). Infertility factors relate to both female and male origin. In addition, PCOS is present in at least 25 % of women who received ART (Norman et al. 1995). These issues are particularly important to consider, when an infertile woman decides to undergo ART treatment. ART success rates have doubled (41.4 %, data from Reproductive Medicine Unit, Adelaide, 2004) since the first introduction of the techniques three decades ago. However, the treatment outcomes are still low and whether changing

some lifestyle factors with regard to healthy eating and weight management can improve ART success has not been studied.

Embryo morphology and growth rate are indicators of embryo quality. The quality of the embryo depends on many factors, such as genetic material, parental age, health and lifestyle, and *in vivo* on growth environment and *in vitro* on culture media. The maternal / paternal age plays an important role in reproduction. The frequency of chromosomal abnormalities proportionally increases with increased maternal age in morphologically normal embryos (Munne et al. 1995). In addition, several animal studies revealed that inadequate nutrition is the second factor impairing the embryo quality and development in cows (Nolan et al. 1998; Yaakub et al. 1999; Siddiqui et al. 2002), rats (Kwong et al. 2000), ewes (Lozano et al. 2003; Peura et al. 2003) and in guinea pigs (Roberts et al. 2001).

Previous publications have shown that weight loss improves endocrine parameters, metabolic status and reproductive function in overweight or obese PCOS women (Clark et al. 1995; Hollmann et al. 1996; Pasquali et al. 1997; Moran et al. 2003). A 5 % - 10 % weight reduction led to improvement of metabolic and endocrine parameters and decreased insulin levels (Pasquali et al. 1989; Kiddy et al. 1992; Foreyt and Poston 1998; Crosignani et al. 2003). In a recent study, a short-term diet (one month) resulted in a significant weight loss and improved reproductive and metabolic parameters in PCOS women (Stamets et al. 2004). Furthermore, animal studies showed the importance of body weight and nutrition on oocyte quality and further embryo development, as increased body weight accumulated by a high caloric intake resulted in an impaired oocyte quality and a low blastocyst yield (Adamiak et al. 2005; Adamiak et al. 2006). However, to the best of my knowledge these studies have not looked at whether improved reproductive function translates to successful live births.

In general, it can be concluded from all the above evidence that some causes of infertility are linked to abnormal body weight, in particular overweight and obese status. The question as

to whether IVF treatment outcome in overweight / obese women can potentially be reversed by the weight management programme using cautiously selected dietary intervention has not been addressed in previous research studies.

The following review will focus on relevant general principles of human reproduction, IVF treatment, nutrition and dietary interventions in overweight / obese women of reproductive age. The aim of this review is to consider the factors and mechanisms influencing the biological and physiological processes with regard to reproductive ability in overweight and obese women and to what extent they can be modulated by energy restriction and weight loss.

1.1 Biological and physiological processes of human reproduction

1.1.1 Human reproduction

Human reproduction is a biological process involving the combination and replication of genetic material of maternal and paternal origin ensuing in a new human individual.

Human reproduction comprises the following structures and stages:

- Patency of the female and male reproductive tracts
- The maintenance of normal oogenesis in women and normal spermatogenesis in men during reproductive age
- Ripening of a quality oocyte and production of quality sperm
- Ejaculation of the sperm in to the woman's reproductive tract and their migration towards the released oocyte
- Fertilisation of the oocyte by spermatozoa
- Transport of the fertilised oocyte (zygote) along the fallopian tube to the uterus
- Synchronised mitotic divisions of the zygote and formation of morula

- Differentiation of the morula into blastocyst
- Establishment of appropriate conditions for embedding the embryo in the uterus
- Implantation of the blastocyst in the wall of the uterus
- Formation of a placenta and maintenance of the fetus in the utero till birth
- Birth of a viable infant (Figure 1.1, page 6)

To carry out these processes certain organs and structures are required in both the male and female. Ovaries and testes are the source of hormones leading to the development of secondary sexual characteristics and production of gametes. The gametes produced must be of sufficient quality to provide a viable embryo. Furthermore, female reproductive organs, such as fallopian tubes and uterus, including their inner layers (endosalpinx and endometrium) must be efficient as they play an important role in:

- a) Transporting the gametes (both female and male origin) and a product of gamete convergence (fertilised oocyte)
- b) Implantation of an early stage embryo
- c) Formation of placenta
- d) Further fetal growth and development and ultimately
- e) Birth of a healthy child and
- f) Expulsion of the placenta.

All of abovementioned processes depend on the normal integrative functioning of the neuro-endocrine and reproductive systems.

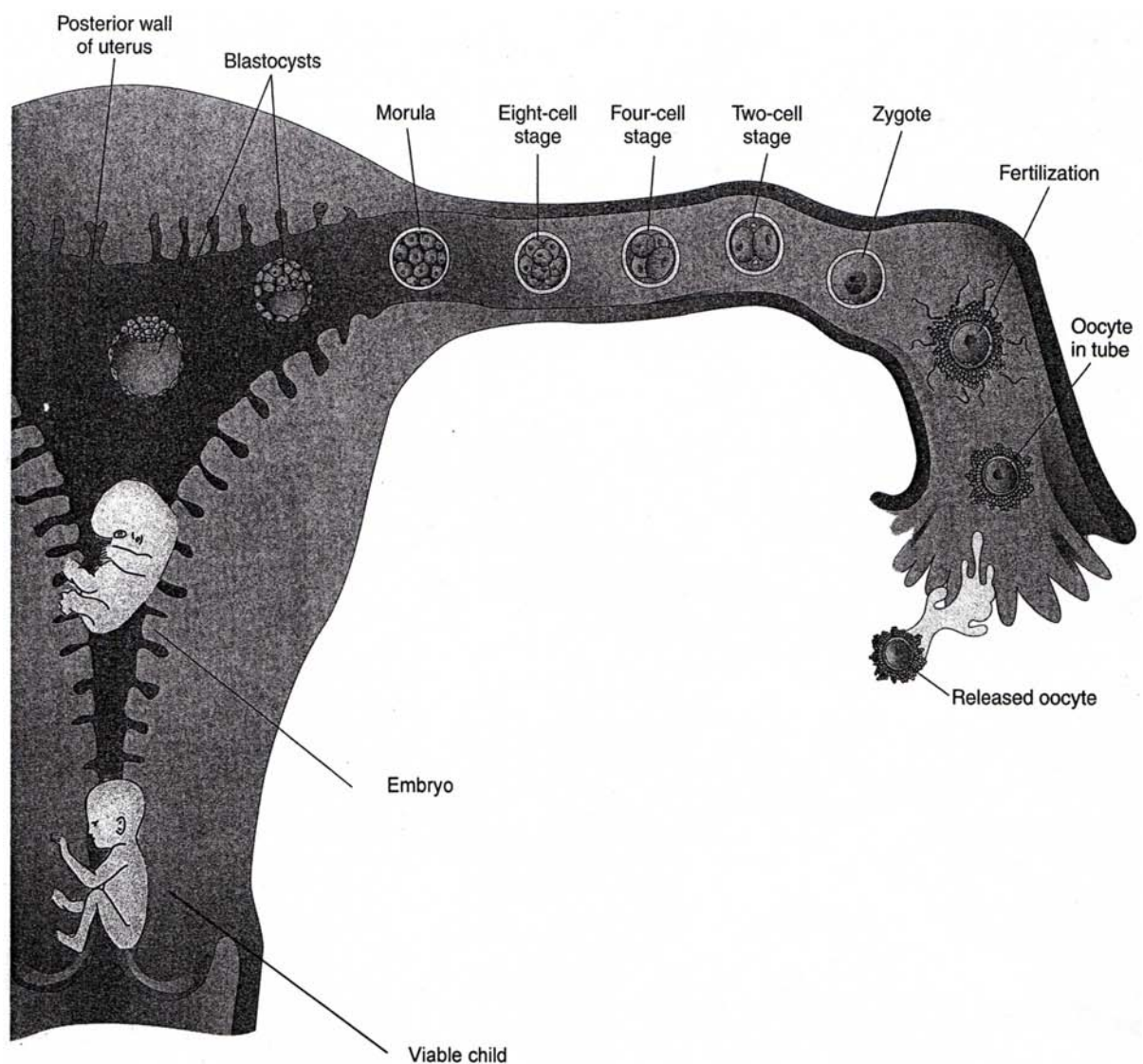


Figure 1.1 Diagrammatic summary of the human reproductive stages: oocyte fertilisation, early embryo cleavage stages, fetal development and birth of the viable child. Modified version of diagram from Moore K.L., and Persaud T. V. N. (1998). *The developing human: Clinically oriented embryology*, 6th Edition. Philadelphia: W. B. Saunders.

1.1.2 Oogenesis

Oogenesis is a process by which the primordial germ cell develops into a mature gamete. This process begins in early fetal life. From the ninth week of embryonic development, primordial germ cells migrate to the fetal ovary from the yolk sac and are referred to as oogonia (Motta et al. 1997). An oogonium proliferates rapidly, replicates its' DNA and differentiates in to a primary oocyte from week 12 – 13 post fertilisation (Gondos et al. 1986) entering meiosis I stage where it remains arrested in the diplotene stage of prophase I until puberty (Anderson and Hirshfield 1992) (Figure 1.2, page 8).

All ~ 6 - 7 million primary oocytes are formed by the fifth month of gestation (Gondos et al. 1986). Different groups of genes are involved in the complex processes of regulation of the ovarian development (Shifren et al. 1993; Bennett et al. 1996). By birth, a layer of follicular cells surrounds the primary oocytes forming primordial follicles. Primary oocytes that fail to proceed to primary follicles are intended to undergo atresia, starting at 5th month of gestation and continue throughout life (Peters et al. 1978; Gondos et al. 1986).

The ovary at birth contains 1 - 2 million germ cells of varying size as a result of prenatal oocyte atresia (Mittwoch and Mahadevaiah 1980). No new oocytes develop after birth during the lifetime of the female. By puberty, the ovary contains approximately 300 000 follicles (Block 1952; Mason 1976) and only 400 - 500 of these become secondary follicles and ovulate during the reproductive life span (A. Henry Sathananthan 2006).

In the absence of fertilisation, the follicle undergoes programmed cell death or apoptosis. There is general agreement that atresia of the follicle is due to apoptosis (Tilly et al. 1991). The vast majority of oocytes undergo apoptosis throughout the women's reproductive lifespan (De Pol et al. 1997).

Normal meiotic division

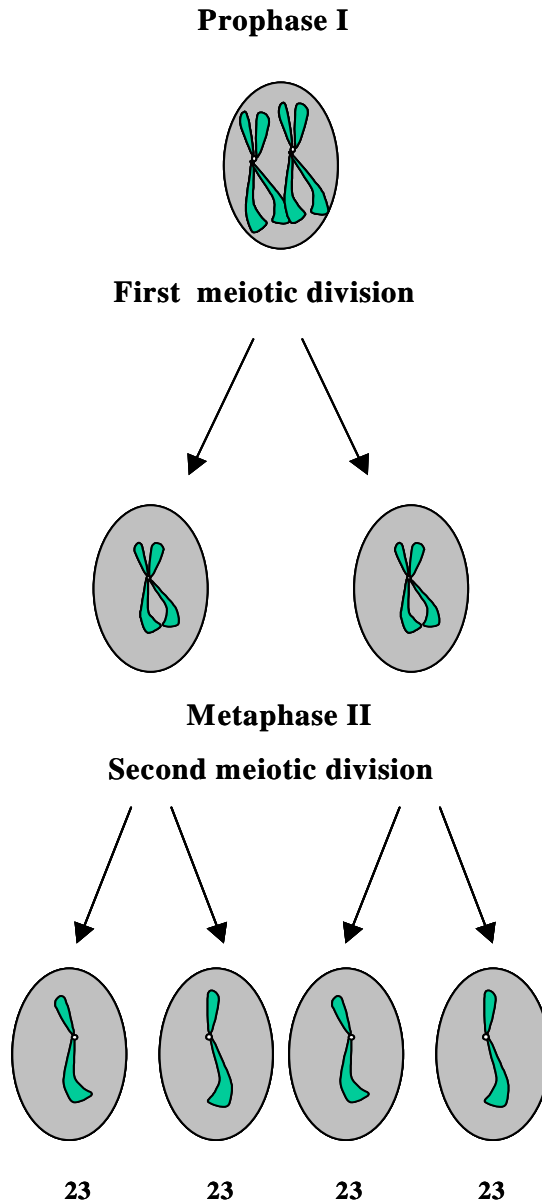


Figure 1.2 Simplified schematic presentation of normal meiotic division preceded by replication of chromosomes and followed by two consecutive cell divisions, Meiosis I and Meiosis II. Meiosis results in production of four daughter cells (gametes) with a haploid number of genetically non – identical chromosomes (23).

Prepubertal girls have a high follicle stimulating hormone (FSH) concentration (Apter 1997), which stimulates further follicular growth and pubertal development (Layman and McDonough 2000). The cumulus cells respond to gonadotrophins and secrete substances that play a role in nuclear and cytoplasmic maturation of oocytes (Chian et al. 1999). Ovarian activation occurs in response to increasing luteinizing hormone (LH) pulses (Spence 1997). After puberty, during each ovarian cycle, a small number of primary oocytes (10 - 43) are stimulated by FSH and LH hormones and undergo further growth and development to secondary oocytes (Figure 1.3, page 11). However, only one dominant follicle is recruited for ovulation from the cohort of secondary oocytes in the first few days of each cycle (Mais et al. 1986). The remainder undergo atresia occurring by alteration in mRNAs required for cell proteins that maintain follicle integrity (Tilly et al. 1992).

The period of approximately three ovulatory cycles or 85 days is required for the follicular growth from secondary follicle (class 1) to preovulatory follicle (class 5 - 8) (Figure 1.4, page 12) (Gougeon 1986; Gougeon 1996). Before ovulation, the first meiotic division is already completed, with unequal division of the cytoplasm, yielding a secondary oocyte and the first polar body, which either degenerates later or may divide during the second meiotic division. The ovulation is a series of complex synchronised processes occurring in the mature oocyte and reproductive organs: extrusion of the secondary oocyte from the ovary and entering the fallopian tube; instigation of the metaphase II and subsequent second meiotic division ensuing in a new arrest of the oocyte. The second meiotic division will be fully completed if the sperm penetration in the ovum occurs. This division is also characterised by unequal division of the cytoplasm, resulting in formation of a mature fertilisable oocyte with a haploid number (23) of chromosomes and the second polar body, which later disintegrates (Figure 1.2, page 8).

It is established that development of the normal haploid gamete depends on the woman's age, health status, lifestyle, nutrition and environmental factors. Amongst of these factors

nutrition is a one of the major factors influencing the reproductive ability of animals (Robinson et al. 1990; O'Callaghan et al. 2000). Any alteration in the genetic material, biological or physiological processes, nutrition or other factors may cause aberrations in gamete structure leading to further abnormal development or cell death.

1.1.3 Abnormal gamete development

Abnormal gamete development can be the result of either chromosomal or morphologic abnormalities. The impact of increasing maternal or paternal age at the time of conception may increase the rate of abnormal meiotic divisions resulting in a new gene mutation (Figure 1.5, page 13). Imbalance in any chromosome can result in non – viable progeny. The older the parents are, the greater the likelihood that germ cells that contain a gene mutation will be passed on to the embryo. The fertility rate of a woman decreases (Menken et al. 1986; Leridon 2004) and likelihood of chromosomal abnormalities increases after the middle of the third decade of life (Kratzer et al. 1992). A study of the human preimplantation embryo showed that 23 % of in vitro fertilised embryos presented with chromosomal abnormalities (Angell, Hillier et al. 1988). The presence of an abnormal karyotype was recorded in about 50 % of spontaneous abortions (Eiben et al. 1990; Gardo and Bajnoczky 1992) where chromosomal trisomy was predominant characteristic (62.1 %) (Eiben et al. 1990). A study showed that ewes fed a high and low (60 % of control) energy intake had similar outcomes with regard to a number, size, quality of collected oocytes and morula formation compared to control group (Borowczyk et al. 2006). However, the cleavage rate and blastocyst formation decreased in low (60 % of control) energy intake group (Borowczyk et al. 2006).

The cause of chromosomal aberration is unknown but could be due to errors arising in spermatozoa or oocyte with regard to the effect of unbalanced nutrition, poor lifestyle, impaired general health, infections and environmental factors, such as pollution, radiation, hot working conditions etc.

NOTE: This figure is included on page 11 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.3 Sequential follicular ultrastructural growth and differentiation proceeding in ovarian cycle. Cross – section of the ovary of woman of reproductive age. Image source: Purves et al., *Life: The Science of Biology*, 4th Edition.

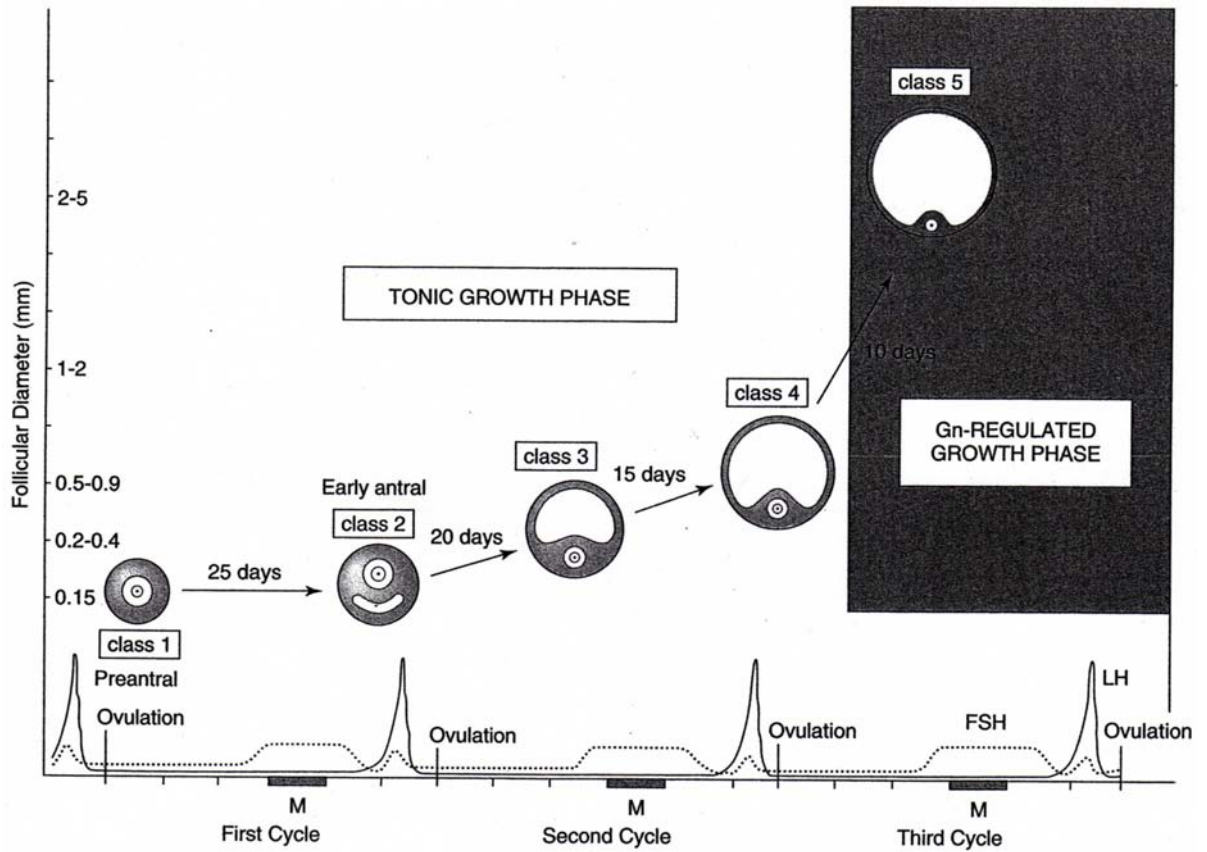


Figure 1.4 This chart shows the period of follicular maturity from preantral stage (class 1) to preovulatory (class 5 – 8). Adapted from Yen S.C., Jaffe R.B. and Barbieri R.L., 1999. *Reproductive endocrinology: Physiology, pathophysiology, and clinical management*, 4th Edition, Philadelphia: W.B. Saunders.

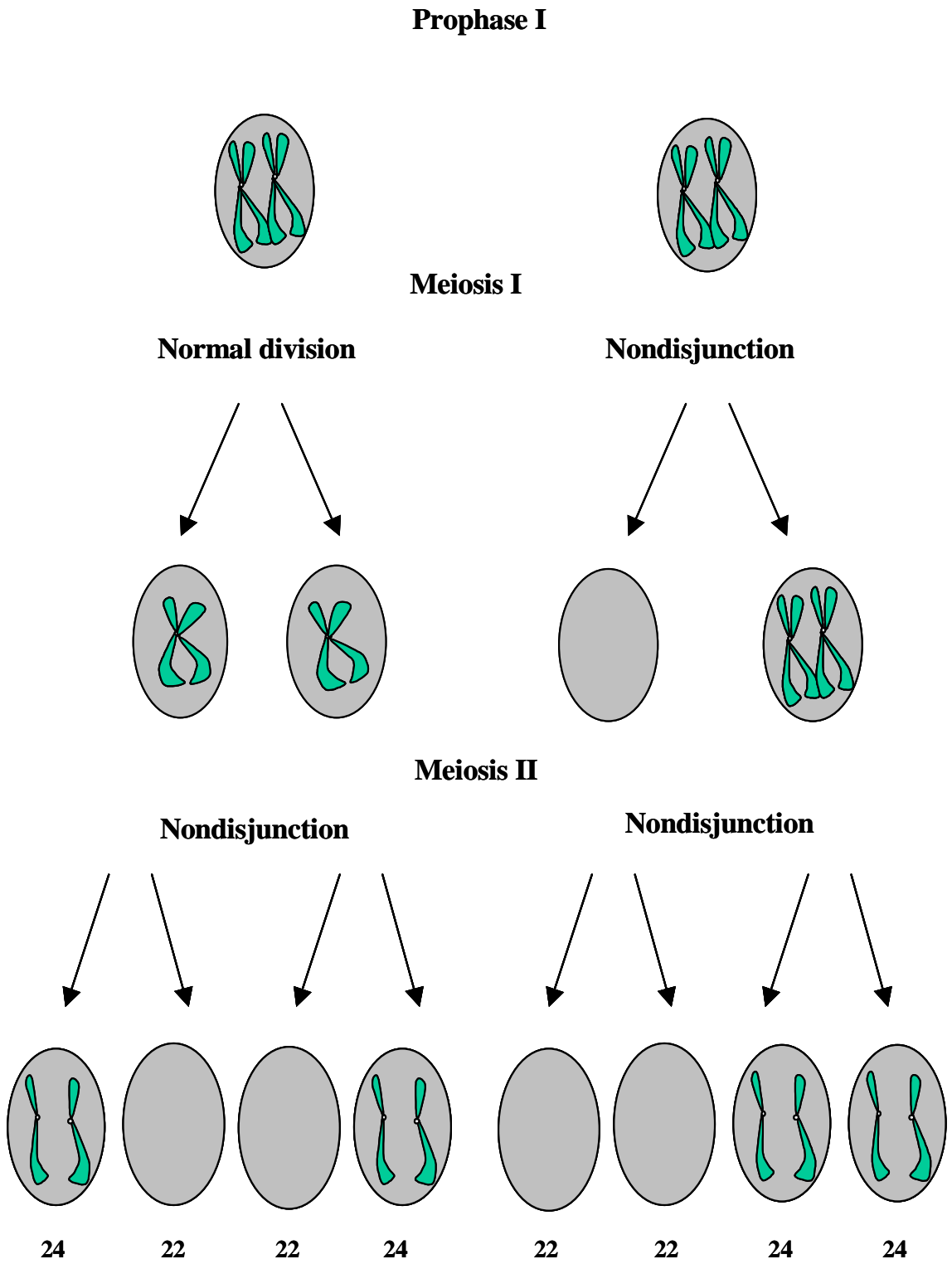


Figure 1.5 Abnormal meiotic segregations.

1.1.4 Ovarian / Menstrual cycle

During the reproductive years, expression of hypothalamic-pituitary hormones and follicular paracrine factors of granulosa and theca cells stimulate ovarian steroid secretion and folliculogenesis, culminating in the cyclic process of follicular maturation and ovulation (Adashi 1994; Canipari 2000). The ovarian cycle consists of the follicular phase (proliferative phase) and luteal phase (secretory phase) (Figure 1.6 (a), page 15).

Throughout the ovarian cycle, the follicles undergo remarkable morphological and ultrastructural changes resulting in sequential development and differentiation (Figure 1.3, page 11). The early follicular phase is initiated at day 7 after immature follicles response to gonadotrophin stimulation and proceeds till day 14 of the menstrual cycle. The ovaries start to synthesize testosterone and most of it converts to oestrogens in the granulosa cells. The presence of FSH promotes follicular growth and the oocyte increases 300 - fold in size, the granulosa cells proliferate and form an antrum, which later develops into the small antral follicle (Layman 2000; Scott et al. 2000) producing oestradiol, the principal ovarian oestrogen. On Day 10 of the cycle an increased level of follicular progesterone can be detected (Yong et al. 1992). In the preovulatory phase, LH levels rise dramatically and enhance antral follicle development to the preovulatory stage (Richards and Bogovich 1982) with ovulation occurring within next 28 - 32 hours (Fanchin et al. 2001) (Figure 1.6, page 15). The luteal phase starts on day 14 and ends on day 28, and includes development of the corpus luteum from luteinisation of the granulosa and theca cells, capillaries and blood vessels, and further regression and replacement by corpus albicans, unless pregnancy occurs. The luteal phase ends in involution of the corpus luteum, which is associated with a fall in both estradiol and progesterone levels (Figure 1.6 (c), page 15). These processes associate with commencement of a menstrual phase proceeding for 4 – 5 days and initiation of a new follicular phase of a next ovarian cycle.

NOTE: This figure is included on page 15 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.6 Menstrual cycle. The schematic presentation of synchronised physiological processes occurring in neuro – endocrine organs and tissues involved in coordination of normal reproductive function during ovarian cycle.

- a) Processes occurring in endometrial tissue during each ovarian cycle
- b) Ovarian response
- c) Hypothalamic - pituitary response and
- d) Temperature fluctuation during menstrual cycle.

Source of unknown origin.

As summarised, ovulation is a complex process controlled by the hypothalamic - pituitary axis, autocrine / paracrine factors and number of hormones transmitting messages between the ovary and the target tissues and coordinating sequential processes within the follicle destined to ovulate. To maintain the normal ovarian cycle, a precise hormone profile is imperative throughout the ovarian cycle.

Factors, including PCOS, obesity and poor lifestyle influence normal activity of the menstrual cycle and are described in the following Sections 1.7 and 1.8, pp 32 - 42.

1.1.5 Role of hormones in ovarian function

Physiological and psychological factors influence hormonal status and may suppress its regularity and consequently impede fecundity. In women of reproductive age, the normal ovary secretes pregnenolone, progesterone, 17 α -hydroxyprogesterone, testosterone, estrone, estradiol, androstenedione and DHEAs (Baird and Guevara 1969; Baird et al. 1974). The gonads and adrenals primarily produce the steroid hormones, androgens, estrogens and progestogens (Hillier 1998). Three following categories of tissues: the ovary, subcutaneous skin and fat, and physiologic and pathologic target sites (hypothalamus and endometriosis cells) secrete estradiol (Bulun et al. 1999). Steroid hormone levels vary in the reproductive cycle and during the reproductive life of the woman (O' Malley 1999). Synchronised processes occurring in the body lead to the cyclic variation of the hormone levels controlling the function of reproductive organs and tissues.

However, these processes can be impaired causing malfunction of particular organs. The most common cause of abnormal ovarian function leading to infertility is chronic anovulation presented by five factors: hypothalamic anovulation, androgen excess, hyperprolactinemia, premature ovarian failure and chronic illness. The causes of anovulation are diverse and relate to physiological and pathological processes that ensue in reproductive target tissues. In 75 % of anovulatory women (Hull 1987; Franks 1995) and in about 23 % of

ovulatory women (Polson et al. 1988; Clayton et al. 1992; Farquhar et al. 1994) the presence of polycystic ovaries (PCO) have been diagnosed by ultrasonography. In addition, polycystic ovaries promote excess secretion of androstenedione, which is a common elevated hormone in PCOS (Kasuga 1980). Furthermore, women with PCOS have a higher level of LH concentration, but low levels of FSH in the early follicular phase compared to women without PCOS (Venturoli et al. 1988; Imse et al. 1992; Hayes et al. 1998). Moreover, DHEAs levels are elevated in 50 % of anovulatory women with PCOS (Carmina et al. 1986). The presence of insulin decreases the serum sex hormone binding globulin (SHBG) concentrations and increases free testosterone levels in PCOS women (Nestler 2000). The PCOS causes are more precisely discussed in Section 1.8, pp 37 - 41. All the above-described research supports an association between impaired reproductive function and abnormal hormonal status. Furthermore, the altered physiological processes and lifestyle may impede hormone production or their normal secretion and circulation in the blood stream in women with normal reproductive function. Other factors, such as stress, excessive physical activities, poor lifestyle, and abnormal BMI are known to be a negative factors affecting hormonal stimulation resulting in the failure of fertility. Some of these factors will be described in details further in Section 1.7, pp 32 - 36. Furthermore, ovarian function and oocyte quality are dependant on the concept of an 'ovarian reserve' in women of reproductive age.

1.1.6 'Ovarian reserve' as a predictor of reproductive endurance

The term ovarian reserve is used to describe the quality and quantity of the oocyte population in ovaries. Ovarian reserve is a predictor of reproductive lifespan. A few methods have been used to establish the capacity of the ovarian reserve and of the most accurate tests is a high-resolution ultrasound (Wallace and Kelsey 2004). Diminished ovarian reserve results in suboptimal ovarian response to induction of ovulation and poor pregnancy rates. Before menopause occurs, there appears to be an acceleration of follicular loss, which is

associated with an increased level of FSH and decrease in inhibin (Lee et al. 1988; Hughes et al. 1990). The loss of oocytes through atresia is a response to changes of many factors, particularly fluctuating gonadotropin levels, autocrine and paracrine factors and diminished ovarian steroid synthesis.

As previously reviewed, the fluctuation of hormone levels plays a pivotal role in ovarian function and perhaps on the capacity to preserve the number of follicles available at birth till late reproductive age. When the pool of primary oocytes is exhausted, it results in the cessation of the menstrual cycles and occurrence of menopause, which is an irreversible process caused by programmed cell death in up to 99.9 % of the cells in the female germ line and leads to infertility and ovarian failure in humans (Tilly 2001). Little is known about how BMI and nutrition affects ovarian reserve.

1.2 Embryology

Knowledge of embryology is essential for better understanding of embryo developmental stages and the causes of congenital malformations. Both are primarily related to health status and genetic characteristics of both maternal and paternal origin. This section will consider normal and abnormal development and growth of the embryo. Any physiological or pathological changes occurring in the maternal - embryo link during embryo development and growth may lead to irreparable consequences, such as congenital malformations and / or abortion of the embryo.

1.2.1 Fertilisation and embryo morphology

Normal embryo morphology and development are related to physiological and pathological processes occurring in the female before and after the time of conception. Fertilisation is defined as the convergence of two gametes, a spermatozoal nucleus of paternal origin with an oocyte nucleus of maternal origin that are competent to converge, undergo meiotic

division resulting in the combined set of chromosomes. The result of fertilisation is a primary nucleus capable to undergo further cellular divisions and form an embryo.

Fertilisation takes place in the fallopian tube within 12 - 24 hours after ovulation (Figure 1.7, page 21). In a normally fertilised oocyte, preparation for mitotic division begins with two haploid pronuclei duplicating their DNA. Within 20 - 34 hours after insemination, the pronuclei congregate and syngamy occurs. This is followed by division for 35.6 hours, which results in the formation of two diploid blastomeres (Trounson et al. 1982; Cummins et al. 1986). A rapid increase of blastomere numbers (daughter cells) occurs by mitotic division during the preimplantation phase. The first blastomere cell division is meridional, with the polar bodies marking one pole (Edwards and Beard 1997), and the second cell division involves equatorial division for one blastomere and meridional for the second blastomere (Antczak and Van Blerkom 1999). Early entry into the first division indicates enhanced embryo viability and higher pregnancy rates (Shoukir et al. 1997).

During this time, the embryo undergoes compaction, the formation of tight junctions and gap junctions, blastomere polarisations, resulting in segregation of two cellular populations outside and inside cells and in communication between blastomeres (Elder and Dale 2000). For the next 2 - 4 days, the fertilised oocyte remains unattached within the tubal lumen, undertakes number of mitotic cell divisions and forms a morula. Afterward, the morula relocates down the fallopian tube, enters the uterine cavity and resides unattached to the endometrium for the subsequent 2 - 3 days. During this time, at the presence of 20 - 30 cells a cavity starts to form within the morula and the embryo is called a blastocyst (Mohr and Trounson 1982).

Blastocyst formation *in vitro* occurs between Day 5 and Day 7 post-insemination (Dokras et al. 1991; Dokras et al. 1993). Cavitation involves the formation of two types of cells: the trophoblast, which forms extraembryonic tissue; and the inner cell mass, which forms the

embryo lineage. When trophectoderm and inner cell mass are distinct and the cavity is visible, the embryo is called an early blastocyst. Further growth and expanding of the cavity forms an expanding blastocyst, which by day 6 or 7 ruptures the zona pellucida and escapes in the uterine cavity. This process is called hatching and the fully escaped blastocyst is called a hatched blastocyst (Figure 1.7, page 21).

1.2.2 Embryo quality

One of the most common morphological features used to assess embryo quality throughout human embryology is embryonic fragmentation, which is the extrusion of the plasma membrane and subjacent cytoplasm of an embryo into the extracellular region. Fragmentation appears in *in vitro* grown human embryos and embryos with 10 – 50% fragmentation on Day 2 showing low implantation rates (Staessen et al. 1993; Giorgetti et al. 1995).

The cell number (Mohr and Trounson 1982; Fong and Bongso 1999) and hatching time (Van Blerkom 1993) are variable within morphologically similar blastocysts. It has been suggested by Shoukir et al. 1998 that good quality blastocysts show well - expanded blastocoel cavity, well - defined inner cell mass and last to day 5 or 6 *in vitro* (Shoukir et al. 1998). Furthermore, animal studies showed that the cleavage rate and development of the blastocyst depends on embryo density in culture (Lane and Gardner 1992; Salahuddin et al. 1995). The following section will address the quality of environment and its effect on embryo development.

1.2.3 Embryonic metabolism

Mammalian embryo metabolism is regulated by amino acids (Barnes et al. 1995; Dumoulin et al. 1997; Devreker et al. 1998), proteins, glucose, pyruvate and lactate (Gardner and Lane 1997).

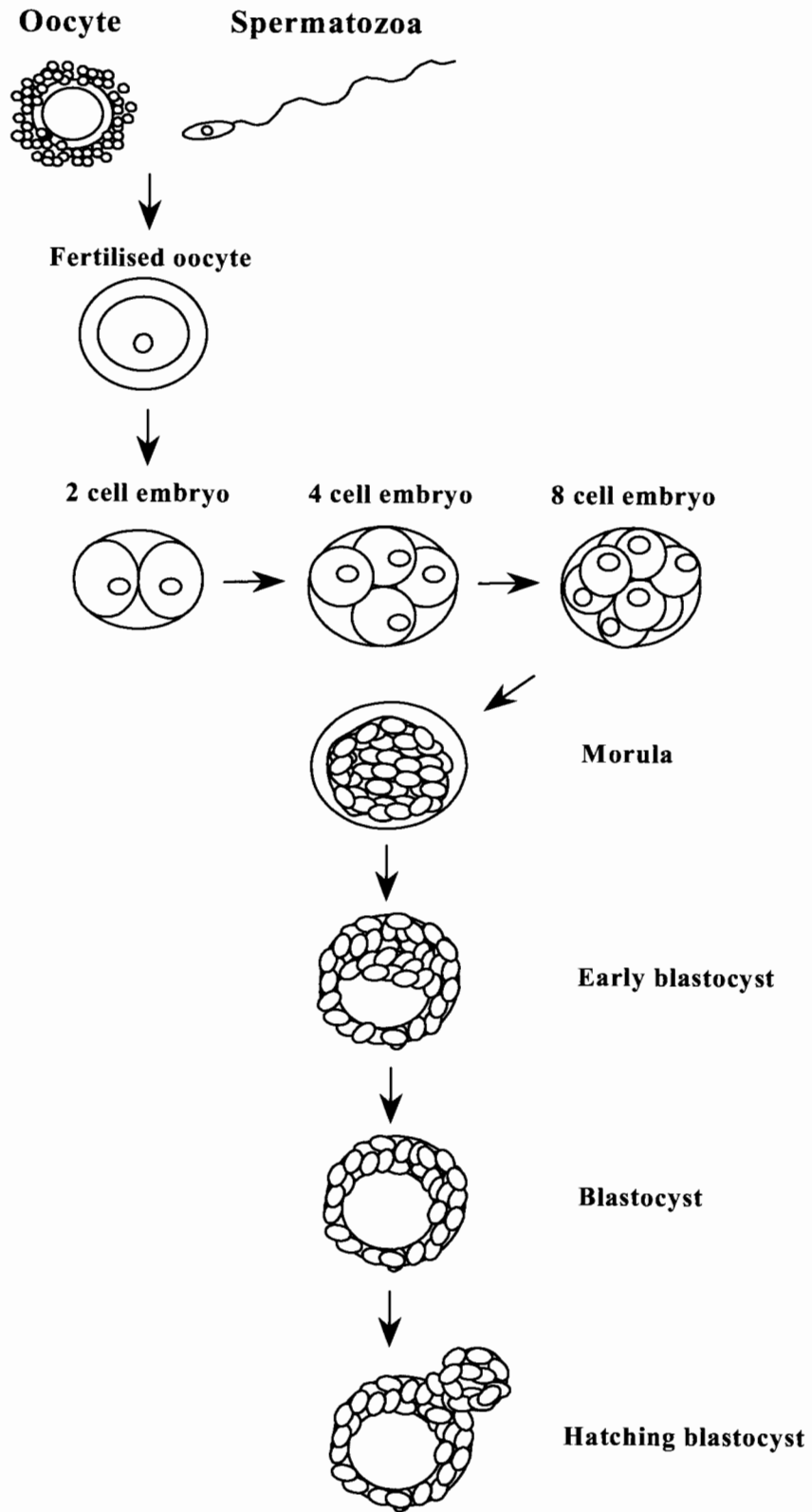


Figure 1.7 Schematic presentation of mammalian oocyte fertilisation and early embryo cleavage stages.

Animal studies have shown that nutrients (inositol, pantothenate (B₅), choline and nicotinamide (B₃)) also are key components of cellular metabolism and play significant roles in embryo quality and the development of cultured embryos of the hamster (Kane and Bavister 1988; McKiernan and Bavister 2000) and mouse (Tsai and Gardner 1994). Amino acids act as buffers of pH in the early embryo and play a role in regulating cell physiology in mouse (Edwards et al. 1998). In the mouse preimplantation embryo, pyruvate is the predominant energy substrate utilized prior to the blastocyst stage (Leese and Barton 1984; Gardner and Leese 1986). Glucose consumption is low at early stages of preimplantation development but increases at the morula stage to become the predominant substrate utilized (Martin and Leese 1995). Elevated glucose levels have been shown to retard the proliferation of the rat blastocyst and trophectoderm cell populations and in the mouse, alter the metabolic state of the embryo (Moley et al. 1996). This suggests that maternal - embryo nutritional communication plays a pivotal role on embryo development, growth and viability. The influence of lifestyle factors on reproductive ability is described in Section 1.7, pp 32 - 36.

1.2.4 Embryonic abnormalities

Poor embryo morphology (embryo development between pronucleate and a 8 – cell stage with irregular shaped blastomeres with severe fragmentation) has been associated with increased chromosomal abnormalities (Almeida and Bolton 1996) and chromosomal mosaicism (Munne and Cohen 1998). Irregular shaped blastomeres and severe fragmentation are associated with poor quality embryos and chromosomal abnormalities in 62 % of in vitro cultured human embryos (Almeida and Bolton 1996). Almeida et al. 1998 found that 63 % of arrested embryos had chromosomal abnormalities between pronuclei and the eight - cell stage (Almeida and Bolton 1998). Furthermore, slow or rapid cleaving embryos showed a higher incidence of chromosomal aneuploidy (Magli et al. 1998). Other studies found that 30 % of arrested embryos were multinucleated (Magli et al. 1998) and 74 % of them were chromosomally abnormal (Kligman et al. 1996). Furthermore, embryo viability was affected

by distribution of the cells between inner cell mass and trophectoderm in mouse (Hardy et al. 1989).

Chromosomal abnormalities (aneuploidy) were significantly increased in embryos from patients with history of recurrent miscarriages (RM) compared to counterparts undergoing IVF treatment with non – abortion history (Simon et al. 1998). Furthermore, the implantation (26 % vs 37 %) and pregnancy rates (21 % vs 29 %) were low in IVF patients with RM compared to counterparts and the results were based on abnormal embryo quality (Rubio et al. 2005). A study on rhesus monkeys showed that individuals with high androgen levels had lower quality oocytes and significantly decreased number of zygotes developed to the blastocyst stage (4 %) (Dumesic et al. 2002).

Apart from chromosomal abnormalities, it has been established that BMI plays an unequivocal role on implantation and pregnancy outcome. Overweight / obese women have a highest rate of miscarriage compared to females with a normal weight (Wang et al. 2000; Koloszar et al. 2002). Fertilisation rates are lower (~ 45 %) in obese women in comparison to normal weight women (Van Swieten et al. 2005). The research area of human obesity and its relation to oocytes quality and embryo formation is a novel area in human reproduction and IVF allows some exploration of this area.

1.3 Implantation

Implantation is the successful establishment and interaction between maternal and embryonic cells (Johnson et al. 1999), which is fully completed 10 days after fertilisation. By the end of the first week of the pregnancy, the human blastocyst enters the uterus, loses zona pellucida following by rapid proliferation of the trophectoderm to form the trophoblastic cell mass, sticks to the uterine wall, breaks down the epithelium and sinks through into the underlying stroma.

Implantation begins 150 hours after the ovulation, when cleavage divisions reach approximately 100 cells. Within this period, synchronized processes occur in the involved parties, the uterus and the embryo, to sustain a new embryo - endometrial relationship. The endometrium undergoes remodelling during the cycle and it must be appropriately prepared for implantation after ovulation in each reproductive cycle. Before implantation, maternal / embryo communication is mediated by the trophoctoderm. After implantation, the blastocyst adheres to and later penetrates the endometrial surface and underlying basement membrane of the endometrium with invasion into the endometrial stroma, where it develops chorionic tissue to facilitate maternal - fetal exchange of nutrients (Rutanen 1993). The endometrial tissue accomplishes transformation (vascularization and adequate intensity) to provide a fetus with essential nutritional support throughout development.

These processes are important to uphold the implantation process and maintain the pregnancy till term. In addition, the hormones (progesterone and human chorionic gonadotropin (hCG)) produced by corpus luteum and trophoblast, respectively are essential until the placenta itself starts to produce progesterone by 6 - 7 weeks after fertilisation. Failure of one of these steps or processes, leads to vasomotor changes, tissue loss and commencement of the menstrual phase with further regeneration of endometrium under the oestrogen stimulation and initiation of the new ovarian cycle.

Initially, the question regarding the embryo quality must be addressed on a basis of environment where the germ cells of maternal and paternal origin produced and matured. The question of the effect of BMI in oocyte-donated treatment on uterine receptivity has been addressed previously and no differences were noted (Wattanakumtornkul et al. 2003). However, Bellver et al. 2003 found that the obese state plays an unequivocal role in infertility treatment and triples the rates of spontaneous abortion (38.1 %) in oocyte donated treatment compared to a normal BMI (13.3 %) (Bellver et al. 2003). A recent study examined the correlation between implantation rate and donated oocytes in women in

different BMI categories and no effect of BMI on implantations and pregnancy rates (Styne-Gross et al. 2005). Compared to the above studies of oocyte donation, an increased BMI was associated with a lower number of collected oocytes and lower embryo survival rate resulting in increased pregnancy loss after IVF / ICSI treatments (Fedorcsak et al. 2004). These studies suggest oocyte quality may be impaired in women who undergo infertility treatment through an ART clinic. The following section will address the processes occurring in the uterus for further embryo development.

1.3.1 Intrauterine environment

The uterus provides the ideal environment for embryonic development and normal growth. Oestrogens and progesterone play key roles in female reproductive tract function. In the proliferative phase of the menstrual cycle, oestrogenic hormones stimulate growth of the uterine glands and increase the thickness of the endometrium. In the late luteal phase, physiological, biochemical and immunological changes occur in the uterine endometrium to allow the blastocyst to implant. The endometrium undergoes vascular remodelling by condensing vascularization (Gambino et al. 2002) and the uterine glands start to secrete carbohydrates and glycogen into the lumen. These complex processes are controlled at the embryo-maternal interface by factors from decidualized endometrium and the trophoblast itself (Duc-Goiran et al. 1999).

Under these distinct biochemical and morphological processes, the uterus, in particular endometrium is prepared for the establishment and maintenance of pregnancy. Oestrogen stimulates proliferation of the uterine epithelium and induces the progesterone receptor. Progestins induce the stromal cell proliferation and differentiation and oppose the oestrogen-induced epithelia cell proliferation.

Human pregnancy can coincide with an array of physiological and pathological conditions that can impede fetal growth and induce intrauterine growth restriction (IUGR). Examples

are genetic anomalies, multiple pregnancies, hormonal insufficiencies, fetal and maternal cardiovascular diseases, infections, maternal nutritional deprivation and “placental vascular insufficiency”. These conditions are not reviewed in this thesis.

1.3.2 Endometrial receptivity

The endometrium is an innermost layer of tissue in the uterus. It lines the uterine cavity and becomes continuous with the lining of the cervical canal. In the late follicular phase, the presence of oestrogen prevailing in endometrial cells (Hoozemans et al. 2004) and oestrogen receptor expression reaches a maximum (Lessey et al. 1988; Snijders et al. 1992). During the early luteal phase, oestrogen receptor expression declines after ovulation and then increases in the mid luteal phase under the influence of the developing corpus luteum. Progesterone is a determining hormone of the luteal phase and acts through the progesterone type A receptor (Wang et al. 1998; Bergeron 2000).

Ovulation occurs in mid ovarian cycle and the necessity of remodelling of the endometrium arises for the further reception and embedding of the fertilised oocyte. The endometrial receptivity is an ability of endometrial epithelium to adhere the blastocyst. This is a self - limited period occurring between day 19 and 24 of ovarian cycle and called a ‘window of receptivity’ (Navot et al. 1991; Wilcox et al. 1999). It is defined as postovulatory stage with various lengths and regulated by the number of synchronised processes dependant on massive changes in metabolic, morphologic, biochemical structures and steroid levels in women. Within this period, an implantation may occur and will be supported by increased progesterone and oestrogen levels deriving from the growing corpus luteum. Establishment and maintenance of the endometrial tissue and communication between embryo and mother at the implantation site ensure a successful pregnancy.

Endometrial thickness plays an important role in implantation of the embryo. A study showed that clinical pregnancy rate was higher (77 %) in patients with the endometrial lining

of $\geq 16\text{mm}$ compared to $\leq 9\text{ mm}$ (53 %) after two blastocyst transfer during IVF cycle (Richter et al. 2007). In a similar study, 67 % of pregnancies miscarried in women with endometrial lining between the range of 7 and 14mm (Weissman et al. 1999). During the window of receptivity integrins are cyclically expressed in the endometrium (Lessey et al. 1992; Gonzalez et al. 1999) and any alteration in the expression impairs endometrial receptivity and leads to infertility (Lessey et al. 1995; Lessey 1997).

Administration of Clomiphene Citrate (CC) to fertile women led to desynchronised expression of integrins (over-expressed α_1 , α_v and β_3 - ESC integrins and under-expressed β_3 - EEC subunit) during the window of endometrial receptivity (Gonzalez et al. 2001) and lowered a pregnancy rate (Kovacs et al. 2003). Furthermore, studying the endometrial receptivity, Quezada et al. 2006 found that epithelial cells had a greater expression of progesterone receptor and lower β_3 -integrin expression in PCOS patients than in counterparts (Quezada et al. 2006). In conclusion, many studies have been conducted with regard to endometrial receptivity, however little is known about the effect of obesity on the 'window of receptivity'.

Alterations in the extent of progesterone, oestrogen and prostaglandins or other pivotal processes, including nutritional status may delay ovum transport and / or successful implantation and maintenance of the pregnancy. If implantation does not occur for some reason, the endometrium returns to a non-receptive status resulting in involution of the corpus luteum and commencement of the new menstrual cycle.

1.4 Implantation and Assisted Reproductive Technology (ART)

Implantation is a complex process of the embedding of an early embryo into endometrial lining. Various authors report that embryo scoring techniques are an essential feature to evaluate the cell number, fragmentation, cytoplasmic pitting, blastomere regularity and expansion and presence of vacuoles (Ziebe et al. 1997; Van Royen et al. 1999; Desai et al.

2000; Kahraman et al. 2000). Another important factor is culture media. The constant development and expansion of embryo culturing techniques result in increased embryo implantation rates in ART. Moreover, in *in vitro*, the embryo quality and growth rate are dependent on the quality of primary cells and later on the culture media and culture conditions.

Pregnancy and implantation rates were better after transferring the embryos on day 3 of culture compared to zygotes transferred on day 1 (Jaroudi et al. 2004). Levron et al. 2002 showed that transferring day 3 embryos has an advantage of day 5 transfer and results in enhanced implantation (38.7 % vs 20.2 %) and clinical pregnancy rates (45.5 % vs 18.6%), correspondingly (Levron et al. 2002).

However, other studies obtained different results suggesting that transferring the day 3 embryos or hatching stage blastocysts had comparable pregnancy and implantation rates (Utsunomiya et al. 2004). Furthermore, similar results were observed in transferring day 3 embryos or blastocyst stage embryos (Coskun et al. 2000; Milki et al. 2002). In addition, the embryo transfer on day 5 was more beneficial than on day 6 (Khorram et al. 2000). Menezo et al. 1995 showed that an embryo transfer at the compacting morula stage resulted in poor pregnancy outcomes (Menezo et al. 1995).

The bias in the above results might be explained by selection of more viable or genetically complete embryos or vice versa. As a result, comparison of the stages used for the transfer and correlation of the embryo quality with regard to developmental rate, blastomere fragmentation, perinuclear and nucleolar behaviour, and multinucleation have been used widely in ART and are still ambiguous due to an absence of a standard worldwide grading system.

1.5 Postimplantation

1.5.1 Age and miscarriage

Early pregnancy loss is defined as cessation of pregnancy in the first 12 weeks of gestation (Farquharson et al. 2005). Spontaneous pregnancy loss occurs in approximately 15 – 20 % of all pregnancies (Brigham et al. 1999; Farquharson et al. 2005). The probability of a viable conception per menstrual cycle is only 30 % (Edwards 1986). Several factors play an important role in infertility. First is the importance of age, which affects fertility and also applies to the prevalence of spontaneous abortion. Approximately 60 % of first trimester abortions are related to the chromosomal abnormalities (Hassold 1980). The incidence of spontaneous abortion increases with advanced age (Gindoff and Jewelewicz 1986; Jacobs et al. 1990; Scott et al. 1993). Miscarriage occurs in 12 % of women younger than 20, and in 26 % of women older than 40 years of age (Warburton 1987). Implantation failures and approximately 50 % anomaly rate are encountered in spontaneously aborted fetuses. A recent study reported that the incidence of spontaneous abortion increased 4 - fold (22 %) in women aged 40 and older compared to 5.3 % in women \leq 30 years of age after IVF cycle (Spandorfer et al. 2004). Most of the patients using ART facilities are in their mid to late thirties or an early forties and apart from increased age with its known negative outcome in infertility treatment, the abnormal weight also adds negative statistic to it and outcomes are described in following section.

1.5.2 BMI and miscarriage

Prepregnancy body weight, defined as underweight (Helgstrand and Andersen 2005) or overweight is an independent risk factor for early pregnancy loss (Hamilton-Fairley et al. 1992; Wang et al. 2002; Bellver et al. 2003). Fedorcsak et al. 2000 showed that obese patients have fewer oocytes collected, higher pregnancy loss rate during the first 6 weeks of pregnancy and lower live-birth rate (Fedorcsak et al. 2000). Miscarriage rates were higher in

women with BMI $> 25 \text{ kg / m}^2$, than in women with normal weight (Loveland et al. 2001). Moreover, obesity in women with PCOS increases miscarriage rates and requires a larger dosage of gonadotropin stimulation (Hamilton-Fairley et al. 1992). Later studies documented that obese women demonstrated a fourfold increase in the risk of miscarriage compared to the normal population (Wang et al. 2002; Bellver et al. 2003). However, in contrast to the above studies, one study reported an unchanged effect of different ranges of weight (underweight, normal, overweight and obesity) on uterine receptivity and embryo quality after standardized hormonal support in women who underwent ICSI (Wattanakumtornkul et al. 2003). Roth et al. 2003 attained similar outcomes where BMI did not affect the rate of spontaneous pregnancy loss and ongoing pregnancy (Roth et al. 2003). The abovementioned studies outcomes are results of different study designs and controversy does support the idea of that this area of interest with regard to abnormal weight and reproduction needs further clarification.

1.5.3 Nutrition, miscarriage and birth defects

A vital source of a healthy pregnancy outcome is appropriate nutrient intake during periconception. A study of high intake of vitamin A showed teratogenic effect on fetal formation (Rothman et al. 1995). Moreover, low folate intake during pregnancy was correlated with an early spontaneous abortion (George et al. 2002) and preterm delivery (Scholl et al. 1996). More than two decades ago, Czeizel et al. 1992 confirmed the importance of periconceptional intake of the trace – elements and multivitamin supplements to reduce the incidence of fetal malformations (Czeizel and Dudas 1992).

1.6 Infertility and ART

Infertility is defined as diminished fertility and inability of a couple to achieve a pregnancy after 1 year of unprotected intercourse. Factors contributing to infertility are linked to anatomical dysfunction of certain organs; abnormal function or processes in neuro -

endocrine and immune system, gametogenesis, reproductive tract infections, exposure to toxins, malnutrition and unexplained manifestations in both males and females. More than 1 % of people worldwide are infertile (Wilks and Hay 2004). According to the World Health Organization (WHO) report, 51.2 % of infertility is due to a male factor in infertile couples (WHO 1987). The incidence of infertility has increased substantially and diagnoses and appropriate management is an essential constituent of reproductive health services.

Since the first IVF and intracytoplasmic sperm injection (ICSI) were introduced (Van Steirteghem et al. 2002) the range of therapeutic options available from reproductive health units for infertile patients has grown. ART has brought many options to assist infertile couples (Thornton 2000), such as intrauterine insemination (IUI), the most common procedure in vitro fertilization (IVF) and embryo transfer (ET), intracytoplasmic sperm injection (ICSI), gamete intra - fallopian transfer (GIFT), cryopreservation and frozen embryo transfer (FET). Clinical effectiveness of ART treatment is continuously improving and results in a greater opportunity for infertile couples to be parents.

1.6.1 Implications of ART

Successful outcomes of assisted reproduction depend on the technology and the general health of the couples using this alternative for infertility treatment. The major reasons for using ART are tubal factors 58 % (Hubacher et al. 2004), male factor infertility 45 % (Franco et al. 2002), ovulatory dysfunction 36 % (Collins et al. 1986), pelvic endometriosis 10 % (Wheeler 1989; Franke et al. 2000; Vigano et al. 2004) and unexplained infertility 15 % - 24 % (Templeton and Penney 1982; Guzick et al. 1994).

The success of assisted reproduction depends on females' age (Abdalla and Thum 2004). Women aged 35 - 39 years are 31 % less fertile than 20 - 24 year olds (Menken et al. 1986). In 2003, US Fertility clinics National report revealed that pregnancy rates were 2.5 - fold higher in women aged < 35 (43 %) compared to age > 41 years (18.5 %) (CDC 2005).

A prospective study of success rates of ART showed that treatment cycles for male infertility factor had higher success compared to female factors or unexplained infertility, and the overall pregnancy rate was 35.2 %, while the live-birth rate was 25.1 % (Rizvi et al. 2004).

Australia is one of the leading countries successfully using ART. The IVF and ICSI procedures are the most commonly used methods in ART (Bryant et al. 2004). The viable pregnancy rate after ART increased from 15.9 to 17.9 per 100 embryo transfer cycles between 1999 and 2000, and the number of births increased by 11.5 % from 1998 to 1999 in Australia & New Zealand (Hurst and Lancaster 2000). The latest statistics on the viable pregnancy rate increased to 21.1 % in Australia & New Zealand, 2003 (Waters et al. 2006). The distribution of infertility causes in Australia & New Zealand were 23.7 % related to male factor, 26.1 % to the female factor, combined factors were associated with 30.5 %, unexplained – 15.5 % and 4.1 % were related to the not stated infertility causes (Waters et al. 2006).

Statistics from the South Australia (2004) showed that the pregnancy rate after the single embryo transfer was 41.4 % in women aged less than 38 years (obtained from www.repromed.com.au). However, the factors underlying successful and failed ART are likely to be complex and evidence suggests that this involves some unexplained matters.

1.7 Influence of lifestyle factors on fecundity

1.7.1 Negative effect of smoking on female's fertility

Cigarette smoking plays an unambiguous role in the aetiology of female infertility. Zenzes et al. 1996 found tobacco metabolites in the follicular fluid of smokers (Zenzes et al. 1996). For smokers compared to non-smokers, almost twice the number of IVF cycles were required to become pregnant (Feichtinger et al. 1997). Furthermore, smoking significantly reduced ovarian reserve and response to ovarian stimulation (El-Nemr et al. 1998), increased risk of

early pregnancy loss (Cooper et al. 1995; Winter et al. 2002) and resulted in 'poor quality' embryo(s) (Winter et al. 2002).

A survey assessing women's knowledge regarding smoking and health risks concluded that most women were unaware of the specific risks relating to smoking: risk with infertility (22 %) early menopause (17%), spontaneous abortion (39 %), ectopic pregnancy (27 %) and cervical cancer (24 %) (Roth and Taylor 2001). Periconceptional smoking reduced the number of retrieved oocytes during IVF and GIFT procedures (Klonoff-Cohen et al. 2001), increased the risk of spontaneous abortion (Maximovich and Beyler 1995) and significantly reduced livebirth rate (Lintsen et al. 2005). In addition to tobacco, use of cocaine pre- and postconception enhances risk of spontaneous abortion (Ness et al. 1999).

1.7.2 Alcohol intake and fertility

Two decades ago, two studies obtained controversial results regarding the relationship between the alcohol intake and females fertility. The moderate alcohol consumption has been suggested as not harmful to women trying to become pregnant (Zaadstra et al. 1994) and there is weak evidence between infertility status and alcohol intake (Olsen et al. 1997). However, Grodstein et al. 1994 demonstrated a close relationship between dosages of alcohol intake (moderate or heavy) and increased to 50 % risk of the endometriosis in women having fertility problem (Grodstein et al. 1994).

Latest studies confirmed Grodstein's result and showed that spontaneous abortion rates are proportionally increased with advanced consumption of alcohol in both males and females at the time of conception (Klonoff-Cohen et al. 2003; Henriksen et al. 2004). Furthermore, Henriksen et al. 2004 showed that spontaneous abortion occurred in 15 % of non - drinkers, 30 % of alcohol intake 1 - 4 times / week and 40 % of cases were related to alcohol intake more than 10 times / week (Henriksen et al. 2004).

1.7.3 Negative influence of caffeine, emotional and physical stress on reproduction

It has been established that caffeine consumption also has a negative effect on fertility and increases risk of spontaneous abortion (Curtis et al. 1997; Cnattingius et al. 2000). A recent study showed that caffeine intake > 50mg / day by both genders undergoing IVF / GIFT procedures was associated with livebirth rate and gestational age of infants (Klonoff-Cohen et al. 2002).

In addition, infertility status is associated with a high prevalence of depressive symptoms (Kee et al. 2000) and 50 % of women rated IVF treatment as the most traumatic experience in their life (Freeman et al. 1985). Women with a 2 – 3 year history of infertility similar to women with known causes of infertility experience higher rates of depression (Domar et al. 1992). Moreover, women experiencing a high level of physiological stress had a prolonged menstrual cycle (> 35 days) and increased rate of spontaneous abortion (Hjollund et al. 1999). Estradiol blood levels were significantly lower in women with depression (Young et al. 2000). Furthermore, women with hypothalamic amenorrhea demonstrate hypercortisolism, which was suggested as the pathway by which stress interrupts reproductive function (Biller et al. 1990; Chrousos and Gold 1992; Meczekalski et al. 2000). Amenorrhoea is also associated with intense exercise (Williams et al. 2001) and Bullen et al. 1985 showed that the majority of women regained ovulation after a reduction in stress and cessation of physical exercise (Bullen et al. 1985).

1.7.4 Detrimental effects of abnormal body weight on woman's fertility

An increased BMI is associated with the atypical variability of steroid hormone levels affecting normal neuro - endocrine processes. Three decades ago, Hemsell et al. 1974 hypothesised that an increased level of circulating oestrogen may be produced by the increased mass of the adipose tissue and skin in obese women (Hemsell et al. 1974). Production of another hormone, dihydrotestosterone (DHT), in the skin plays a role in the

aetiology of hirsutism (Azziz et al. 2000), which is also closely associated with PCOS. Women with central obesity have a greater adrenal activity with increases in adrenocorticotrophic hormone (ACTH) and cortisol secretion (Pasquali et al. 1993). Ukkola et al. 2001 showed that BMI is an important predictor of testosterone levels (Ukkola et al. 2001).

Obesity is associated with anovulation (Hartz et al. 1979; Reid and Van Vugt 1987), amenorrhoea, increased risk of infertility (Bolumar et al. 2000; Moran and Norman 2002; Homan 2006) and spontaneous abortion (Wang et al. 2002). Moreover, clinical characteristics of obese women include a combination of insulin resistance and androgen excess. Insulin resistance and compensatory hyperinsulinemia frequently accompany intra - abdominal fat (Carey et al. 1996). The combination of obesity and PCOS is associated with more severe degrees of insulin resistance in obese than in non - obese PCOS women (Campbell and Gerich 1990; Jahanfar et al. 1995; Morales et al. 1996). Among all other problems, overweight anovulatory women with androgen excess have an android type of body fat distribution or increased waist - to - hip ratio (Peiris et al. 1989; Kirschner et al. 1990; Pasquali et al. 1991). In conclusion, it has been documented that a woman's fecundity is primarily related to abnormal BMI, in particular to the overweight / obese state (Wang et al. 2000; Koloszar et al. 2002).

1.7.4.1 BMI and ART success

The prevalence of overweight and obesity in women of the reproductive age in Australian population is 24 % and 16 %, correspondingly (Cameron et al. 2003). In ART practice, 34% of IVF patients of reproductive age are significantly overweight or obese (Callaway et al. 2006). It has been shown that pregnancy rates are reduced whether conception is spontaneous or by ovulation induction and IVF in ovulatory obese women (Hamilton-Fairley et al. 1992; Zaadstra et al. 1993; Wittemer et al. 2000). Furthermore, 31 % of IVF cycles

were cancelled due to the insufficient response to treatment in overweight patients (Mulders et al. 2003). The response to gonadotropin induction of ovulation is inversely related to BMI in women with PCOS (Hamilton-Fairley et al. 1992; Crosignani et al. 1994). Loveland et al. 2001 established that the duration of stimulation, gonadotropin requirements and spontaneous miscarriage rates were slightly higher in patients with a BMI > 25 kg/m² while basal rate of FSH, implantation and pregnancy rates were significantly lower (Loveland et al. 2001).

1.7.4.2 BMI a predictor of general health issues

There is a strong curvilinear relationship between BMI and relative body fat mass (Gallagher et al. 2000). BMI provides an acceptable measurement for assessment of total body fat and is determined as weight (kg) divided by height squared (m²). According to the World Health Organization (WHO) classification (2000), BMI ≤ 18.5 kg/m² defines underweight condition, BMI between 18.5 and 24.9 kg/m² is a normal weight, BMI > 25 < 29.9 kg/m² is overweight condition and obesity is BMI ≥ 30 kg / m², Table 1.1, page 37 (WHO 2000).

The prevalence of increased body weight has doubled over the past two decades and a definite trend to escalation is observed (Cameron et al. 2003). Approximately 52 % of Australian females (aged 25 years and over) are overweight or obese (Cameron et al. 2003). A BMI greater than 30 kg/m² is related to a higher risk of reduced reproductive function, infertility, insulin resistance, cardiovascular diseases, including hypertension and adverse cholesterol-lipoprotein profiles (Lapidus et al. 1984) and mortality in women, compared to women with lower BMI. Independently from BMI, there are other measurements for body fat assessment, such as waist circumference and waist-to-hip ratio, which have been widely used in other studies. Waist circumference has been found as a better characteristic of central abdominal fat compared to waist-to-hip ratio measurement (Pouliot et al. 1994; Dos Santos et al. 2005) and compared to BMI is a significant predictor of morbidity in obesity related health risks (Janssen et al. 2004).

Weight Classification	BMI (kg / m²)
Underweight	< 16
▪ Severe thinness	< 16
▪ Moderate thinness	16 – 16.9
▪ Mild thinness	17 – 18.49
Normal range	18.5 – 24.9
Overweight	≥ 25 – 29.9
Obese	≥ 30
▪ Obese class I	30 – 34.9
▪ Obese class II	35 – 39.9
▪ Obese class III	≥ 40

Table 1.1 BMI classification.

Adapted and combined from World Health Organisation (1995, 2000 and 2004).

1.8 Polycystic Ovarian Syndrome (PCOS)

PCOS is a heterogeneous condition and one of the most common endocrinological disorders affecting 4 – 10 % of women of reproductive age (Diamanti-Kandarakis et al. 1999; Lobo and Carmina 2000). PCOS is the result of a complex processes involving pathologic mechanisms in target tissues, abnormal steroidogenesis and steroid action of various endocrine and subcutaneous tissues. The latest criteria for PCOS diagnosis was revised on ESHRE workshop (2003) and new definition includes the following criteria: oligo - or anovulation, clinical or biochemical presence of hyperandrogenism and presence of polycystic ovaries (excluding presence of: Cushing's syndrome, congenital adrenal hyperplasia and androgen secreting tumours) (Casimirri et al. 1989).

In clinical practice, women with PCOS present with infertility (40 %)(Franks 1989), menstrual irregularity (dysfunction bleeding 29 %; amenorrhea 51 %), hyperandrogenism (59 %) (Isik et al. 1997). Pregnancy in patients with polycystic ovaries (PCO) is more likely to terminate in miscarriage (Sagle et al. 1988; Regan et al. 1990; Tulppala et al. 1993; Clifford et al. 1994). Patients who miscarry have a higher androgen levels than women with an ongoing pregnancy (Tulppala et al. 1993). Furthermore, high levels of LH and androgens have been related to poor reproductive performance (Regan et al. 1990; Okon et al. 1998) and approximately 55 – 75 % of women with PCOS have elevated LH (Barnes et al. 1989; Kalro et al. 2001). LH pulse amplitude increased in PCOS non-obese women and is normal in overweight PCOS women (Arroyo et al. 1997). Alteration in LH secretion might be caused by hyperinsulinism in PCOS women (Dunaif 1997). FSH administration decreases the incidence of apoptosis in granulosa cells in follicles of normal ovaries compared to follicles from polycystic ovaries (Mikkelsen et al. 2001). Moreover, oocyte maturation and implantation rates were improved after FSH administration prior to harvesting of immature oocytes in *in vitro* maturation (IVM) process in PCOS patients (Mikkelsen and Lindenberg 2001).

In summary, PCOS is characterised by hyperandrogenism, abnormal menstrual cycles, chronic anovulation and polycystic ovaries, with an increased risk of diabetes mellitus and potentially, cardiovascular diseases. Moreover, PCOS is also associated with changes in the ovarian morphology, menstrual abnormalities, chronic anovulation, hyperinsulinemia, insulin resistance and subfertility or infertility (Correa and Jacoby 1978; Frisch 1989; Lake et al. 1997) in women of reproductive age. The aetiology of PCOS remains unclear. One theory is based on the role of insulin resistance and hyperinsulinemia. Some PCOS women also experience several metabolic abnormalities, including central obesity, abnormal glucose metabolism and abnormal blood lipid levels, similar to the metabolic syndrome or Syndrome X.

1.8.1 Relationship between PCOS, obesity and insulin resistance

In fact, obesity, PCOS and insulin resistance together or in separate perspective are linked to a number of identical disorders, in particular glucose intolerance, reproductive disturbances, metabolic abnormalities and cardiovascular diseases. Approximately 50 % of women with PCOS are overweight or obese, reflecting primarily visceral adiposity (Gambineri et al. 2002; Silfen et al. 2003). Furthermore, PCOS occurs in at least 25 % of women who received ART treatment (Clark et al. 1995). It should be emphasised that the major factor contributing to insulin resistance and hyperinsulinemia in PCOS is obesity (Campbell and Gerich 1990; Morales et al. 1996). Norman et al. 1995 found a significant correlation between increased BMI, insulin resistance and hormone imbalance in a subgroup of PCOS women compared to lean PCOS women (Norman et al. 1995; Dravecka et al. 2003). Wang et al. 2002 showed that the increased miscarriage rate was proportionally increased with BMI; women with normal BMI had 18 % of miscarriage rate; overweight women – 27 % and obese women – 31 % of all pregnancies, which is almost twice the rate for early miscarriages in women with normal BMI (Wang et al. 2002). Insulin and androgens together influence the pattern of body fat distribution (preferential abdominal body fat localization) in overweight

women (Pasquali et al. 1991; Ditkoff et al. 1995). Insulin regulates ovarian function including steroidogenesis. Carey et al. 1996 demonstrated that abdominal adiposity is a major determinant of insulin resistance in women (Carey et al. 1996) and a BMI > 25 kg/m² is a predictor of gestational diabetes (Turhan et al. 2003).

Insulin resistance is characterised by an exaggerated insulin response to maintain a normal glucose response to ingested carbohydrate (Reaven 1988). A study confirmed that insulin resistance is more common in obese PCOS women (Dunaif 1997) and women with PCOS are considered at risk of gestational diabetes (Lanzone et al. 1995). Insulin resistance, hyperinsulinemia, obesity (presented as central obesity) may be the key factors linking obesity and PCOS together or vice versa.

1.8.2 Weight loss and insulin resistance

Commonly, insulin resistance results in hyperinsulinemia leading to abnormal ovarian and adrenal androgen secretion (Nestler and Jakubowicz 1996; Diamanti-Kandarakis et al. 1998; Nestler et al. 1998; Glueck et al. 1999; Gordon 1999; Kahn and Gordon 1999; Moghetti et al. 2000) associated with abnormal LH and FSH release and subsequent menstrual disturbances. However, insulin resistance improves simultaneously with energy restriction, even before significant amounts of weight loss have occurred. Plasma insulin concentrations reduced concomitantly under dietary restriction and weight loss (Pasquali et al. 1989; Guzick et al. 1994; Clark et al. 1995). Some studies have shown that improved insulin sensitivity resulting from weight loss, leads to recovery of spontaneous ovulation and other reproductive parameters (Hamilton-Fairley et al. 1992; Glueck et al. 2003; Moran et al. 2003). Low fat - high carbohydrate diet accentuated hyperglycaemia and hyperinsulinemia in patients with hypertension (Parillo et al. 1988).

Whether short - term energy restriction impacts on fertility treatment outcomes are not known. Moreover, cardiovascular risk is associated with increased impaired fasting glucose,

BMI, waist circumference and hypertension in women prior to presence of gestational diabetes mellitus (Pallardo et al. 2003). Two and a half decades ago, weight loss was promoted as a first priority to improve insulin resistance and reduce risk of cardiovascular disease (CVD) (Wild et al. 1992).

1.8.3 Insulin lowering drugs, dietary interventions and woman's fecundity

In the last decade numerous studies (Morin-Papunen et al. 1998; Glueck et al. 1999; Moghetti et al. 2000; Glueck et al. 2001; Glueck et al. 2004) have assessed the effects of insulin sensitising agents suggesting their potential use in treating the spectrum of reproductive, endocrine and metabolic abnormalities in women with PCOS.

Management of PCOS includes the use of medications to induce ovulation, decrease circulating androgen levels and improve insulin sensitivity in target tissues. Insulin-lowering drugs tend to reduce circulating androgen concentrations and promote ovulation in non-obese and obese PCOS women (Dunaif et al. 1996; Nestler and Jakubowicz 1996; Nestler et al. 1998). Furthermore, insulin-sensitising drugs such as metformin and clomiphene citrate reduced hyperinsulinism, facilitated ovulation in 40 – 67 % of cases and 69 % of ovulating PCOS patients conceived (Heard et al. 2002).

Numerous studies have revealed positive effects of metformin on PCOS treatment outcomes, such as improvement of insulin sensitivity (Diamanti-Kandarakis et al. 1998; Glueck et al. 1999), reduction of hyperinsulinemia (Velazquez et al. 1994), resumption of menstrual cycles (Velazquez et al. 1994; Morin-Papunen et al. 1998; Moghetti et al. 2000), ovulation (Diamanti-Kandarakis et al. 1998; Nestler et al. 1998), achieved weight loss with the reversal of infertility problems (Crave et al. 1995; Glueck et al. 1999) and pregnancy (Diamanti-Kandarakis et al. 1998). Using metformin in obese infertile patients has also resulted in improved reproductive performance (Batukan and Baysal 2001; Song et al. 2002; Glueck et al. 2003) presumably through its effect on insulin sensitivity.

Another study by Glueck et al. 2001 established a significant reduction of weight, insulin and cholesterol levels, and central obesity in morbidly obese patients after the introduction of metformin (Glueck et al. 2001). The same author (Glueck et al. 2004) showed a correlation between age, body weight and therapeutic approaches were established in the most recent study, where the pregnancy outcomes in women on metformin were better at a younger age (≤ 30 years) and with BMI $< 35 \text{ kg/m}^2$. Metformin together with dietary intervention (High Protein – Low Carbohydrate) has been used in teenage girls with PCOS and resulted in the resumption of normal ovulatory menstrual cycles, hormonal status and reduced weight (Glueck et al. 2001). In addition, an advantage of using metformin together with a high protein diet (1500 or 2000 kcal/day) during preconception in PCOS women, which led to weight loss, decreasing of insulin and testosterone levels, improved insulin resistance and diminished development of gestational diabetes in the fetus (Glueck et al. 2004).

1.9 Role of weight loss for overweight / obese women undergoing ART

Animal studies have shown that energy restriction suppresses LH pulsatility and a single ad libitum meal restores stimulation of LH pulses within 24 hours in food-restricted female rats (Bronson and Heideman 1990), ewes (Foster et al. 1989) and gilts (Booth et al. 1996). As discussed previously, the hormonal imbalance causes reproductive disturbance. Weight loss has been strongly promoted as one of the most effective yet simple means of increasing fertility in overweight and obese women (Clark et al. 1998). The effect of different diet regimes on the reproductive performance of infertile women, including the time required and composition of the diet has not been thoroughly studied. The literature clearly demonstrates benefits of short or long-term diets in overweight and obese women. Improvement observed are increased pregnancy and ovulation rates, and a reduction in menstrual and metabolic abnormalities (especially decreases in insulin levels, lipid profiles and weight loss) in overweight and obese women with or without PCOS (Hollmann et al. 1996; Pasquali et al. 1997; Crosignani et al. 2003; Moran et al. 2003).

1.9.1 Weight loss and reproduction

Previous studies have focused on a various forms of weight loss using diet and exercise in obese patients (Hamilton-Fairley et al. 1992; Hollmann et al. 1996). Physical activity has many benefits, such as reduction in blood pressure and improved insulin sensitivity, regardless of the amount of weight lost (Miller 2001). Clark et al. 1998 found a close correlation between weight loss and improved ovulation rate in obese anovulatory women (67 women, 90 % resumed ovulation after weight loss and 78 % conceived) (Clark et al. 1998). A recent study that has looked at short-term effects of two hypocaloric diets, high protein (HP) and high carbohydrate (HC) on weight loss in 26 women with PCOS found that a significant weight loss paralleled significant improvement in reproductive and metabolic abnormalities (Stamets et al. 2004). Moran et al. 2004 showed that a small degree of weight loss after a short – term (8 weeks) of dietary intervention restored reproductive function in overweight PCOS women (Moran et al. 2004). Furthermore, many studies have found that a 5 – 10 % reduction in weight can result in significant improvement in reproductive function (recovery of spontaneous ovulation followed by natural conception), hormonal and metabolic abnormalities (Casimirri et al. 1989; Hamilton-Fairley et al. 1992; Huber-Buchholz et al. 1999; Crosignani et al. 2003; Van Dam et al. 2004). Weight loss in obese, hyperandrogenic, anovulatory women appears to reduce insulin and non - SHBG T concentrations, and increase SHBG, which may lead to resumption of ovulation (Guzick et al. 1994) in obese women including women with PCOS (Pasquali et al. 1997).

1.9.2 Energy restriction and effects on reproductive outcomes

There is good evidence that acute energy restriction, independent of weight loss, improves insulin sensitivity in subjects with type II diabetes as well as normoglycemic overweight subjects (Heilbronn et al. 1999; Noakes and Clifton 2000). Few studies have looked on dietary restriction and its effect on embryo growth during pregnancy. Early studies suggested that the calorie restriction (1200 kcal/day) induced lesser maternal weight gain and lighter

birth weight in newborns (Campbell and MacGillivray 1975). In contrast to the above findings a later study of restricted calorie intake to 1800 kcal/day reported that diet did not affect embryo intrauterine growth during pregnancy in women (Gregory and Rush 1987). Weight loss and caloric restriction improves excessive androgen production and restores ovulatory cyclicality in 50 – 80 % obese PCOS patients (Pasquali et al. 1989). This evidence suggests that a short-term diet strategy aiming at maximising energy restriction but is nutrient replete may be a possible therapeutic strategy in infertility treatment to improve conception and livebirth rates.

1.9.3 Outcomes of using a very low calorie diet

VLCD are commercially prepared meal replacement formulae of about 800 kcal/day that are nutritionally complete and replace all daily food intake for several weeks (short - term) or months (long - term). More than 80 studies have been conducted with using VLCD. The study of 4026 morbidly obese patients of both genders using Optifast VLCD for 14 weeks revealed that 25 % of participants were unable to adapt to the regimen and dropped out within the first 3 weeks (Kirschner et al. 1988). The remaining women lost an average 22kg (1.4 kg / week) (Kirschner et al. 1988). Long - term (6.25 months) administration of VLCD increased LH levels and decreased estradiol concentration in one week (Van Dam et al. 2004). Moreover, levels of fasting insulin, glucose and free testosterone decreased, whereas SHBG levels increased after the weight reduction (≥ 10 % of initial weight) in obese PCOS women (Van Dam et al. 2004).

Previous studies have shown that 84 % of the participants could persist on a VLCD (330 or 405 kcal/day) longer than 5 weeks (Shapiro et al. 1989). Similarly, the VLCD has been used in a group of diabetic patients for 12 weeks without any adverse reactions (Guzick et al. 1994). In a randomised controlled trial of dietary energy restriction in the management of obese women with gestational diabetes, there were no adverse effects of a 30 % energy restriction during pregnancy (Lidor et al. 2000).

Administration of VLCD for a short - term (one week) decreased fasting glucose and insulin levels, serum testosterone and leptin after a small weight reduction (Van Dam et al. 2002). Extreme dietary energy restriction (Loucks and Heath 1994) and extreme exercise energy expenditure (Loucks et al. 1998) has disrupted LH pulsatility in women. A case study of the use of VLCD in a woman with type II diabetes led to weight loss, reduction in glucose levels, lowered hypertension and achieved pregnancy after a fourth trial of intrauterine insemination (Katsuki et al. 2000). However, there is no data on the efficacy of this approach in randomised trials.

1.10 Thesis Hypotheses

This research aims to clarify the role of the short - term use of an energy restriction resulting in weight loss during ART treatment in overweight or obese women on successful IVF outcomes. These results may assist in understanding the physiological processes occurring at the time of ovulation and fertilisation in overweight / obese women.

Whether short-term energy restriction impacts on fertility treatment outcomes is not known and following hypotheses were proposed:

1. The short - term use of a nutritionally complete low kilojoule diet may improve the success of IVF in overweight or obese women in terms of pregnancy and livebirth outcomes.
2. Short - term energy restriction will improve ovulation and fertilisation rates and blastocyst quality in obese female mice.

1.11 Thesis Aims and Significance

The following aims were proposed in accordance to the hypothesis:

1. In order to assess the first hypothesis, we proposed to perform a pilot clinical study with the following aims:
 - a) Examine the feasibility of VLCD during IVF treatment.
 - b) Evaluate IVF treatment outcomes after using a particular dietary regime.

2. To examine second hypothesis, we proposed to perform an animal study with the following aims:
 - a) Assess ovulation and fertilisation rates in overweight / obese model mice after a short term weight loss.

 - b) Evaluate blastocyst quality.

The significances of the proposed studies are seen to be:

1. To increase knowledge on reproductive processes occurring in females body after a rapid weight loss.

2. To improve the treatment efficacy of IVF cycle by implying a short term of weight management in women with a weight dilemma.

3. Combination of treatment with weight management will minimize the stress levels and repetitions of infertility treatment in ART.

4. To enhance the cost effectiveness of management.

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CHAPTER TWO

Effect of a very low calorie diet on in vitro fertilization outcomes

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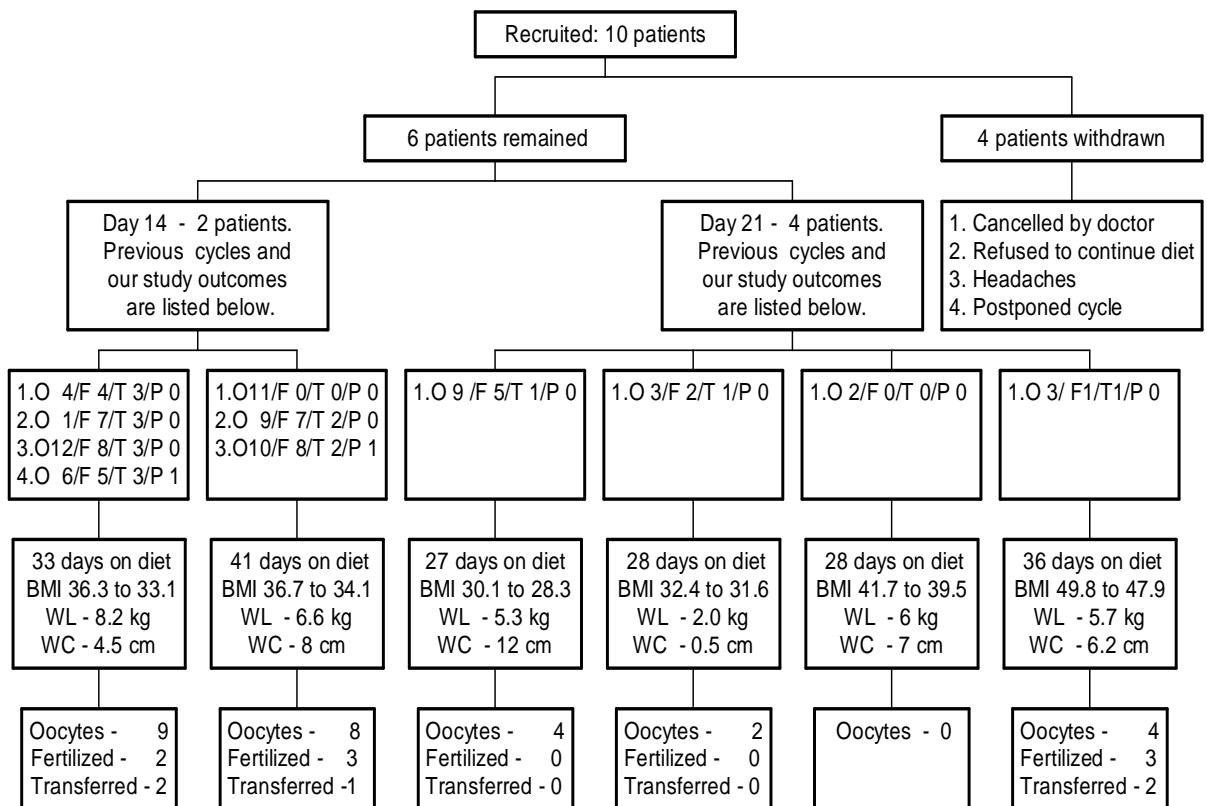
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Table 2.1 Clinical characteristics of recruited patients.

Patients n = 10	Original values	Mean values \pm SD
Age (years)	29 – 38	34 \pm 2.9
Weight (kg)	75.2 – 144	99.2 \pm 20.2
BMI (kg/m ²)	28.9 – 50	36.1 \pm 6.4
Waist circumference (cm)	91 – 144	102 \pm 16.4
Blood pressure (mmHg)	110 / 70 – 140 / 85	125 / 80 \pm 10.7 / 7.1
Duration of infertility (years)	3 – 5	3.2 \pm 1.2
No. of previous IVF cycles	1 – 8	3.1 \pm 1.8

Table 2.1 shows the original and mean values of clinical characteristics of 10 recruited patients prior the study commencement.



Acronyms: BMI – body mass index; WL – weight loss; WC – waist circumference loss
 O – oocytes collected; F – oocytes fertilized; T – embryo transferred
 P – pregnancy outcomes.

Figure 2.2 Study sequence after using a VLCD for a short-term before and during IVF treatment. This chart presents patients preference of dietary regimes, withdrawals, body weight changes and previous and current IVF treatment outcomes for each patient.

CHAPTER THREE

Mechanisms of obesity and short term weight loss on reproductive processes

3.1 Introduction

It has been shown that increased energy intake for long period decreases follicle numbers, impairs total cell numbers in the embryo (Yaakub et al. 1999), and reduces embryo numbers in cows (Siddiqui et al. 2002). According to Peura et al. 2003 a long period of high nutrition (1.3 maintenance level of high calorie and high nutrient) intake before the superovulation treatment increased the number of ovulations and recovered oocytes; and at the end of the gestation the number of transferred embryos were higher compared to ewes exposed to low nutrition intake (0.7 x maintenance level) (Peura et al. 2003). Maternal overnutrition led to increased insulin concentrations and correlated negatively with placental and lamb birth weight (Wallace et al. 1997).

Furthermore, precise quantities of macronutrients in dietary intake also are important. Excess dietary protein intake (19 %) in lactating cows (Butler et al. 1996) and increased intake (50 %) prior to conception decreased fertility rate (Elrod and Butler, 1993). Furthermore, 73 % of excess protein intake decreases the number of transferable ova (Blanchard et al. 1990). Another study showed, that a diet containing protein excess (23 %) intake decreased the conception rate by alteration of minerals (magnesium, urea, potassium, phosphorus and zinc) in uterine secretions during oestrous in cows (Fan et al. 2003). Maternal intake of a low protein diet (50 % of the control group, nutrients adjusted to the normal dietary intake) during the preimplantation (0 - 4 days after mating) period reduces cell numbers in embryos, reduces insulin and amino acid levels and increases glucose levels in the rat (Kwong et al. 2000). The embryos cultured in high glucose media showed retarded development (Moley et al. 1996). The effect of different macronutrients on reproductive health has not been well studied in obese human females.

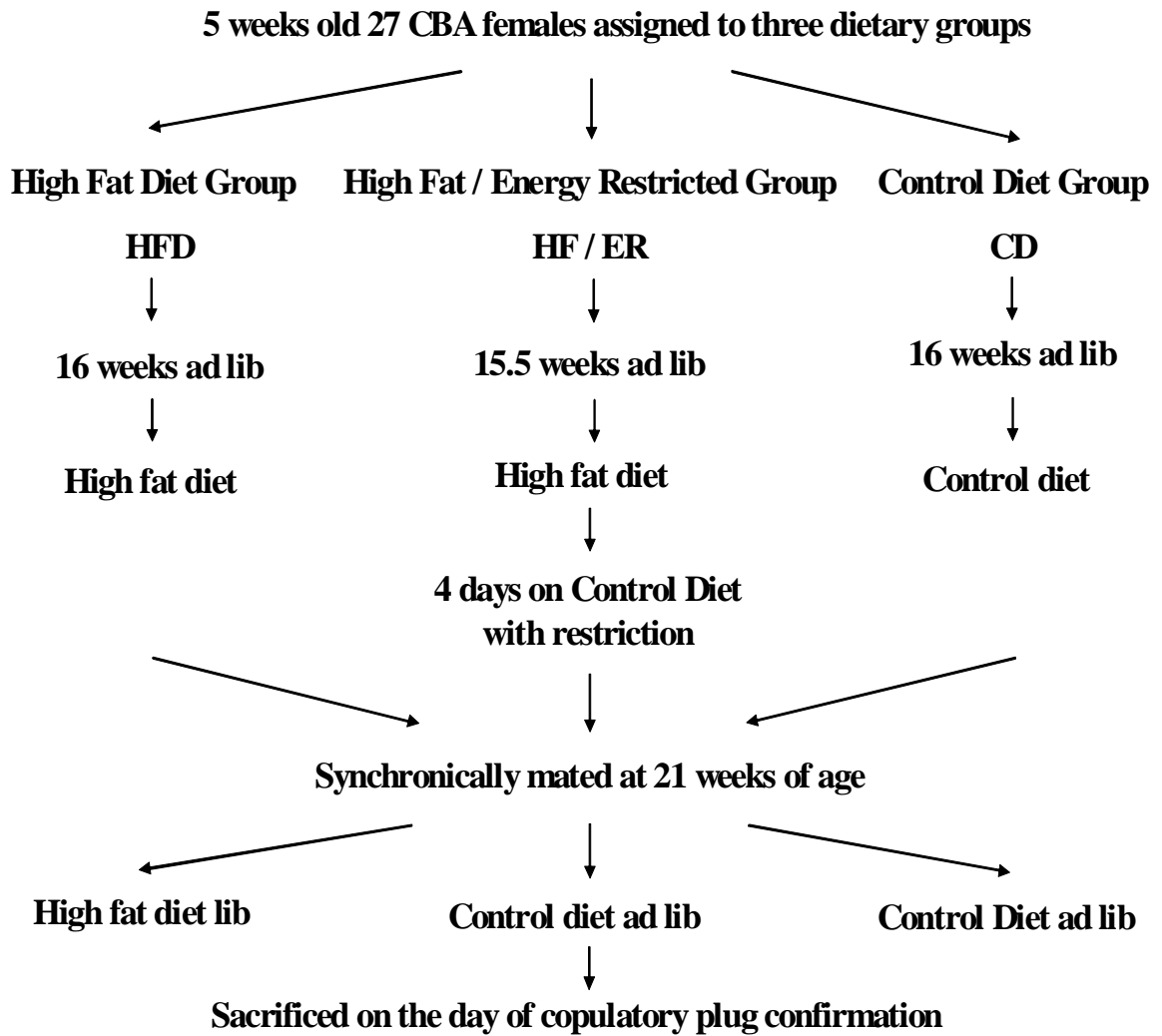
Despite the established relationship between reproductive disturbances and the overweight / obese state in both human and animal models, less is known about appropriate diet including

composition duration and caloric intake, which may improve the reproductive health of the female. Nolan et al. 1998 showed the beneficial effect of low caloric food intake to high caloric intake during superovulation resulted in the high follicle number and total cell number at day 8 (Nolan et al 1998).

There have been no studies of the use of calorie restriction for a short period prior to mating in animal models with induced obesity and this issue can be effectively addressed in the mouse model. Use of a low calorie diet complete with recommended daily nutrients prior to mating may have a positive influence on the oocyte quality and later on the embryo development in obese mice.

In view of the importance of the quality of preconception nutrition, we hypothesised that short-term calorie restriction (70 % of normal food intake) prior to mating will improve embryo quality and implantation rate. To test this hypothesis, we have compared the quality of blastocysts of nutritionally induced obese female mice, obese female mice who lost weight as a result of energy restriction and a control group of mice with normal body weight.

3.2 Schematic study design



3.3 Materials and methods

3.3.1 Experimental animals maintenance and handling

Laboratory mice (CBA strain, 27 females and 14 males, 5 weeks of age) were obtained from the breeding stock held at The University of Adelaide Laboratory Animal Services. For experimental purposes, all animals were maintained in a room with a standard temperature of 20⁰C and artificial illumination with 10 hours dark and 14 hours light cycle at The Queen Elizabeth Hospital Animal House.

Females were randomly assigned to three groups, n = 9 in each group, and fed three different types of dry food (12 mm size pellets). The dry atherogenic high fat diet comprising food ‘SF00 – 219’ (equivalent to a “Western type of diet”) and matched to it, the low fat control diet ‘SF04 - 057’ were purchased from Specialty Feeds Ltd., Australia. The standard rodent chow ‘Animal joint stock II’ was supplied by The Queen Elizabeth Animal House and purchased from Ridley AgriProducts Pty., Ltd, respectively. Dietary food caloric content and composition is described in Appendix II, pp 119 - 121.

Animals were housed separately according to gender, and maintained as nine females per standard cage (size 25cm x 39cm x 16.5cm) and one male per standard cage (size 14cm x 27cm x 12cm) with the high top wire lids. The cages were replaced twice a week. All animals were provided with unrestricted access to food (unless otherwise stated according to the study design) and fresh tap water, which was supplied by glass bottles attached to the front of each cage and changed twice a week.

3.3.2 Animal Ethics

All experiments were approved by The University of Adelaide Animal Ethics Committee and The Institute of Medical and Veterinary Science IMVS Animal Ethics Committee,

Adelaide, and performed under the Ethics approval numbers M – 043 – 2005 and 69 / 05, correspondingly. Mice (females and males) were tagged by using the ear puncture method.

All procedures were performed in viral and bacterial free atmosphere using surgical gloves. Sufficient amounts of food and the quality of water were available at all time. The animals were humanely sacrificed by cervical dislocation. Experiments were performed with regard to the legislation and the requirements of The Australian Code of Practice for the Care and Use of animals for scientific purposes, 2004 and the South Australian Prevention of Cruelty to animals Act 1985.

3.3.3 Chemicals and solutions

Chemicals were purchased from Sigma - Aldrich Chemical Company, USA, unless otherwise stated. Solutions were prepared using a Milli - Q water system (Millipore Corporation, Australia). All chemicals were weighed by using the balance (Shimadzu AUW 220P, Shimadzu Corporation, Japan). Solution osmolality was measured by the freezing point method on The Advanced TM Micro Osmometer (model 3300, USA) and pH levels assessed by pH meter (Ecoscan pH 6, Eutech Instruments Pty., Ltd). All the chemicals, the recipes and storage procedures for solutions are listed in Appendix I, page 116 along with the required technical procedures.

3.3.4 Biological materials

Bovine serum albumin (BSA) and Guinea pig serum (GPS) were kindly provided by Dr Michelle Lane and purchased from Pacific Vet and Lyppards Holding Ltd, correspondingly.

3.3.5 Equipment

The microscope Nikon SMZ 1500 (model C – DSS230, Nikon, Japan) was used in *in vitro* experiment. Collected zygotes were cultured in a 5% O₂ – 6 % CO₂ incubator (model MCO – 175M, SANYO Electric Biomedical Co., Ltd, Japan). A confocal microscope (Nikon eclipse

TE 2000 – E motorised focus, with Photometrics, CoolSnap Es, Nikon, Japan) was used for the assessment of the quality of *in vitro* cultured and differentially stained preembryos. Images were captured on the microscope OLYMPUS VANOX (AHBT3, AH3 – RFCA, by using Olympus Optical Co., Ltd, utilising Photometrics, CoolSNAPTM_{cf}, Roper Scientific Photometrics and Scanalytics IP lab V36 Software. Blood samples were spun using the centrifuge 5415D (Eppendorf, Crown Scientific Pty, Ltd).

3.4 Study protocol and general procedures

3.4.1 Mice feeding protocol

In the present study, we investigated the effects of prolonged weight gain (16 weeks) and rapid weight reduction after a short term of calorie restriction (5 days) on blastocyst quality. The experiment was conducted in early spring 2005 and females were randomly assigned to three – Control (CD), High fat diet (HFD) and High fat diet / Energy restricted (HF / ER) experimental groups (9 ♀ in each group). The HFD group received a high fat / + cholesterol comprising food HF (SF00 – 219; 21 % fat and 6 % cholesterol; 19.4 kJ/g, 4.64 kcal/g) whereas the CD group received a low fat control diet LF (SF04 – 057; fat 6 % and no cholesterol - corresponding to the high fat diet; 16.1 kJ/g 3.85 kcal/g). Dietary food caloric content and composition is described in Appendix II, pp 119 - 121. All groups were fed *ad libitum* for the duration of the experiment (unless otherwise stated). In the HF / ER group, HF food was replaced by LF food with simultaneous calorie reduction to 70 % for a short period of 5 days on Week 15.5 and thereafter, HF / ER group continued *ad libitum* LF food for the rest of experiment. At week 16, all females were synchronically mated with intact CBA males. Mated animals were nourished *ad libitum* on dry food according to the female (s) dietary group affiliation, unless otherwise stated. All animals had unlimited access to water. The calculation of the energy restricted feeding in the HF / ER group was based on the daily records of food intake in CD group recorded for 10 days prior to actual restriction in the HF / ER group.

3.4.2 Weight assessment

The animal's weight was assessed and recorded weekly at the same time in the morning for 16 weeks. On the first day of the experiment, weight was recorded as Week 0 weight in the chart. The female was gently picked up by the tail, placed in the plastic box and weighed using a calibrated balance (Sartorius 1216MP, GMBH Göttingen, Germany). The two extra weight assessments were performed on days prior to overnight fasting for the blood sampling. Animal identification was ensured throughout procedures during the experiment.

3.4.3 Mating

14 CBA males (5 weeks old) were housed in separate cages for 17 weeks and their fertility proven by mating with non-experimental females. After 16 weeks of different dietary regimes, all females (CBA, 21 weeks old) were placed with a stud male in 2 : 1 ratio. Every female was inspected daily in the morning for 12 consequent days. Mating was confirmed by the presence of a copulatory plug and female considered as Day 1 pregnant. On day 12 after pairing, the remaining females were separated from males.

3.4.4 Tissue collection and storage for histological analyses

Female mice were humanely sacrificed by cervical dislocation and tissues of interest (abdominal fat, ovaries, liver, pancreas, kidneys, spleen and heart) removed, cleaned of blood and connective tissue and weighed.

3.4.5 Animal length assessment

Two measurements of the animal length were taken, nasal – anal (NA) and nasal – stretched tail (NT). The mouse was placed on the experimental table face up and the length measured by the standard ruler from the tip of the snout to the base of the tail (NA), and from the tip of the snout to the end of the tail (NT).

3.5 Embryo collection and in vitro culture

3.5.1 Embryo zygote collection and culture

Females were sacrificed about 12 hours after confirmed mating and the oviducts dissected and immediately transferred into 5ml clear plastic tubes containing 2 ml of warmed 37 °C G - MOPS medium supplemented with BSA (4mg / ml). A warmed Petri dish (size 35mm) was placed on the microscope's warm plate (37 °C) and the content of 5 ml clear plastic tube was poured into the dish. The oviduct ampullae were assessed and opened with a 26-gauge needle and fine watch – maker's forceps and the cumulus freed into the medium. Using the fine hand pulled glass pipette, fertilized eggs (presumptive zygotes) were released and separated from the cumulus cells, transferred and washed twice into prepared warmed G-MOPS medium before being relocated to the G1 culture medium droplets (up to 10 zygotes in 20 µl of medium) placed under mineral oil and incubated in atmosphere of 5 % O₂ – 6 % CO₂ at 37°C in the incubator for up to 48 hours.

On Day 3, using fine hand pulled glass pipettes, preembryos were transferred from G1 to G2 medium and in the midst of those two, washed once in 20µl droplets of prewarmed G2 culture medium under mineral oil and incubated in the same conditions of 5 % O₂ – 6 % CO₂ at 37°C for next 48 hours. Preembryo cleavage stages were assessed and recorded on Day 3, Day 4 and Day 5 of culturing. On Day 5, cleaved blastocysts were stained by blastocyst differential staining method (Appendix III, page 122).

3.5.2 Assessment of the developmental stage of the cultured embryos

Cultured embryos were assessed on Day 3, Day 4 and Day 5 after the culturing procedure by embryo cleavage scoring to determine *on time* development using “Embryo culturing and daily assessment protocol” (Appendix III, page 123).

3.6 Embryo quality assessment

3.6.1 Blastocyst quality assessment

To assess embryo cell numbers and quality, on Day 5 after embryo culture, blastocysts were stained by differential staining methods (Appendix III, page 122) and visualization and cell counting analysed by epifluorescent microscope at 200 x magnification. Differentially stained blastocysts were transferred in 20µl droplets of pure glycerol (2 blastocyst per droplet) placed on the microscope slide and then covered with a glass coverslip (size 22mm x 22mm). For the total nuclear cells count a UV filter – 2A, excitation filter - 330-380nm, dichromatic mirror - 400nm, barrier filter – 420nm was used. The trophectoderm cells were counted under an excitation filter – 540 / 25 nm, dichromatic mirror – 565 nm, barrier filter – 605 / 55 nm. inner cell mass (ICM) number was counted as subtraction of number of trophectoderm cells from the total cell number.

3.7 Statistical analysis

Statistical significance between three treatment groups was calculated by a one-way analyses of variance (ANOVA) test followed by the Pairwise Multiple Comparison Procedures using the Tukey test and by a one-way ANOVA t-test within the each treatment group (SigmaStat Version 2.0, Software, USA). The results were considered significant at $P < 0.05$.

3.8 Results

The objective of this study was to examine the effects of a short – term calorie restriction prior to mating on preembryo development and quality in CBA type mice. At the commencement of the experiment at 5 weeks of age, the initial body weights of all animals were the same. Three groups (CD, HFD and HF / ER) of females were fed different calorie content diets for 16 weeks and Figure 3.1 (page 103) illustrates weekly body weight fluctuations across the study. Females consuming the HF diet at week 14 (HFD and HF / ER groups) and week 16.5 (HFD group), correspondingly, developed marked diet - induced

obesity compared to the age matched CD group maintained on the control LF diet ($P < 0.05$). As expected from the experimental design, a calorie restriction for a short period of 5 days induced a significant body weight reduction by 8.1 - 14.0 % (mean 12 %) in the HF / ER group ($P = 0.001$). This data is presented in Figures 3.1 and 3.2 (pp 103 - 104). Furthermore, the weight loss in the HF / ER group resulted in weight normalisation with the CD group females (difference less than 0.8 %), and the markedly heavier HFD group became significantly different from the both HF / ER and Control group by 10.4 % and 12.6 %, respectively ($P < 0.05$, data is illustrated in Figures 3.3 and 3.4 (pp 105 - 106). During the pairing with males for 12 days, the body weight of females in CD and HFD groups decreased by 1.04 g (3.55 %) and 1.78 g (5.4 %), correspondingly, whereas HF / ER group showed the tendency to weight escalation by 0.28 g (0.95 %). However, all females were fed the same food according to the group allocation. The post - mortem body weight did not differ between the treatment groups. Conversely the body weight of non - pregnant females from the HFD group was drastically different from the CD group females ($P = 0.018$), see Figure 3.4, page 106.

In addition to the weight fluctuation, the females' length is presented for two groups of females (pregnant and non - pregnant), Table 3.1, page 99. The mean nasal - tail length of pregnant females was significantly different between the CD group and corresponding females from HFD and HF / ER group ($P < 0.001$), however, the length in non - pregnant females was similar (18.7 ± 0.1 cm). Interestingly, the nasal - anal length was not different between pregnant and non - pregnant females within the treatment group and did not differ between the treatment groups (Table 3.1, page 99).

In the present study, results of internal tissue weights are also expressed for two groups of females, pregnant and non - pregnant and presented in Table 3.2 (a) and Table 3.2 (b), pp 100 - 101, correspondingly. The female was identified as pregnant Day 1 by the presence of a copulatory plug and fertilised eggs in oviduct, and non - pregnant definition applied to

the female who failed to get pregnant within 12 days after pairing with proven males. The mean weight of the abdominal fat pad and spleen were significantly different between pregnant females from HFD and HF / ER groups compared to their counterpart females from CD group, correspondingly ($P < 0.05$), whereas no appreciable differences existed with regard to other internal tissues (heart, pancreas, kidney, ovaries and liver). The data is presented in Figures 3.5 (a) and 3.5 (b), page 107. There was no significant difference between the weights of internal tissues in non - pregnant females. Results are illustrated in Table 3.2 (b), page 101.

The reproductive characteristics of the CBA mice, which were fed a different diet and maintained on different dietary regimes are summarised in Table 3.3, page 102. The experimental results showed that there was no difference in the number of days (between 1 - 10 days (median ~ 4.7 days)) required for achieving pregnancy between the treatment groups ($n = 9$ per group). The number of animals that ovulated tended to be higher in the HFD group (55.6 %) than in CD and HF / ER groups (44.4 % in each, respectively). However, the number of retrieved oocytes did not differ between the dietary groups (22, 20 and 20 in CD, HFD and HF / ER group, correspondingly). Moreover, there was a difference in the number of females with ovulated / retrieved oocytes and the number of females with fertilized oocytes (Table 3.3, page 102). This is recorded as a percentage of the total number of females that ovulated and shows that all females that ovulated from the HF / ER group had fertilised eggs, whereas this number was decreased to 75 % and 40 % in CD and HFD groups, correspondingly. Therefore, the fertilization rate was recorded as a percentage of total recovered oocytes and tended to be higher in the HF / ER group compared to CD and HFD groups, 80 % to 45.5 % and 55 %, respectively ($P < 0.05$), Figure 3.6, page 108.

The embryo developmental stages were recorded on Day 3, Day 4 and Day 5 during in vitro culture. On Day 3 of embryo culture, females exposed to the HF and HF / LF dietary feeding demonstrated delayed embryonic cell division and this tendency was observed to the end of the experiment. The proportion of embryos developing to the blastocyst stage was recorded

as a percentage of the total number of fertilized oocytes, and CD and HF / ER groups showed an equal outcome (50 %) of formed blastocysts compared to the HFD group (27.3 %), Figure 3.6, page 108. The number of embryos at different cleavage stages on Day 3 are presented in Figure 3.7, page 109, where all embryos from the HFD group and partial number from the HF / ER group are showing a slow developmental pattern in *in vitro* culture. The quality of embryos' that accomplished the blastocyst stage was not affected between the treatment groups (Figure 3.8, page 110).

3.9 Discussion / Conclusion

The present work has attempted to determine how a short period of energy restriction prior to mating influences ovulation and fertilisation rates, and embryo quality in overweight or obese CBA type mice. In this study, three groups of animals were fed two types of diet (HF and LF) and maintained on three dietary regimes: HF and LF diets for 16 weeks; and HF diet for 15.5 weeks with following replacement by LF + 70 % of energy restriction for 5 days. As expected, females nourished on HF diet (either HFD or HF / ER group) presented with significant diet induced obesity after 15.5 weeks of feeding. Furthermore, calorie restriction (70 % of energy requirements) within one reproductive cycle resulted in significant weight loss (mean 12.2 %) in the HF / ER group, and the achieved weight in this group became similar to the weight of females from the CD group.

The number of oocytes retrieved did not differ between the treatment groups. However, the oocytes from the HFD group were of lower quality compared to CD and HF / ER groups, as evidenced by lower number of females with fertilised oocytes, despite the number of ovulated females (n = 5) amongst treatment groups. While there was no difference in the percentage of fertilised eggs in the HF / ER group (100 %), the CD and HFD group showed less fertilised eggs (75 % and 40 %, respectively). Furthermore, there was an interaction between the level of feeding and blastocyst developmental rate, which was expressed as a proportion of fertilised oocytes and the HFD group presented with the lowest number of

cleaved to blastocyst stage embryos during in vitro culture between the treatment groups (27.3 % vs 50 % (CD), 50 % (HF / ER)). In addition, blastocyst hatching in vitro was not observed in embryos of HFD group. In both CD and HF / ER groups on time blastocyst hatching were detected on day 5 of culture.

Furthermore, with regard to the oocyte quality, the negative relationship was detected between collected oocytes and blastocyst development. Animals, who had less than 4 oocytes collected, did not form any blastocyst. Females with more than 7 oocytes collected had quality blastocyst yields. This expressed as a proportion of the number of fertilised oocytes for each animal (62.5 % and 50 % in CD; 57.1 % from HF / ER and 37.5 % in HFD groups). The data could not be statistically calculated, as initially, we had low number of animals with oocytes collected and consequently low number of blastocyst yield. In addition, the quality of remaining blastocyst was unaffected between the treatment groups. Our data interpretation has been affected by a number of factors, such as number of 'pregnant' females, number of collected and fertilized oocytes and number of blastocysts at the end. Consequently, some of the results were insufficient and the statistical data (P values) could not be assessed in many cases, especially with regard to the embryo development and quality.

In conclusion, this pilot study tried to elucidate the effects of different levels of nutrition on embryo quality. Our results are inconclusive, although this study laid a basis for the need of further detailed and appropriately powered studies in this area of interest. A higher energy intake resulted in a greater number of retrieved oocytes. However postfertilisation development was diminished, which suggests a lower quality of oocytes. It would be of interest to investigate further, how different dietary regimes with regard to different macronutrient composition, length of feeding (days / weeks), different levels of calorie restriction (90 %, 80 %, 70 % of daily energy restriction) and length of experiment might affect the oocyte quality, embryo development and pups number at different time points of mice lifespan.

3.11 Image capture

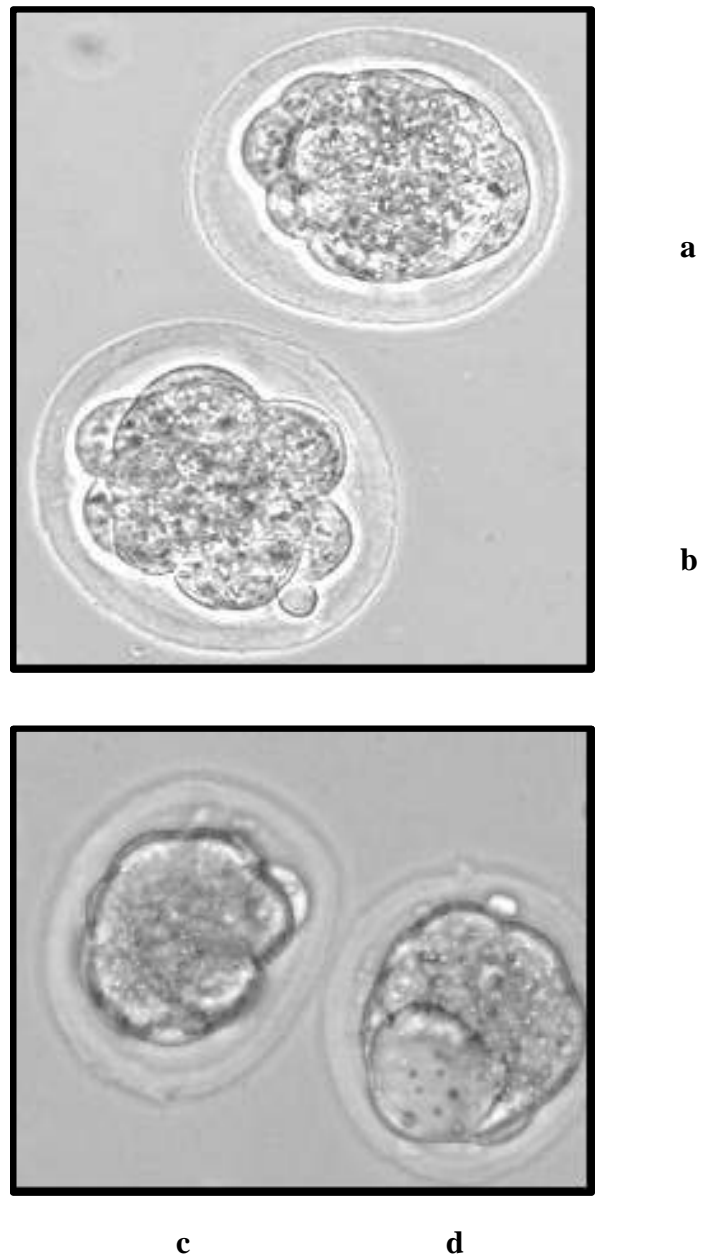


Image 1. Mouse embryos on Day 3 of *in vitro* culture.

The image ^a shows compacting embryo to the morula stage

^b - embryo starting a compaction stage

^c - compacted morula and ^d - an early blastocyst formation

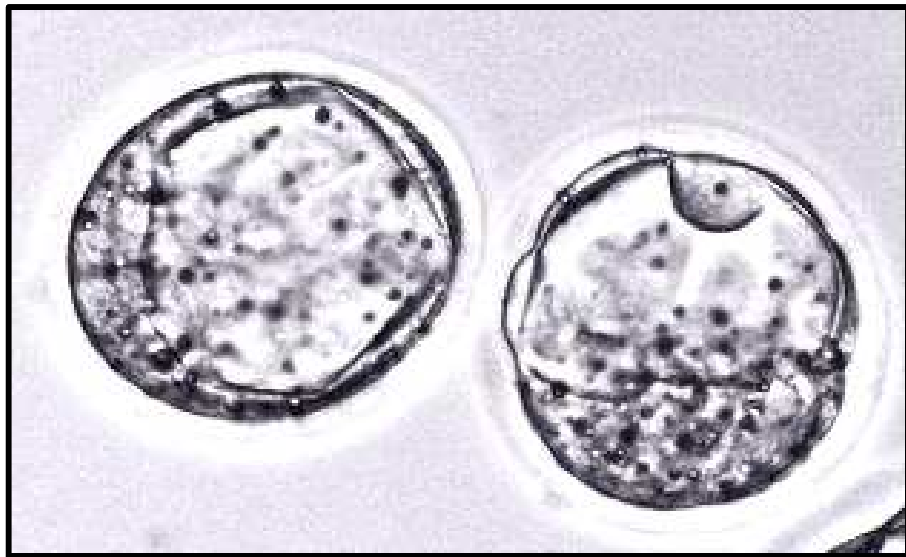


Image 2. In vitro cultured embryos on Day 5.

The image ^a shows the blastocyst stage embryo with blastocoel cavity (up to 50%), ^b – expanded blastocyst and ^c – blastocyst.

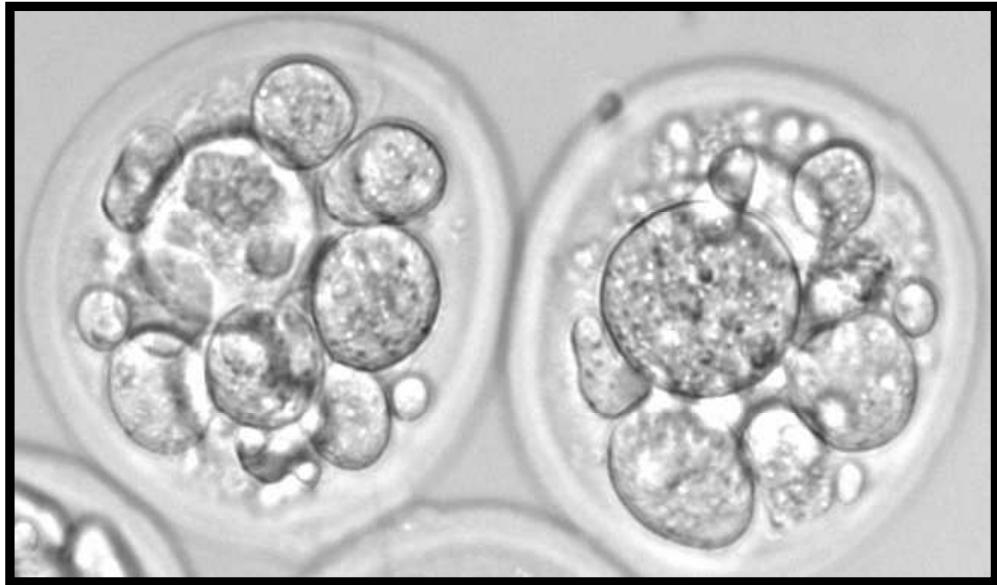


Image 3. Fragmented embryos on Day 3.

This image was taken on Day 3 of *in vitro* culturing and showing embryo defragmentations.

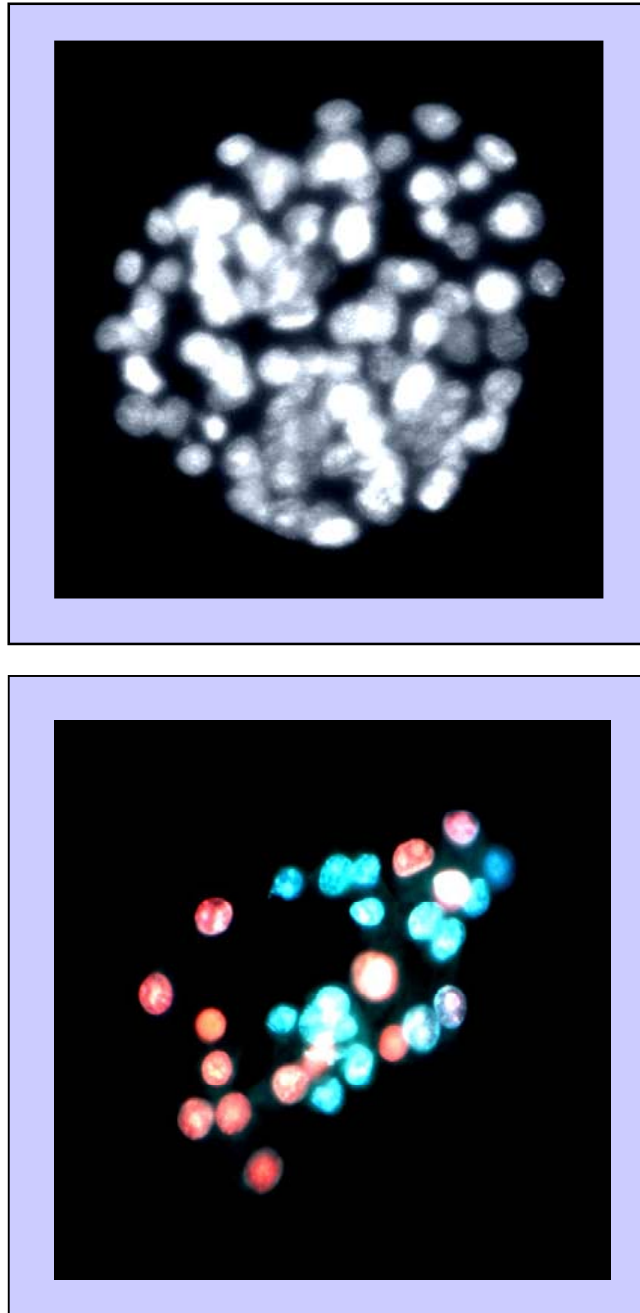


Image 4. Blastocyst on Day 5.

Photos of blastocyst have been taken on Day 5 of *in vitro* culturing and the density of cells shows the presence of inner cell mass surrounded by trophoblast cells.

3.11 Tables and Figures

Table 3.1 The effect of the dietary intake on absolute length of CBA females.

Length	CD	HFD	HF / ER
All	n = 9	n = 9	n = 9
N / A	9.7 ± 0.13	9.7 ± 0.07	9.5 ± 0.10
N / T	18.7 ± 0.15	18.5 ± 0.09	18.5 ± 0.13
Pregnant	n = 5	n = 7	n = 6
N / A	9.6 ± 0.16	9.6 ± 0.07	9.6 ± 0.13
N / T	18.6 ± 0.24	16.5 ± 0.09*	16.2 ± 0.19**
Non – pregnant	n = 4	n = 2	n = 3
N / A	9.7 ± 0.23	9.8 ± 0.25	9.5 ± 0.13
N / T	18.8 ± 0.19	18.8 ± 0.20	18.6 ± 0.00

Two different lengths (cm) were recorded at the end of experiment: nasal – tail (N / T) and nasal – anal (N / A). This table presents the mean length of females in each treatment group and separately in groups for the pregnant and non – pregnant females. * and ** denotes are presenting a detected significant differences of the pregnant females length in HFD and HF / ER groups, respectively compared to the pregnant control CD group ($P < 0.001$). Values are expressed as absolute mean length of females (cm) ± SEM and analysed by one-way ANOVA using the Student – Newman – Keuls test.

Table 3.2 (a) Absolute weight of tissues from ‘pregnant’ females.

Internal tissues	CD group n = 5	HFD group n = 7	HF / ER group n = 6
Ovaries	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Abdominal fat pad	1.64 ± 0.30	2.56 ± 0.22 ^a	2.43 ± 0.23
Liver	1.36 ± 0.09	1.64 ± 0.08	1.49 ± 0.07
Spleen	0.06 ± 0.01	0.07 ± 0.00	0.08 ± 0.01 ^b
Pancreas	0.14 ± 0.01	0.17 ± 0.01	0.15 ± 0.01
Kidney	0.28 ± 0.01	0.27 ± 0.01	0.25 ± 0.03
Heart	0.09 ± 0.01	0.09 ± 0.00	0.10 ± 0.00

Female was considered pregnant after the incidence of a copulatory plug and at the same day identified as a pregnant. Values are presented as absolute weight mean (g) ± SEM. On Week 16 of different dietary intake, females from CD, HFD and HF/ER groups were mated and internal tissues dissected from Day 1 pregnant females. ^{a,b} - denotes a significant difference of the abdominal fat pad and spleen in HFD and HF / ER groups, correspondingly, compared to the CD group (P < 0.05).

Table 3.2 (b) Absolute weight of the internal tissues from non – pregnant females.

Internal tissues	CD group n = 4	HFD group n = 3	HF / ER group n = 2
Ovaries	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
Abdominal fat pad	2.58 ± 0.55	3.07 ± 0.99	2.20 ± 0.26
Liver	1.54 ± 0.15	1.94 ± 0.25	1.63 ± 0.06
Spleen	0.07 ± 0.00	0.07 ± 0.00	0.08 ± 0.01
Pancreas	0.17 ± 0.02	0.23 ± 0.06	0.14 ± 0.01
Kidney	0.28 ± 0.12	0.29 ± 0.01	0.29 ± 0.01
Heart	0.09 ± 0.00	0.10 ± 0.01	0.09 ± 0.00

Female was considered non – pregnant, if copulatory plug was not observed within 12 days after the pairing with proven male. Values are presented as absolute weight mean (g) ± SEM and there are no significant difference detected.

Table 3.3 Reproductive outcomes following the different caloric intake / or regime for 16 weeks in CBA females.

Reproductive outcomes	CD group	HFD group	HF / ER group
No. of animals mated	9	9	9
No. of days required to achieve pregnancy (Day 1)	2 - 8 (~ 4.5)	1 - 10 (~ 4.6)	2 – 9 (~ 4.5)
No. of observed plugs	5 (55.6 %)	7 (77.8 %)	6 (66.7 %)
No. of ovulating females	4 (44.4 %)	5 (55.6 %)	4 (44.4 %)
Total no. of oocytes collected from ovulating females	22 (3 – 8)	20 (2 – 9)	20 (1- 8)
Number of females with fertilized oocytes	3 (75 %)	2 (40 %)	4 (100 %)
Total no. of fertilised oocytes	10 (45.5 %)	11 (55 %)	16 (80 %)
No. of animals whose embryos reached to the blastocyst stage	1 (33.3 %)	1 (50 %)	2 (50 %)
Total no. of blastocyst	5 (50 %)	3 (27.3 %)	8 (50 %)

This table shows reproductive outcomes of three groups (n = 9 / per group) maintained on different dietary regimes (HF, LF and HF / LH + 70 %) for 16 weeks and afterwards mated. Day when the copulatory plug was observed called Day 1 of pregnancy and females were sacrificed, the reproductive tract removed, the oviduct flushed and by the presence of oocytes females were identified as ovulating females. Oocytes were collected, cultured for 5 days and fertilisation rate and blastocyst number were assessed.

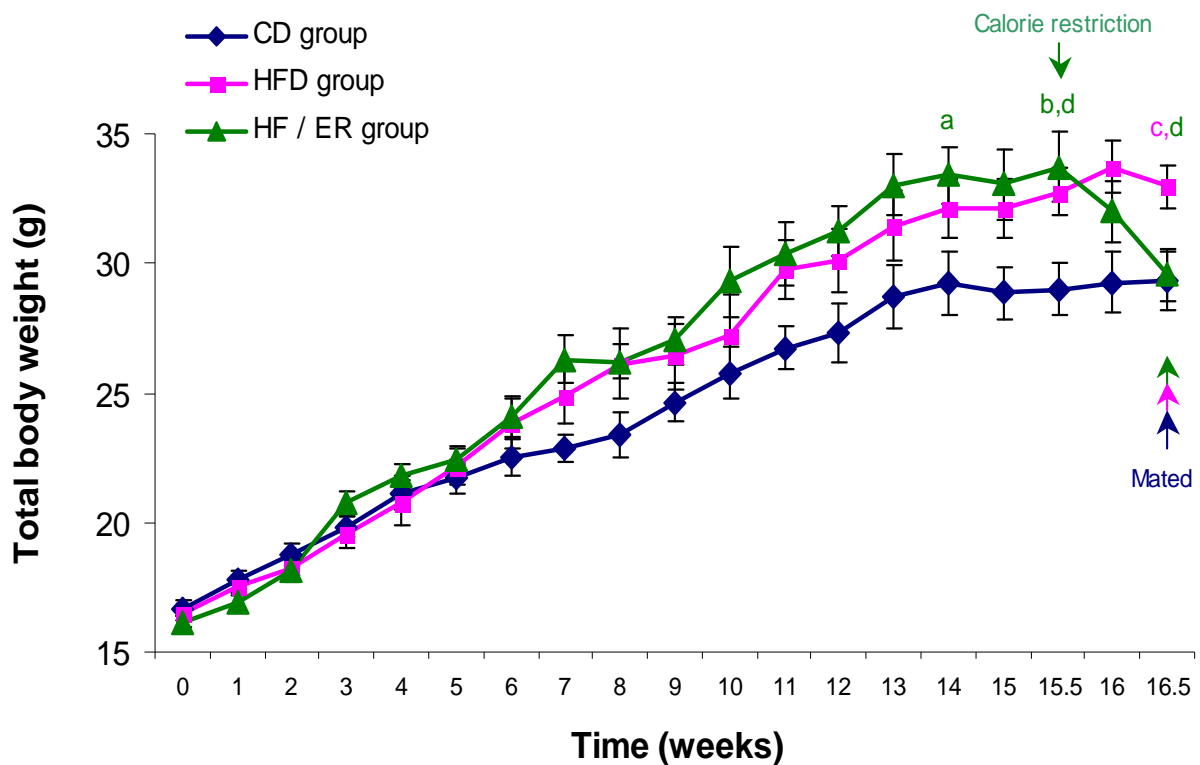


Figure 3.1 Female mice growth curves during the experiment. The total body weight (g) of CBA female mice fed either a control diet LF (CD group) or high fat diet HF (HFD group) or HF / LF diet (HF / ER group) was recorded for 16.5 weeks (n = 9 / per group). On Week 15.5, HF diet in HF / ER group was replaced with LF diet and restricted to 70 % of ad libitum for 5 days and afterwards fed LF *ad libitum*, whereas all groups were fed *ad libitum* at all other time. On Week 16.5 females were mated and final weight recorded. ^{a, b} and ^c denotes are showing significant difference (P < 0.05) in HF / ER and HFD groups compared to the females in control CD group at Weeks 14, 15.5 and 16, respectively. ^d – indicates significant difference between the mean body weight in the HF / ER group prior to (Week 15.5) and after (Week 16.5) calorie restriction to 70 % of ad libitum for a short period of 5 days (P > 0.05). Data are expressed as means of absolute body weight means (g) ± SEM and analysed by a one-way analysis of variance test ANOVA followed by Pairwise Multiple Comparison using Tukey test.

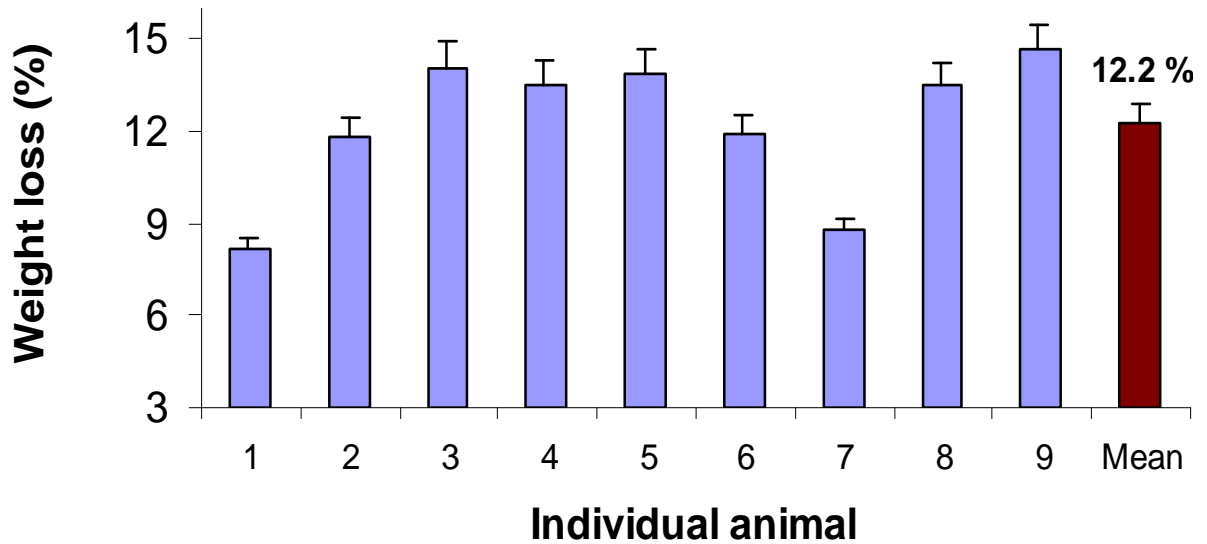


Figure 3.2 A sample graph of cumulative weight loss during a short period of energy restriction in HF / ER group. CBA females received a high fat diet for 15.5 weeks and thereafter, a regular diet (low fat food) with a reduced daily energy intake to a 70 % for 5 days. Calculation of the energy restriction / low caloric consumption was based on daily food intake in CD group for 2 weeks prior restriction in the HF / ER group. Mean weight loss (%) attained to 12.2 % and presented for nine animals. Data is presented by individual animal weight loss (%) within 5 days and their mean value (%). Error bars represent values for a single animal.

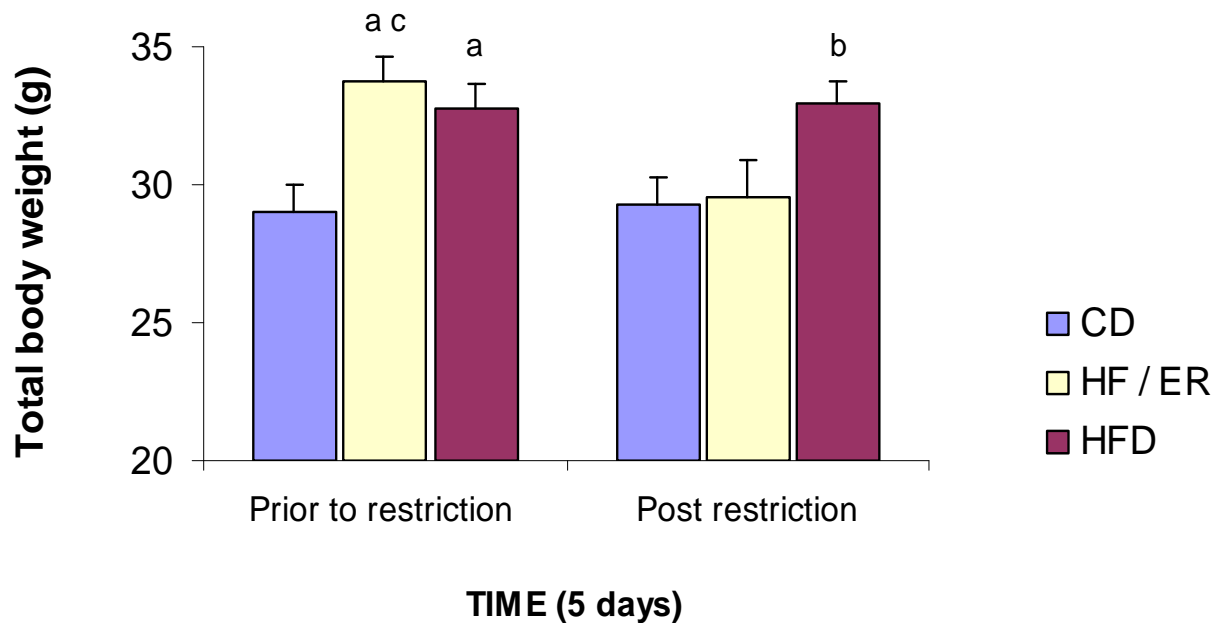


Figure 3.3 The difference of body weight in mature females (21 weeks old CBA mice) were recorded prior to and after energy restriction for 5 days. Data expressed as a mean value of body weight (g) \pm SEM, n = 9 / per group and analysed using a one-way ANOVA t-test to compare the outcome within each treatment group and a one way analysis of variance test ANOVA followed by Pairwise Multiple Comparison using Tukey test for multigroup assessment. Denotes ^a and ^b are showing a significant difference between the HF / ER and HFD treatment groups prior to and after calorie restriction, compared to females in CD group, correspondingly (P < 0.05). Whereas ^c is a detected difference in the body weight prior to and post energy restriction within the HF / ER treatment group (P = 0.001).

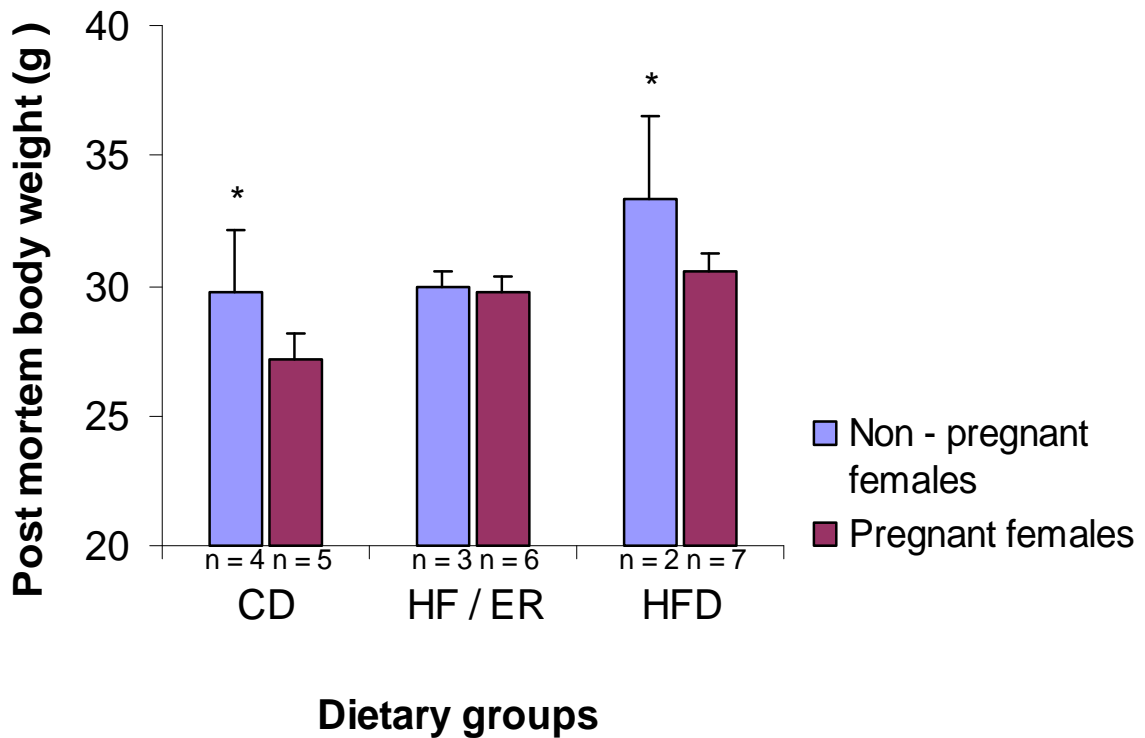


Figure 3.4 The body weight disparity within each dietary group at the end of experiment. This graph shows the body weight of females who were identified as pregnant or non – pregnant females at the end of experiment and the weight distribution was not significant within the treatment groups. However, the weight of non – pregnant females in the HFD group was significantly different from the non - pregnant females in the CD group and the difference is showed as * ($P = 0.018$). Data are expressed as relative means (g) \pm SEM. Values were analysed by using ANOVA t- test within the each treatment group and a one-way analysis of variance test ANOVA followed by Pairwise Multiple Comparison using Tukey test for the multigroup assessment.

Weight of internal tissues of pregnant females

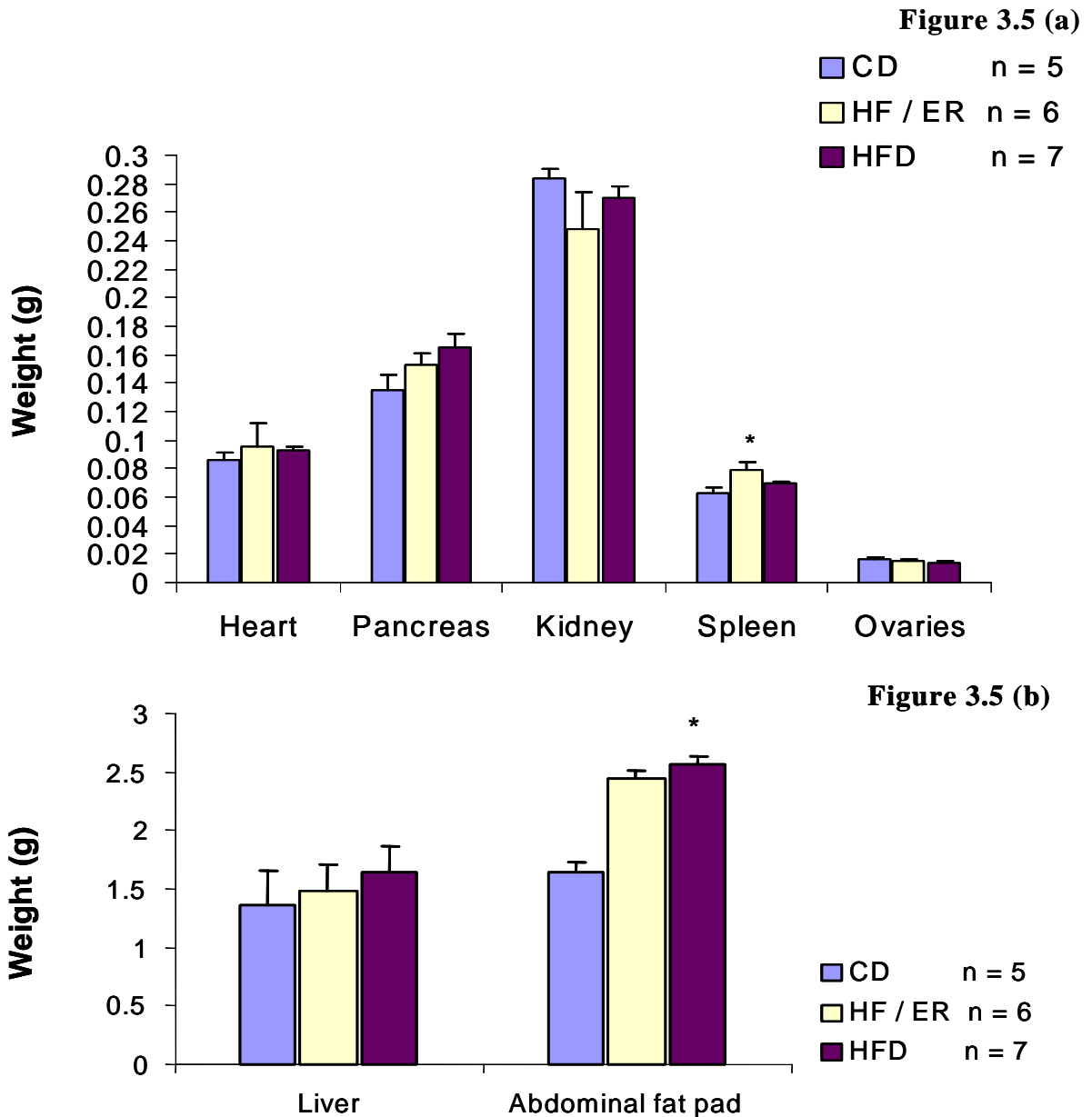


Figure 3.5 (a, b). The effect of different dietary regimes on internal tissues weight in three dietary groups - CD, HFD and HF / ER. According to group affiliation females received control diet, or high fat diet or high fat diet replaced by control diet with energy restriction for 16 weeks and then mated. Graphs represent data expressed as relative means (g) \pm SEM and assessed by one way analysis of variance ANOVA followed by the Pairwise Multiple Comparison procedures using Tukey test. * - indicates significant effect of various dietary food intake on weight of spleen and abdominal fat in HF / ER and HFD groups ($P < 0.05$), respectively compared to the CD group.

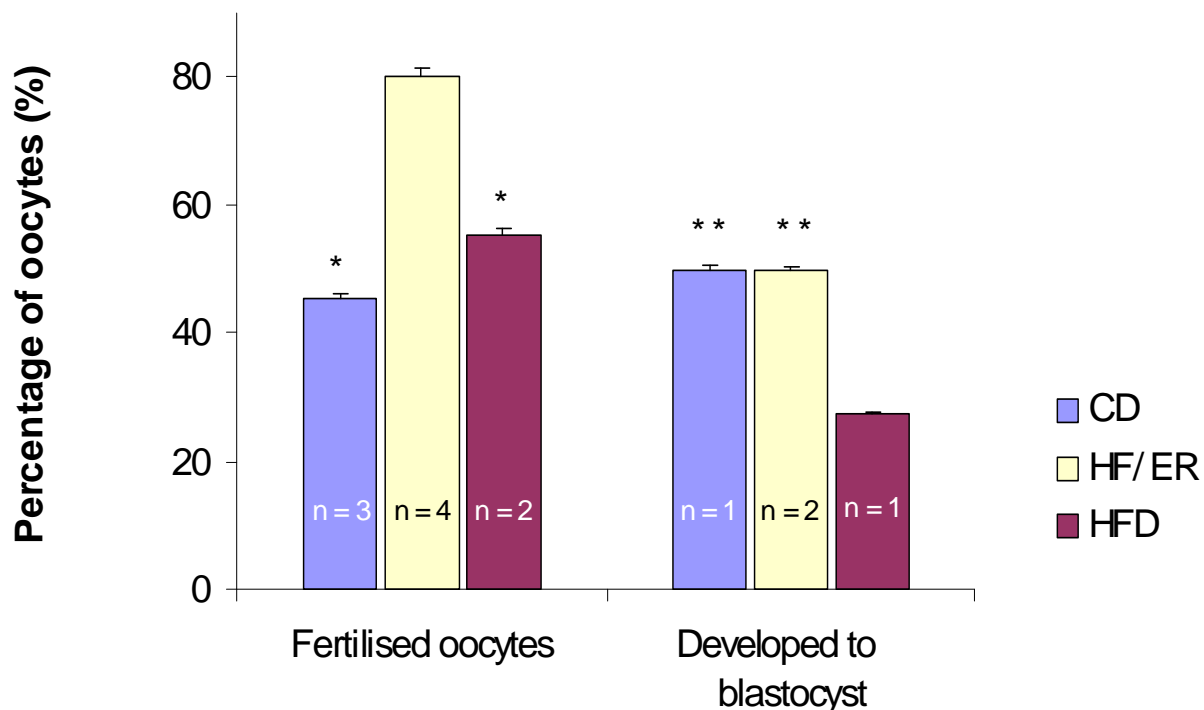


Figure 3.6 Graph represents the percentage of fertilised oocytes and oocytes developed to the blastocyst stage. Number of fertilised oocytes collected from HF / ER group was higher (80%) compared to CD (45.5 %) and HFD (55 %) groups. The proportion of embryos developed to the blastocyst stage were equal in CD and HF / ER groups (50 %) compared to HFD (27.3 %). * - indicates a difference in number of fertilised oocytes between HF / ER group and CD and HFD groups. ** - indicates a difference in number of embryos developed to the blastocyst stage between HFD group and HF / ER and CD groups. However, the significant difference cannot be detected due to a low number of animals.

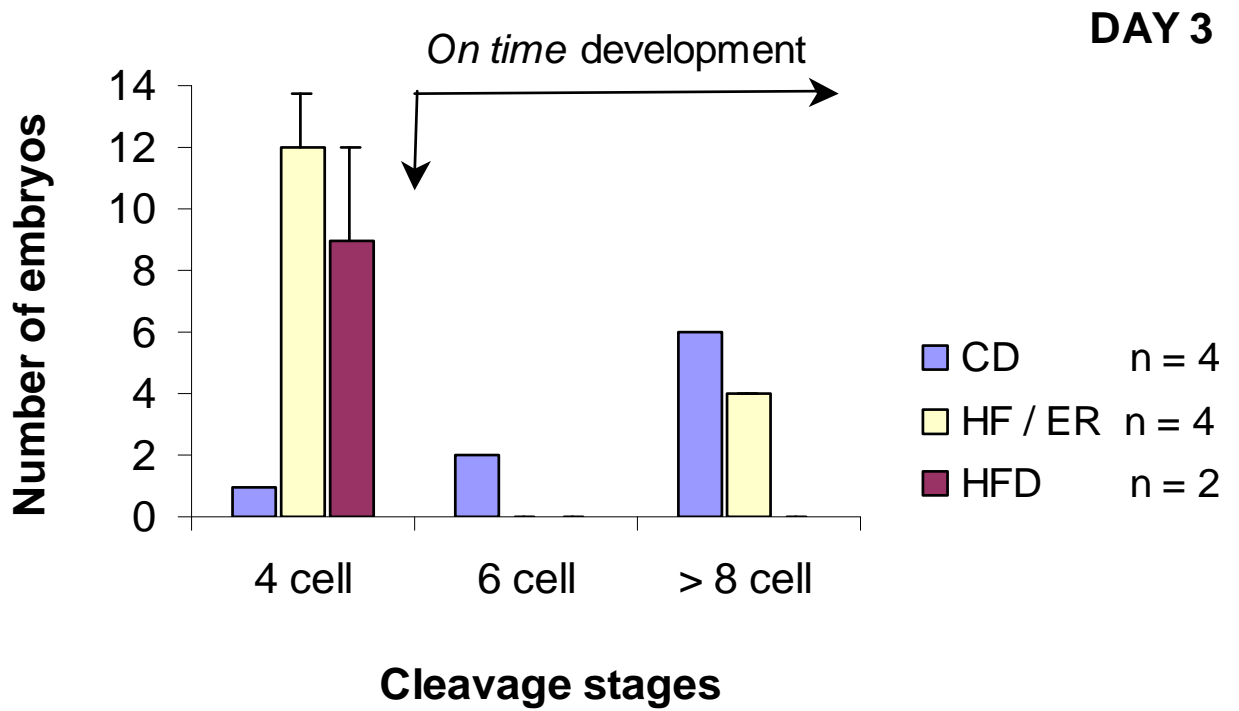


Figure 3.7 Postfertilisation *in vitro* development on Day 3. This graph presents the number of embryos at different cleavage stages. All embryos from the HFD group and partial number from the HF / ER group are showing a slow developmental pattern on Day 3 of *in vitro* culture.

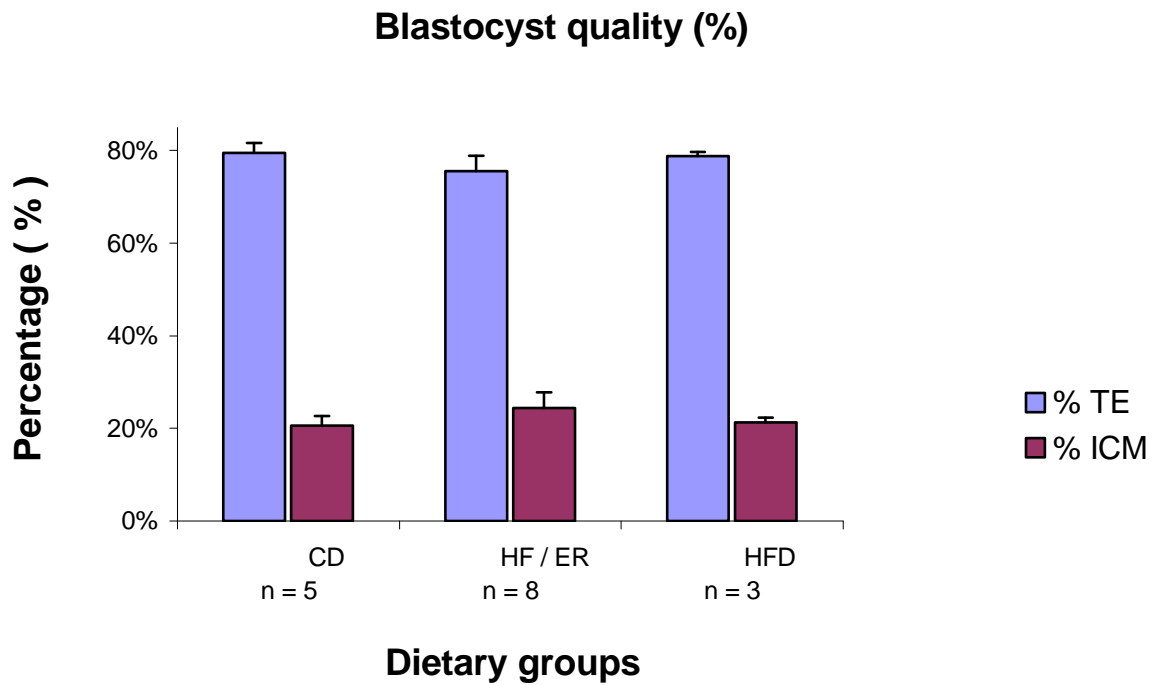


Figure 3.8 Blastocyst qualities. The qualities of blastocysts, as trophectoderm cell numbers (%) and inner cell mass numbers (%) were assessed under the fluorescent microscope and all three groups were not statistically different.

3.13 References

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CHAPTER FOUR

Summary and future work

4. Summary and suggestions for the future work

Numerous clinical and animal studies in various health disciplines have established the detrimental role of excessive body weight on general health. The tendency to human obesity is apparent worldwide with further dramatical escalation each year. Obesity / overweight is associated with increased rate of mortality and morbidity, metabolic syndrome, cardiovascular diseases, diabetes, insulin resistance, hypertension and cancer. Furthermore, it is well established in the literature, that obesity impairs reproductive function and decreases the chances of female's of reproductive age to fulfil their wish to be a mother. One third of patients attending the ART clinic and seeking infertility treatment are overweight / obese and it was shown that abnormal weight lowers treatment outcomes.

Previous publications showed improved metabolic and hormonal parameters, and reproductive function followed by weight reduction, resulting from different dietary or exercise regimes in overweight / obese women (Clark *et al.*, 1995; Moran *et al.*, 2003). Various dietary combinations (protein vs carbohydrate, high dietary fibre or vegetables intake, monounsaturated fat vs polyunsaturated fat and etc.), different dietary regimes and caloric intakes (very low calorie, low calorie or moderate calorie intake) have been used to achieve the weight loss and improve general health in the last few decades. However, the 'golden dietary formula' and 'golden weight loss program' remains uncovered yet.

The work presented in this dissertation has attempted to shed light on two proposed questions: the first, how the short – term dietary intervention (VLCD diet) prior to fertilisation will impact on IVF treatment outcomes in overweight / obese women. To enlighten this question, we proposed to perform a pilot study as initial trial to assess the feasibility and tolerability of dietary regime alongside IVF treatment. The second question was related to the blastocyst quality and how a short term of dietary intervention prior fertilisation in the overweight / obese female might affect embryo development. This study was performed in the mouse model due to ethical aspects relating to the human embryos.

The clinical pilot study outcomes illustrated that the dietary weight management is feasible alongside IVF treatment and women preferred to select a week shorter duration (from Day 21 of their treatment) with a fully caloric restriction to a graduate energy restriction term (Day 14 of their treatment). Furthermore, both groups achieved a significant weight loss independently of their dietary preference. As number of women who completed the study was low and thus, negative IVF outcomes were difficult to evaluate on this base. However, adverse effects of acute energy restriction were not established.

The animal study showed that even within the small number of animals, it is clear that oocyte quality is dependent on the level of nutrition and calorie intake. Limitations of this study were the number of dropouts in the clinical study and the small sample size in both clinical and animal studies. In conclusion, the both clinical and animal work suggests that the dietary regime or length or calorie intake should be selected cautiously prior to fertilisation in overweight / obese female.

The work described in this thesis laid a basis for the future research and the longer-term energy restricted approaches may be a possible option for the overweight / obese patients prior to addressing to ART alternatives, as it will allow gradual metabolic / endocrine adaptation. The results of these studies highlight the need to enhance understanding of the potential effects of a very low calorie diet in the short – term prior to fertilisation on physiological processes and underlying mechanisms with respect to oocyte quality, blood chemistry and interactions on cellular level. Further detailed studies are required in this area of research.

Based on the initial pilot study results, we commenced a randomised clinical study and will assess the effects of a moderate calorie restriction along with gradual physical exercise during IVF treatment on embryo quality and pregnancy outcomes. As this study is ongoing, the results cannot be assessed and not presented in this dissertation. The study design and aims are described in Appendix V, pp 134 - 141.

CHAPTER FIVE

Appendices

I.I General solutions

Saline (NaCl 9 %)

Sodium chloride – 9g

Milli Q water – 1000 ml

Store at + 4°C.

Paraformaldehyde (PFA – 4 %)

Milli Q water – 1000 ml

Paraformaldehyde - 40 g

Phosphate Buffer solution (PBS)

80 mM $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$ (Disodium hydrogen orthophosphate 2- hydrate)

20 mM $\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O}$ (Sodium dehydrogen orthophosphate)

$\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$ – 7.12 g

$\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O}$ – 1.56 g

NaCl – 2.92 g

Milli Q water – 500 ml

Store at + 4°C.

I.II Embryo culture medium

i.ii.i G 1 and G 2 mediums

These mediums were kindly donated for our project by Michelle Lane and obtained from embryology laboratory at Repromed The recipe of mediums are available from the book “ *A Laboratory guide to the mammalian embryo*” (edited by David Gardner, Michelle Lane, Andrew J. Watson, 2004).

i.ii.ii G – MOPS - wash medium without protein

Recipe obtained from the book “ *A Laboratory guide to the mammalian embryo*”
(Edited by David Gardner, Michelle Lane, Andrew J. Watson, 2004).

Ingredients for 1 litre of solution:

Milli-Q water must be used at all time.

NaCL	5.283 g	CaCL ₂	0.147 g
Glucose	0.090 g	Lactate	1.950 g
KCl	0.410 g	Non-essential amino acids	10 ml
MOPS	5.800 g	Glutamax	10 ml
Na pyruvate	0.035 g		
NaHCO ₃	0.420 g		
Phenol red	0.005 g		
MgSO ₄	0.440 g		
NaH ₂ PO ₄	0.035 g		

Method:

Weigh the first 9 chemicals separately and pour into 1L volumetric flask, add Milli – Q water (less than half of the flask volume) and dissolve chemicals. Weigh **Lactate** (liquid), add some water to it and pour into the flask. Afterward, separately dissolve **CaCL₂** in 10 ml tube with water and pour into the flask. Next step, add **NEAA** and **Glutamax** (liquids) to flask, fill to appropriate level of 1L with water and pour solution into the empty 1L beaker. Stir and check pH level (normal range of pH = 7.30 - 7.35) and afterward, osmolality (normal range is 255 ± 3). If pH level is too acid add NaOH (4 -5 pellets dissolve in 10 ml of this solution) very carefully, ml-by-ml and check the pH level it every time, until the normal level of the pH is attained. If osmolality level is higher than expected, Milli – Q water can be added. When every parameter is accurate, the solution must be filtered, poured out into the labelled flasks and kept at 4° C for 2 - 3 months.

i.ii.iii G – MOPS medium with protein (Bovine serum albumin BSA)

G-MOPS medium not filtered (see above) - 1L

Add Bovine serum albumin (BSA) - 4 g

Filter and keep at 4°C for 2 –3 months.

I.III Solutions for differential nuclear staining

Wash media – no protein

G-MOPS (no protein) see above.

Pronase solution

Protease 0.5 g

G-MOPS 100 mls

Aliquot 100 μ l

Store at - 20°C for 2 months.

Picrylsulfonic Acid, 2,4,6 –trinitrobenzenesulfonic acid (TNBS)

TNBS 10 μ l

PVP 90 μ l

Store in dark at 4°C.

Anti – Denitrophenyl solution (Anti – DNP)

Aliquot in 10 μ l

Store at - 20°C for 8 weeks.

Polyvinil medium (PVP)

PVP 4 mg

G-MOPS 10ml

Store at 4°C for 2 months.

Guinea Pig Serum (GPS)

Aliquot in 60 μ l

Store at - 80°C

Propidium Iodide stain (PI)

PI 2 g

G - MOPS 100 ml

Store in the dark at + 4°C for 2 - 3 months.

Bisbenzimidazole solution

Bisbenzimidazole 2.5 mg

Absolute Ethanol 100 ml

Cover with aluminium foil.

Store in dark at 4°C for 4 months.

Glycerol

Glycerol is used for embryo mounting.

Mineral oil (M - 5310)

Store at room temperature.

Absolute ethanol

Absolute ethanol is used for embryo wash - 5ml

Anti –Denitrophenyl solution (Anti – DNP)

G - MOPS 90 μ l

Anti - DNP 10 μ l

Complement solution

Propidium Iodide stain (PI) 50 μ l

Guinea pig serum (GPS) 50 μ l

Keep in dark

II.I Atherogenic dry food for mice

ii.i SF00 – 219 (Obtained from specialty feeds Ltd.,www.specialtyfeeds.com)

12 mm diameter pellets

Metabolizable energy - 19.4 MJ / kg

Caloric content - 4.64 kcal /g

Ingredients

Casein - 195 g / kg

DL Methionine -3.0 g / kg

Sucrose - 341 g / kg

Wheat starch - 154 g / kg

Cellulose - 50 g/kg

Clarified butter (Ghee) - 210 g / kg

Calcium carbonate - 17.1 g / kg

Sodium chloride - 2.6 g / kg

Potassium citrate - 2.5 g / kg

Potassium sulphate -1.6 g/kg

Potassium dihydrogen phosphate - 6.9 g/kg

AIN93G trace minerals - 1.4 g / kg

Choline chloride (65%) - 2.5 g / kg

SF00 – 219 vitamins - 10 g / kg

USP cholesterol -15 g / kg

Etoxyquin (66%) - 0.04 g / kg

Amino acids

Valine - 1.2 %

Leucine – 1.7 %

Isoleucine - 0.8 %

Threonine - 0.7 %

Methionine - 0.8 %

Cystine – 0.05 %

Lysine – 1.5 %

Phenylalanine – 0.9 %

Tyrosine – 1.0 %

Tryptophan – 0.3 %

Minerals

Calcium – 0.58 %

Phosphorus – 0.30 %

Magnesium – 0.09 %

Sodium – 0.11 %

Chloride – 0.16 %

Potassium – 0.4 %

Sulphur – 0.22 %

Iron – 90 mg / kg

Fat - 21 %

Protein -19 %

Cholesterol - 0.15 %

Crude fibre - 4.7 %

Acid detergent fibre - 4.7

Copper 6.7 mg / kg

Iodine – 0.22 mg / kg

Manganese – 20 mg / kg

Zinc – 40 mg / kg

Selenium – 0.15 mg / kg

Chromium – 2.0 mg / kg

Lithium – 0.1 mg / kg

Boron – 0.7 mg / kg

Nickel – 0.55 mg / kg

Vanadium – 0.10 mg / kg

Vitamins

Vitamin A – 10 000 IU / kg

Vitamin D3 – 1 100 IU / kg

Vitamin E – 62 mg / kg

Vitamin K – 12 mg / kg

Vitamin C – 700 mg / kg

Vitamin B1 – 11 mg / kg

Vitamin B2 – 11 mg / kg

Niacin – 50 mg / kg

Vitamin B6 – 11 mg / kg

Pantothenic acid – 33 mg / kg

Biotin – 200 mg / kg

Folic acid – 1 mg / kg

Inositol – 55 mg / kg

Vitamin B 12 – 18 mg / kg

Choline – 2 5000 mg / kg

Fat composition

Saturated fats C 12 :0 or less – 2.4 %

Myristic Acid 14 : 0 – 2.3 %

Palmitic Acid 16 : 0 – 6.1 %

Stearic Acid 18 : 0 - 1.9 % Arachidic

Acid 20 : 0 – 0.5 %

Palmitoleic Acid 16 :1 – 1.0 %

Oleic 18 : 1 – 5.6 %

Gadoleic 20 : 1 – trace

Linoleic 18 : 3 n3 – 0.8 %

Cholesterol - 0.15 %

Linoleic Acid 18 : 3 n3 – trace

ii.ii SF04 – 057 (Control diet, matched to SF00 – 219 diet)

Obtained from Specialty feeds Ltd., www.specialtyfeeds.com

12 mm diameter pellets

Metabolizable energy - 16.1 MJ / kg

Caloric content - 3.85 kcal /g

Acid detergent fibre - 4.7 %

Fat - 6 %

Protein -19 %

Crude fibre - 4.7 %

Ingredients

Casein - 195 g / kg

DL Methionine -3.0 g / kg

Sucrose - 341 g / kg

Wheat starch - 306 g/kg g / kg

Cellulose - 50 g / kg

Canola oil - 60 g / kg

Calcium carbonate - 17.1 g / kg

Sodium chloride - 2.6 g / kg

Potassium citrate - 2.5 g / kg

Potassium sulphate -1.6 g / kg

Potassium dihydrogen phosphate - 6.9 g / kg

AIN93G trace minerals - 1.4 g / kg

Choline chloride (65%) - 2.5 g / kg

SF00 – 219 vitamins - 10 g / kg

Etoxyquin (66%) - 0.04 g / kg

Manganese – 20 mg / kg

Zinc – 40 mg / kg

Selenium – 0.15 mg / kg

Chromium – 2.0 mg / kg

Lithium – 0.1 mg / kg

Boron – 0.7 mg / kg

Nickel – 0.55 mg / kg

Vanadium – 0.10 mg / kg

Vitamins

Vitamin A – 10 000 IU / kg

Vitamin D3 – 1 100 IU / kg

Vitamin E – 62 mg / kg

Vitamin K – 12 mg / kg

Vitamin C – 700 mg / kg

Vitamin B1 – 11 mg / kg

Vitamin B2 – 11 mg / kg

Niacin – 50 mg / kg

Vitamin B6 – 11 mg / kg

Pantothenic acid – 33 mg / kg

Biotin – 200 mg / kg

Folic acid – 1 mg / kg

Inositol – 55 mg / kg

Vitamin B 12 – 18 mg / kg

Choline – 2 5000 mg / kg

Amino acids

Valine - 1.2 %

Leucine – 1.7 %

Isoleucine - 0.8 %

Threonine - 0.7 %

Methionine - 0.8 %

Cystine – 0.05 %

Lysine – 1.5 %

Phenylalanine – 0.9 %

Tyrosine – 1.0 %

Tryptophan – 0.3 %

Minerals

Calcium – 0.58 %

Phosphorus – 0.30 %

Magnesium – 0.09 %

Sodium – 0.11 %

Chloride – 0.16 %

Potassium – 0.4 %

Sulphur – 0.22 %

Iron – 90 mg / kg

Iodine – 0.22 mg / kg

Copper 6.7 mg / kg

Fat composition

Myristic Acid 14 : 0 – trace

Palmitic Acid 16 : 0 – 0.3 %

Stearic Acid 18 : 0 - 0.1 %

Arachidic Acid 20 : 0 – trace

Palmitoleic Acid 16 :1 – trace

Oleic 18 : 1 – 3.6 %

Gadoleic 20 : 1 – trace

Linoleic Acid 18 : 3 n3 – 0.6 %

A Linoleic Acid 18 : 2 n6 – 1.1 %

Arachidonic 20 :4 n6 – trace

EPA 20 : 5 n3 – trace

ii.i.iii Animal joint stock II (Animal house - Standard rodent chow)

Obtained from Ridley AgriProducts PTY Ltd, www.agriproducts.com.au

Food presented as a 12 mm diameter dry pellets.

Ingredients

Min. Crude protein - 21 %

Min. Crude fat - 5 %

Max. Crude fibre -10 %

Max. salt - 1 %

Vitamins

Vitamin A - 22 000 I.U / kg

Vitamin D3 - 4000 I.U / kg

Vitamin E - 135 mg / kg

Vitamin B2 - 10 mg / kg

Vitamin K - 8.3 mg / k

Niacin - 18 mg / kg

II.II Dietary product and CSIRO booklet

ii.ii.i Optifast VLCD (Novartis International AG, www.novartisnutrition.com)

VLCD is scientifically formulated powder, presented in sachets (40g)

Metabolizable energy / per sachet - 638 kJ

Caloric content - 152 cal

Protein - 17.3 g

Carbohydrate total - 15 g

Carbohydrate Sugars - 11.8 g

Fat - 2.3 g

Recommended daily intake is three sachets - 1914 kJ or 456 cal.

Vitamins

Vitamin A - 1100 IU

Thiamin (Vitamin B1) - 0.53 mg

Riboflavin (Vitamin B2) - 0.66 mg

Nicotinamide - 6 mg

Pantothenic Acid (Vitamin B5) - 2.67 mg

Biotin (Vitamin H) – 67 mcg

Pyridoxine (Vitamin B6) - 0.66 mg

Cyano – cobalamin (Vitamin B12) – 1 mcg

Folic acid – 133 mcg

Ascorbic acid (Vitamin C) – 25 mg

Cholecalciferol (Vitamin D3) – 66.67 IU

Phytomenadione (Vitamin K1) – 33 mcg

Dl – alpha Tocopherol (Vitamin E) – 4.4 IU

Minerals

Calcium Total – 300 mg

Chromium – 33 mcg

Copper – 833 mg

Iodine – 50 mcg

Iron – 5 mg

Magnesium – 116 mg

Manganese – 1 mg

Molybdenum – 67 mcg

Potassium total – 668 mg

Selenium – 17 mcg

Sodium total - 332 mg

Zinc – 5 mg

ii.ii.ii “The Total Wellbeing Diet”

TWD booklet can be downloaded from the website www.themainmeal.com.au.

Further information related to the diet can be obtained from www.csiro.au

III.I Blastocyst differential staining

The blastocyst differential staining protocol to be completed requires two days. On the first day, five Petri dishes with an appropriate solution was prepared and placed on to a warm plate (37 °C) or in the fridge (where applicable) and covered with aluminium foil (where applicable) at least for 60 minutes prior staining procedure. Prepared solution were placed as 20 µl microdrops on to the marked dish and covered with 5 – 6 ml of mineral oil. Up to 10 embryos were transferred in each drop of solution. Dishes with the complement solution and TNBS were covered with aluminium foil during the differential staining procedure.

Embryos at the blastocyst stage were transferred into pronase and left for 2 - 5 minutes till the zona pellucida dissolved. Disappearance of the zona was checked under the microscope and embryos immediately were transferred into wash drops. Afterwards, embryo were incubated for 10 minutes in TNBS solution at + 4 °C and then washed two or more times if required, in the wash media. Washed embryos were transferred and incubated in anti - DNP solution for 10 minutes at 37 °C and then washed in the fresh wash drops.

Thereafter, embryos were transferred in the complement solution and the dish covered with aluminium foil and placed at the warm plate (37 °C) for 5 minutes. Staining efficiency was checked under the green UV filter of the confocal microscope. Stained embryos were placed in the Nunc well with 800 µl of bisbenzimidazole, covered with aluminium foil and kept in the fridge at + 4 °C overnight.

About 24 hours later, embryos were transferred into the absolute alcohol with further relocation and mounting, 2 embryos in each glycerol microdrop placed on the microscope glass slide. Thereafter, each microdrop was covered with a glass cover slip and embryos cell nuclei counted by using a confocal microscope. A timer was used at all times during the procedure.

Nuclear staining procedure was based on the protocol from the book “ *A Laboratory guide to the mammalian embryo*”, edited by David Gardner, Michelle Lane and Andrew J. Watson, 2004.

III.II Embryo culturing and daily assessment protocol

Days	Time	Procedures	Assessment
Day 0	> 4pm	Prepare dishes with G1 and MOPS (no protein) mediums as drops in each half of the dish and cover with paraffin oil, consequently. Place dishes in incubator (lids off) and leave no longer than 18 hrs.	None
Day 1	10am	Collect fertilised eggs in warmed to 37 ⁰ C MOPS (no protein) medium, then transfer and wash in prepared on Day 0 dishes, first in MOPS (no protein) medium and then, wash few times in G1 medium (within the drop). Assess embryos and culture them (lids on) in incubator for 48 hrs.	Zygotes
Day 2	> 4pm	Prepare dishes with G2 and G-MOPS (no protein) mediums in each halve of the dish as drops and cover with paraffin oil, consequently. Place the dishes in incubator (lids off) no longer than 18 hrs.	None
Day 3	9am	Embryos changeover. Transfer embryos from G1 medium to G 2 medium by washing them in prepared on Day 2 dishes, two times in G-MOPS medium (no protein) and then place into G2 medium. Return dishes in the incubator and culture (lids on) for 48 hrs.	≥ 8 cell
Day 4	4pm	Score the embryo developmental stage and return dishes in the incubator (lids on).	≥ Compaction
Day 5	9am	Embryo cleavage assessment. Quality of the embryo can be assessed by differential staining procedure (page 131).	≥ Blastocyst

Procedures used during embryo culture and assessment:

The Pasteur pipette was pulled in advance from glass capillary tubes using the flame at the gas burner.

- Petri dish was numbered (each droplet) and marked (line dividing the dish into halves) from outside.
- New pipette tips were used for each new medium drop. The tip first was rinsed with the medium and then, microdrops placed next to the marked number on the dish.
- The lids of the Petri dishes were removed, where applicable and placed next to the dish by setting an angle with the plate and left for appropriate time in incubator.
- Embryos were cultured in incubator in atmosphere of 5 % O₂ – 6 % CO₂ at 37 °C.

In the animal study, for the assessment / scoring of the *on time* embryo developmental stages we have used a sequential daily assessment as described in the book: “ *A Laboratory guide to the mammalian embryo*” (edited by David Gardner, Michelle Lane and Andrew J. Watson, 2004).

IV.I Clinical Pilot study

**PATIENT INFORMATION SHEET**

Title: A pilot study to assess acceptance of a short-term very low calorie diet (VLCD) on overweight women who are undergoing IVF treatment.

Introduction

You have been invited to participate in a research pilot study. Before agreeing to participate in the study, it is important that you read and understand the study design.

The ovary produces eggs and different hormones. As eggs mature in the ovary, surrounding cells start developing and producing hormones to help the egg mature. Increased body weight is associated with impaired treatment outcomes such as miscarriage, high blood pressure and a decreased pregnancy rate. We hope that using a very low calorie diet for a short term will lead to weight loss that may improve these outcomes. There is enough evidence that women with weight problems show a poor response to infertility treatment and we would like to find better ways of improving these outcomes.

What is the study about?

In this study we are trying to assess your acceptance of a dietary meal replacement along with your IVF treatment. There will be 2 groups of dietary intervention with different regimens.

How does the study work?

After you have consented to participate in this study you will be allocated to one of the two groups of your choice:

- Group 1 will take an approved and tested meal replacement 2 times per day from Day 14 of the menstrual period, and one week later 3 times per day for the rest of the study, up to the evening prior to egg collection (oocyte pick up OPU) procedure.
- Group 2 will take the meal replacement 3 times a day for the whole study, from Day 21 of menstrual period to the day before the egg collection (oocyte pick up OPU).
- The dietary intervention stops the evening before the day of egg collection. After the OPU procedure you will be asked to follow advice on the less restrictive weight loss plan using normal foods (see CSIRO booklet) for 2-3 days before the embryo transfer procedure.

We will also measure your weight, waist circumference and blood pressure on six occasions, when you visit Repromed.

Additionally, you will be asked to complete a simple diary of your daily activities during the study. Also, we would like to get your comments about your experience during the treatment and suggestions that may improve treatment in the future.

We will record and compare the dietary and infertility treatment outcomes between the two groups.

What are the risk and/or discomfort involved in this study?

- This study poses no risk or discomforts other than those that apply to routine infertility treatment, including blood and egg collection procedures, such as, occasionally pain and/or bruising at the collection site.
- The meal replacement is a tested and approved product in Australia (see attached product information) and should not cause any harmful outcomes if used according to the user instructions.
- Patients in both groups will need one extra visit to Repromed on Day 14 of their cycle for measurements (weight, waist circumference and blood pressure) to be taken.

What will I get out of this study?

There may be no specific, immediate benefits for you.

What happens to the results?

The results of this study will be published in research papers. We hope the results of the study will increase our understanding of the potential benefits of short-term dietary intervention on infertility treatment. Also, this may change our patient information handouts and improve our services. Your personal details will not be identified in the analysis.

What happens if I say No?

Your involvement in the study is entirely voluntary and saying no will not affect your treatment at Repromed in any way. Also, you are free to change your mind at any time and withdraw from the study; you may do so freely and without prejudice to any future treatment at the centre.

What if I have a question about the study?

If you have any questions about the study, please speak to

Professor Robert Norman ph: 8222 6788

Dr Manny Noakes (Senior Dietitian / Research Scientist) ph: 8303 8827

Dr Victoria Tsagareli ph: 8222 8720

You may also speak to your doctor or one of the nurses at Repromed.

The research described in this paper has been approved by the Women's and Children's Hospital Research Ethics Committee. As a part of its professional responsibility, this committee may need to access medical records from time to time. Any such access will be made in manner that respects and protects your privacy.

If you have questions regarding the conduct of the study or other ethical aspects, please contact Brenda Penny, Research Secretariat at the Women's and Children's Hospital, on: 81616521.

Thank you for taking the time to read this information sheet.

Information of a dietary product of Optifast VLCD in the pilot study

Optifast VLCD (very low calorie diet) is a dietary product designed by Novartis Nutrition Company (USA) and has been effectively used for the last twenty years in the USA, Australia and other countries.

More than 1000 000 patients have used Optifast and achieved good results. Optifast meets very high manufacturing standards and uses an established diet regime by reducing daily calorie intake to 456 kcal / 1914kJ for rapid weight loss and also improves metabolic conditions such as raised insulin resistance, cholesterol, glucose levels, blood pressure and gynaecological hormonal disorders.

Optifast VLCD overview

Optifast VLCD is designed to replace your normal daily food intake and contains moderate levels of carbohydrates, essential fatty acids (low in fat, especially in animal fat), high quality proteins that help to preserve body mass (muscle), rich in fibre and the recommended dietary intake (RDI) of vitamins, minerals and trace elements. Optifast VLCD is completely made from food products. The diet is a nutritionally adequate product, which has been tested and approved in Australia. This product should not cause any harmful outcomes if used for the purpose of this study and according to the user instructions. It is free of any stimulants or drugs.

- Use of Optifast VLCD requires medical supervision. Optifast VLCD is intended for use as a part of management of overweight patients and associated medical problems, like type II diabetes, gynaecological disorders, hypertension and osteoarthritis.
- Individuals receiving any medications should check the patient information sheet or seek medical advice from a nurse or other medical staff at Repromed
- Alcohol should be avoided when on Optifast VLCD dietary plan.
- Drink at least two litres of water or other calorie-free liquid (water, mineral unflavoured water, soda water, diet soft drinks or diet cordials, decaffeinated coffee or tea) per day while on Optifast VLCD dietary plan.

What does the Optifast VLCD dietary plan mean

Optifast dietary plan means, different combinations of Optifast VLCD sachets (milkshake or soup) for daily intake + what you are allowed to eat or drink extra per day.

Additionally,

- Up to two cups (250 ml) per day of green and low starch vegetables, raw or cooked without fat or oil. (See additional allowances.)
- At least two litres of energy free liquids (water, soda water or mineral water) must be taken every day.
- Up to 4 cups of decaffeinated tea or coffee per day.
- Up to 2 cups of diet soft drink or diet cordials per day.
- Up to 2 cups of diet jelly.

How to take Optifast

For patients in **Group 1**, from Day 14 to Day 21 you will have your usual breakfast and after midday, i.e. instead of lunch and dinner you will choose and take one of the three options of Optifast VLCD sachets per day + additional allowances.

Options:

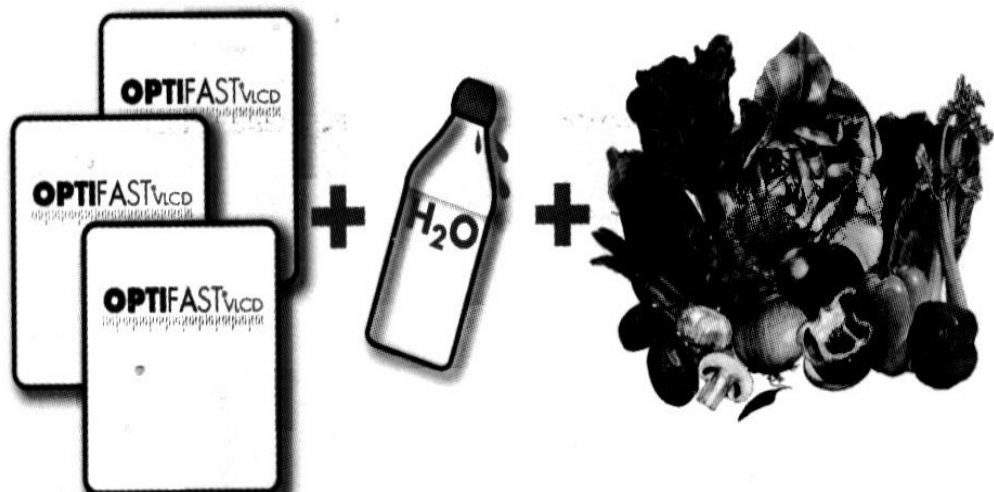
- Milkshakes alone: **2** sachets/day or
- Combination: **1** sachet of Milkshake + **1** sachet of Soup/day or
- 1** sachet of Milkshake + **2** sachet of Soup/day

For all patients (Group 2 and Group 1), from Day 21 of your cycle you will replace all your meals, i.e. breakfast, lunch and dinner with one of three options of Optifast VLCD per day + additional allowances, till the day of egg collection procedure.

Options:

- Milkshakes alone: **3** sachets/day or
- Combination: **3** sachets of Milkshake + **1** sachet of Soup/day or
- 2** sachets of Milkshake + **2** sachet of Soup/day

Mix each sachet with 200 ml cold or warm water to provide the essential nutrients required by the body as you lose weight. Stir, blend or shake well. Supplement this with at least 2 litres of calorie free liquids.



3 Optifast sachets

2 litres of water

2 cups low starch vegetable

How to complete the diary

1. We would like you to complete the attached diary each day.

2. Please, write correct time, as much as you are able to.

For example: Get up time – 7.50 a.m Activities (gardening) ~ 40 min.

Lunchtime – 12.10 p.m (20 min) or 12.10 - 12.30 p.m

Shopping ~ 1h 50 min or 7.00 p.m - 8.50 p.m

3. Please, list ALL not allowed food and the amount eaten during the day.

For example: Meat -100gr or

Fruit - half an apple

4. Please, record the duration of activities you have done on that day.

For example: parties, going to the movies, BBQ, gym, walking or other exercises etc.

5. In section of ‘my feelings’ could you describe your feelings and emotions related to food on that day (e.g. not hungry, hungry, very hungry, bad mood, diet improved your mood, do you have any cravings, Do you feel hungry from chosen dietary combination today, etc.).

ADDITIONAL ALLOWANCES CHART

NOTE: This chart is included on page 130 of the print copy of the thesis held in the University of Adelaide Library.

Obtained and modified from 'Optifast VLCD clinical treatment protocol', page 7.

Your treatment / study schedule

Days of my menstrual cycle	Date	Events	Notes
1st day of my period Day 1	...2004		Please fill in the date.
Day 14		Extra visit to Repromed	<u>Measurements:</u> <ul style="list-style-type: none"> • <u>weight (W)</u> • <u>waist circumference (WC)</u> • <u>blood pressure (BP)</u> <u>Commence Optifast 2 sachets /day</u>
Day 21		Visit to Repromed	1. Blood test as routine IVF treatment. <u>2. Measurements as above.</u> <u>3. Optifast: 3 sachets /day</u>
Day 24		Start Synarel (nasal spray)	
Day 10 –14 of Synarel (your period will start during this time)		Visit to Repromed	1. Blood test to check hormones <u>2. Measurements as above</u> <u>3. Continue Optifast 3 sachets /day</u>
Day 2 of IVF cycle		Nurse will contact you	1. Continue Synarel 2. Start FSH injections <u>3. Continue Optifast 3 sachets /day</u>
Day 9 of IVF		Visit to Repromed	1. Scan and blood test <u>2. Measurements as above</u> <u>3. Continue Optifast 3 sachets / day</u>
Day 11 – 17 of IVF		Egg collection procedure (OPU)	<u>1. Measurements as above</u> <u>2. Cease Optifast night before</u> <u>3. Follow CSIRO brochure of healthy food intake for 2-3 days</u>
2 – 3 days after OPU		Embryo transfer	Measurements as above

NOTICE: Underlined information relates to our study events.

Non-underlined events relate to routine IVF treatment

<u>Date:,, 2004</u>	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Wake up time (record time)							
Breakfast time (record time)							
Lunchtime! Yummy Optifast!							
First cup of Vegetables							
Did I have some fruit? Which one's?							
Soup time.							
Dinnertime! Yummy Optifast!							
Second cup of Vegetables?							
Low calorie jelly.							
Did I have 2 litres of water?							
Diet soft drink or cordial.							
How many cups of decaff. coffee or tea?							
Some alcohol (please specify)							
Other food or drink intake.							
Please record my mood or feelings today relating to food, hunger etc.							
Did I have a rest, eg. watching TV, reading or other activities eg. walking, gardening, cooking or special events BBQ's, parties, picnics' etc.							
Bedtime. Good night diary. See you tomorrow.							

Diary of daily food intake and activities.



WOMEN'S & CHILDREN'S HOSPITAL RESEARCH ETHICS COMMITTEE

CONSENT FORM

I _____
hereby consent to my involvement in the research project entitled:

Acceptance of a short-term very low calorie diet (VLCD) on women who are overweight and undergoing IVF treatment.

1. The nature and purpose of the research project described on the attached Patient Information Sheet has been explained to me. I understand it, and agree to take part.
2. I understand that I may not directly benefit by taking part in this study.
3. I acknowledge that the possible risks and/or side effects, discomforts and inconveniences, as outlined in the Patient Information Sheet, have been explained to me.
4. I understand that while information gained in the study may be published, I will not be identified and information will be confidential.
5. I understand that I can withdraw from the study at any stage and that this will not affect medical care or any other aspects of my relationship with this hospital.
6. I understand that there will be no payment to me for taking part in this study.
7. I have had the opportunity to discuss taking part in this research project with a family member or friend and/or have had the opportunity to have a family member or friend present whilst the research project was being explained by the researcher.
8. I am aware that I should retain a copy of the Consent Form, when completed, and the Patient Information Sheet.

Signed:Dated:.....

Full name of patient:

I certify that I have explained the study to the parent and consider that he/she understands what is involved.

Signed:..... Title.....

Dated:.....

V.I Introduction

The objectives of the randomised controlled study are connected to the results of the Pilot study which showed no specific benefit of rapid weight loss in improving IVF outcomes in overweight / obese patients. We hypothesised that the lack of beneficial outcomes may be partially related to the severity of energy restriction in these patients. We subsequently planned a randomised controlled study to assess the effects of a short - term moderate kilojoule restriction along with exercise prior and during IVF on pregnancy outcomes.

V.II Study aims

This study pursued the following aims:

- (i) To assess pregnancy rate after a moderately energy restricted diet and exercise regime.
- (ii) To identify the benefit of weight loss on hormonal and metabolic changes in overweight / obese patients undergoing IVF treatment.

V.III Primary and Secondary Outcomes

Primary outcomes are:

- (i) Pregnancy rate (%)
- (ii) Livebirth rate (%)
- (iii) Changes in weight and waist circumference before and after intervention

Secondary outcomes are:

- (i) Number and quality of oocytes
- (ii) Number and quality of embryos
- (iii) Hormone profile
- (iv) Changes in glucose, insulin and lipids before and after intervention.
- (v) Economic analysis
- (vi) Quality of life

V.IV Study population / Inclusion Criteria

Overweight / obese and infertile women undergoing IVF / ICSI treatment will be recruited by the Research Centre for Reproductive Health of The University of Adelaide based at Adelaide Fertility Centre, Adelaide. Women of reproductive age (18 – 40 years old) with $BMI \geq 28 \leq 45 \text{ kg/m}^2$ and who had previously undergone at least one assisted reproductive treatment (ART) with regard to in vitro fertilisation (IVF), intracytoplasmic sperm injection (ICSI), frozen embryo transfer (FET) procedures were included in the study. Women with severe diseases and currently following the dietary management will be excluded. All women fulfilling the inclusion criteria will be provided with adequate information through the “Patient information sheet” (not included in this thesis) and recruited after initial medical consultation and discussion with their clinician to understand the implication of the research.

V.V Exclusion Criteria

Women with following diseases and conditions:

- Acute cerebrovascular or cardiovascular disease
- Renal disease, hepatic disease, diabetes, gall bladder diseases and those on lithium therapy
- Malabsorbtive disorder

Women, who are currently following a weight loss programme (diet).

V.VI Human Ethics

The following study was approved by the Women’s and Children’s Hospital Human Research Ethics Committee, approval number REC 1601 / 6 / 2007, CSIRO Ethics Committee and Adelaide Fertility Centre Scientific Advisory Committee, Adelaide. The research was performed with state and commonwealth legislation and all the requirements set out in the National Health and Medical Research Council (NH&MRC) in the relevant document “National Statement on Ethical Conduct in Research Involving Humans”.

Furthermore, this trial was registered under the name of “The effects of moderate energy restriction combined with exercise on in vitro fertilisation (IVF) outcomes in infertile overweight women” in the Australian Clinical Trials Registry, registration number ACTRN 012606000114549.

No drugs are involved in this study, other than those used routinely in IVF. This study involves no use of radiation. Advice from CSIRO Health Science and Nutrition indicates that the CSIRO brochure “The Total Wellbeing Diet” is nutritionally adequate and safe for the purpose of this study. Exercise will be ceased on the day prior to oocyte pickup procedure. Signed consent forms and the records will be kept on file, to verify the willing participation of study subjects and confidential, observing Adelaide Fertility Centre’s, CSIRO’s and NHMRC privacy guidelines. Results and analysis will be statistically analysed, suitably reported and used for the future, better understanding of infertility treatment in overweight/obese women. All subjects will be treated in non-identifying manner and confidentiality will remain outside the Adelaide Fertility Centre and CSIRO city clinic.

V.VII Informed consent

- Provide the patient with the patient information sheet (not included in this thesis). Explain to the patients’ the study objective, process and potential impact, as outlined in the patients information sheet.
- Eligible patient will be recruited prior to their booking on for an IVF cycle.
- Offer a counselling session with a counsellor or a consultation with a doctor.
- Obtain from the recruited patients a signed and dated consent form, which must be witnessed i.e. signed and dated by the investigator or his/her delegate.
- The obtaining of the consent should be documented in patient’s notes and copy of the patient information and consent form will be given to the patient.
- Once the consent is given, a sticky label provided in the trial documentation will be placed on patients tracking sheet for that cycle to identify the trial status of this patient.

V.VIII Withdrawal of Consent

All subjects will be entitled to withdraw without prejudice to their future treatment.

- Participants will be informed about their right to withdraw from the study at any time.
- Intention of withdrawal by the participants can be communicated verbally or in writing to any one of the following people: the study coordinator, the medical, nursing, laboratory or counselling staff in the centre.
- The identity of patients who has withdrawn will be revealed to her doctor and other staff immediately so patient may continue the standard care.
- A counselling session will be offered to participants who withdraw.
- A sticky label provided in the trial documentation will be placed on the patients tracking sheet to indicate withdrawal.

V.IX Recruitment and randomisation

a) Assignment of subject number

- A patient recruitment log (register) will be used to record patient identification.
- Patients will be assigned a number that is patient specific.
- Number will be assigned from the patient register when consent is signed.
- Dietary intervention study I.D of patients that have withdrawn will not be reissued.

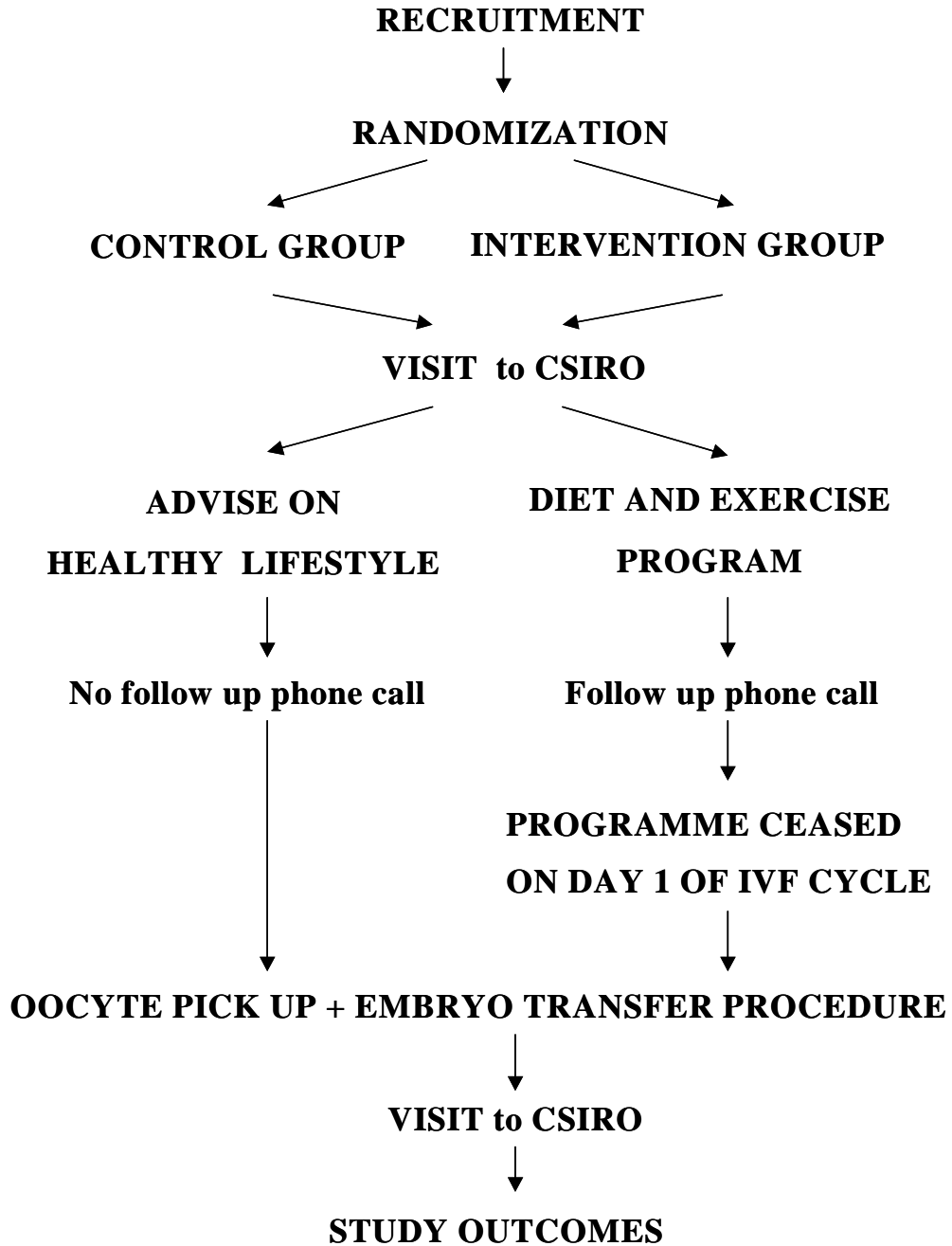
b) Method of Randomisation

- Pre-randomised envelopes will be used and stored in the unit (see page 141).

c) After consent has been signed

- Make an appointment to see dietitian at CSIRO
- Perform randomisation (Dietplus or Control group).
- Notify CSIRO office about recruited patient: RMU number and Group (Dietplus or Control) allocation of the recruited patient.

V.X Schematic study design



V.XI Study Design

Recruitment: all women fulfilling the inclusion criteria will be approached after their initial medical consultation and before the commencement of their IVF cycle. Patients will be provided with a patient information sheet, consent form and given opportunity to discuss any matters with nursing / medical staff.

1. Randomisation will be carried out by computer-generated sequence of group allocation (**Control Group** and **Dietplus Group**) after giving written informed consent.
2. Subjects will be blinded to their treatment group and this is reflected in the generality of the Information Sheet. We believe this is critical in avoiding any bias due to subject knowledge of their control or treatment status.
3. Recruits will be divided in the two groups, the Control Group and Dietplus Group.
4. The Control Group will receive Repromeds' standard information on lifestyle factors influencing fertility and this will be provided by dietitian at the CSIRO.
5. Subjects in Dietplus Group will also see the CSIRO dietitian, who will explain the dietary regime and exercise program.
6. Under the dietary regime, we assume one meal replacement with Optifast VLCD sachet (replaces lunch) and two meals from CSIRO brochure per day.
7. Subjects will be supplied with a CSIRO "The Total Wellbeing Diet " booklet, Optifast sachets as well as a brochure illustrating the exercise program at CSIRO Health Sciences and Nutrition as soon as they have consented to participate in the trial.
8. This dietary regime will provide 5500 Kilojoules (1300 Calories) and is adequate in all micro and macronutrients for this group. Follow up nutrition and exercise advice will be provided by telephone for the Dietplus group only.
9. Subjects will cease Optifast VLCD on first day of their menstrual cycle, which will commence during IVF treatment. After that day, they will continue only CSIRO TWD diet and exercise intervention until oocyte pick up procedure.

10. Dietitian, who will be trained by professional instructor, will introduce the exercise program to participants.
11. Exercise program:

Dietplus group will participate in a home-based progressive physical conditioning and walking program. Subjects in this group will be instructed to perform 8 exercises in a circuit-type resistance exercise training fashion three times per week, using rubber tubing for resistance. The progression of the exercise program (see attachment) will be as follows: week 1, 2 sets of 15 repetitions (reps) of each exercise; week 2, 2 sets of 15 reps; week 3, 3 sets of 15 reps; week 4 to conception, 3 sets of 20 reps. The exercise stops day before OPU procedure.
12. Subjects in the both groups will be provided with standard advice as well as a multivitamin/mineral supplement (Elevit) as soon as they have consented to participate in the trial.
13. Subjects in both groups will have following measurements: 1) a fasting blood sample to assess reproductive hormone profile, glucose, insulin, triglycerides, total cholesterol, HDL-cholesterol, adiponectin, ghrelin and leptin on 4 occasions and 2) weight, waist circumference and blood pressure on 5 occasions.
14. All patients will be advised to limit alcohol intake to no more than 2 standard drinks per week.
15. Women will undergo all infertility treatment procedures as normal and their treatment process will be recorded as usual practice. Records will be kept confidential, observing Repromed and NHMRC privacy guidelines.
16. Subjects will be asked to complete Dietary Questionnaire, General Health Questionnaire (GHQ -12) and Habitual Physical activity questionnaire (see attachments) on two occasions: before the commencement of study at CSIRO city clinic and before the OPU procedure.

17. Results and analysis will be statistically analysed and used for the future, better understanding of infertility treatment in overweight/obese women.

18. All patients, who will complete the study, will be offered a follow up appointment with the CSIRO dietician to discuss any lifestyle issues arising from the study.

V.XI Total number of subjects and power analysis

Based on previous work, we expect weight loss can improve the livebirth rate by increasing the response to stimulation and increased chance of pregnancy and reduced risk of spontaneous abortion following the achievement of pregnancy. The overall pregnancy rate is expected to be about 40 %. Wang et al. 2002 showed that the pregnancy rate in overweight women was half that of the pregnancy rate in women with normal weight.

In randomised study our expectation of the pregnancy rate is:

- (i) In non-dieting group 15 %
- (ii) In dieting group 40 %

Using these figures as the base, we need 120 patients (60 patient in each group) to have 80% power to detect a significant difference at 5 % level (P value). This sample size will also allow us to detect the possible changes in pregnancy rate, hormonal and metabolic changes as well.

V.XII Questionnaires

Patients in the both groups will be asked to complete a Food Frequency Questionnaire, General Health Questionnaire (GHQ -12) and Habitual Physical activity questionnaire on two occasions (before the commencement of study at CSIRO city clinic and on day of the OPU procedure). The questionnaires are not included in this work.

V.XIII Randomisation method

In the randomised controlled study, we will use the stratified randomization method. Numbers (32) will be randomised in four blocks and the derived sequence used in each group of patients stratified by age. The importance of the age stratification was considered by the specifics of IVF treatment outcomes in the women in various age groups. Patients are divided into three groups based on the age category: from 18 – 30 years old, 31 – 34 years old and 35 – 40 years old (inclusive). Each group (Control and Intervention) is equally randomised using the computer randomisation method (www.randomization.com).

Randomisation numbers or in case of this study, randomised groups (Control group and Intervention group) are placed in the numbered from 001 to 032 envelopes for each age category group and sealed. Three packs of numbered and age-marked envelopes are kept in the separate box. Each patient will be blindly randomised after the informed consent obtained in one of the two treatment groups. A sealed envelope will be opened by the Investigator in the presence of other qualified person or nominee and the patient's randomisation assured. All the details (patient's identification number, date of birth and treatment group allocation) will be double confirmed and recorded.

* * *