The Investigation of the Effect of Nasal Packing Materials on the Healing of the Nasal Mucosa of Sheep after Full Thickness Injury.

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Abstract

Rhinosinusitis is a common condition that is chronic in up to 18% of the general population. If intensive medical treatment fails, then surgery may be required. Currently the accepted form of surgery is endoscopic sinus surgery. This technique involves opening the natural ostia of the sinuses to restore aeration and mucociliary drainage.

The most frequent complication of this surgery is the development of postoperative adhesions. Their formation represents a failure of the healing process. To prevent the formation of adhesions, nasal packing is often used. To date the effect of nasal packing on the healing process has not been studied with rigorous scientific control.

The sheep has been chosen as the most suitable animal model to investigate the healing process. The sheep is suitable in terms of size, histology, physiology, and pathology. The sheep's nasal cavity is also suitable for nasal endoscopy and surgery. Research conducted previously in the sheep model has demonstrated that unpacked full-thickness wounds take longer than three months to heal. The research in this thesis sought to determine if the use of different packing materials influenced healing.

This thesis has compared the healing process that follows the use of three different nasal packing materials. These are a polyvinyl acetate based pack, a hyaluronic acid-based pack, and the hyaluronic acid-based pack with Insulin-like growth

factor-I incorporated into it. Assessment was made using light microscopy, immunofluorescence, and electron microscopy.

The results demonstrate that the use of a polyvinyl acetate sponge and a dissolvable hyaluronic acid based pack confers no significant benefit to the healing process when compared to controls. However, the incorporation of insulin-like growth factor-I into the hyaluronic acid based pack resulted in a statistically significant ($p<0.05$) improvement in re-epithelialisation at day 28 (89% for Insulin-like growth factor-I versus 44% for controls).

Attempts to assess the effect of this pack on the rate of adhesion formation was unsuccessful due to the inability to produce a replicable animal model of adhesion formation.

It is concluded that the use of Insulin-like growth factor-I in the hyaluronic acid based packs confers an important benefit to the healing process after full-thickness injury.

Statement of Originality.

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 and, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made.

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

David McIntosh

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I. Statement of Aims

I. To perform an extensive literature review on the pertinent topics related to this work. This includes the following:
   a. History of nasal disease and its treatment, including surgery.
   b. Nasal structure and function.
   c. Disorders of nasal structure and function and assessment thereof.
   d. Treatment of disorders of nasal structure and function.
   e. General aspects of wound healing and manipulation thereof.
   f. Specific aspects of wound healing of ciliated epithelium and manipulation thereof.
   g. General aspects of adhesion formation and prevention thereof.
   h. Specific aspects of adhesion formation in the nose and prevention thereof.
   i. Utilisation and efficacy of packing materials in general.
   j. Utilisation and efficacy of packing materials in the nose.

II. To develop and characterise an animal model suitable to study wound healing after endoscopic sinus surgery.
   a. Literature review.
   b. Sheep anatomy.
   c. Sheep physiology.
   d. Sheep pathology.
   e. Sheep radiology.

III. To screen for potential growth factors which stimulate respiratory epithelial growth by means of:
   a. Literature review.
   b. Cell culture.

IV. To determine the suitability of the sheep for the use of Insulin-like growth factor I.
   a. Human versus sheep IGF-I.
   b. Presence of IGF-I receptors in sheep nasal mucosa.
   c. Primary culture.

V. To develop a nasal pack containing Insulin-like growth factor I.
   a. Incorporation of IGF-I into a dissolvable nasal pack.
   b. Demonstration of the release of IGF-I from the pack.

VI. To determine the effect of nasal packing on the healing of the nasal mucosa of sheep after endoscopic surgery.
   a. Merocel.
   b. Merogel.
   c. Merogel and IGF-I.

VII. To determine the effect of nasal packing on the development of adhesions in sheep after endoscopic surgery.
   a. Development of an adhesion model.

II. Historical Overview

The study of medicine provides great insight into the progress of knowledge and skills that have culminated into the modern day techniques that we have today. Through studying different civilisations and the interplay between religion, science, and chance observations, it becomes appreciable how pioneers of yesteryear have advanced the cause to achieve remarkable leaps forward in the fields of general and specialised medicine and surgery.

Ancient Egyptian writings provide information on the practices of thousands of years ago [1,2]. In records that have been dated to around 1550 BC, there is a description of remedies, spells, and incantations implemented in attempts to heal the sick. The Egyptian records would suggest that rhinology is possibly the oldest of specialties with evidence of relevant practices dating back to 3500 BC [2]. In an important Egyptian record, the Edwin Smith Papyrus, there is a description of how to treat a fractured nose, including instructions for the nose to be "packed with strips of linen saturated with grease and honey" [2]. As part of the mummification process, the brain is removed through the nose and sawdust is instilled in the empty skull [3]. Historically, this was probably the first "procedure" to be done via the endonasal route.

The contributions of the ancient Greek medical practitioner, Hippocrates are significant. His application of scientific method and logical reasoning has contributed to rhinology with a description of a method of treatment for nasal polyps [2]. This technique involved tying a piece of string to some sponges. The string

was then pushed back through the nose by a probe into the pharynx. Then the string is pulled out through the mouth, pulling the sponge through the nose, hopefully also with the polyps [2]. The records of ancient Hebrew society include a reference to the diagnosis of nasal polyps, which showed themselves by a 'bad smell of the nose' [2].

The use of herbs and plant extracts in the treatment of ailments is renown to have been developed by the practitioners of Chinese medicine. Their traditional medicine dates back to at least 2700 BC. Their contribution to the speciality of rhinology is identifiable in the use of a particular herb, known as Ma huang, which contains the alkaloid substance ephedrine [4]. Ephedrine is still used in many of the nasal decongestant preparations available today.

In the twelfth century, a surgeon called Ruggiero Frugardi published the work titled "Chirurgia magistri Roderi" [5]. This significant piece of literature formed the basis for education in surgery through out Europe. The authors had reached the conclusion that wounds could not heal properly without the formation of pus. In the later part of the twelfth century, a disciple of Frugardi, Theoderic, challenged the belief regarding the role of pus in healing and stated that it was not necessary for pus to occur for wounds to heal, and in fact, that it prolonged illness and hindered healing [1]. Theodoric was a historical pioneer of antisepsis in the advancement of surgery [1].

In 1316, the first anatomical textbook [2] was produced but it was the artistic skill and curiosity of Leonardo da Vinci in the 1400's [6] that is still famous today. His works feature in many medical texts as a graphical representation of historical medical

achievements. He was the first to depict the maxillary and frontal sinuses, as well as the heart, and ventricles of the brain [2].

Throughout history various observers have attributed many functions to these paranasal sinuses, including being the reservoir of grease for eyeball lubrication, and to allow a means for the brain to drain its evil spirits [7]. In the 1500's, Andreas Vesalius, the historical father of anatomy, further described the maxillary, frontal and sphenoid sinuses, and stated that he believed they contained air [2].

Before the middle ages, Galen explained nasal catarrh as a purging of the brain [2]. Such beliefs were held until they were corrected in 1660 when Conrad Victor Scheider [2,6] published a work on catarrh in which he stated that it was not from the brain but from the mucosal membranes. Furthermore he said that the production of nasal fluid was a normal process and that it was not until it was produced in excess that it became obvious as catarrh.

Advances that were made in the field of rhinology in the 1600's to the 1800's were driven by a desire to drain pus from the sinuses, and in particular, the maxillary sinus [3]. The first important observation was in 1651, when it was noted that objects could be passed into the maxillary antrum after extraction of an infected upper tooth [4]. In 1675 a trephine was used to enter this sinus [4]. In 1677, Johannes Ham discovered cilia [8,9] the surface projections of cells that are the machinery behind the movement of surface mucus. In the 1700's William Cowper noted that medications instilled in the maxillary sinus through the upper molar alveolus came out into the nasal cavity [10].

Also in the 1700's, Jourdain attempted to cure maxillary sinus suppuration by irrigation of this sinus via its natural ostium [6] and Lamorier described an alternative approach through the canine fossa [3]. In 1886 Mikulicz-Radecki opened the maxillary sinus via the inferior nasal meatus using a trocar which Krause modified into a cannula to allow irrigation of the sinus via the same puncture wound [4]. It was much later (1912) that Siebemann developed a technique for draining the maxillary sinus via the middle meatus [10,11].

A landmark technique of entering the maxillary sinus via an anterior approach, and then removing the sinus mucosa and forming a window in the inferior nasal meatus became standard practice for many decades. It was described independently by Caldwell in 1893 and Luc in 1897 [6,12]. This approach is quite aggressive as it involves the removal of considerable amounts of the lining mucosa. In the early 1920's moves towards more conservative surgery with less tissue removal were being advocated [4]. An important reason for advocating such a change was that researchers were demonstrating that removal of large areas of mucosa resulted in scar formation, which subsequently interfered with sinus drainage [13]. Such an outcome is obviously counterproductive to the intention of the original treatment. However, several decades passed before conservative methods of sinus surgery were actually described.

The history of the surgical management of frontal sinus disease is also interesting. Ogston reported trephination of the frontal sinus in 1884 [14]. Intranasal procedures were attempted in the early 1900s but were abandoned due to a high mortality rate from the blind procedures described [14]. This led to a focus on surgical modalities

that were external in their approach. This includes external frontoethmoidectomy procedures developed by Knapp in 1908, and an external ethmoidectomy combined with resection of the frontal sinus floor described by Lothrop in 1914 [14]. In 1921 Lynch and Howarth described an external approach via a medial periorbital incision, which became popularised and modified by many different surgeons for several decades [14]. This was despite the high failure rate [14]. In the 1960s osteoplastic flap procedures became popularised as an alternative the modified versions of the Lynch-Howarth approach [14]. Obliteration of the frontal sinus with either fat or other material was common practice when this approach was used [14]. There were similar problems of high failure rates with this approach. However, it was not until endoscopic procedures became popularised, that alternative and more conservative surgical interventions could be offered.

The history of endoscopic surgery starts with its use in fields outside the aerodigestive tract, such as the urinary bladder. In 1901 Hirschmann used a cystoscope to examine the nasal sinuses [4,15]. He did this by passing the cystoscope through both an inferior meatal antrostomy and via a canine fossa puncture.

It was not until 1954, however, when Hopkins invented a fibreoptic solid rod lens system that further advances in nasal endoscopy occurred [4]. With improved visualisation and development of surgical tools and techniques, a new approach evolved in the surgical treatment of nasal disease. A pioneer in the new surgical techniques was Messerklinger who first described them in 1967 [4]. In the 1980s Stammberger [16] and Kennedy [17] advocated Messerklinger's theories on functional endoscopic sinus surgery (FESS) in which they endorsed opening the natural ostia

of sinuses to promote a cure [18]. The outcome of this was a shift towards more meticulous surgical treatment with a reduced amount of surgical trauma [19]. This was possible because the improved visualisation of the surgical field has allowed more precise management of disorders of the nasal and paranasal mucosa. Despite the advent of these more meticulous techniques, the assessment of the wound healing process has been mostly overlooked.

III. Nasal Structure and Function

Nasal gross anatomy

The paranasal sinuses are four paired pneumatized and ventilated spaces located within the facial and skull bones [20]. They are the maxillary, ethmoid complex, frontal, and sphenoid. These are lined by pseudostratified columnar ciliated (respiratory) epithelium. The paranasal sinuses are in continuity with the nasal cavity. There are recognised variations of normal with respect to their shape and size. Some of the anatomical configurations of the sinuses predispose towards obstruction of the sinus ostia and the development of chronic sinusitis [21,22].

Respiratory mucosa

The upper respiratory tract is lined by pseudostratified columnar ciliated epithelium [23,24], which transports a mucous blanket [23]. This functional epithelium is found lining the nose and paranasal sinuses [25]. The mucus traps inhaled particles and the ciliary mechanism removes them from the nasal cavity and sinuses [24], This protects the airway from injury against inhaled substances, microbiological colonisation, and infection [23,26]. In addition, sneezing due to stimulation of sensory nerves [24,27] and coughing aids this defence mechanism [28].

Apart from the ciliated cells of the nasal respiratory epithelium, other cell types have been described [29]. These include basal cells, intermediate cells, goblet cells, seromucinous gland cells, and lymphocytes [30]. The goblet cells and seromucinous gland cells are important in the production of mucus [30,31]. The composition of the mucus produced by these cells is mostly water (ninety eight percent) with albumin,

glycoproteins, globulins, and salts composing the remaining two percent [28,32-35]. Changes in this composition can affect ciliary function [23,24,28,36]. The presence of immunoglobulins in the mucus provides an extra defence mechanism for the airways [37], as do a variety of proteolytic intranasal enzymes [38].

Cilia

Cilia are the mobile surface organelles of the respiratory epithelium that confer upon it the ability to move mucus along its surface [28]. Approximately 80% of the airway epithelial cells are of the ciliated type [39]. Within the human, light microscopy has demonstrated the presence of cilia in the paranasal sinuses, the nasopharynx and larynx, the lower airways, the efferent ductules in men, and the fallopian tubes and adjacent uterus in women [31].

The structure of cilia

The ultrastructure of cilia has been studied in great detail. The transmission electron microscope has allowed the characterisation of the normal ultrastructural pattern of cilia [31,40-43]. Between fifty to three hundred cilia can reputedly be found on the surface of each appropriate cell [28-30,34]. These cilia are protrusions of the cell membrane [28] that possess a surrounding cell membrane layer [28,44,45]. Their length is measured at about 3-8 micrometres [28], with the average length changing from about 6 micrometres in larger airways, down to 5 in smaller bronchioles [34,46]. It is estimated that their diameter is between 0.1 to 0.33 micrometers [28,47]. At their tips there are 3-7 short 'claws' which are about 25-35 nanometres long [29,34,48] and are thought to be important in propelling the mucus along. The root of each cilium lies

in the cell and provides anchorage for this organelle [34]. Images depicting these features in sheep cilia are presented in chapter 15 (Figure 15-12 and Figure 15-13).

Ciliary movement

The movement of mucus has been observed with the use of dyes [8], and various pathways and the speed, or clearance rates of mucus have been recorded [49-52]. The rate of mucus flow is now termed the mucus, or mucociliary, clearance rate. With modifications to techniques that increased sensitivity and the use of technological advances, including lasers [53,54] the actual rate that cilia move back and forth can be counted. This has become known as the ciliary beat frequency.

Many investigators have examined the influence of various factors on respiratory mucosa and ciliary function [8,15,50-52,55-63]. These include environmental factors such as temperature and humidity, the effects of radiation, the effects of cigarette smoke, and the effects of drugs on ciliary activity. The measurement of such activity has been via the recording of ciliary beat frequency or mucus clearance rates. Many of the findings from this research are relevant to the investigative techniques used today.

Mucus

For descriptive purposes, the protective mucus blanket is considered to be in two layers- a lower sol layer with low viscosity and an upper gel layer of greater viscosity [64]. The goblet cells of the respiratory mucosa produce a protein-rich secretion of complex carbohydrates [65]. The cells of the respiratory mucosa that bear

microvilli regulate the serous content of the mucus, and thus its viscosity [65]. The pH is usually neutral or slightly alkaline [58].

The cilia lie within the lower sol layer of the mucus, with their tips passing through the upper layer [60,64]. Hence, this lower layer is also referred to as the peri-ciliary layer [28,66]. Maintenance of the sol phase is vital for normal ciliary function [65]. Hence, either a decrease in microvillous cells, which would decrease the aqueous content of the sol phase, or an increase in the goblet cells or seromucinous gland cells, which would increase the thickness of the mucus layer would adversely affect mucociliary clearance [65]. As discussed later, chronic sinusitis is associated with a loss of cilia and increase in goblet cells [65]. Together, these factors impair the effectiveness of the mucociliary transport [65]. Furthermore, inhibition of the system results in longer contact times of the mucosa with entrapped viruses and bacteria, possibly leading to repeated infection of the epithelium [64]. This is the basis for the viscous cycle theory of chronic rhinosinusitis [65].

Ciliary Beat Frequency

This is a measurement of the number of times cilia move in a defined period of time. With modern methods, the ciliary beat frequency has been reported to be between 800-1000 beats per minute [24,29,67,68]. It is important to maintain temperature, pH, and osmolality [24] because they have an affect on the ciliary beat frequency. Other factors known to affect the frequency of ciliary beating include adenosine triphosphate concentration, dynein concentration, calcium concentration, and magnesium concentration [24,69]. The viscosity of the medium that the cilia are beating in also influences the ciliary beat frequency [69]. Finally pharmacological

agents can affect CBF, with phenylephrine [70] and dexamethasone [71] both examples of drugs that decrease CBF. This is only a transient effect [72].

Measurement of CBF has been utilized in many pathological processes, including sinusitis [73]. There have been studies on the relationship between disorders of ciliary ultrastructure and CBF. In a rabbit sinusitis model, it was determined that when ultrastructural abnormalities affected 10% of the cilia, CBF decreased [73]. However, it has been demonstrated that even when cilia are normal, the number of total cilia present also affects CBF [74]. In general, the greater the number of ciliated cells present, the better the CBF [74].

Mucociliary Clearance

The outcome of ciliary movement is the transfer of energy from the cilia to the mucus such that the mucus is propelled along [58]. There have been attempts to determine if a relationship exists between mucociliary clearance (MCC) rates and CBF. One study demonstrated a significant correlation between CBF and MCC in normal volunteers [75]. However, there is an important distinction between CBF and MCC. The difference is that CBF is a functional measurement of individual cilia while MCC is a product of all functional cilia [32]. The sum total of the mechanical energy transferred from the beatings of cilia to the mucus is not only the movement of mucus, but the promotion of flow along certain pathways [31,49,76].

IV. Pathology of Nasal Mucosa

The presence of normal functioning cilia is crucial. The classification of the abnormalities of ciliary ultrastructural abnormalities is into congenital (primary) and acquired (secondary). The primary ciliary defects are hereditary, whilst the secondary defects are caused by adverse stimuli such as infection, inflammation, and trauma [31]. The important distinction between the two is that secondary defects are reversible whilst primary changes are not. Chronic rhinosinusitis may result from both primary and secondary ciliary defects. It may also result in secondary ciliary defects.

Chronic rhinosinusitis

Rhinosinusitis implies that there is pathological inflammation of the lining mucosa of the nasal cavity and paranasal sinuses [20]. The aetiology of chronic sinusitis is individual for each patient but is influenced by anatomy and its variants [22], immune dysregulation including immune compromise and allergy [77], infectious processes including viral, bacterial, and fungal agents, congenital ciliary disorders such as Kartagener's syndrome, and mucus disorders such as cystic fibrosis. The disease process is divided into acute (less than 2 weeks), subacute (2 weeks to 3 months) and chronic (greater than 3 months) [78,79], depending upon the duration of symptoms. The symptoms of rhinosinusitis include nasal obstruction, rhinorrhea, postnasal discharge, headache, and facial pains or pressure [78]. Acute episodes may result in systemic symptoms and febrile episodes. The development of a chronic condition is probably related to the inability of the mucosa and immune system to adequately respond to the inciting pathogen. Population interview surveys, have reported that sinusitis is the most frequently reported chronic disease [20]. In a survey study in the

United States in 1997, 63% of the population had consulted their family doctor with symptoms consistent with rhinosinusitis [80]. The economic cost of this was estimated to represent 0.6% of the total budget for consultations (equivalent to 1 of every 160 consultations) and approximately 3% of the total medication costs [80].

The pathological features of chronic rhinosinusitis includes the persistent loss of cilia [23], with each episode of sinusitis resulting in a 10% decrease in the ciliated cell population, epithelial desquamation, squamous metaplasia with increased goblet cells, basement membrane thickening, submucosal oedema, inflammatory cell infiltration, and hypertrophy of submucosal glands [81]. Furthermore, the cilia that regenerate in the chronic condition, demonstrate secondary ultrastructure changes [23]. Hence the potential result after each episode of rhinosinusitis is a reduction in the total number of cilia, with a lesser number of these being functional cilia. This may result in bacterial colonisation with possible further epithelial damage. This can become a very vicious cycle with progressive mucosal damage and chronic symptoms.

The chronic mucosal disease obstructs sinus drainage and impairs mucociliary transport [78]. This is most notable in maxillary sinusitis where the region within the middle meatus, referred to as the ostiomeatal complex, becomes obstructed [82].

The aim of any treatment is to relieve symptoms and prevent recurrence [83,84]. The first line of therapy for this condition is usually medication [83]. The medical therapies used are intended to address the inflammatory process and reduce mucosal oedema, facilitating ostial patency and allowing the drainage of sinus secretions [84]. The

medical therapies include topical and systemic steroid preparations, topical saline preparations, anti-histamines, mast cell stabilisers, and leukotriene inhibitors.

Surgery

Medical therapy for chronic rhinosinusitis is aimed at dealing with infectious agents, inflammatory and allergic processes, and restoration of mucus rheology. By addressing these factors, disease resolution is often achieved. However, if this should fail, surgical treatment is often offered to the patient.

Surgery, itself, results in tissue injury above that already caused by the processes described above. The wounds that result vary in size and thickness for each individual. This section introduces the selected reported research on mucosal injury due to physical trauma. The specifics of surgical techniques are reviewed in the background discussion on surgery.

The most important pioneer in the field of research into the effect of surgical interference of the epithelium of the sinuses was Hilding [85-87]. Amongst his findings was the characterisation of the normal mucociliary pathways of the sinuses and demonstration that removal of bands of epithelium results in scar formation that interferes with mucociliary transport [85].

More recently, studies into the effect of removal of nasal and sinus mucosa demonstrate that the mucosa is able to regenerate [88]. However, in this trial it was noted that this mucosa did not seem to be functional because MCC was absent. The author raises the concern that removing the mucosa may replace one pathological

process with another. In a different study of human patients treated surgically, the investigator demonstrated that MCC at 3 weeks post-operatively had not improved [89]. This author suggested that the healing process of nasal and sinus epithelium after surgery can take weeks to months but made no other suggestion as to why the MCC had not improved. This author also believed that minimising mucosal trauma was important, given this apparent lack of function following the removal of significant amounts of mucosa.

V. Wound Healing

After injury, a series of events are set into motion to repair the defect created. These processes include cell mobilisation, new cell regeneration, and the formation of fibrous connective tissue [37,90-93]. These processes are complex and carefully orchestrated events that involve sequential alterations in extracellular matrix proteins, release of growth factors, and secretion of migration-stimulating cytokines [90,94,95]. The outcome of these processes may be either replacement of injured tissue with newly regenerated cells or with scar tissue formation [96,97]. The balance between these two is the result of the interaction between intrinsic ability of the injured tissue to be replicated, the rate of the cell proliferation and migration of regenerated tissue, and cell-matrix interactions. Importantly, the presence of a basement membrane is essential for cell migration, proliferation, and polarity. The processes involved in wound healing are pivotal to this thesis. Hence, the relevant aspects related to the control mechanisms involved in cell growth and differentiation, as well as the formation of fibrous connective tissue will be discussed.

The repair of the nasal mucosa after injury has not been extensively researched. Hence, an overview of the wound healing process based upon knowledge of dermis and lower respiratory mucosa is provided. Whilst the same type of epithelium lines the nasal cavity, sinuses, and respiratory system conduits, there are some important differences. The most significant of these is that whilst some of the epithelium of the nasal cavity lies on a bed of soft tissue and cartilage, as does the respiratory conduits [98], the remainder of the nasal and all of the sinus epithelium lies on bone. The relevance of this is that the underling vasculature is different, and hence, the potential source of a provisional matrix, infiltrative cells, and ongoing nutrition may

be less in these areas. Where relevant details pertaining specifically to nasal mucosa have been elucidated, they will be highlighted.

Wound healing can be divided into different stages [96]. In the adult, the first of these stages is haemostasis [93,99]. When an injury to a tissue occurs, it tends to involve the local vasculature system. There are protective mechanisms that are activated to reduce the amount of blood loss [99]. These include vasoconstriction, platelet aggregation, and blood coagulation [99]. This results in not only haemostasis but also the deposition of a fibrin rich matrix [93]. The newly formed blood clot is a temporary protective barrier. It consists of a variety of blood-derived molecules and cells, but fibrin and platelets are the prominent constituents. This clot undergoes a transformation over the following days. This process is common to dermis and nasal epithelium.

The next stage in wound healing is known as inflammation [93,96,99-101]. Inflammation involves a cellular response that begins with the infiltration firstly of polymorphonuclear neutrophils, and is then followed by the appearance of macrophages and lymphocytes [96,99,102]. These cells are responsible for defending against bacteria and other injurious agents as well as in the secretion of numerous growth factors, cytokines, and extracellular matrix components [99]. The macrophages in wounds are derived from circulating monocytes that have been attracted to the wound [96]. Macrophages are crucial cells involved in the coordination of wound repair [99]. Their purpose at this stage is to neutralise and remove the injurious agent, remove dead cells, and to lay down a connective tissue framework to promote cell and tissue regeneration. Cell and tissue regeneration is known as the proliferative

phase [96]. The events currently described occur within the first few weeks after injury.

The proliferative phase involves the multiplication of fibroblasts [100,101], endothelial cells, and epithelial cells to regenerate new tissue [99]. This is associated with replacement of the original proteoglycan rich fibrin matrix with collagen [99,101]. With the establishment of new tissue, the next stage is remodelling [96,100], otherwise known as maturation [93]. This involves alterations in the architecture of the newly generated tissue so that it resembles the native state. Furthermore, the collagens present in the wound become cross-linked to form a scar [99].

The initial matrix of fibrin and fibronectin, which is derived from the haemostatic process and the macrophages [96], is replaced with a matrix consisting mostly of proteoglycans and glycosaminoglycans, which is then followed by a matrix mostly composed of collagen [93]. Collagens are glycoproteins and are derived from fibroblasts [96]. In all there are at least 18 types of collagen, which are divided into 4 families [96]. The important matrix proteins synthesised and secreted during wound healing are types I, III, and IV [96,97]. Type I and III belong to the group of fibrillar collagens with uninterrupted triple helices and provide tensile strength to wounds [96]. Type IV is an important component of the basement membrane [96]. The contribution to this collagen matrix by the different classes of collagens also changes with time. In normal, intact dermis, there is predominantly collagen I (80 to 90%) and III (10 to 20%) [93]. However, in the early stages of tissue healing, collagen III is laid down first in quantities greater than normally present (30%) [103]. As remodelling occurs, this level decreases to between 5 [104] and 10% in a mature scar [93] and at the same

time, type I levels increase [96,97,103]. When there is an abundant collagen matrix, synthesis and secretion stop, and fibroblasts change into myofibroblasts, which are able to contract the wound [96]. The formation of the components of the matrix by fibroblasts is known as fibroplasia [96]. These synthesised proteins are assembled to form either basement membrane or interstitial matrix.

An important group of extra-cellular matrix proteins found in the healing wound are the glycoproteins and proteoglycans. The glycosaminoglycans play a vital role by facilitating entry of cells to the injured area and contributing to the orientation of the fibrous component of extracellular matrix [100]. An important property of glycosaminoglycans is that they are extremely hydrophilic and exist in a highly hydrated state [96]. This facilitates easy penetration of cells [96]. An important glycosaminoglycan is hyaluronic acid [96]. Most cells, including fibroblasts [103], produce hyaluronic acid [96]. It consists of repeating n-acetyl glucosamine-glucuronic acid disaccharides [96]. Just as cells have integrin receptors for fibronectin, so too, they possess receptors for hyaluronic acid [103]. There are also interactions between it and other matrix molecules [103]. The importance of hyaluronic acid in wound healing is evident from studies of foetal wounds. Early gestational foetuses heal without scarring. In these wounds, hyaluronic acid is in abundance.

There are extrinsic agents that may influence cell growth, they are simply known as 'growth factors' and belong to the general class of cell signalling agents called cytokines. They are derived from cells and are released into the extracellular environment. They are recognised by the target cell by receptors [97]. The nature of the interaction between the source and target cell is categorised in relation to the

distance that the signalling agent has travelled. There are three schemes: (1) autocrine, where there is an interaction between mediator and the cell that has produced it; (2) paracrine, where there is close proximity of the source and target cells; and (3) endocrine, where a significant distance between the source and target cell exists [90].

Apart from interacting with cells, the matrix proteins and cytokines also interact with each other [97,105]. This includes: (1) the binding of growth factors to extracellular matrix proteins; (2) growth factors influencing the production of extracellular matrix proteins and their receptors; (3) extracellular matrix molecules influencing growth factor production or receptor expression [105]. The extracellular matrix molecules themselves may be mitogenic or influence response of cells to growth factors [105]. The binding of growth factors to extracellular matrix proteins influences the bioavailability of growth factors but also protects them from degradation; this results in a local store of growth factors [97,105].

Healing of respiratory mucosa

The processes or stages of the repair of respiratory epithelium are similar to skin in that there is proliferation, migration, and differentiation [37,97,106-109]. The reparative fibrotic sequence of the healing of the underlying lamina propria is also comparable in respiratory and cutaneous epithelia [106]. Also akin to skin, intercellular junctional complexes between the epithelial cells need to be re-established to form a barrier against the diffusion of exogenous injurious agents [107,110]. These stages of healing of respiratory epithelium of humans are not well understood [107]. The time course taken for these events to transpire is substantially longer than that described for skin

healing [97]. For example, it has been suggested that they may continue for up to six months after sinus surgery [97]. Others have indicated an even longer time course than this [111]. The following will present the information currently available on these stages of healing.

Different healing responses occur in response to traumatic insult to the nasal mucosa [37]. For example, the respiratory mucosal wounds caused by viral and bacterial infection, leads to the loss and destruction of ciliated cells and cilia [112] with the greatest evidence of injury in the first week of viral infections [113]. Within the first few days after this injury, there is evidence of the beginning of healing [86] and for viral infections the epithelium appears normal by 10 weeks [113,114]. This is in contrast to bacterial infections, however, which lead to a reduction in ciliated cell numbers, which is marked, and larger compared to that of viral injury [112]. The reasons for these differences are unknown but may possibly be related to differences in local interferon and IgA production [37], as well as the differences in the finer details of the inflammatory response to these different pathogens. Whilst the epithelium is able to eventually recover, the newly regenerated epithelium may not necessary be normal, particularly if the process causing epithelial damage is persistent or chronic.

Proliferation

The first premise of wound healing by regeneration is that there is a source of new cells. There is almost constant turnover of the nasal mucosa cells [30], which implies that there must be a source for new cells. Attempts to gauge the normal duration of the life cycle of cells of the nasal mucosa is complicated by unavoidable factors

such as noxious agents in the inhaled air [37], the trauma from nose picking, and infections. This is because all of these events increase the rate of normal epithelial loss [37]. This probably accounts for the disparity of quoted figures for the normal cell cycle, with estimations starting at seven and a half [37] to fourteen days [115], and go up to four to eight weeks [47,116].

An important source of replacement of lost cells is probably from the basal layer of cells of the nasal epithelium [37,47,97,117-119]. This is supported by a study of tracheal cells, where the basal cells were isolated and then grafted onto an area of the trachea that had been denuded of epithelium [120]. After one week, the cell coverage was poor but then improved between the second to fourth weeks [120]. Furthermore, there was evidence of ciliary differentiation of these new cells at this stage [120]. In trying to further clarify the source of new cells, specifically in the case of trauma, a study has demonstrated, using a culture of human nasal mucosa, that the cells nearest to a wound showed both the highest mitotic and migration activity [109].

After wounding, the extent or depth of the injury is important [97]. The reason for this is that the presence of cells able to replicate is a prerequisite for wound healing to successfully occur. However, given that stem cells are yet to be properly identified, it is unknown how widely dispersed amongst the mucosa they are and if they reside in particular areas more so than others. Given that some of these are potentially removed in full thickness mucosa injuries, it seems probable that in those areas, healing will be compromised. A significant difference between the rates of re-epithelialisation of full and partial thickness nasal mucosal injuries has been observed [121]. The importance of the maintenance of the basement membrane is

highlighted by the fact that if the basement membrane is intact, the epithelium regains its normal height in a few days [97]. However, if the basement membrane is injured, the restitution of the epithelium takes several weeks with the formation of a squamous or 'transitional' epithelium [97,122]. Also, once the wound is covered by an epithelium, the cells tend to be one cell thick in the middle and several cells thick at edges [37]. These features suggest that measuring the epithelial thickness may be an additional indicator of epithelial restitution.

Migration

Whilst the importance of proliferation has been highlighted above, it is cell migration that is usually the first process that occurs in an effort to re-establish an epithelial covering [97]. In nasal mucosa, it commences with the first few hours of injury [37,123] and occurs at an estimated rate of 4μm/hour [124]. The wounds heal concentrically [97].

Differentiation and Ciliogenesis

The outcome of proliferation and migration of the respiratory epithelium should be closure of the wound. However, this epithelium has a functional active role as well as being a protective barrier. To be able to achieve appropriate mucociliary function, the new cells must generate cilia. This specialisation of the cells is known as differentiation. The formation of new cilia is referred to as ciliogenesis [125].

The stages of the ciliogenesis process have been recorded by electron microscopy [126]. The evidence from this study is suggestive of organisation of components of cilia and the formation of cell membrane blebs as precursor events to the cilia

projecting from the cell surface. The stimuli for the promotion of cilia formation remain elusive.

VI. Adhesions

Adhesions are fibrotic connections between tissue surfaces [127]. The need to reduce adhesion formation in the nose is an important clinical problem. Despite the common nature of nasal adhesions, most of the scientific investigation on adhesion formation has been related to the abdomen and pelvis. This is because intra-abdominal adhesions result in significant morbidity such as abdominal pain and intestinal obstruction [128], while intra-pelvic adhesions are associated with infertility. Prevention of adhesion formation is important because there is evidence of adhesion recurrence in the abdomen and pelvis after adhesionolysis [129,130]. There are similarities in the predisposing and aetiological factors in adhesion formation in the abdomen and pelvis and the intranasal cavity. Hence, research findings in the abdomen and pelvis should be applicable to intranasal adhesion formation.

The pathogenesis of adhesion formation is as simple as it is complex. The basic principles involved in their aetiology have been identified but the cellular and molecular biological events that are involved are still not completely understood. The chronology of adhesion formation can be summarised as tissue injury, resulting in bleeding and inflammation [131], which leads to the deposition of fibrin monomers [131] that polymerise into a fibrin gel matrix, which is not removed due to inadequate fibrinolysis, allowing fibroblast invasion and proliferation resulting in local collagen synthesis [132]. The formation of adhesions seems to be quick with studies showing the presence of adhesions at 5 days after injury [132]. While these series of events have been studied in the abdomen and not the nose and sinuses, the sequence should be similar. An understanding of the above stages has resulted in different attempts to

interrupt or manipulate the processes involved in adhesion formation in the abdomen [132]. This should also be equally applicable in the prevention of intranasal adhesions.

Wounding

Tissue injury is a prerequisite for adhesions to form. It is important that the wounded tissues are in apposition for fibrin bridges to form between them [128,133]. After injury, the structures can adhere to each other within 3 hours [134]. Hence, the abdomino-pelvic cavity would seem to be designed in such a way that even meticulous technique is not enough to prevent adhesions [129].

In the nasal cavity, the close anatomical relationship of prominent structures to each other and the walls of the nasal cavity are evident during endoscopic assessment [135]. This is especially so for the relationship between the middle turbinate and the lateral nasal wall [135]. It is also important to note the relationship of one side of an ostium to the other side of the same ostia. Particularly during antrostomy, there may be circumferential injury to the lining of sinus ostia, and hence, these locations are also at possible risk of adhesion formation. In the paediatric population, the incidence of adhesions may be higher, and this is thought to be related to the closer relationship of the intranasal structures [136].

Bleeding

The presence of blood between two injured surfaces is a prerequisite for adhesion formation [128]. This is because fibrin is derived from the blood that extravasates from a wound. The development of fibrin polymer bridges requires the initial presence of

fibrin monomers. Fibrin is present in the circulating blood and bleeding at a local site will result in extravasation of fibrin into wounded areas [129,131]. Fibrin is also present in exudates of tissue affected by inflammation due to various pathologies other than surgical wounding [137]. The role of fibrin in the circulating blood is to act as a haemostatic agent at sites of blood vessel wall injury. Fibrinogen is converted to fibrin through the action of specific enzymes. In particular is the action of thrombin [128], which itself circulates in an inactive form known as thrombinogen. Hence, the minimisation of trauma during any procedure is highly emphasized, as less bleeding should then occur. In nasal surgery, the use of decongestant sprays pre-operatively, as well as vasoconstrictor agents such as cocaine and adrenaline are important to reduce blood loss.

Since blood loss is almost unavoidable, the next important principle is to ensure adequate haemostasis at the end of the procedure performed as well as removing excess blood clot from the surgical field. Unfortunately, even these measures would not seem to be enough to achieve the absolute avoidance of adhesion formation. For this reason, packing materials have been researched [128,133]. Packing the surgical field is thought to achieve two important objectives- the first is to promote haemostasis, and the second is to act as a physical barrier between two injured surfaces [128]. An extra potential benefit of some types of packing is that they may promote healing of the wounds.

Inflammation

If tissue damage is minimised, then the inflammatory response should also be reduced. If haemostasis and removal of excess clot from wounded areas is also

achieved, then a significant proportion of the circulation-derived factors within the blood clot that promote inflammation will also be removed. There will always be some form of inflammatory event and for this reason, various therapies have been trialled that target the mediators and pathways of inflammation. For example, a study using the non-steroidal anti-inflammatory drug (NSAID), indomethacin, showed a reduction in the rate of adhesion formation [132]. The use of these medications in nasal surgery is probably limited by the fact that a proportion of patients having surgery for nasal polyposis may be sensitive to NSAIDs and related compounds.

Despite this haemostatic system, fibrin will be deposited in wounds before haemostasis is achieved because this action is not immediate. Hence, blood will make its way into wounded areas before damaged blood vessels can be sealed. With fibrin present within the wounds, the monomers may polymerise and form stable matrix structures [128,129]. This matrix is referred to as the fibrin gel matrix [128]. The process of formation of these fibrin matrices also involves an interaction with fibronectin and a series of amino acids [128]. Cells and debris can then become trapped within the sticky gel [128]. The process of fibrin exudation from a wound continues for several days following the injury [128]. The value of the formation of the fibrin gel matrix in wound healing has been discussed. The interposition of this gel between two mucosal surfaces is the basis for future adhesion formation if the matrix is not removed [128].

In the abdomen and pelvis, attempts to reduce adhesion formation have included the utilisation of physical barrier packing agents. This packing may be either a gel [138] or

liquid [139] that coats the surface of the abdominal contents, or a solid packing material that dissolves. Successful reduction in adhesion formation in the abdomino-pelvic cavity has been demonstrated using a gel that consists of modified hyaluronic acid combined with carboxymethylcellulose [138]. With regards to dissolvable packing, chemically cross-linked hyaluronic acid when interposed between surgically handled tissues also reduces adhesions [140,141]. These packs have also been used as vehicles to deliver pharmacological agents to wounds to either promote wound healing and/or fibrinolysis [142].

The use of packing materials is not without concerns. These include a potential of increased infection [143] and an allergic inflammatory response to the packing material [144]. This inflammatory response comes about because packing materials are foreign materials. This problem has been reported with Seprafilm [144], which is made of chemically modified hyaluronic acid and carboxymethylcellulose (CMC).

Fibrin degradation

The process of fibrin deposition, discussed above, is balanced and usually countered by the process of fibrinolysis. This is clearly an important mechanism because it is necessary for clots to be removed from blood vessels once they have served their purpose of haemostasis and from wounds once they have aided cell migration and healing. Aberrant clotting of vessels without degradation of this clot is the basis of certain diseases such as coronary artery disease and cerebrovascular disease. In the case of fibrin within wounds, if it involves two distinct surfaces, then adhesions may form if the fibrin polymerises between these surfaces [128]. The main mechanism

by which the fibrin gel matrix is broken down is by the action of the proteolytic enzyme plasmin [131,145]. This enzyme is derived from plasminogen [131,145]. The catalyst for the conversion of the inactive plasminogen to the active plasmin is another group of enzymes called plasminogen activators [145] [131,142]. The presence of either plasminogen activator inhibitor I or II favours adhesion formation [131,146]. In the abdomen, adhesion formation is favoured by two events related solely to the processes involved in fibrin degradation- a reduction in fibrinolytic enzymes and an increase in inhibitors of enzymes that promote the activation of fibrinolytic enzymes [131]. The effects of this are exacerbated by the fact that inflammation of the peritoneum results in an outpouring of fibrin [134]. It is important to realise that despite the potential deficiencies of the fibrinolytic system, it is in fact very capable with the majority of adhesions being transient [134].

Collagen synthesis

Collagen is present in healing wounds as well as mature adhesions. In fact it is the presence of collagen within an adhesion that designates it as being mature [131,134,137]. The reason for this is that the presence of collagen gives great strength to adhesion bands. Following the removal of fibrin by the invading granulation tissue, fibroblasts activity results in the production of collagen bundles [129]. By as early as day 5, these bundles became organised [129]. With further maturing time, these bundles eventually become a strong fibrous band [129].

VII. Hyaluronic Acid

Hyaluronic acid is a connective tissue glycoaminoglycan [147] that is made by fibroblasts and other cells [148]. It is composed of repeating disaccharide units arranged in a linear polymer [148-150]. Hyaluronic acid production is stimulated by epidermal growth factor (EGF), platelet derived growth factor (PDGF), tissue growth factor beta (TGF-β), and insulin-like growth factor-I (IGF-I) [148].

Tissue cells have receptors that recognise hyaluronic acid [148]. This interaction facilitates cell locomotion [148,149], which is especially important during tissue repair following injury [151]. The promotion by hyaluronic acid of cell migration and differentiation during tissue formation and repair [147,152] has been studied as a possible topical treatment of cutaneous wounds [104,153]. Its use in this way has been shown to reduce post-operative scarring [153], promote tissue regeneration without excess inflammatory processes [104], and accelerate the healing process of deep cutaneous wounds [104].

Animal research has shown that foetal fibroblasts have a greater density of hyaluronic acid receptors compared to adult fibroblasts [152]. The important role of hyaluronic acid in minimising scarring in foetal wounds was demonstrated by the addition of a hyaluronidase to a foetal injury. This resulted in increased numbers of inflammatory cells and collagen deposition [154]. Furthermore, the addition of hyaluronic acid to fibroblasts, *in vitro*, results in a decrease in fibroblast proliferation [95]. Another possible benefit of hyaluronic acid is that it has varied bacteriostatic effects [147].

A disadvantage of unmodified hyaluronic acid is that it is fluid in nature [155] and, hence, does not persist in a particular site for long [156]. To overcome this problem, chemical modification of hyaluronic acid by esterification has been successfully achieved [104,149,156]. These esterified formulations have been shown to be stable and suitable for clinical use. Their biological properties related to healing are maintained despite these modifications to its physical properties [155,156], as is their biocompatability [149,157,158]. Clinically, hyaluronic acid esters have shown possible benefit in the treatment of problems such as chronic venous leg ulcers and chronic wounds requiring skin grafting [159].

The esterified form of hyaluronic acid can be processed into fibres, meshes, and membranes [104,157]. These structures have been assessed as a means of acting as a scaffold in cell culture. They have proven to be successful, for example, in the propagation of keratinocytes, fibroblasts, endothelial cells, and bone marrow cells [157].

It has been suggested that a limiting factor in the success in clinical efficacy of growth factors in wound repair is limited retention of the growth factors to the site of injury [160]. Efforts have been made to overcome this and this includes the incorporation of cytokines in a stable matrix that permits retention to the site of the wound [95,160]. Esterified hyaluronic acid is such an example of a potential matrix and has been used in combination with cytokines [161], with the benefit to healing demonstrated [95].

VII. Cytokines and Insulin-like Growth Factor-I

Cytokines

Cytokines are substances present in the wound environment due to either the leakage of serum, or release from platelets, macrophages, mast cells, and epithelial cells [94,162]. They are potent chemicals with only a small quantity of molecules needed to achieve a biological effect. The understanding of this biological effect is incomplete because of the complexity involved in their differing actions. They have what is referred to as pleiotropic actions [163,164], which means one cell may respond in one way to a particular cytokine (for example the cytokine may be synergistic to cell function), but a different cell may respond completely the opposite (for example the same cytokine is antagonistic to a different cell's performance). Also, the effect of two different cytokines may overlap [165]. Furthermore, the response of different cells may be dependant on the local cytokine concentration [93].

Wounding initiates the expression of growth factors [162]. Their activity is via complex and cascading interactions with receptors of the target cells. This ligand-receptor recognition results in initiation of specific cell transduction pathways that ultimately influence gene expression and protein synthesis and, hence, alter cell behaviour [162]. An important example of the cascade mediated by the action of cytokines is the effect they have on endothelial cells. When these cells are appropriately stimulated, they express special receptors called surface adhesion molecules [165]. These adhesion molecules recognise complementary surface molecules on circulating blood cells. The binding of the circulating cells to these molecules facilitates cell migration across the blood vessel wall and into the

extravascular tissue [164]. This transendothelial migration of inflammatory cells is important in wound healing and the attractive force that the stimulatory cytokines induce is known as chemotaxis [90]. Once, these inflammatory cells enter a wound, they themselves also release their own cytokines. These stimulatory effects for cell migration are also involved in allergy responses [165].

Apart from chemotaxis, cytokines play various roles such as the stimulation of the proliferation of epithelium [166] and native tissue. However, cytokines do not only promote cell growth but may also inhibit it [167,168]. They are also implicated in scar tissue formation [169,170].

The importance of cytokines in wound healing is well established. As the role of certain cytokines began to be appreciated, a theory developed that either adding exogenous cytokines or blocking the activity of endogenous cytokines may modify the healing process *in vivo*. This could be in both a positive and adverse way.

Wound repair involves interactions between growth factors and the extracellular matrix. The use of extracellular matrix analogues, such as hyaluronic acid, in combination with a growth factor has been suggested as a suitable synthetic agent for wound manipulation [95]. The proposed benefit of the two agents relates to the fact that the extracellular matrix components mostly promote cell adhesion and migration whilst the growth factors promote cell growth [95]. An additional proposed benefit of hyaluronic acid analogues is that through their local degradation by hyaluronidases, there would be an associated release of growth factors locally [95]. Examples of growth factors that have been incorporated into hyaluronic acid include

fibroblast growth factor and platelet derived growth factor, both of which enhanced fibroblast replication [95].

Given that the nasal mucosa has been demonstrated to heal slowly after surgical wounding, a window of opportunity exists for manipulating this process. The combination of an extra-cellular matrix analogue with a wound-healing promoting cytokine was thought to offer the greatest likelihood of success. A review of the literature showed that there are many cytokines implicated in nasal physiology, pathology, and wound healing. These include tissue necrosis factor-alpha, transforming growth factor-beta, and epidermal growth factor, and insulin-like growth factor-I.

Epidermal growth factor (EGF)

EGF promotes proliferation of epidermal cells [162] and stimulates hyaluronic acid production [171]. A number of growth factors have been shown to promote skin wound repair. In particular, EGF increases keratinocyte migration by 2.5-4.5 times the normal rate in cell culture [151]. This effect is concentration dependent. Concentration-dependant enhancement of migration was also seen with IGF-I [151]. However, this migration required the presence of collagen IV or fibronectin [151]. Importantly, the addition of an antibody against the EGF receptor blocked effect of EGF but not IGF, indicating separate mechanisms of receptors for these growth factors [151].

In vitro, the presence of EGF in culture media is detrimental to ciliation of respiratory epithelium [172]. The appearance of respiratory epithelium cultured in the

presence of EGF tends to be more squamous [173]. I*n vivo*, the effect of EGF on tracheal and bronchial epithelium is to promote proliferation of basal cells, but this benefit is offset by the promotion of sloughing of ciliated and mucus producing cells [174].

Insulin-like Growth Factor II.

This cytokine is a single chain polypeptide, 67 amino acids long [175], which is homologous to proinsulin and is involved in cell regulation [176]. The liver is the main source of this growth factor [177 178]. IGF-II plays an important role in mammalian growth, exerting an influence upon foetal cell division [178]. The biological activity of IGF-II is mediated mostly via the IGF-I receptor, with the IGF-II receptor involved in IGF-II degradation. IGF-II is expressed in greater amounts in foetal than adult life [175] and, hence, much more is known about the biological activity of the related growth factor, IGF-I.

Insulin-Like Growth Factor I.

IGF-I was discovered in 1957 [179]. It is a single chain anabolic polypeptide [90,92,127] of 70 amino acids, crosslinked by 3 disulfide bridges, [180] with approximately 50% homology with pro-insulin [180,181]. The IGF-I gene is located on chromosome 12 and consists of at least 5 exons and 4 introns [182]. It is an important cytokine because it modulates the effect of growth hormone [92,127,183]. The IGF-I receptor has intrinsic tyrosine kinase activity [184].

The production of IGF-1 is dependant on growth hormone [184] and performed mostly by the liver [92,181]. There is also local production [179,181,185] or release of IGF-I. The evidence for this includes the findings that it is released by platelets [91,92], is synthesised by intestinal cells [184], and that macrophages express IGF-I mRNA [91,92,96]. Interestingly, one of the stimuli for IGF-I production by macrophages is the recognition of hyaluronic acid by the macrophage receptor known as CD44 [186]. Given that IGF-I is produced by the liver and locally, its actions may be endocrine, paracrine, or autocrine, depending on the circumstances [183].

In the circulation, IGF-I is bound (99%) to carrier proteins known as insulin-like growth factor-binding proteins (IGFBP) [90,127,179]. These binding proteins modulate the activity of IGF-I [92,127,179]. The binding proteins may play an important role in wound healing, with research demonstrating that the addition of IGF-I together with IGFBP-I results in a significant increase in wound breaking strength compared to IGF-I alone [187]. The level of circulating IGF-I is related to the age, gender, nutritional status, and is regulated by growth hormone, prolactin, thyroid hormone, and sex hormone levels [90].

IGF-I is implicated in wound healing for several reasons. These include demonstration of increased concentrations in wounds after injury, expression of IGF-I mRNA by wound macrophages, and transient expression of IGF-I mRNA by epithelial cells during reepithelialisation [187].

The actions of IGF-I include the promotion of cell proliferation, differentiation, and inhibition of apoptosis [181]. It specifically targets epithelial and fibroblast cells,

which are stimulated to proliferate [91,92], and it is involved in the promotion of angiogenesis [91,127]. Such actions are beneficial to improving the healing process and use of exogenous IGF-I has been shown to increases the rate of re-epithelialisation of non-respiratory epithelial wounds [91,184]. *In vitro*, it enhances keratinocyte migration in a concentration dependant manner [151]. This migration is dependant upon the presence of a matrix framework [151]. This is important because, *in vivo*, full thickness wounds result in the removal of the basement membrane and, hence, unless an artificial matrix is provided, the effect of cytokines that favour re-epithelialisation will be limited until a new basement membrane is synthesised.

Despite the benefits of increased migration of epithelial cells, IGF-I is also chemotactic for fibroblasts [91] and stimulates collagen synthesis by these cells [91,127]. For this reason it is considered a fibrogenic cytokine [91]. To investigate if the fibrogenic effect of IGF-I was detrimental to wound healing, a study has been performed using a rabbit model [188]. Injection of IGF-I into the edges of wounds created in the ears of the rabbits showed significant improvements in healing but no increase in granulation tissue formation. This suggests a beneficial effect on wound healing without the formation of excess scar tissue. The reasons for this are not presently understood.

Glucocorticoids inhibit IGF-I expression in the liver and result in decreased levels of wound IGF-I [189]. The detrimental effect on corticosteroids on wound healing can be reversed when supplemental IGF-I is administered [189]. IGF-I levels are also decreased in diabetic wounds. The addition of IGF-I to these wounds also improves the wound healing process [190].

The application of IGF-I to non-cutaneous wounds has also proven to be beneficial. For example, superficial injuries to the gut heal by restitution due to rapid migration of epithelial cells from adjacent areas [184]. IGF-I enhances this migration as well as increasing cell proliferation [184]. IGF-I also resulted in increased mucosal mass [184]. These results are consistent with a similar study looking at the influence of IGF-I on gut growth [191]. These differential actions on cell migration and proliferation are related to the stimulation of different cell receptor types and post-receptor pathways [183]. Another example of the use of IGF-I in a non-cutaneous site includes the demonstration of IGF-I resulting in faster healing and with less fibrosis than control in the treatment of muscle injury [192]. IGF-I is normally expressed at the site of skeletal muscle injury by satellite cells [187].

Whilst the above studies described the benefits of local application of IGF-I, parenteral administration has been suggested [181]. This is an important route, as the use of parenteral IGF-I is safe. An example of parenteral administration of IGF-I is intranasally [193]. This route was selected for a study investigating the effect of IGF-I on cerebrovascular stroke [193]. These investigators have demonstrated that the instillation of IGF-1 intracerebroventricularly protected against stroke in rats. They suggested that intranasal administration offered a non-invasive method of bypassing the blood-brain-barrier. Their results showed that IGF-I significantly decreased the volume of infracted brain when the middle cerebral artery was occluded. This was associated with lesser neurological deficit on testing. Studies have shown that IGF-I is strongly expressed at the site of neuronal injury by glial cells [187].

IGF-I and the insulin like growth factor binding proteins have been detected within the mucus of the nose [194]. There are variations in the local concentrations of IGF-I in the nasal mucus with it being greater in the mucus in the areas of the olfactory epithelium than the in mucus covering the respiratory epithelium [194]. The reason for this difference is speculative but an interesting finding is that the IGF-I concentration is lower in the mucus over the olfactory epithelium in some patients with degenerative neurological disease [194]. It has been be suggested that IGF-I is important in maintaining the health of the olfactory apparatus.

There are no studies that have investigated the effect of IGF-I on the healing of nasal respiratory mucosa. There is, however, some work that has studied bronchial respiratory epithelium. In the repair of airway epithelium, migration of cells to cover areas of injury is thought to be important [195]. A study of bronchial epithelial cells showed that IGF-I was chemotactic for the cells and was 1000 times more potent in this action than was insulin [195]. Lung fibroblasts produce IGF-I [195]. Alveolar macrophages produce an IGF-I-like peptide [182,195]. This IGF-I-like molecule is produced when the macrophages are activated and it is a signal that is recognised by the fibroblasts and results in them replicating [182]. These findings suggest that IGF-I plays a role in airway epithelial repair.

IX. Functional Endoscopic Sinus Surgery and Packing Materials

The revolution in sinus surgery came about through the development of endoscopes [135]. The pioneers of endoscopic sinus surgery were Wigand and Meserklinger who advocated differing techniques. Wigand advocated opening up all sinus cavities and removing the middle turbinates [196,197] whereas Messerklinger advocated minimal opening of the osteomeatal tract at the anterior ethmoid sinuses [196]. Although the techniques differed, both attempted to improve aeration and drainage of the sinuses [196]. In the 1980s, Stammberger [16] and Kennedy [17] advocated the endoscopic sinus surgery techniques of Messerklinger that allowed the opening of the sinuses via their natural ostia. Kennedy coined the term "functional endoscopic sinus surgery" as this technique attempts to restore function by restoring the natural physiology. The decision on the appropriateness of surgical intervention is based on the conglomerate of patient history, findings on physical examination, and the results of CT scanning [16].

There are several advantages of endoscopic surgery compared to previously utilized techniques. These include the ability to use the endoscope to monitor post-operative results [198-200] and perform post-operative toileting of the surgical field [198,200]. The avoidance of external skin incisions is also favourable [201]. The most important advantage is restoration of the normal physiology [202].

The evidence supporting the value of this surgery includes the demonstration of an improvement in the mucociliary clearance rates in people who were treated

surgically for chronic sinusitis [201,203] as well as improvements in the ciliary beat frequency [19]. Importantly, studies have shown a high success rate in the resolution of patient symptoms [199,204,205]. Such improvements mean that the use of functional endoscopic sinus surgery for the treatment of chronic sinusitis is now in common use [196].

Surgical complications are divided into minor and major [206]. The major complications of sinus surgery include orbital injury and intracrainial penetration. The formation of post-operative adhesions is an example of a minor complication. It is also the most frequent overall complication of sinus surgery, with a quoted incidence of between 5-40% [88,135,136,196,198,200,207-209]. These adhesions may require further surgery to be divided [196,200].

Adhesions can potentially form between any two mucosal surfaces with adverse effect on mucus clearance resulting. The use of nasal packing has been advocated as a possible means of keeping mucosal surfaces separate from each other, and, hence, preventing adhesion formation [135]; Wormald, #176; Messerklinger, 1978 #109; Nayak, 1998 #219; Toffel, 1995 #637]. The following is a summary of the types of nasal packs available and the research related to them.

Packing

Pre-operative

Nasal packing is used as part of the pre-operative preparation for sinus surgery [210]. The packs are soaked with various types of agents in order to facilitate vasoconstriction and analgesia [210]. The use of these nasal packs is not for the

prevention of adhesion formation. The use of these nasal packs may cause mucosal trauma [211]. The documented risks of nasal packing include nasal injury, aspiration of the pack, infection, respiratory compromise, and cardiac arrest via vagal nerve reflexes [212].

There are several types of pre-operative nasal packing materials available. Ribbon gauze has been used to pack the nose for many years [213] and more recently neuropatties have been used. The effect of pre-operative packing with these has been assessed, with both ribbon gauze packing and neuropattie packing resulting in significant loss of the ciliated surface of the mucosa when compared with the control group [211]. There was a trend showing less mucosal loss with the neuropatties compared to ribbon gauze but this difference was not statistically significant ($p>0.25$).

Post-operative packing

The use of post-operative packing with an occlusive, non-adherent material has been advocated as a means of improving surgical outcomes by reducing adhesion formation [97]. Many types of post-operative packing materials have been utilised. Unfortunately, there is a paucity of research looking at the efficacy of these packs [135,196,213]. One concern about the use of traditional packs or splints in the paediatric population is the potential need for another anaesthetic [214].

Amongst the different types of physical barriers that have been tried in the nose are polyurethane [135], a combination pack of a plastic film and cotton [213], and expandable polyvinyl acetate packs (Merocel®) [196]. This last pack is the most commonly used

in clinical practice at the moment. The manufacturers of this nasal pack advocate its use for epistaxis and post-operative nasal surgery as a means of achieving haemostasis [215]. It has been advocated for use as a stent between the lateral nasal wall and middle meatus [196]. While these packs may act as a physical barrier between mucosal surfaces, they need to be removed in the post-operative period. The act of removing the pack may lead to further mucosal injury at the site of operation and new mucosal damage to other sites. This may increase rather than decrease the propensity towards adhesion formation. Another concern regarding the use of this type of packing is that it may occlude the sinus ostia, potentially resulting in bacterial growth [216]. It is important to note that this dressing has no antimicrobal properties [215]. The most frequent problem with this type of nasal packing comes at the time of removal which can be the most unpleasant experience associated with the operation and may be associated with haemorrhage [217].

A recent advancement in nasal packing is the development of a hyaluronic acid ester woven fibre pack. This newly developed nasal pack is commercially known as Merogel®. This pack becomes a gel on exposure to fluid [218]. The hyaluronic acid gel may act as a framework for cell migration. This may be advantageous in the promotion of wound healing. Another possible benefit is that, given that they are dissolvable, they do not require removal. This avoids mucosal trauma and, therefore, may potentially be more comfortable for patients, making them particularly suitable for paediatric patients. The degradation of the packs takes about two weeks [218].

The idea of using hyaluronic acid in the nose is not unique to this type of packing material. A clinical trial of the application of a gel containing hyaluronic acid after endoscopic sinus surgery has been undertaken [219]. This study looked at mucosal wound healing in 56 patients recovering from endoscopic surgery. The hyaluronic gel was compared to a control group of patients. The hyaluronic acid group demonstrated less crust formation during the first week of wound healing. Importantly from a product safety perspective, there were no adverse reactions.

In contrast to the gel, there is little evidence of the efficacy of the hyaluronic acid ester woven fibre pack in nasal epithelial wound healing. There is also no scientific evidence regarding its effect on adhesion formation after endoscopic sinus surgery. In addition, there is no supporting evidence as to the effect of the pack on haemostatic control. This is important because hyaluronic acid may interfere with platelet-platelet interactions due to physical impedance [220]. This is the same property that is proposed to reduce adhesions. Hence, research is required to determine whether the use of these packs has any benefit in post-operative recovery.

A potential advantage of a dissolvable nasal pack that consists of hyaluronic acid based compounds is that a combination of a complex matrix interwoven with cytokinetic agents may provide an improved environment for wound healing to proceed [103]. The two week time period that the hyaluronic acid ester packs take to dissolve, theoretically means that the impregnated cytokines would be released over this time period [95]. This would provide a prolonged local source of supplementary growth factors.

The normally slow healing process after endoscopic sinus surgery offers an opportunity to manipulate the rate of healing. The unknown effects of the polyvinyl acetate, hyaluronic acid ester, and hyaluronic acid ester- growth factor combination nasal packs on the wound healing rate form the basis for this thesis.

X. The Sheep as an Animal Model for Endoscopic Sinus Surgery

Animals that have been used to study sinus surgery include rabbits, dogs, pigs [221] and sheep [10,222-224]. There are various problems with each of these animal models. For example, rabbits have the disadvantage that it is very difficult to ensure that their sinuses are free from Pasturella infection [225]. The disadvantage of dogs is that they are not readily available and with regards to the sheep, they are large animals that need a suitably sized holding pen when at the site of investigation, and an adequate grazing area when in between experimental interventions.

However, despite the disadvantages, the sheep have certain attributes that make them most suited for endoscopic sinus surgery research. The anatomy of the sheep's nasal cavity is similar to that of human's [224] and the sinuses are in approximately the same orientation. In fact, the use of sheep for endoscopic surgery has been suggested as suitable for training in various endoscopic techniques [224]. The sheep is also suitable in terms of histology (similar nasal respiratory epithelium) [226], physiology (similar mucociliary drainage), and pathology [106,224,226,227]. It is possible to carry out long-term serial measurements, including mucus clearance, ciliary function, and mucus rheology [227]. From a research perspective, another advantage of sheep is that they are not inbred, hence, maintaining genetic diversity and not selecting for a particular phenotype.

While conferring these benefits of similarity, it is also necessary to be aware of some important differences. This includes the fact that the dimensions of the sheep's nasal cavity contrasts to the human nasal cavity [226]. In the human nose the average surface area of the lining mucosa is 150-181 cm^2 [31] whereas it is 327 cm^2 in

the sheep [226]. There is also a difference in the volume, with the sheep's nasal cavity measured at 114 millilitres compared to 19 millilitres in the human [226]. An important difference, for technical reasons, is that the sheep's nasal cavity is 18cm long, compared to 8cm in humans [226]. This difference requires special surgical instrumentation to carry out endoscopic procedures. Another difference in the comparative anatomy of humans and sheep is that the two halves of the nasal cavity are only incompletely separated by the nasal septum in sheep [228]. This is compared to the complete separation offered by the human nasal septum.

For technical reasons relating to access, the sheep model undergoes total middle turbinectomy. This does not significantly alter either the mucociliary clearance rate or histology on the lateral nasal wall at 3 weeks following the operation in the sheep [229]. This is consistent with Waguespack's study of partial middle turbinate resection in humans [47]. The middle turbinate of the sheep needs to be removed because it is extensive and almost fills the nasal cavity. Other interventions studied include the effects of different drugs [211], intubation and anaesthesia [230], and pre-operative packing [211].

Studies have also been conducted assessing the healing process of sheep respiratory mucosa after the application of various injurious stimuli [231]. This includes healing after full and partial thickness mucosal injuries [121], whereby the slow healing process after injury was demonstrated, with only 50% cilia return at day 84 post-injury in the full thickness wounds.

XI. Screening for Potential Growth Factors that Stimulate Respiratory Epithelial Growth

Several growth factors known to be beneficial to wound healing in the skin were assessed for their potential effect on wound repair in the nasal mucosa. Insulin-like growth factor-I (IGF-I), insulin-like growth factor II (IGF-II), and epidermal growth factor (EGF) were trialled using a cell based wound healing assay.

Cell culture

An immortalised human respiratory (tracheal) epithelial cell line was utilised for the experiments (courtesy of Dr David Parsons, Women's and Children's Hospital, Adelaide). This cell line is known as 1 Human Airway Epithelia (1HAE), and was generated from human trachea epithelium.

These cells were grown in polystyrene tissue flasks ($75cm^2$) in 12ml of media consisting of Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL), 10% foetal calf serum (JRH Biosciences), 100ul/ml penicillin, 100μg/ml streptomycin (Penstrep) (Gibco BRL) in a humidified 5% CO_2 incubator at 37°C. The media solution was changed every 2-3 days.

When the replicating cells had grown to confluence, the media was removed and the cells dissociated using 3ml of trypsin solution 0.05% (CSL, Australia). Once, dissociated from the flask, 9ml of the culture media referred to above was added to neutralise the trypsin. The dissociated cell suspension was transferred to a centrifuge tube and spun at 1200rpm for 5 minutes. The supernatant was aspirated

and the cells resuspended in 12 ml of the media solution before being transferred into 12 well tissue culture plates (1ml per well) and allowed to become confluent again (approximately 2-3 days). When the cells became confluent they were serum starved by changing the media 24 hours before wounding. The media at this stage was the same as above except the foetal calf serum was omitted. This was done to remove the influence of any factors within the foetal calf serum, which may have otherwise confounded the results of experimentation, and to synchronise the cells into the same stage of their growth cycle.

A scratch wound was made in each of the 12 wells to the sheet of confluent cells growing within them. The injury was made using a sterile pipette tip and was approximately 2-4 mm in length and 1-2 mm in width. This is a well reported and accepted method for this type of investigation. The media was then changed with control wells having the serum free media and treatment wells having serum free media with the growth factor added. The trial of each growth factor was performed at the concentration 100ng/ml. This was chosen because it represents a super-maximal concentration necessary to stimulate the appropriate receptor. Using an inverted phase contrast microscope, digital camera, and image analysis software (Image Pro Plus, Media Cybernetics), images were recorded at the time of injury and serially over the next 24 hours at 4x magnification. The same image analysis software was used to measure the sizes of the wound. The sequential wound size was then expressed as a percentage of the original wound area. A student t-test was used to compare results. Any wells in which the scratch wound was not successfully created were discarded.

Results

Insulin-like growth factor-I

The results of the effect of IGF-I in cell culture are represented in Figure 12-1 (see also Appendix to Chapter 12). At a concentration of 100ng/ml, there was a statistically significant ($p<0.05$) improvement in wound closure with IGF-I (n=9) compared to controls (n=12) from 9 to 24 hours. At 24 hours, the IGF-I wounds were 86% closed compared to 68% for controls. Images of representative treatment and control wounds at the time of injury, and 12 and 24 hours after this injury, demonstrate the differences in the rate of wound closure over time (Figure 12-2).

Insulin-like growth factor-II

The results of the effect of IGF-II in cell culture are represented in Figure 12-3 (see also Appendix to Chapter 12). At a concentration of 100ng/ml, there was a statistically significant improvement in wound closure with IGF-II (n=9) compared to controls (n=8) from 12 to 18 hours ($p<0.05$ at 12 hours, $p<0.01$ at 15 and 18 hours). At 24 hours, the IGF-II wounds were 98% closed compared to 93% closure for controls.

Epidermal Growth Factor

The results of the effect of EGF in cell culture are represented in Figure 12-4 (see also Appendix to Chapter 12). At concentrations of 100ng/ml, there was a statistically significant improvement in wound closure with EGF (n=9) compared to controls (n=9) at 9 hours ($p<0.05$), and from 18 to 24 hours ($p<0.05$ at 15 and 24 hours, $p<0.01$ at 18 hours). At 24 hours, the EGF wounds were 99% closed compared to 95% closure for controls.

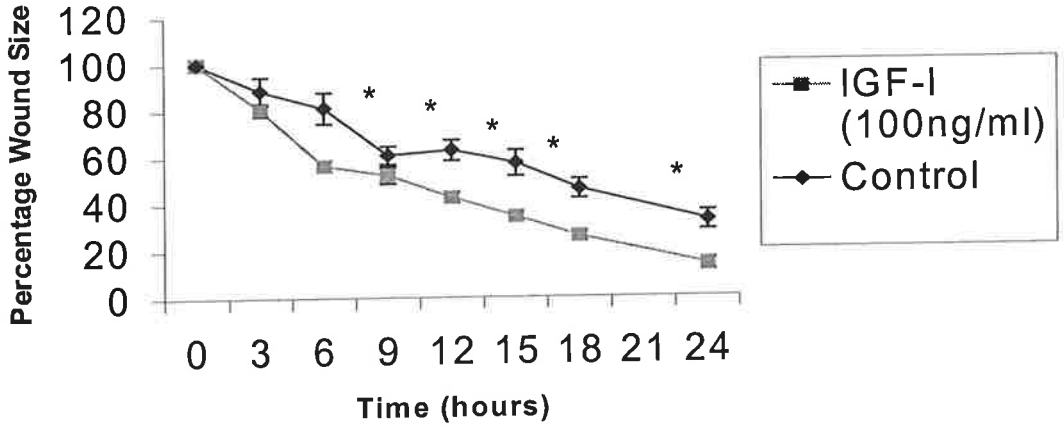

Figure 11-1. Graph of IGF-I cell culture results. Media supplemented with IGF-I (n=9) at 100ng/ml compared to standard media (n=12). *=statistically significant difference control vs IGF-1 ($p<0.05$). Error bars represent standard error of mean.

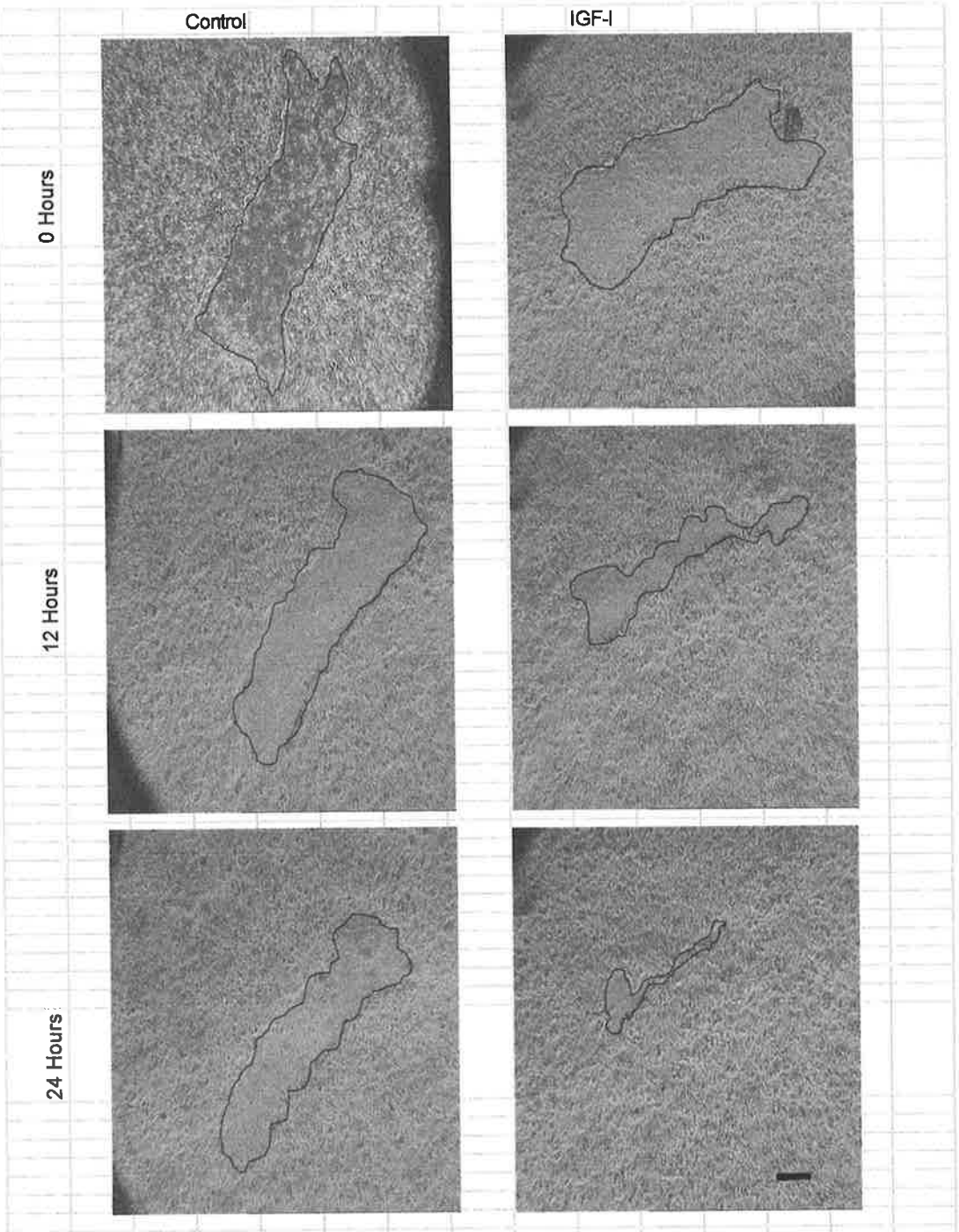

Figure 12-2. Images of representative control and IGF-I treated cells in culture. Time points of the images are indicated. The edge of the wounds have been traced to allow for easier visualisation. Scale bar = 100um.

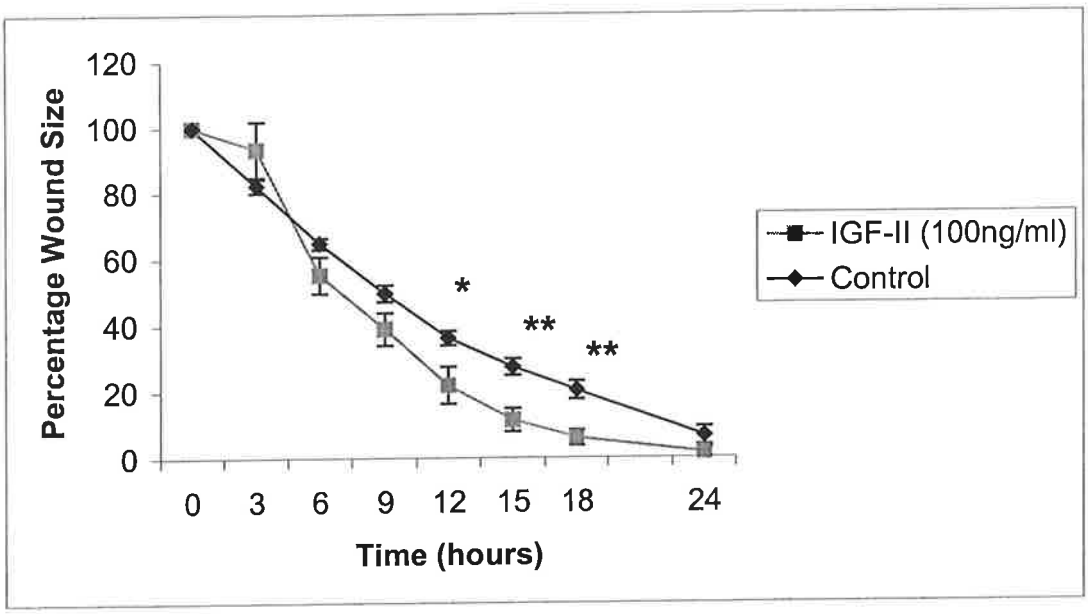

Figure 11-3. Graph of IGF-II cell culture results. Media supplemented with IGF-II (n=9) at 100ng/ml compared to standard media (n=8). *=statistically significant difference between contol and IGF-II 100ng/ml ($p<0.05$), **=statistically significant difference between contol and IGF-II 100ng/ml ($p<0.01$). Error bars represent standard error of mean.

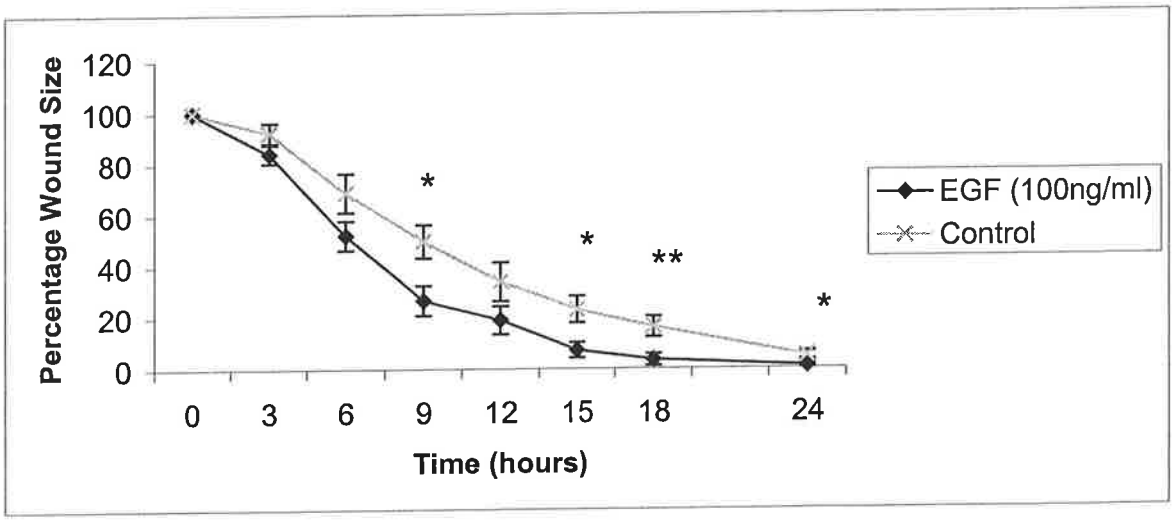

Figure 11-4. Graph of EGF cell culture results. Media supplemented with EGF at 100ng/ml (n=9) compared to standard media (n=9). *=statistically significant difference between contol and EGF 100ng/ml ($p<0.05$), **=statistically significant difference between contol and EGF 100ng/ml ($p<0.01$). Error bars represent standard error of mean.

Discussion

Cell culture is a valuable tool for assessing growth factor function. This trial demonstrated improved wound closure of immortalised human tracheal respiratory epithelial cells when IGF-I, IGF-II, or EGF was added at a concentration of 100ng/ml to a standard media solution.

Selection of Insulin-like growth factor I (IGF-I).

The results support the selection of any one of these growth factors as being suitable for incorporation into nasal packing material. Although EGF significantly improved wound closure *in vitro,* a review of the literature suggested that this growth factor is potentially detrimental to respiratory epithelial wound healing when used *in vivo* because it delays the wound healing process [97,124]. Therefore, it was decided not to use this in the preliminary animal trials. IGF-I or IGF-II could both be potentially useful candidates.

Previous studies have shown that IGF-I greatly improves wound healing. Additionally, IGF-I receptors are expressed in nasal mucosa and have been demonstrated to increase after injury [232]. Therefore, IGF-I was selected as the first growth factor to be incorporated into the nasal packing material.

Since the animal to be utilised as a representation of wound healing after endoscopic sinus surgery is the sheep, the next important step was is to determine the suitability of using IGF-I in this model.

XII. Assessment of the Suitability of the use of Human Insulin-Like Growth Factor-I in Sheep.

Human versus sheep IGF-I.

Insulin-like growth factor-I is a ubiquitous growth factor, found in many species [233]. The structure is well preserved between species [233]. The structure of human IGF-I varies to the sheep by one amino acid. The difference is a substitution in the sheep of alanine at residue 66 for prolene [233]. With respect to biological activity, the human and sheep IGF-I are virtually equipotent in growth related bioassays [233]. This would imply that human IGF-I should be bioactive in sheep.

Presence of IGF-I receptors in sheep nasal mucosa.

The presence of IGF-I receptors in human nasal mucosa has been demonstrated [187]. These receptors are upregulated during wound healing [187]. There are no previous reports of the demonstration of the presence of IGF-I receptors in sheep nasal mucosa.

An immunohistochemical technique was utilised to ascertain whether the sheep nasal mucosa expressed IGF-I receptors. Specimens were collected from the nasal cavity of 12 sheep sedated with intramuscular xylazene (4mg) under endoscopic control using topical anaesthesia (copheylcaine spray) and endoscopic biopsy forceps. The specimens were fixed in 10% formalin for 4 hours and then preserved in 70% ethanol until embedded in paraffin. The paraffin blocks were sectioned at 4μm thickness and placed onto snowcoat slides.

The sections were dewaxed through a series of xylene and graduated ethanol solutions and then pre-treated with boiling target retrieval solution (Dako) for 12 minutes. The solution was then cooled to 50°C and rinsed in Phosphate Buffered Solution (PBS) before being placed into a pre-warmed solution (37°C) of PBS and trypsin (Sigma) (0.0625g/250ml PBS) for 3 minutes. The samples were rinsed in PBS and then incubated for 30 minutes at room temperature with 100µl of 3% normal horse serum (in PBS) covering each section. After 30 minutes, the solution washed off with PBS. A solution of 1:100 anti-human IGF-I receptor antibody (Gropep Ltd, South Australia) in 3% normal horse serum was applied to each section (100µl/section) and incubated overnight in an airtight container at 4°C. A control sample was also prepared following the same protocol but omitting the anti-human IGF-I antibody . The next day, excess solution wash washed off with PBS and 100µl per section of a secondary biotinylated antibody, 1:200 anti-IgG (**Vector**) in PBS, was added and incubated for 60 minutes at room temperature in the airtight container. The excess solution was then washed off with PBS and then 100µl per section of Streptavidin Cy3 (**Sigma**), 1:200 in PBS, was incubated for 45 minutes in an airtight dark box. The solution was washed off with PBS and a fluorescent mounting medium (**Dako**) was used (1 drop/section) to allow examination under a fluorescent microscope, with image analysis software (Image Pro Plus, Media Cybernetics) used to measure the intensity.

IGF-I receptors were observed in the nasal mucosa of the sheep (Figure 13-1). They are located in the epithelial and underlying basal tissue. The results of this method were qualitative rather than quantitative due to the variable staining of different sections collected from the same sheep. This was thought to be related to specimen

quality and affinity of the primary antibody (anti-human) for the sheep IGF-I receptors.

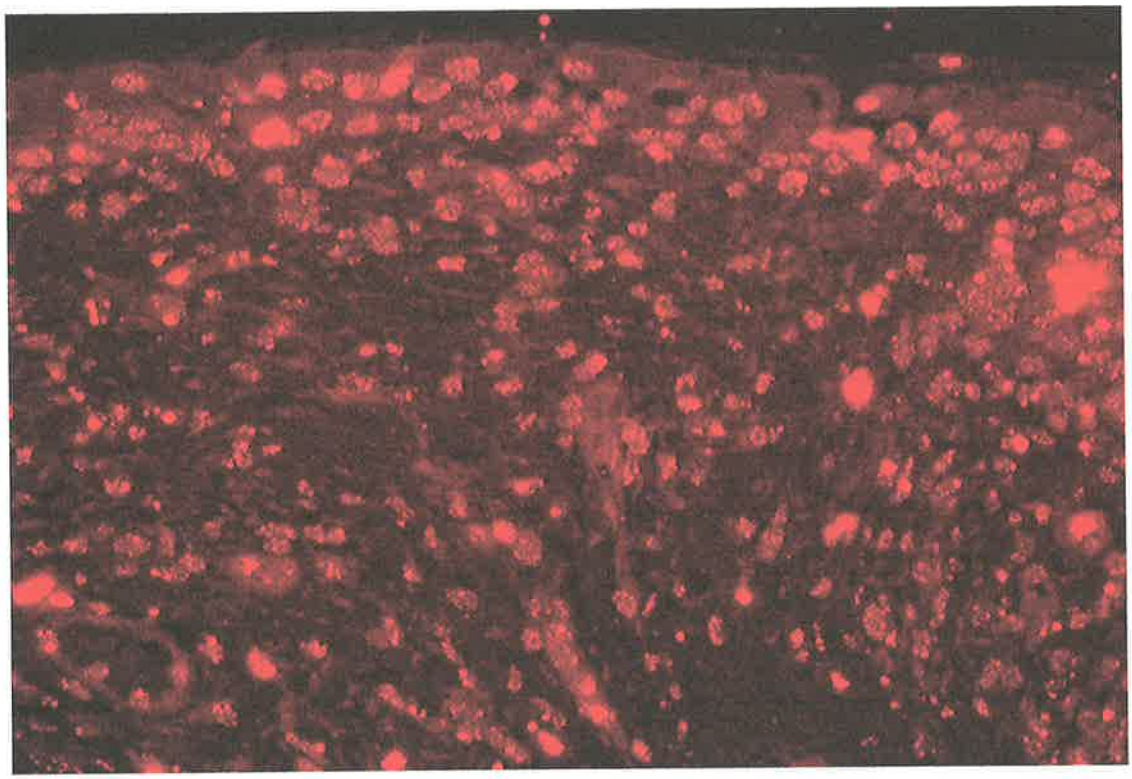

Figure 12-1. Immunostaining of IGF-I receptors in sheep nasal mucosa. Surface epithelium is at the top of the image. Receptors indiated by red staining.

Primary culture of sheep nasal mucosa

With the successful demonstration of IGF-I receptors, it was hoped that an *in vitro* biological effect of human IGF-I on sheep nasal mucosa could be demonstrated using cell culture techniques. In this instance, both an organ culture and primary cell culture method were attempted.

Organ culture entails removal of intact tissue and maintenance of this extracted material in media. Sheep nasal mucosa (full thickness) was harvested from the middle turbinate of freshly sacrificed animals under direct vision with scissors and a scalpel. The full thickness mucosa was then laid flat on a sterile surface and a round

section of tissue obtained using a 6mm skin biopsy punch (Stiefel). A 19G needle was then dipped in Indian ink before making a central circular wound in the round sections. The use of the ink allowed for easy identification of the wounded area on repeat inspection. The tissue was then transferred to transwell plate with the epithelium orientated upwards. The specimens were kept viable in culture media consisting of DMEM with 10% foetal calf serum, 100u/ml penicillin, and 100µg/ml streptomycin. The tissue was incubated at 37°C in 5% CO_2. The media was added to a depth such that basolateral supply to the tissue occurred with an air-liquid interface maintained at the surface. Survival of the tissue was confirmed by observing cilia motion using light microscopy. Despite the success in maintaining organ viability using this method, tissue shrinkage could not be avoided. Hence, measurement of wound size was not representative of healing. Primary culture was therefore explored as an alternative method for *in vitro* testing.

Mucosa was harvested from recently sacrificed sheep. The specimens were cleaned to remove blood and mucus. They were then incubated at 4°C with protease 0.1% (Sigma) overnight to dissociate cells from each other. Foetal calf serum 10% in PBS was added to neutralise the protease. The solution was centrifuged, the supernatant aspirated, and the cells resuspended in culture media consisting of DMEM with 10% foetal calf serum, 100u/ml penicillin, and 100µg/ml streptomycin. The suspension was transferred to a culture flask and incubated at 37°C in 5% CO_2. When the cells replicated to become confluent in the flask, the cells were dissociated and transferred into 12 well culture plates and allowed to become confluent again. 24 hours before intended wounding, the cells were serum starved. This was done so that the growth factors within the serum would not be present at

the time of injury and healing. This was thought to be important because confounding factors would otherwise be present during the period of observation of the wound healing process. Unfortunately, the cells did not tolerate these conditions and rapidly died before wound scratch experiments could be performed. This suggests that the viability of the cells in primary culture is dependant upon the stimulation provided by the factors present within the serum. Given that the level of these factors is not standardised, and that there are potential interactions between these factors and the factors of interest, confounding variables could not be controlled.

Hence, the studies described in this section were successful in demonstrating the presence of IGF-I receptors in the sheep nasal mucosa. Unfortunately, efforts were unsuccessful in demonstrating the *in vitro* effect of exposing sheep nasal mucosa to human IGF-I. However, given the structural homology between sheep and human IGF-I, it was decide to proceed to incorporate the IGF-I into the hyaluronic acid ester pack for animal trials given the support provided from the immortalised cell line used in the screening process.

XIII. Development of a Nasal Pack Containing Insulin-like Growth Factor-I.

Incorporation of IGF-I into a dissolvable nasal pack.

The nasal packing material utilised as a carrier for the IGF-I is the recently developed woven fibre hyaluronic acid ester pack available commercially registered under the name Merogel® (Xomed Medtronic). This pack consists of hyaluronic acid esterified with an alcohol moiety. The specifications of incorporation were that the IGF-I was present in a dose of 1μg/ml of packing material. The dimensions of the packing material are 4cm x 4cm x 0.1 cm (volume = 1.6 cm^3). Hence, it is estimated that there would be 1.6 μg of IGF-I per pack (1 ml = 1 cm^3). The packs were transported in plastic petri dishes from Italy to our animal laboratory.

Demonstration of the release of IGF-I from the pack.

To be biologically active the IGF-I must retain its structure during the incorporation process and the pack must allow it to dissociate during the dissolving of the pack. To ensure that the IGF-I had maintained its structure and that it was released from the pack, a radioimmunoassay test for IGF-I was performed.

The first step in this process is to dissolve the packs in sterile water. A piece of the IGF-I hyaluronic acid pack (9 separate packs in total) measuring 0.6 cm in diameter and 0.1 cm thick (volume = 0.028 cm^3) was placed in 1 ml of phosphate buffered saline in a 37°C water bath for 72 hours. As a control, three hyaluronic acid packs without IGF-I incorporated in them were also subject to the same protocol. This was sufficient time for the packs to dissolve. These were centrifuged and the supernatant aspirated and retained. Based on the above assumption, this portion of the pack

should have contained 0.028 μg of IGF-I (1ml = 1 cm^3). Hence, the theoretical maximum amount of IGF-I in each sample would be 0.028 μg or 28,000 pg. Since this was further diluted down 1 in 10 when used in the experimentation, 2,800 pg is the theoretical expected maximum amount of IGF-I that would be present if all the IGF-I was intact and successfully leached out.

Quantification of the IGF-I leached out of the packing material was assessed by radioimmunoassay (RIA). This technique relies on competitive reaction with an anti-IGF-I antibody between a known concentration of radioactive IGF-I and the IGF-I from the hyaluronic acid ester packs. In brief, 100 μl of the sample was added to 150 μl of assay buffer [30 mM NaH_2PO_4, 0.2 g/l protamine sulphate, 10 mM EDTA, 3.8 mM NaN_3, 0.05% (v/v) Tween-20, pH 7.5]. Thereafter, approximately 25 000 cpm of [^{125}I]-IGF-I in 50 μl was added. Finally, 50 μl of anti-human IGF-I (GroPep Pty Ltd) diluted to a final concentration of 1:80 000 was added. Samples were incubated for 16-18 h at 4°C after which 10 μl of sheep anti-rabbit immunoglobulin (Silenus Laboratories, Australia; final concentration 1:200) was added. After 30 min incubation at 4°C, 1 ml of 5 % (w/v) polyethylene glycol 6000 in 0.9% NaCl was added, and left for 10 min on ice. The supernatant was aspirated after centrifugation at 4000 x *g* for 30 min at 4°C and the radioactivity of each pellet measured using a LKB 1261 multigamma counter and RiaCalc software.

The results are demonstrated in Table 14-1. These results confirm that the IGF-I is released when the hyaluronic acid ester packs are dissolved for 72 hours in sterile water at 37°C. The calculated ranges span 4.11 to 14.57% of total calculated IGF-I release, with an average of 8.52%. The results for hyaluronic acid ester packs free of

Pack Type	IGF-I Amount (pg/tube)	Percentage of expected
HA-IGF-I	408	14.57
HA-IGF-I	222	7.93
HA-IGF-I	164	5.86
HA-IGF-I	394	14.07
HA-IGF-I	328	11.71
HA-IGF-I	115	4.11
HA-IGF-I	169	6.04
HA-IGF-I	116	4.14
HA-IGF-I	232	8.29
HA	5	N/A
HA	9	N/A
HA	11	N/A
Average		8.52

Table 13-1. Calculated percentage of IGF-I release from hyaluronic acid ester pack. Measurements made used radio-immunoassay.

IGF-I are low enough to be considered within the error range of the test and hence, it can be concluded that they did not demonstrate any significant cross-reaction with the anti-IGF-I antibodies. The positive results also allow for the inference that the process of incorporation of IGF-I into the hyaluronic acid pack and the dissolution of this pack does not affect the structure of IGF-I. Hence, the bioavailability of IGF-I from these packs was demonstrated using this technique.

Discussion

The calculated ranges of IGF-I released are between 4.11 to 14.57% of the total calculated (or maximum predicted) IGF-I release, with an average of 8.52%. With such a low rate of release, it would be reasonable to be concerned that much of the IGF-I that was incorporated is either not active or not released. There are many potential causes of error in these calculations. This includes lesser impregnation of IGF-I than assumed and/or denaturing of IGF-I during the impregnation process. Unfortunately, due to lack of disclosure by the manufacturer of whether they have any information regarding these two issues means that they can only remain speculative. Another possible source of low recordings is that the IGF-I is not released in its entirety when the hyaluronic acid ester is dissolved in sterile water for 72 hours. If this were the case, then greater amounts would be expected to be released *in vivo*, due to the presence of enzymes, which would break down the hyaluronic acid ester. Another possibility, and potentially the source of greatest error, is that the IGF-I hyaluronic acid ester packs were transported in plastic containers. When these packs were removed for use, a residue was always present on the plastic surface. Discussions with Gropep, the manufacturers of the IGF-I, have revealed that IGF-I has an affinity for plastic.

XIV. The Assessment of the Effect of Nasal Packing on the Healing of the Nasal Mucosa of Sheep After Endoscopic Surgery.

The use of an animal model in researching new therapeutic measures requires suitable care of the animal concerned. All research performed in the sheep was approved by the animal ethics committees of the Queen Elizabeth Hospital and the University of Adelaide.

Methods

1. Statistical power analysis

An improvement in the time for epithelial and ciliary regeneration (greater than 25% when compared to the normal healing process) would be considered clinically significant. Assuming nasal packing alters the healing process by greater than 25%, a power study showed that a paired sample size of 12 would give 80% power, with a significance level of 0.05 ($p<0.05$, 2 tailed).

2. Surgical techniques

Middle turbinate removal

The nasal cavity of the sheep is obviously longer that that of humans. To enable endoscopic surgery to be performed, specially modified (elongated) instruments were manufactured, as represented by examples in Figures 15-1A and 15-1B. A cystoscope was often used in the place of nasal endoscopes (Figure 15-1C).

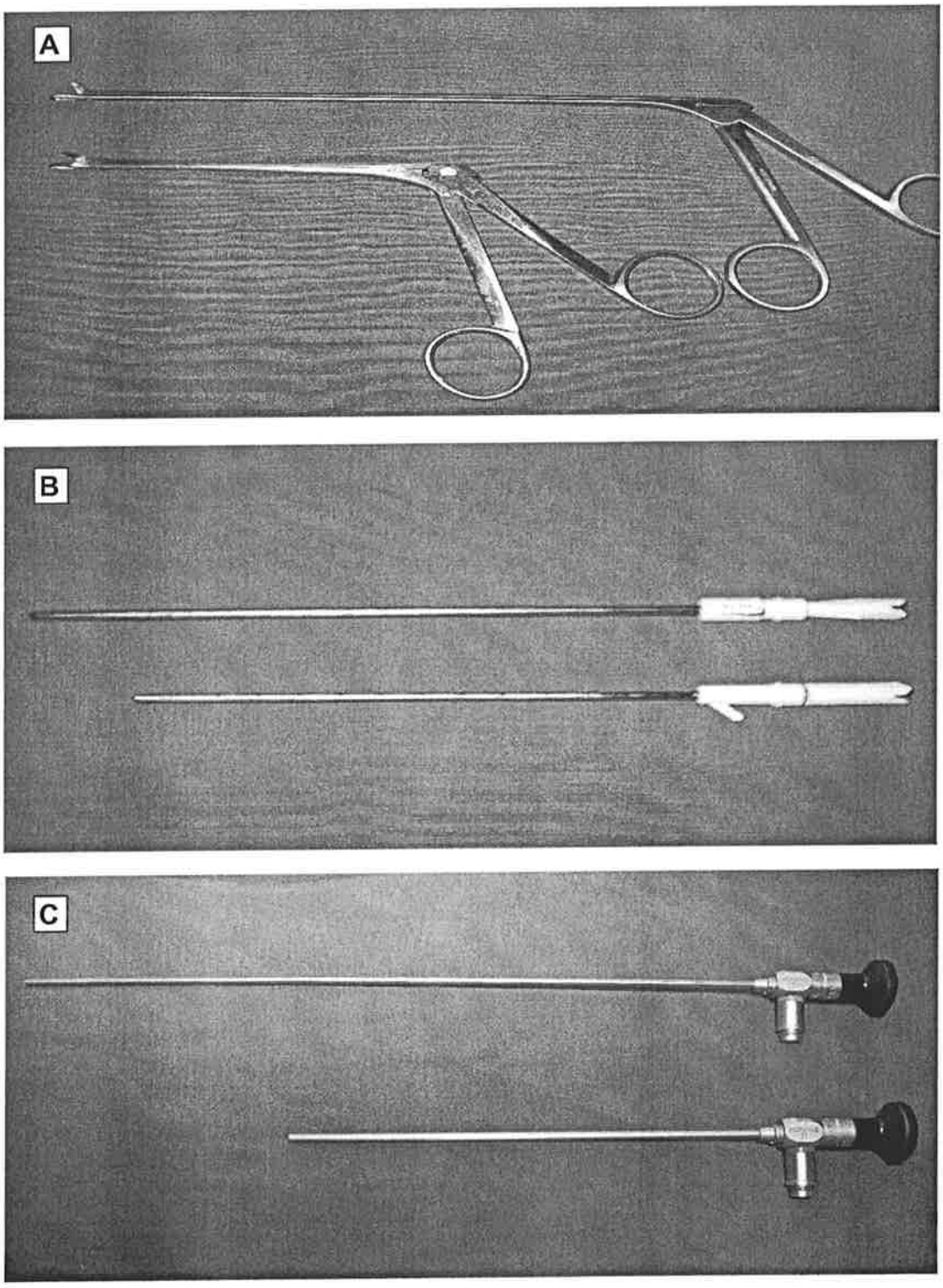

Figure 14-1. Representative demonstration of modifications made to standard instrumentation. A) Biopsy forceps, B) Micro-debrider blade, C) Cystoscope compared to standard sized endoscope. In A) and B), the modified instruments are above the standard human instruments. In C), the cystoscope lies above the sinoscope.

The sheep used in this study were provided as laboratory grade. The important aspect of this is that they have been "dipped" with drugs designed to reduce *Oestrus ovus* infestation. Any sheep that demonstrated evidence of this parasite (Figure 15-2) at the time of middle turbinate removal were "dipped" again before being used in the wound healing trials. The prevention of this infestation is important because the purpose of this study was to investigate the healing process that follows trauma to healthy mucosa. Inflammation related to this parasite would have been a confounding variable.

The middle turbinates of the sheep occupy a significant proportion of the nasal cavity. This is demonstrated in (Figure 15-3) the coronal and axial computerised tomographic scans of the nasal cavity. A computer-generated image (Figure 15-4) demonstrates the room available in which to operate once these are removed. The removal of these structures allows easy access to the lateral nasal wall where the wounding studies are conducted. This procedure requires general anaesthesia.

The general anaesthesia was induced with sodium pentobarbitone (720mg) IV into the jugular vein. A cuffed endotracheal tube was inserted and subsequent anaesthesia was maintained by inhalation of halothane 1.5 - 2%. Application under endoscopic control of neuropatties soaked with topical 10% cocaine solution decongested the middle turbinate. Infiltration under endoscopic control of 2% lignocaine, 1:80,000 adrenaline was performed at the anterior face of the middle turbinate and at the posterior attachment to the lateral nasal wall via the inferior surface. This is the location of the sphenopalatine artery. Using endoscopic scissors under endoscopic visualisation with a 0° cystoscope, the middle turbinate was

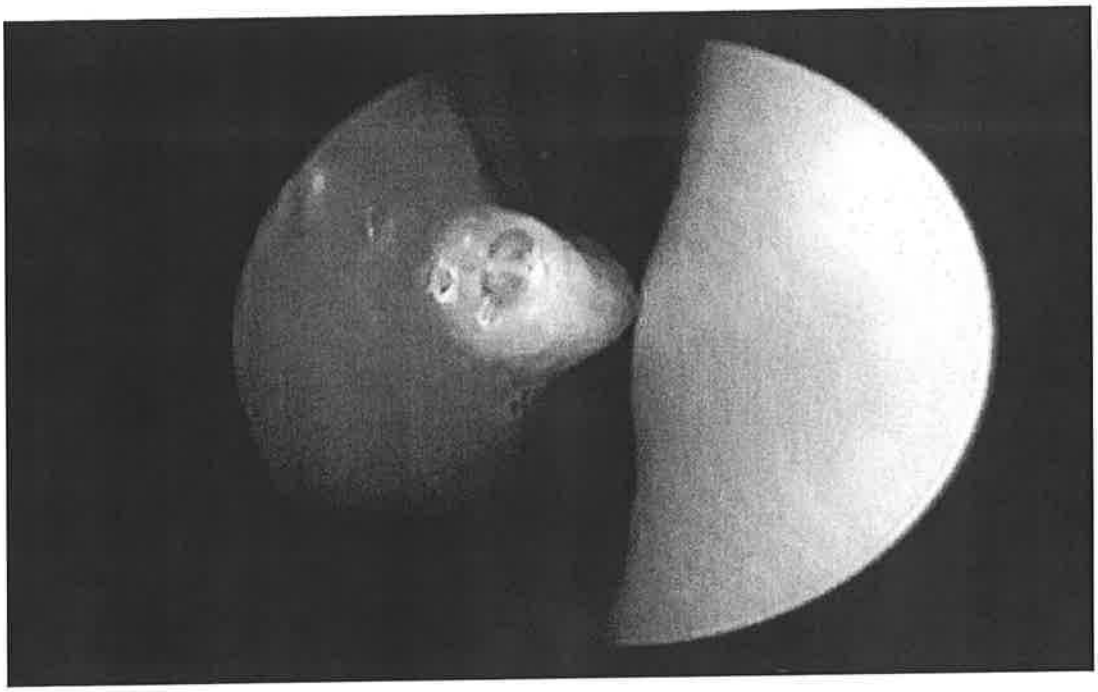

Figure 14-2. Demonstration of the presence of a nasal bot fly (*Oestrus ovus*) seen within the nasal cavity using an endoscope.

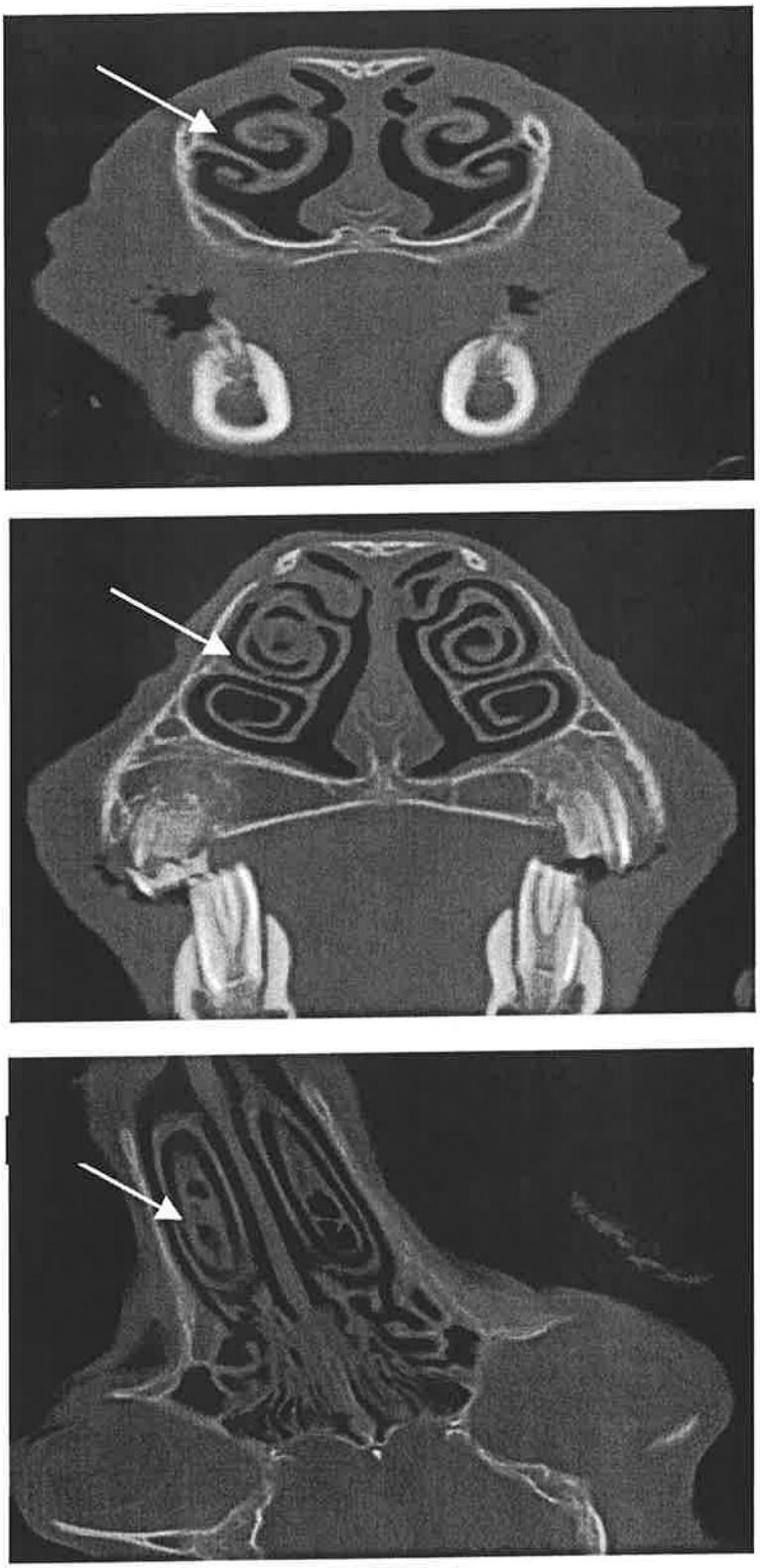

Figure 14-3. CAT-scan (CT) images of the sheep nasal cavity and paranasal sinuses. Middle tubinate indicated by arrow.

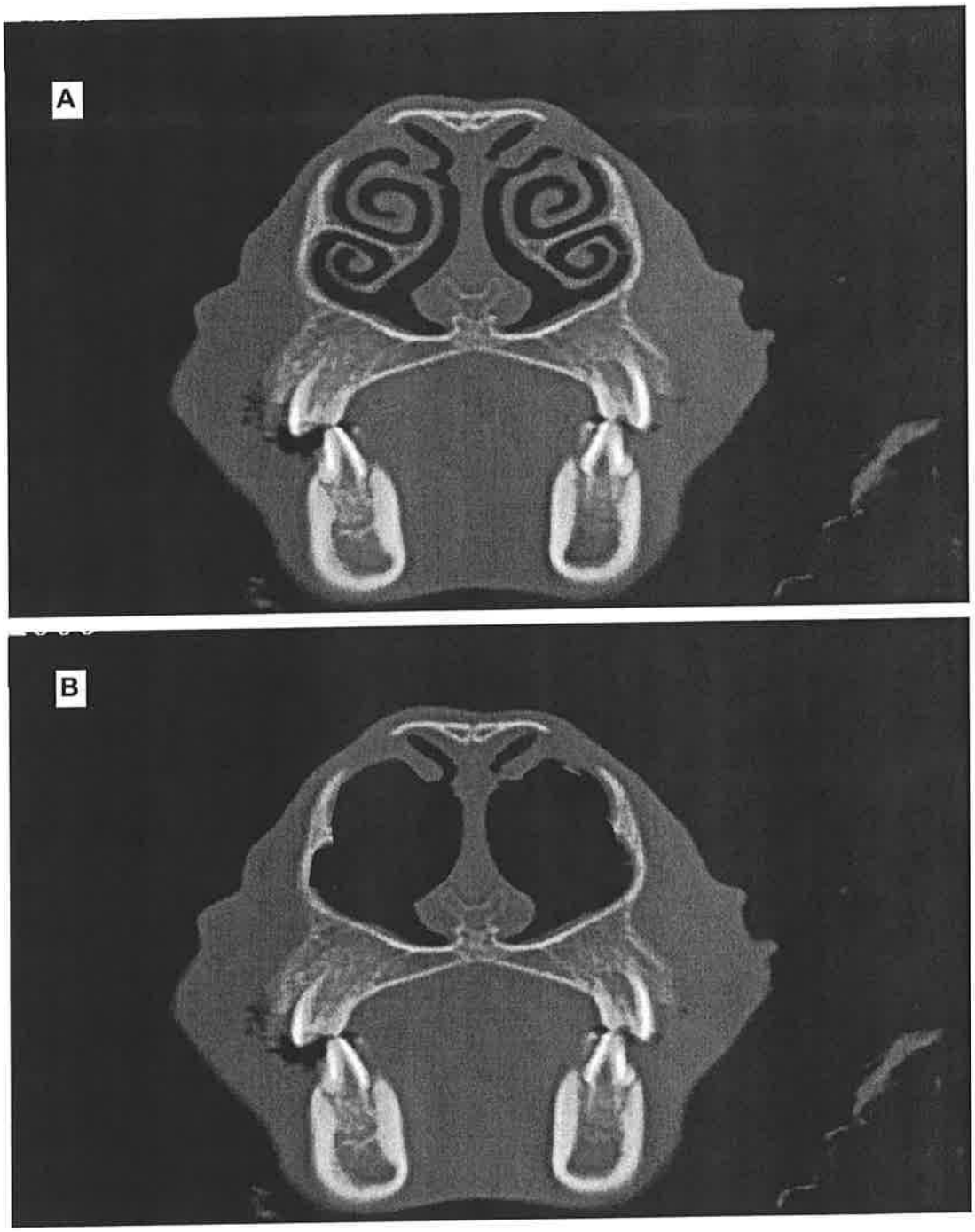

Figure 14-4. Computer generated CT-scan image demonstrating effect of middle turbinate removal. A) Before removal, B) After removal.

detached from the lateral nasal wall. Haemostasis was achieved with 10% cocaine soaked neuropatties and diathermy when required. The pharynx was cleared of blood clot before anaesthesia was withdrawn and the sheep allowed to gain consciousness. Post-operatively, the sheep were observed for any signs of distress. Protocols for post-operative care included the use of intra-muscular analgesia if thought to be required (xylazene). This procedure was well tolerated and no sheep required this injection.

The effect on the removal of the middle turbinate on nasal function has been studied by other members of our group [229]. There is no effect on mucociliary function, histological appearance, or electron microscopic assessment.

Following middle turbinate removal, the sheep were returned to the paddocks for at least four weeks before wounding was performed. This was to ensure that any inflammatory agents elicited due to the removal of the middle turbinate would have dissipated at the time of formal wound creation.

Wound creation

The removal of the middle turbinate exposes the lateral nasal wall. The majority of this mucosa overlies the medial surface of the nasal bones of the sheep. The injury created in the lateral nasal wall was in two adjacent areas, separated only by the ridge of bone that remains after middle turbinectomy. These injuries are made under general anaesthesia using the same anaesthetic protocol described above for middle turbinectomy.

Under endoscopic control with a 0° cystoscope, an anterior mucosal incision is made slightly posterior to the osseo-chondral junction using a Freer's dissector. This is a constant point in the lateral wall of the sheep and is easily identified on repeated endoscopy. Superior and inferior mucosal incisions are made in a posterior direction behind the osseo-chondral junction. The guide to the extent of these incisions on the superior aspect of the lateral nasal wall is the anterior face of the ethmoid turbinate. The landmark for the inferior lateral nasal wall injury is the posterior limit of the middle turbinate ridge. The mucosa to be removed as part of the creation of the wound is elevated in its full thickness as a muco-periosteal flap. This tissue was used to provide baseline specimens for comparison to subsequent investigations. Some tissue was collected at the time of injury for transmission electron microscopy assessment. The size of the wound was standardised to be the same. In each section the wound was 4 by 1 cm. Hence, with two separate wounds on each side, a total area of 4 by 2 cm, or $8cm^2$ was created. A special measuring device developed for wound size assessment was used to ensure uniformity (Figure 15-5). This was also used to enable accurate recording of the location of the wound in relation to fixed landmarks. The anterior face of the ethmoid turbinate superiorly, and the posterior extent of the middle turbinate ridge inferiorly were the landmarks used as reference points. The base of the wound was then debrided using a powered microdebrider under endoscopic control to ensure complete mucosal and periosteal removal. Computer generated images in Figure 15-6 are representative of the area of injury creation, and packing application.

At the time of injury, the nasal packing was inserted on one side. The side on which this occurred was computer randomised. The other side was left unpacked, hence,

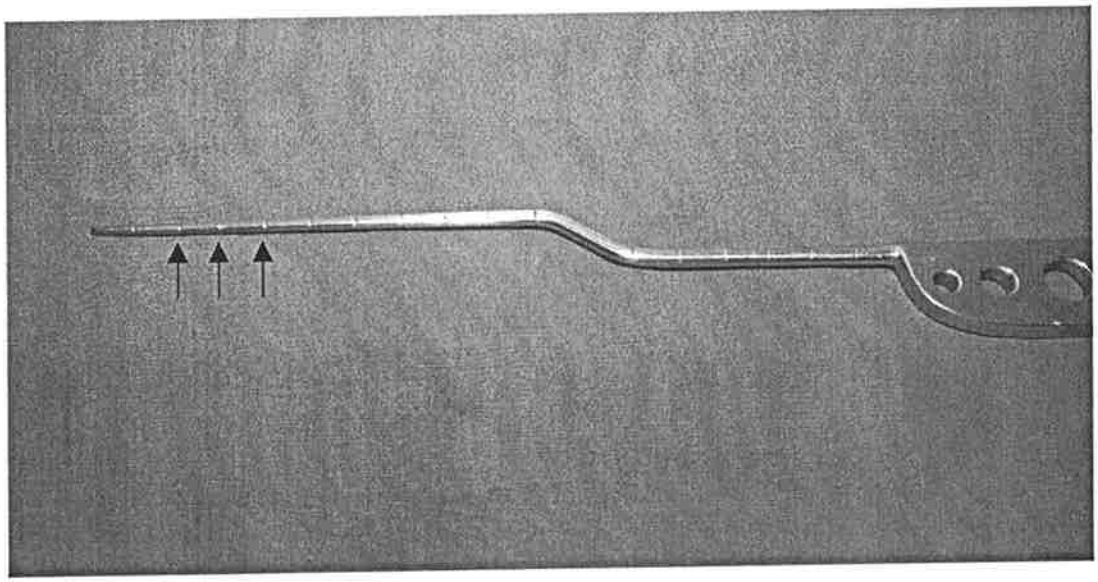

Figure 14-5. Nasal measuring device. Arrows indicate notches on measuring device, each spaced 1 cm apart.

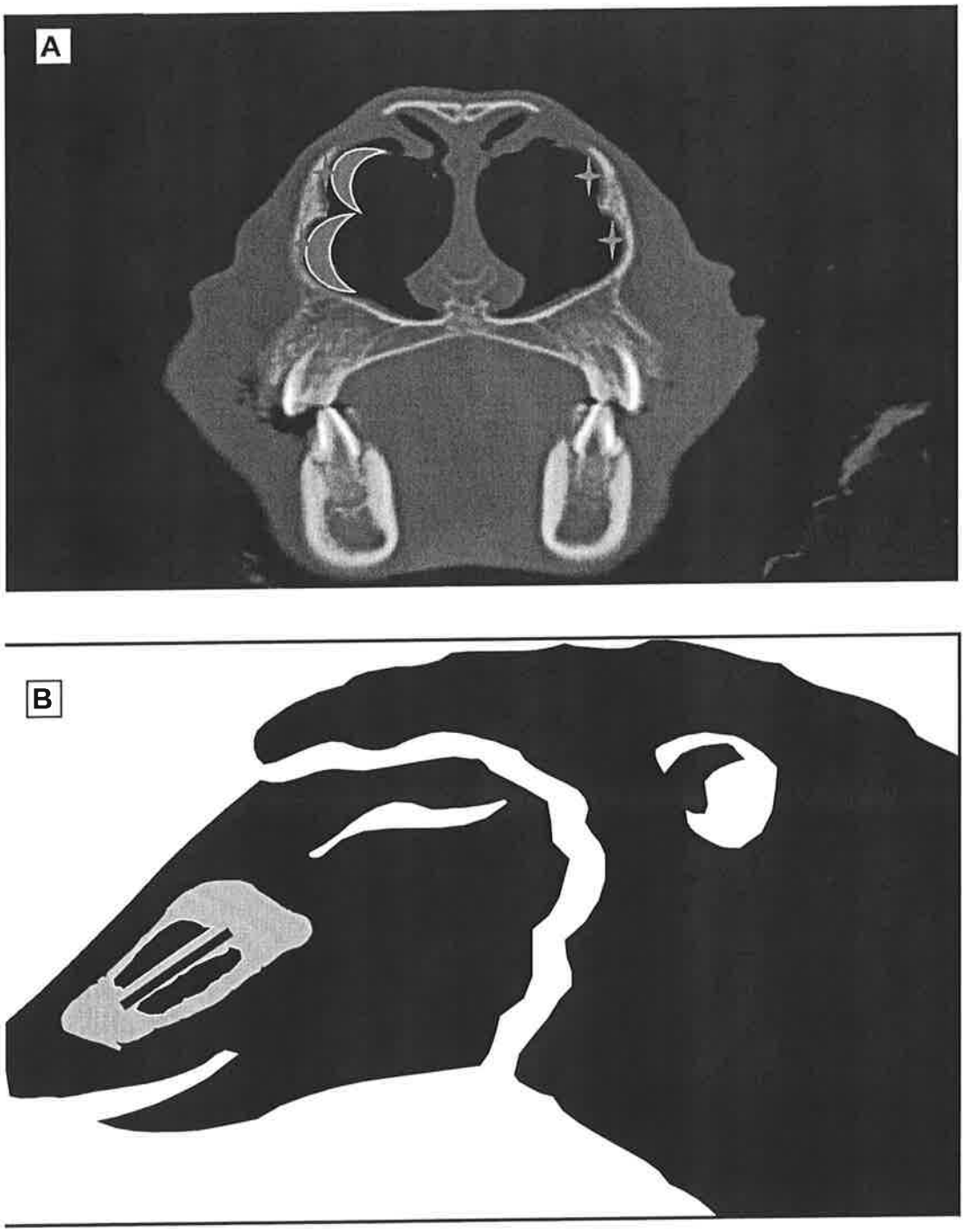

Figure 14-6. Computer generated images indicating lateral nasal wall injury and packing material insertion. A) CT-scan with stars indincating areas of injury to lateral nasal wall and packing on one side, B) Two injuries on lateral nasal wall seperated by remnant crest of bone of middle turbinate.

allowing each sheep to act as its own control. The packing was inserted under endoscopic guidance. Three different types of nasal packing were used. They are a polyvinyl acetate sponge, a hyaluronic acid woven fibre, and the same woven fibre with IGF-I impregnated. The first pack requires removal, and this was done on day five post-operatively. The other two packs are dissolvable, and, hence, do not require removal. If polyvinyl acetate packs were to be used, then two such sponges were inserted into the nasal cavity of the side randomised for packing. They covered the superior and inferior wounds on that side. They were expanded using 10ml of sterile saline (5ml each pack). The presence of these packs did not cause the sheep any distress. Furthermore, the removal of these packs also did not cause any distress. The other two types of packing were also inserted as two separate pieces to cover the wound superiorly and inferiorly. The normal dimensions of these packs are 4 by 4 cm, hence, the choice of 4 cm as the maximum length of the wounded area. The packs were hydrated with sterile saline solution (5 ml each), which is conducive to the packs becoming gelatinous. The sheep were monitored for signs of discomfort or distress after this procedure. Again, no undue distress was observed.

Specimen collection

The sheep had biopsies taken from the wounded area at days 28, 56, 84, and 112 post-injury. The location for each biopsy was standardised. The day 28 biopsy was taken from the anterior half of the superior wound, day 56 from the posterior half of the superior wound, day 84 from the anterior half of the inferior wound, and day 112 from the posterior half of the inferior wound. These time points were chosen because previous work showed that earlier time points yielded friable specimens unsuitable for analysis, and that specimens taken within 4 weeks of each other

showed little change [121]. Furthermore, at day 84, previous research has demonstrated incomplete wound healing [121]. The records obtained from the time of injury that represented the location of the wound were used to guide this process. At each time point, the sheep was sedated with intramuscular xylazene (4mg) and topical anaesthesia was achieved using copheylcaine spray. Under endoscopic control, a mucosal flap was elevated and a biopsy measuring approximately 5 mm by 4 mm was obtained. Two such biopsies were taken from each side at each time point. As mentioned above, baseline specimens were also taken at the time of injury to allow for comparison of the data obtained at these later time points. On day 112 (16 weeks), the sheep were sacrificed by anaesthesia overdose with 6500mg of sodium pentobarbitone IV.

The specimens taken at day 0, 28, 56, 84, and 112 were used for light microscopy and immunohistochemical studies. Specimens at day 56 and 112 were also used for electron microscopy assessment.

3. Light microscopy assessment

Specimen handling and processing

Biopsies for light microscopy studies were fixed in 10% formalin and then preserved in 70% ethanol before being embedded in paraffin wax using a Tissue-Tek® VIP processor. 4μm sections were stained with H&E and studied using light microscopy and image analysis software (ImageMaster Pro).

Measurement of re-epithelialisation of wounded mucosa

The return of the epithelium is an important feature of wound healing. Digital images of the H&E sections were obtained using a digital camera, ImageMaster Pro image analysis software, and a normal light microscope. The percentage re-epithelialisation was determined by measuring the length, in cross-section, of the nasal mucosal surface and the length of this surface that had an epithelial covering.

Epithelial height as a marker of epithelial maturation

The epithelial height has been noted to vary during the healing process of respiratory epithelium [97,122]. The same digital images obtained for re-epithelialisation measurements were also used to measure the average epithelial height. This was the average distance between the basement membrane surface and apical surface of the epithelium.

Cell density and size

The respiratory epithelium is a pseudostratified epithelium. The epithelial height is a marker for epithelial volume. This is based on the following equation:

Epithelial volume = Epithelial height x Epithelial surface area (1)

If the surface area is considered to be a constant, then,

Epithelial volume $\propto$ Epithelial height

Furthermore, it can be used in a calculation of average cell size thus:

Epithelial volume = Total number of cells x Average cell size (2)

Combining (1) and (2):

Epithelial height x Epithelial surface area

= Total number of cells x Average cell size (3)

And rearranging,

Average cell size =

Epithelial height x Epithelial surface area / Total number of cells (3a)

Furthermore,

Epithelial surface area = Epithelial length x Epithelial width (4)

Therefore,

Average cell size =

Epithelial height x Epithelial length x Epithelial width / Total number of cells

(5)

Rather than count the entire number of cells, we can count a number of cells in a field of view and measure the length they occupy. Since, the width of each section is constant, we can infer average cell size (expressed as an index rather than an absolute due to this assumption) by measuring the epithelial height, the length of epithelium, and the number of cells that occupy this length. We can then relate the cell size back to the baseline measurements to determine whether the cell density index is changing over time as the wound heals.

Hence, in the same slides used to measure epithelial height, a count of epithelial cell nuclei and the length they occupied was recorded. These variables were then used to calculate the index to average cell size for each time point. These numbers were also expressed as a ratio to baseline to establish if there were relative changes over time.

4. Immunostaining

Immunostaining was performed for measuring collagen I, collagen III, and IGF-I receptors. As discussed in the section on the assessment of using IGF-I in the sheep animal model, the staining of IGF-I was not reproducible and hence not pursued. The following describes the technique for measuring collagen I and III.

Due to the intense labour involved in imunostaining, only four sheep from each of the three arms were selected. The only criteria for selection of appropriate sheep from each group was the demonstrated histological suitability of both treatment and control specimens at all time points, as determined initially by H&E staining. The specimens were sectioned from the embedded paraffin blocks onto Snow coat X-tra slides. The sections were dewaxed using xylene and graduated ethanol solutions. The slides were then pretreated with boiling target retrieval solution (Dako) for 12 minutes. The solution was then cooled to 50°C and rinsed in PBS before being placed into a pre-warmed (37°C) solution of PBS and trypsin (0.0625g/250ml PBS) for 3 minutes. The specimens were rinsed in PBS and then incubated for 30 minutes at room temperature with 100µl per section of 3% normal horse serum in PBS, which is then rinsed off with PBS and followed by overnight incubation at 4°C with 100µl per section of the primary antibody (rabbit anti-collagen I (Rockland) 1:200 in PBS or rabbit anti-collagen III (Rockland) 1:200 in PBS) in an airtight container. The next day, the primary antibody solution was rinsed off with PBS and 100µl per section of the secondary biotinylated antibody (anti-rabbit IgG (Sigma) 1:200 in PBS) was added and incubated for 60 minutes at room temperature in the airtight box. Then Streptavidin cy3 (Sigma) 1:200 in PBS was added and incubated in an airtight black box for 45 minutes before being rinsed off with PBS. A flourescent-mounting medium (Dako) was used to allow examination under a fluorescent

microscope with image analysis software (Image Pro Plus, Media Cybernetics) used to measure the intensity. The integrated optical density (IOD) was calculated for each specimen by dividing the average (mean) intensity by the area measured. The ratio of collagen I to III was then calculated as a means of assessing the maturation of the collagen profile.

5. Electron microscopy

Development of methods

Several different techniques were employed before the final protocol was derived. Initially trial specimens were fixed in a solution of 4.0% paraformaldehyde and 1.25% glutaraldehyde in phosphate buffered saline, pH 7.2. These specimens were then examined using environmental surface electron microscopy. This SEM technique has the advantage of being quick, with specimens not requiring any special processing. A representative image is evident in Figure 15-7. The quality obtained was thought to be substandard and hence, conventional SEM was trialled. The specimens for this trial were fixed using the same solution as above. A representative image is evident in Figure 15-8. The problem identified with this technique was excess surface mucus and blood obscuring the view. Hence, the next trial specimens were again fixed as described above but then put into an agitator known as a "sonicator" in an attempt to remove this surface blood and mucus before specimen processing. A representative image is evident in Figure 15-9. Again, whilst deemed an improvement, the problem was still present. However, given the improvement, it was decided to sonicate the specimens twice. A representative

image is shown in Figure 15-10. Unfortunately, the effect of two sonications of the

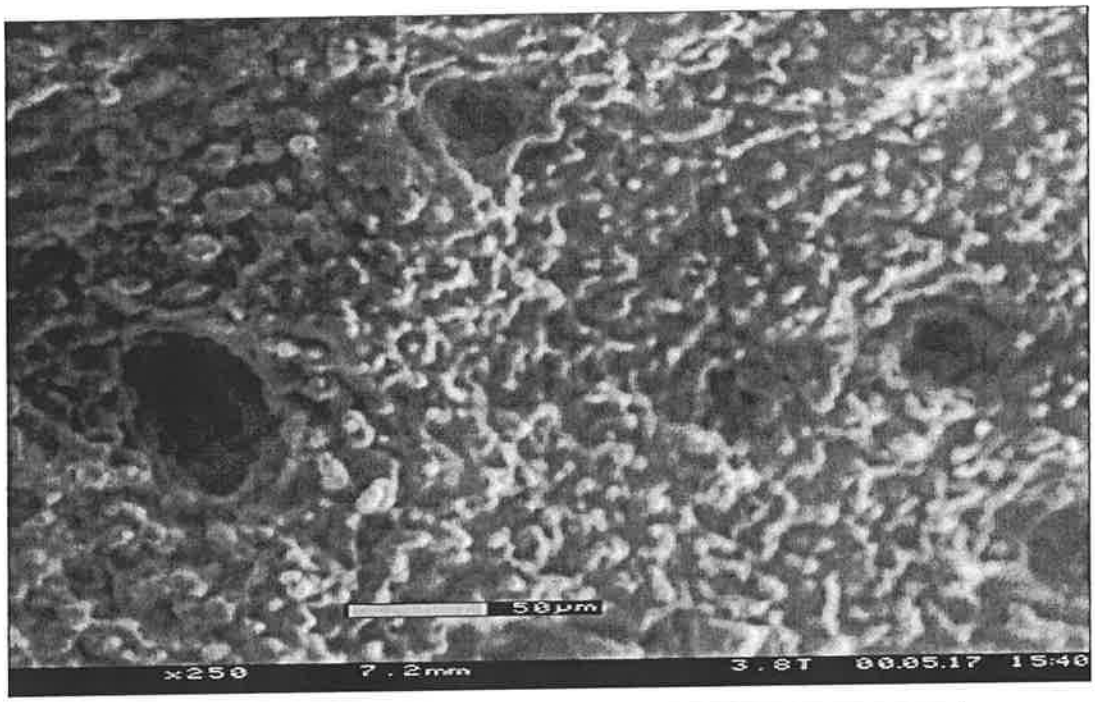

Figure 14-7. Representative image of demonstration of cilia using Environmental surface electron microscopy.

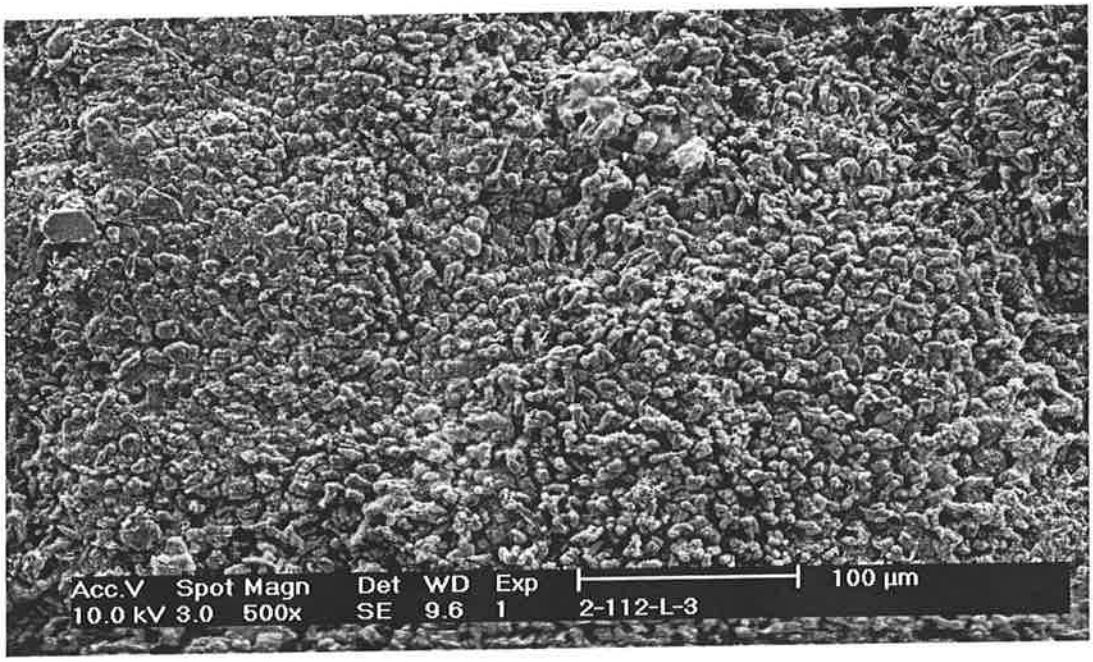

Figure 14-8. Surface electron microscopy demonstration of cilia without previous sonication. Mucus obscures the view.

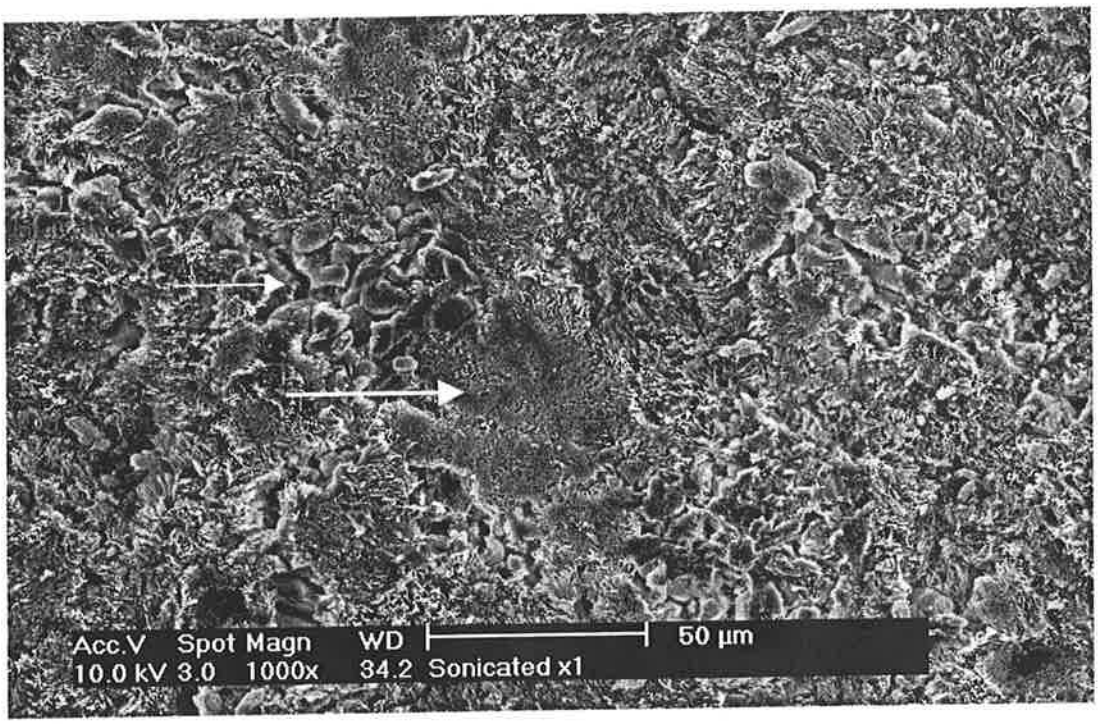

Figure 14-9. Surface electron microscopy demonstration of cilia after sonication once. Cilia (large arrow) are seen with some mucus (small arrow) obscuring the view.

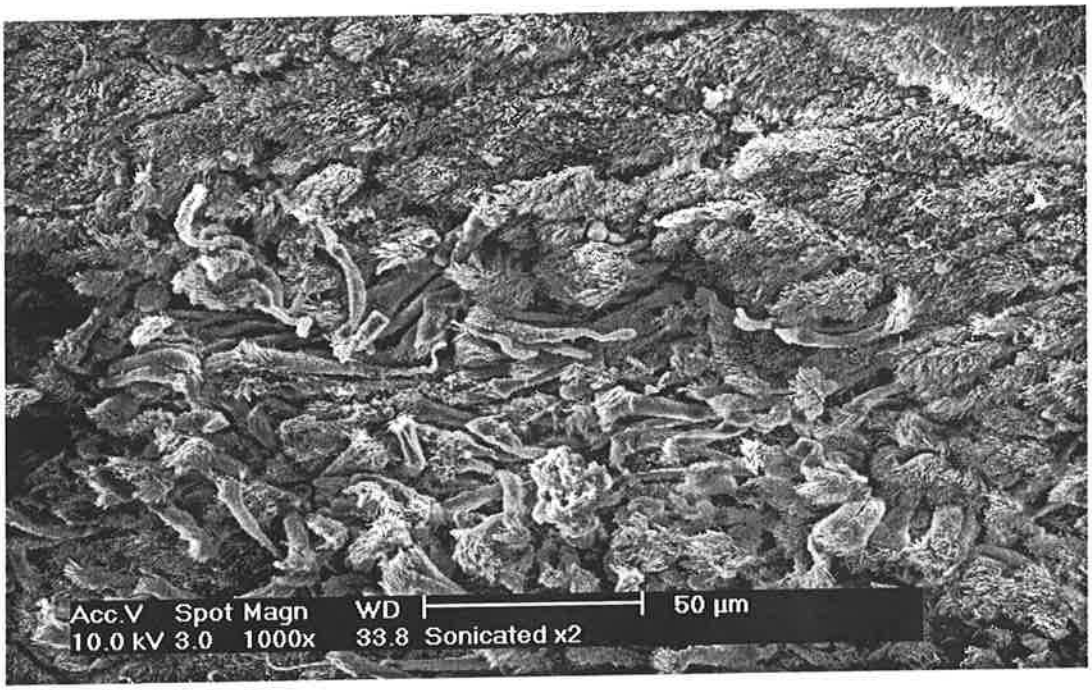

Figure 14-10. Surface electron microscopy demonstration of cilia after sonication twice. The lower half of the image demonstrates cell dislogdement from the basement membrane.

specimen was to dislodge the epithelial cells from their basement membrane attachment. As an alternative, the specimens were not fixed before sonication but rather after it. A representative image at 500x and 2000x magnification is demonstrated in Figure 15-11. This was a more successful approach and, hence, was adopted. It is described presently.

Specimen handling and processing

Specimens were taken at day 56 and 112 for electron microscopic assessment of cilia. They were initially placed in phosphate buffered solution and then attempts to remove surface blood or mucus using sonication were made. The specimens were then fixed in a solution of 4.0% paraformaldehyde and 1.25% glutaraldehyde in phosphate buffered saline, pH 7.2 and stored at 4°C until processed.

The specimens were removed from the fridge and fixed using an automated processor. This involves specimen dehydration using serial strengths of ethanol and acetone. The processing of the samples involved a sequence of treatments with different media. These media were Osmium tetroxide, 70% ethanol, 90% ethanol, 95% ethanol, 100% ethanol, a half and half mixture of 100% ethanol and 100% acetone, and finally 100% acetone. After this, the specimens were dried using a carbon dioxide critical point dryer. They were then mounted on EM stubs and subsequently coated with gold and carbon.

Ciliary measurements

Surface images were obtained using the Philips field emission scanning electron microscope Excel 30 and saved in TIF image format. The images were taken at both

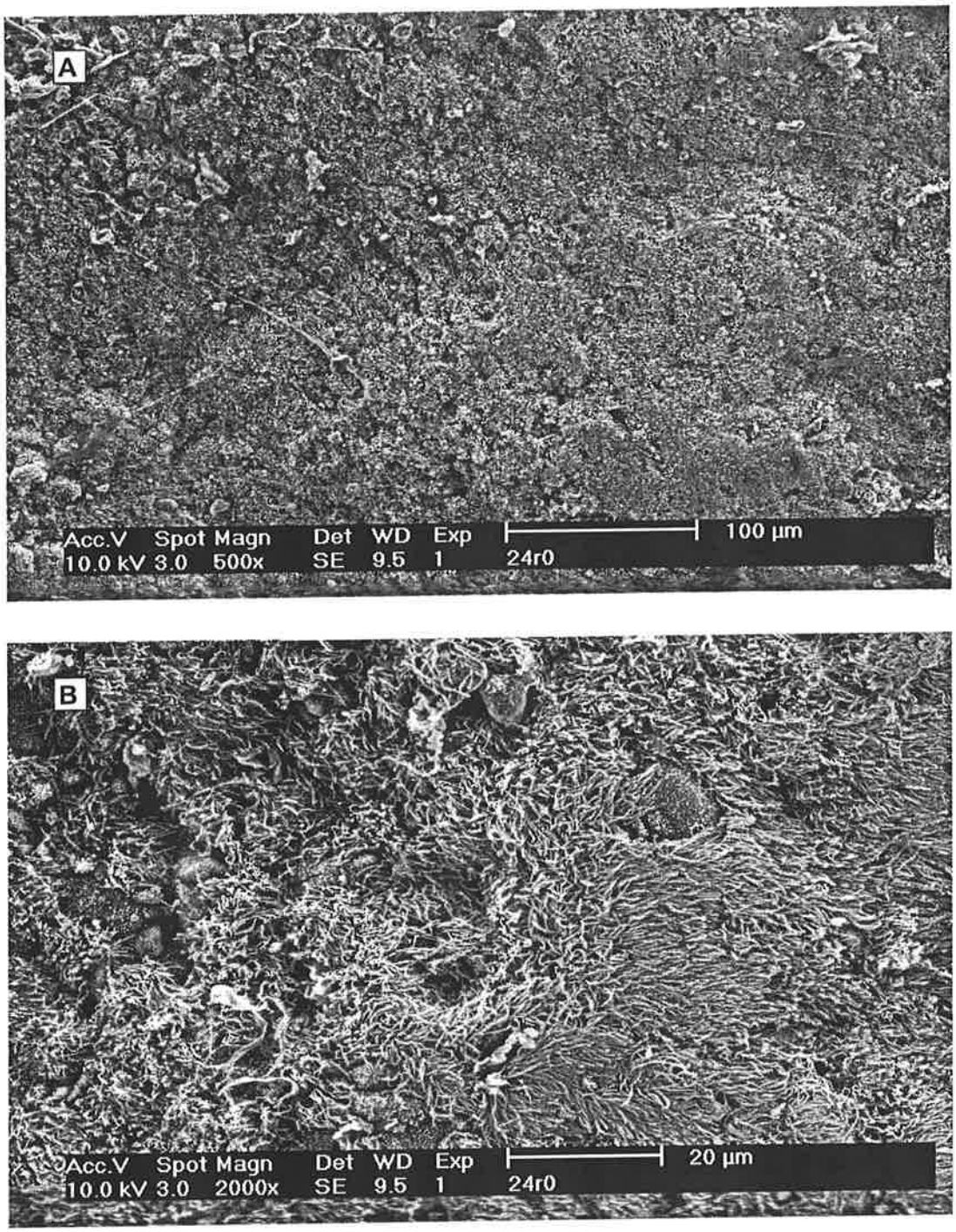

Figure 14-11. Surface electron microscopy images of cilia sonicated before tissue fixation. A) 500x magnification, B) 2000x magnification.

500x and 2000x magnification for all suitable specimens. Where possible, 4 images were obtained at each magnification. In this study we used AnalySIS Pro® (Soft Imaging Systems GmbH) (version 3.00). This software allows digital images to be assessed on an intensity scale. This is advantageous as cilia are electron dense and appear bright compared to the dark background. Hence, the cilia can be differentiated from the background by the software. The image analysis software was used to measure the area on the biopsy covered by cilia. Specimens that were rejected as being unsuitable had surface blood and/or mucus still obscuring the visualised field. Some specimens were excluded due to fungal overgrowth, which obscured the field.

The percentage of surface cilia coverage was measured on specimens at 500X magnification using image analysis software (analySIS- Software Imaging Solutions). This was done based on the difference in intensity of ciliated and unciliated epithelial surfaces.

The technique of combining SEM and image analysis for calculating cilial coverage was validated. This was done by independent measurement by two blinded investigators (David McIntosh and Dr Leslie Shaw, Queen Elizabeth Hospital) on two different occasions. Dr Shaw provided the specimens. The results for both observers are summarised in the appendix to chapter 15.

The difference between the 1st and 2nd assessments for each observer was very small (mean 6.335 %, Standard deviation (SD)= 9.191, median 3.245%; mean 6.08 %, SD=5.02, median 4.13 %, respectively). In addition, the correlation between the 1st

and 2nd assessments for each observer (LS, DM) was high and statistically significant ($p<0.05$) (Pearson correlation coefficient $r = 0.533$, $p = 0.023$; $r = 0.549$, $p = 0.018$, respectively). Therefore, there was little intra-observer variability for each blinded observer's assessment. When comparing the results between the observers, the difference between the observer's assessments was small (mean of the differences between the observer's assessments 7.47 %, SD= 5.67; median 5.13 %). The two blinded observers assessments were found to have a high degree of correlation and the correlation is significant at the 0.05 level (Pearson correlation coefficient $r = 0.508$, $p = 0.031$).

Transmission electron microscopy

This method of electron microscopy has also been used to assess cilia. Traditionally it has been most useful to investigate and characterise ciliary abnormalities. The purpose for its use in this trial was simply to characterise the ciliary ultrastructural details of the sheep cilia and compare these properties to the documented human ultrastructure. Specimens obtained for this study were obtained at the time of initial injury in the first three sheep (two per sheep).

These six specimens were fixed immediately in a solution of 4% paraformaldehyde, 1.25% glutaraldehyde in PBS, with 4% sucrose, pH 7.2. After 24 hours, the specimens were washed in a solution of PBS and 4% sucrose before being further fixed in a solution of 2% osmium tetroxide for 1 hour. They were then dehydrated using serially concentrations of ethanol (70%, 90%, 95%, and 100%) for 3 x 20 minute immersions at each concentration, with an additional 60 minutes of 100%

ethanol. They were then resin infiltrated by immersion in a solution of half 100% ethanol, half TEM resin overnight followed by 3x 8 hour immersions in 100% resin. They were then embedded in fresh resin and polymerised in a 70°C oven for 24 hours. The representative images from this trial are evident in Figures 15-12 and 15-13.

As can be seen in Figures 15-12A and 15-B, the cross-section of cilia is difficult to display in a perfect perpendicular plane. However, as highlighted in this Figure 15-12C (arrows) the cilia ultrastructure conforms to the '9+2' arrangement. Furthermore, the arrow in Figure 15-13 demonstrates the presence of short claws at the tip of the cilium. It also shows the longitudinal presence of the ultrastructural components along the length of the cilium.

From the perspective of this research, it was decided not to use TEM in the assessment of wound healing and ciliogenesis. The reasons for this include the small area of sample size represented by these images, which may not be representative, and that it is very difficult to obtain sections in the exact plane required for meaningful interpretation.

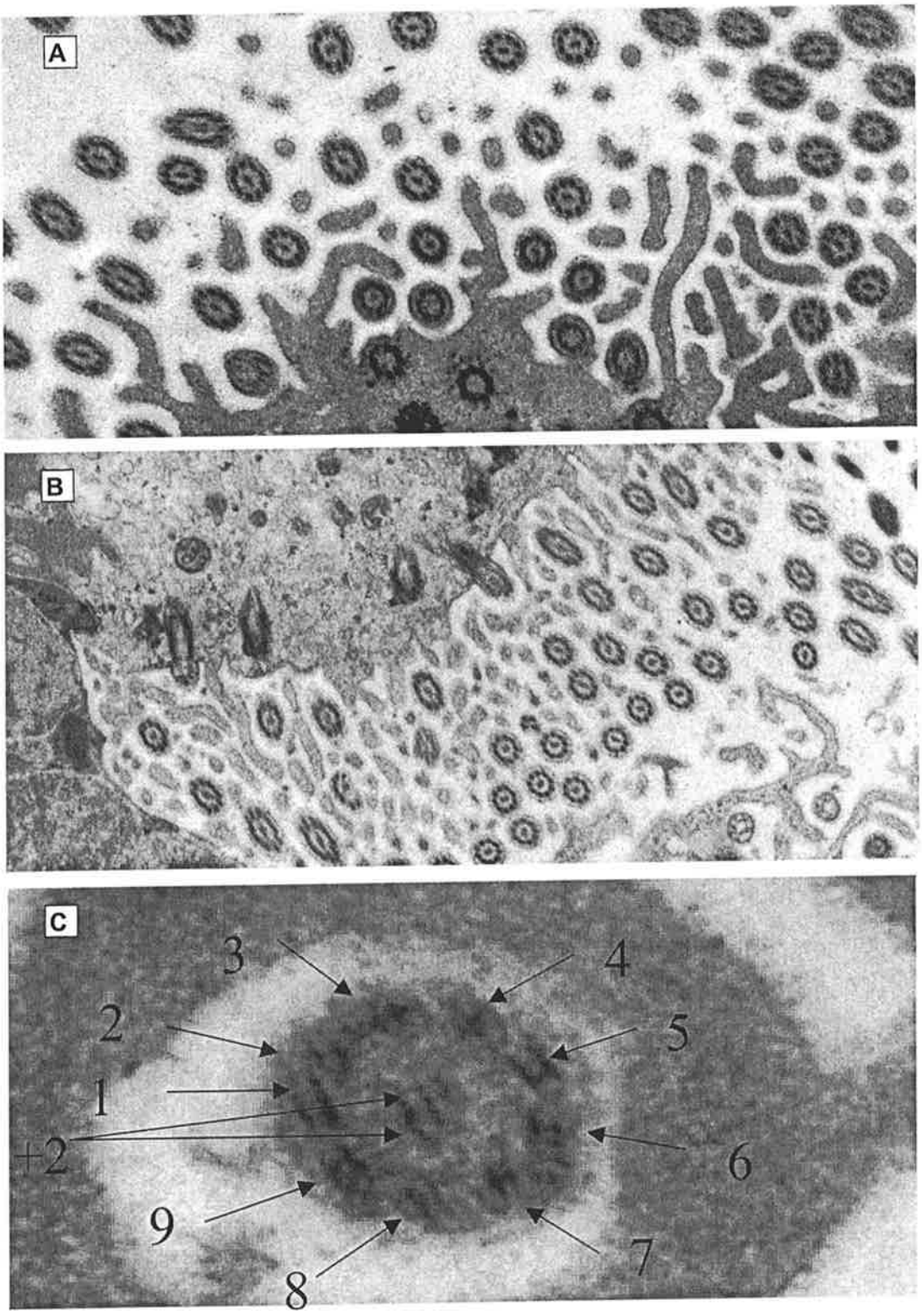

Figure 14-12. Transmission electron microscopy cross-sections of cilia. A) and B) cilia in cross-section, with C) demonstrating '9+2' pattern.

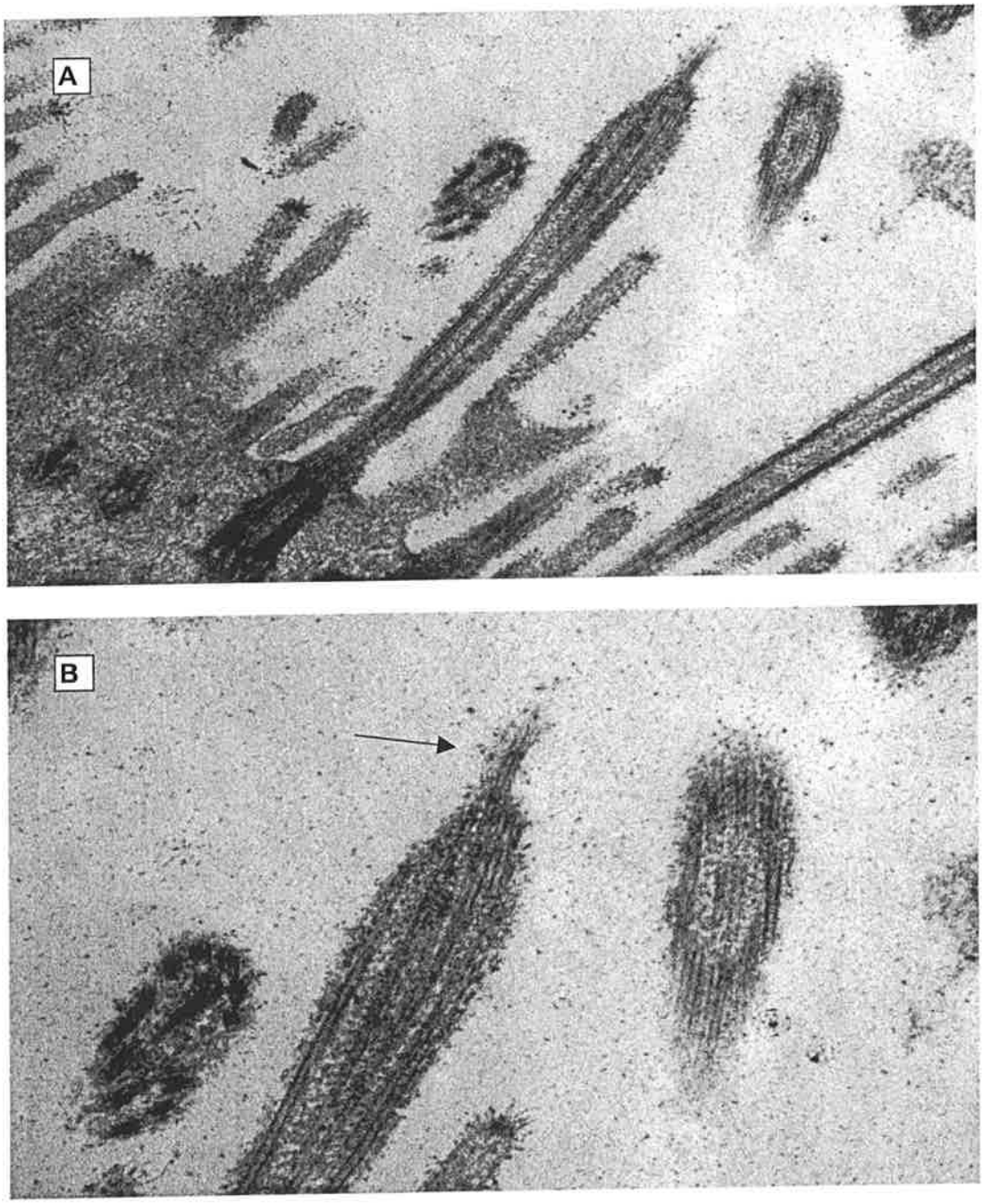

Figure 14-13. Transmission electron microscopy of longitudinal section of cilia. A) Cilia demonstrated in logitudinal section, B) Magnified view of (A) with demonstration of presence of 'claw' at tip of cilium (arrow).

Results

All data pertaining to these results is summarised in the Appendix to Chapter 15.

1. Merocel nasal packing versus control

There were 12 sheep in this trial initially but one died of natural causes during the course of the experimentation, and hence, there are a total of 11 sheep that were used in this arm. Measurements of re-epithelialisation, epithelial height, average cell volume index and ratios were performed for all animals in suitable specimens at all time points. Reciliation was recorded using SEM at days 56 and 112. Collagen I and III measurements were performed in 4 sheep that had suitable H&E sections at all time points. This number was chosen due to the high labour intensity associated with this technique. There was no bias in the selection of these 4 sheep other than prior determination of their suitable H&E stained sections.

Light microscopy

The results of re-epithelialisation are shown in Figure 15-14 (see also Appendix to chapter 15). This demonstrates a slow rate of re-epithelialisation in both the packed and unpacked wounds. The rates of re-epithelialisation were found to be similar. Even at day 112 the percentage of re-epithelialisation was incomplete for both the polyvinyl acetate packed wounds (88%, standard error of mean 6.03%) and for the control (86%, standard error of mean 5.52%) wounds.

Epithelial height was measured as a marker of epithelial maturation. The height of the epithelium was measured at all time points and expressed as a relative value of

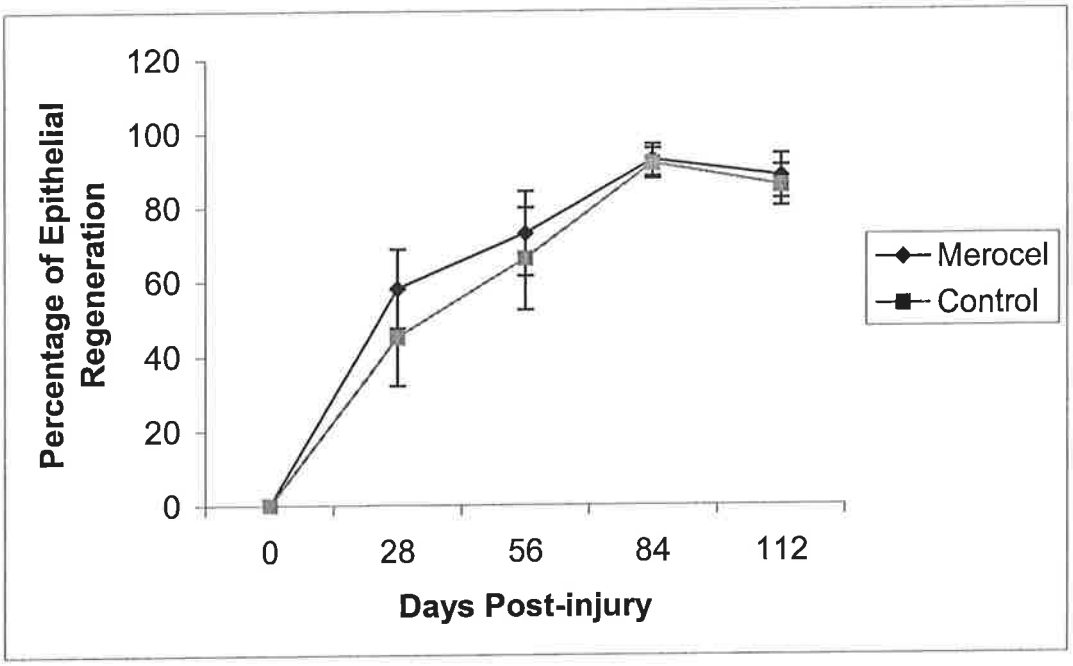

Figure 14-14. Graph of epithelial regeneration over time. Comparison of Merocel packed wounds to control wounds. Error bars indicate standard error of mean.

the original baseline height (adjusted to equal 1) (Figure 15-15) (see also Appendix to chapter 15). The relative heights of the control wounds were approximately 1.5 times the original epithelial thickness throughout all time points (day 28= 1.45, day 56= 1.42, day 84= 1.27, day 112= 1.67). The relative epithelial heights of the polyvinyl acetate packed wounds were higher than controls from day 28 to 84, and similar at day 112 (day 28= 2.30, day 56= 2.12, day 84= 1.96, day 112= 1.64) (see also Appendix to chapter 15). The difference between the relative heights of the packed and control wounds was not significantly different at any time point ($p>0.05$). Representative pictures of nasal mucosa at day 0 and 28 show this increase in epithelial thickness (Figure 15-16).

Average cell size

Measurement of epithelial height and cell density allows the calculation of an index of cell size. By comparing this index at each time point to the baseline it is possible to determine if there is a change in the cell numbers in the healing wound. The result for this measurement is demonstrated in Figure 15-17 (see also Appendix to chapter 15). At day 28, the index was similar for control (1.45, standard error of mean 0.43) and packed wounds and (1.44, standard error of mean 0.37). At day 56, these ratios were 1.51 for control wounds (standard error of mean 0.49) and 2.08 for packed wounds (standard error of mean 0.43). These ratios fell at day 84 to 0.90 for control wounds (standard error of mean 0.15) and 1.27 for packed wounds (standard error of mean 0.30). At day 112 the ratios were 1.66 for control wounds (standard error of mean 0.58) and 1.36 for packed wounds (standard error of mean 0.35). There was no significant difference between packed and unpacked wounds.

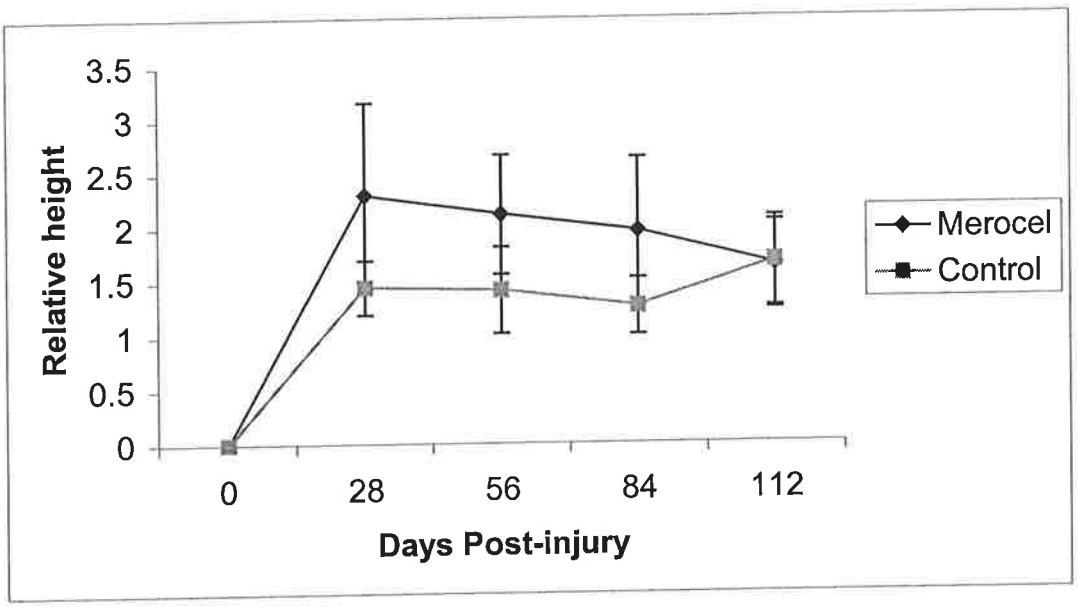

Figure 14-15. Graph of relative epithelial height over time. Comparison of Merocel packed wounds to control wounds. Heights adjusted relative to baseline (designated to equal 1). Error bars indicate standard error of mean.

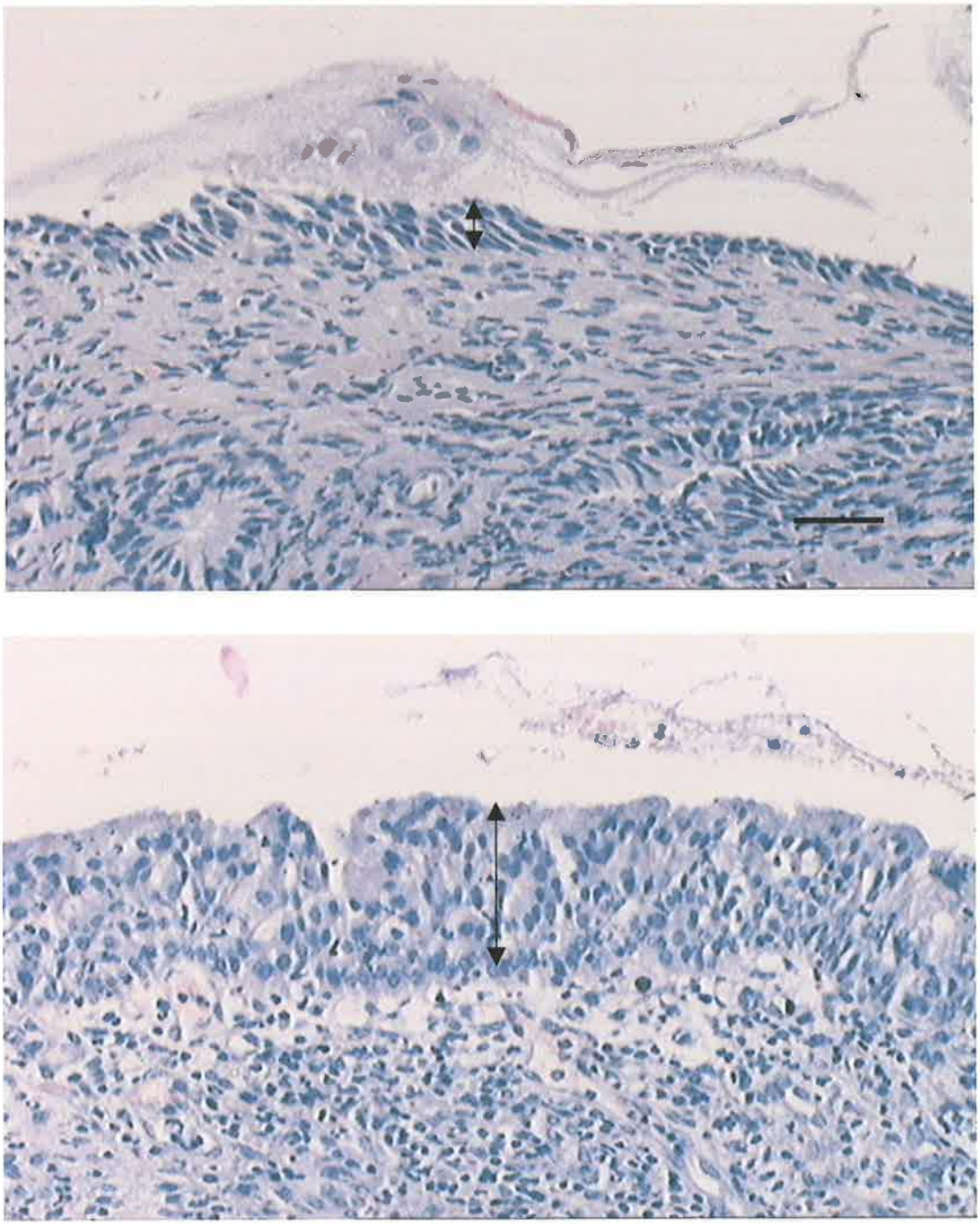

Figure 14-16. Representative pictures of nasal epithelium demonstrating changes in epithelial thickness. A) Normal epithelium at time of injury, B) Day 28 regenerated epithelium in Merocel packed wounds. Differences in epithelial height are indicated by the arrows. Scale bar = 50 um.

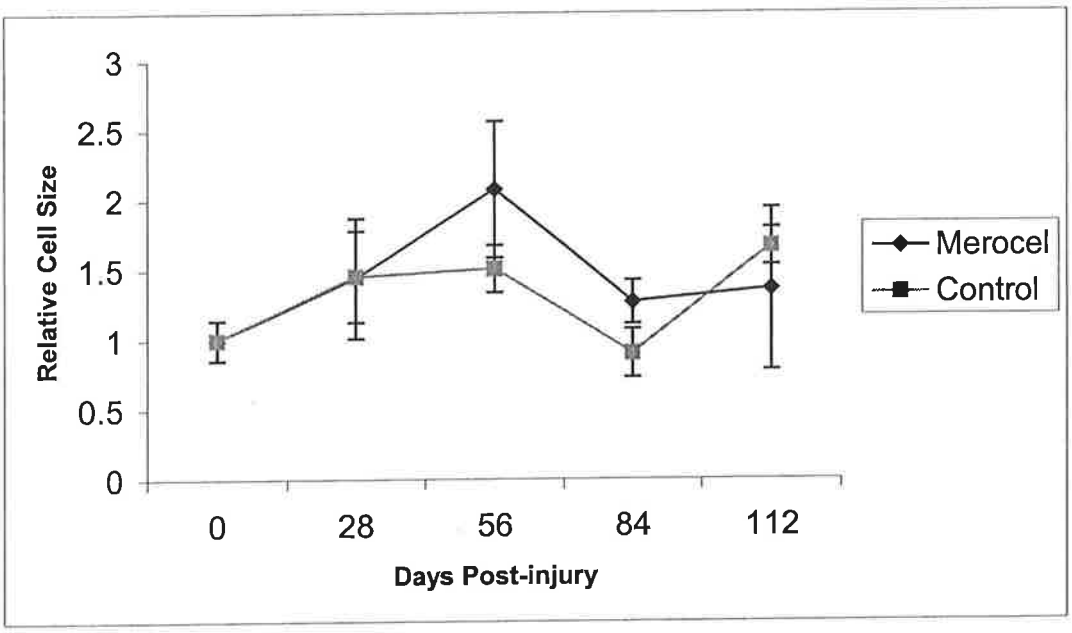

Figure 14-17. Graph of relative average cell size over time. Comparison of Merocel packed wounds to control wounds. Cell size related to baseline (designated to equal 1). Error bars indicate standard error of mean.

Immunostaining

Immunostaining for collagens I and III was performed. The integrated optical density was calculated and expressed as a ratio of collagen I:collagen III. These ratios were compared to the baseline ratio (relative to a baseline of 100). An increase in the relative measures would be representative of early predominance of collagen I compared to collagen III, which would be indicative of increased submucosal healing. The result is demonstrated in Figure 15-18 (see also Appendix to chapter 15). There was no significant difference between packed and unpacked wounds. At day 28 the relative ratio was 92.87 for packed wounds (standard error of mean 24.23), compared to 173.53 for control wounds (standard error of mean 54.51). At day 112, it was a 68.62 for packed wounds (standard error of mean 18.41) and 76.50 for control wounds (standard error of mean 22.09).

SEM

Scanning electron microscopic analysis of cilia present on the epithelium was performed at days 56 and 112 post-wounding at 500x. A representative scanning electron microscopic picture of cilia from both packed and control wounds taken at 500X magnification at day 112 is shown in Figure 15-19. The results of the cilial regeneration are summarized in Figure 15-20 (see also Appendix to chapter 15). There was no significant difference in total amount of cilia regeneration between packed and unpacked wounds. SEM confirmed incomplete (71% packed, 73% control) regeneration of cilia at day 112 in both groups.

An estimation of cilial maturity was made at 2000X magnification by identifying the short or stubby cilia [234] (Figure 15-21) (see also Appendix to chapter 15). At day

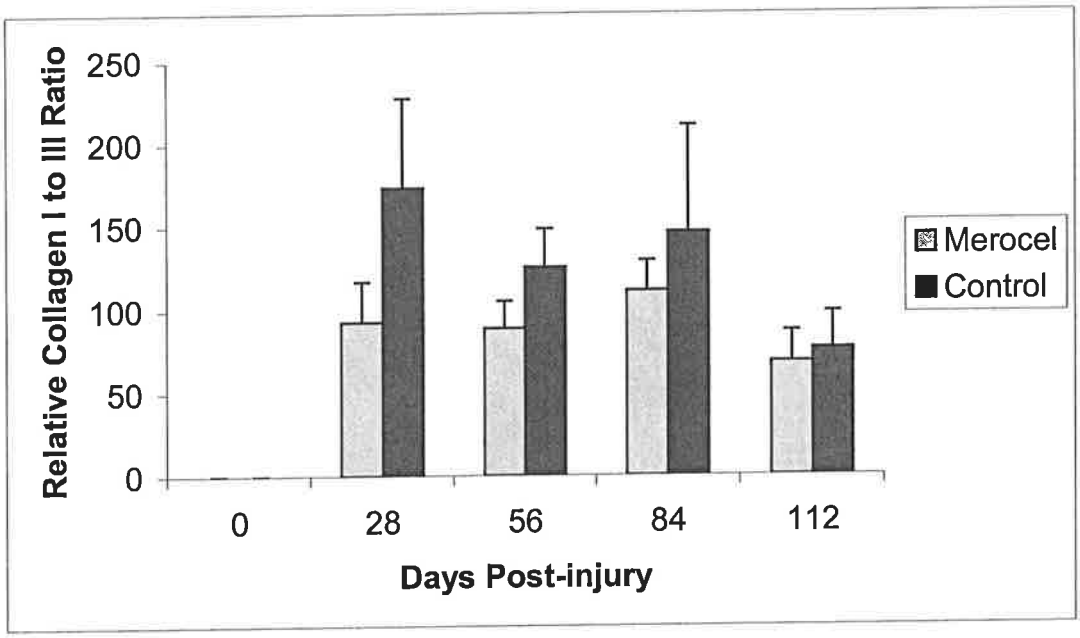

Figure 14-18. Graph of relative collagen I to collagen III ratios over time. Comparison of Merocel packed wounds to control wounds. Error bars indicate standard error of mean.

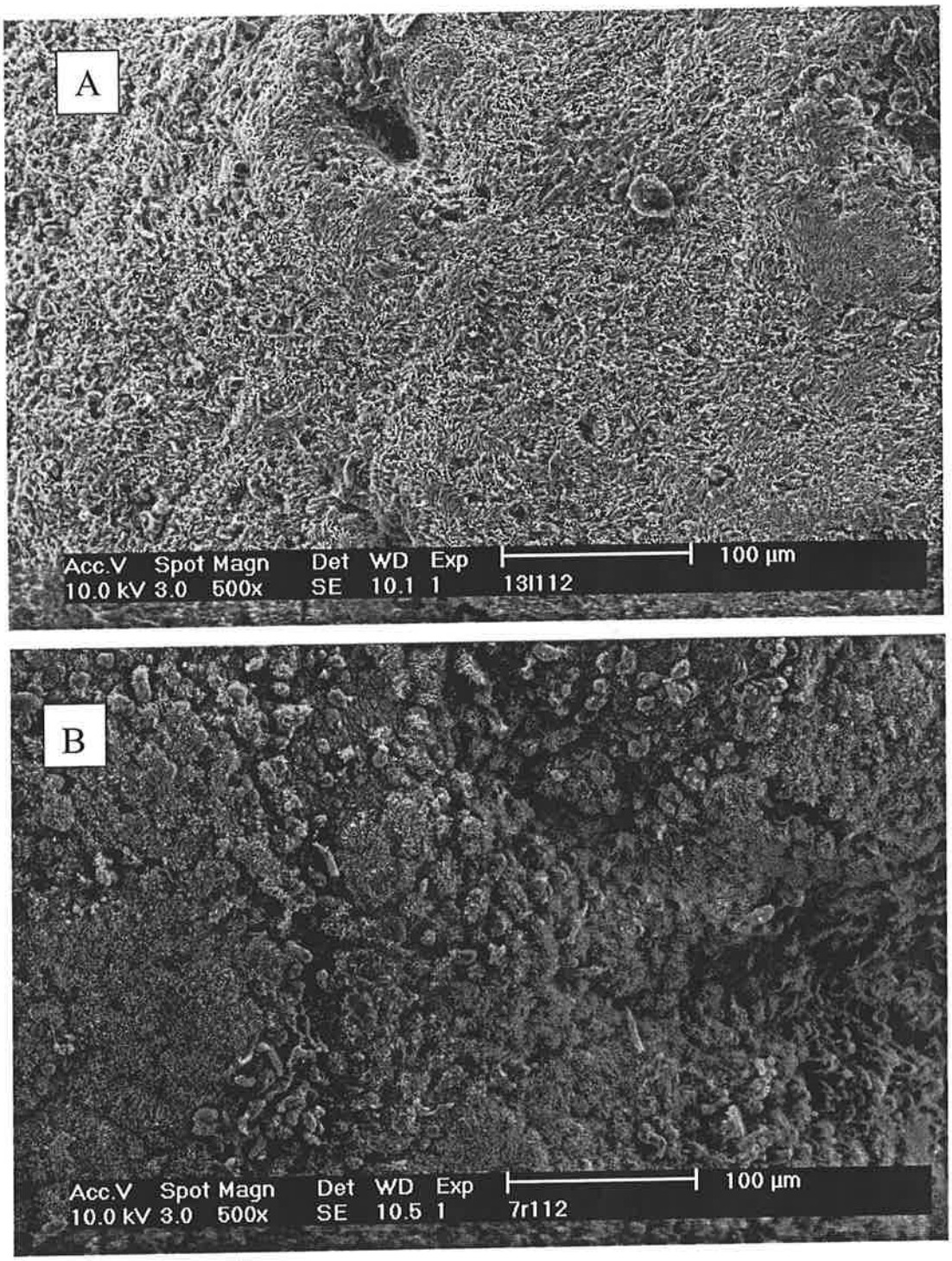

Figure 14-19. Surface electron microscopy images of Merocel packed and control wounds at day 112 at 500x magnification. A) Control wound, B) Merocel wound.

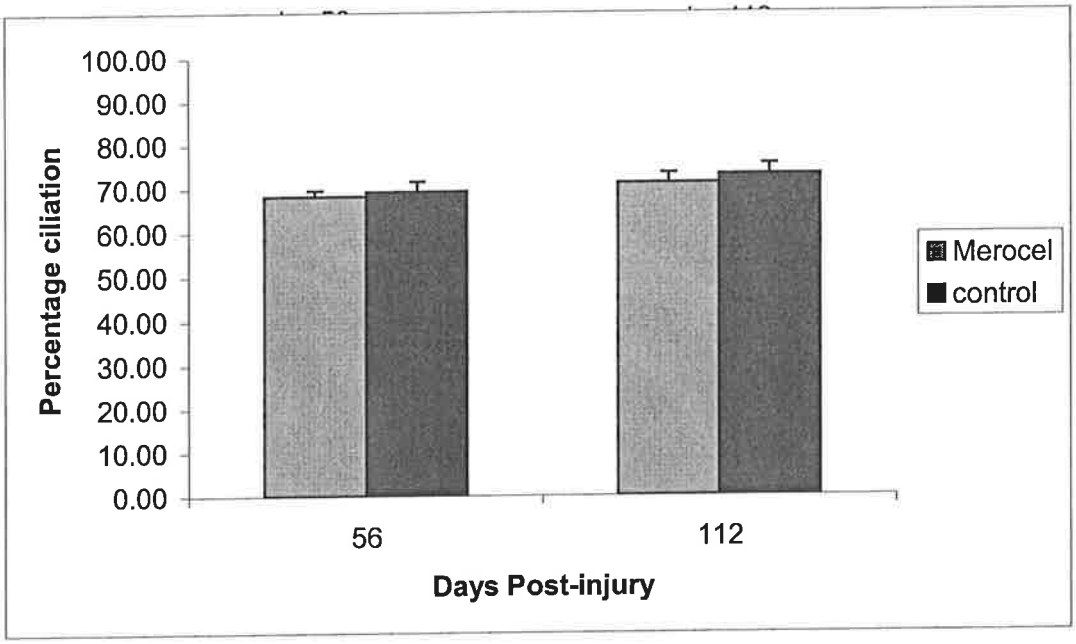

Figure 14-20. Graph of reciliation over time. Comparison of Merocel packed wounds to control wounds. Error bars indicate standard error of mean.

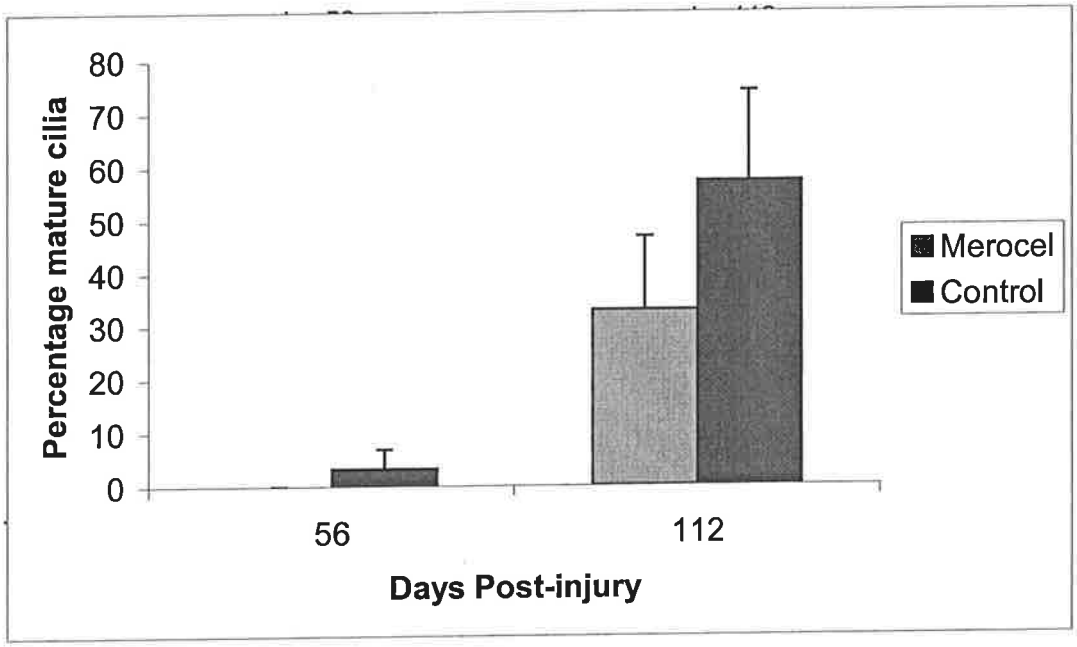

Figure 14-21 Graph of cilia maturity over time. Comparison of Merocel packed wounds to control wounds. Error bars indicate standard error of mean.

56 the percentage of mature cilia in the polyvinyl acetate packed wounds was 0% compared to 3% for unpacked wounds, which was not significant. There was no statistical difference at day 112, where 33% of the cilia seen were mature in the polyvinyl acetate nasal packed side compared to 57% in the control groups.

2. Merogel nasal packing versus control

There were 13 sheep in this trial arm. Measurements of re-epithelialisation, epithelial height, average cell volume index and ratios were performed for all animals in suitable specimens at all time points. Reciliation was recorded using SEM at days 56 and 112. Collagen I and III measurements were performed in 4 sheep that had suitable H&E sections at all time points. This number was chosen due to the high labour intensity associated with this technique. There was no bias in the selection of these 4 sheep other than prior determination of their suitable H&E stained sections.

Light microscopy

The percentage of the nasal mucosa that had re-epithelialized at 0, 28, 56, 84 and 112 days post wounding was histologically assessed. At day 84 there was a statistically significant difference ($p<0.05$) between the re-epithelialisation of control (83.39%, standard error of mean 4.23%) and packed (94.33%, standard error of mean 2.22%) wounds (Figure 15-22) (see also Appendix to chapter 15). There was no statistically significant difference in the rate of re-epithelialisation between the side of the nasal cavity packed with the hyaluronic acid based pack compared to control wounds at the other time points.

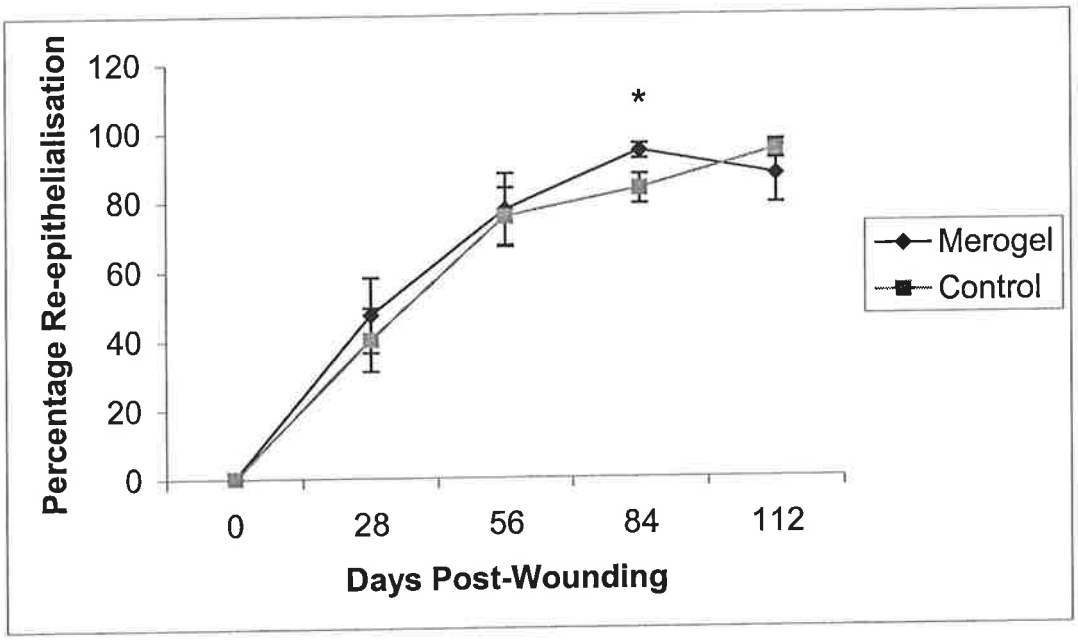

Figure 14-22. Graph of epithelial regeneration over time. Comparison of Merogel packed wounds to control wounds. Error bars indicate standard error of mean. *=$p<0.05$

Epithelial height was measured as a marker of epithelial maturation. The height of the epithelium was measured at all time points (Figure 15-23) (see also Appendix to chapter 15). The epithelial height is expressed as a relative value of the original baseline height. The relative height of the epithelium on the side packed with the hyaluronic acid based Merogel® (1.75, standard error of mean 0.38) was statistically significantly increased when compared to the percentage height of the epithelium in the control (0.81, standard error of mean 0.14) ($p<0.05$) at day 28 (Figure 15-23). There was no statistically significant difference between the measured relative heights at the latter time points.

Average cell size

Measurement of epithelial height and cell density allows the calculation of an index of cell size. By comparing this index at each time point to the baseline it is possible to determine if there is a change in the cell numbers in the healing wound. The result for this measurement is demonstrated in Figure 15-24 (see also Appendix to chapter 15). There was no statistically significant difference between packed and unpacked wounds. At day 28 the relative cell size was 1.41 for packed wounds (standard error of mean 0.33), compared to 0.63 for control wounds (standard error of mean 0.19). At day 112, it was a 1.17 for packed wounds (standard error of mean 0.16) and 1.07 for control wounds (standard error of mean 0.19).

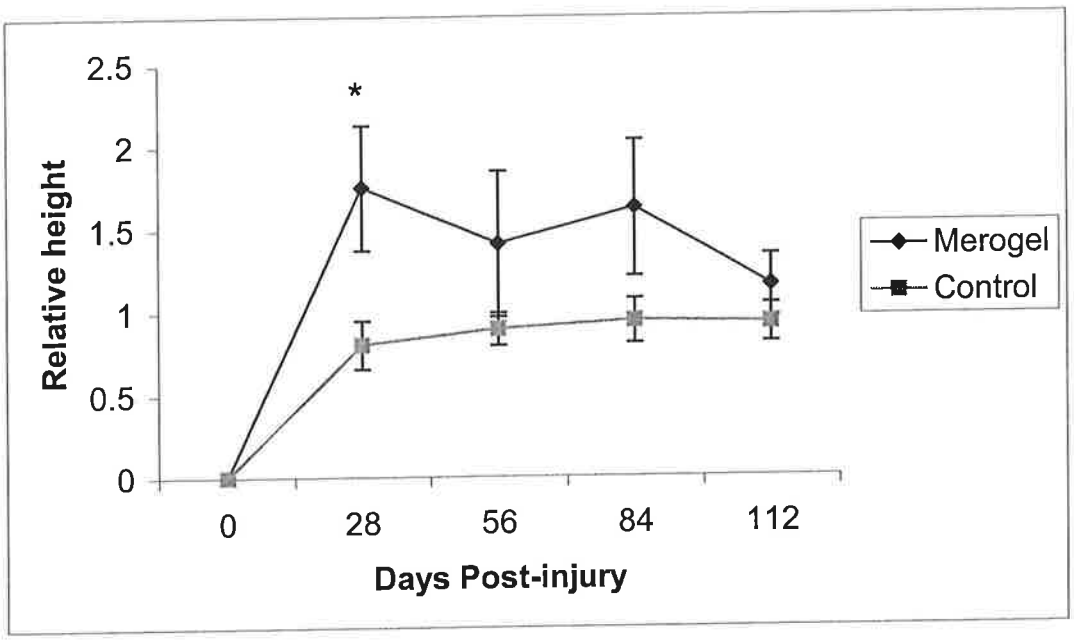

Figure 14-23. Graph of relative epithelial height over time. Comparison of Merogel packed wounds to control wounds. Epithelial height related to baseline (designated to equal 1). Error bars indicate standard error of mean. *=p<0.05

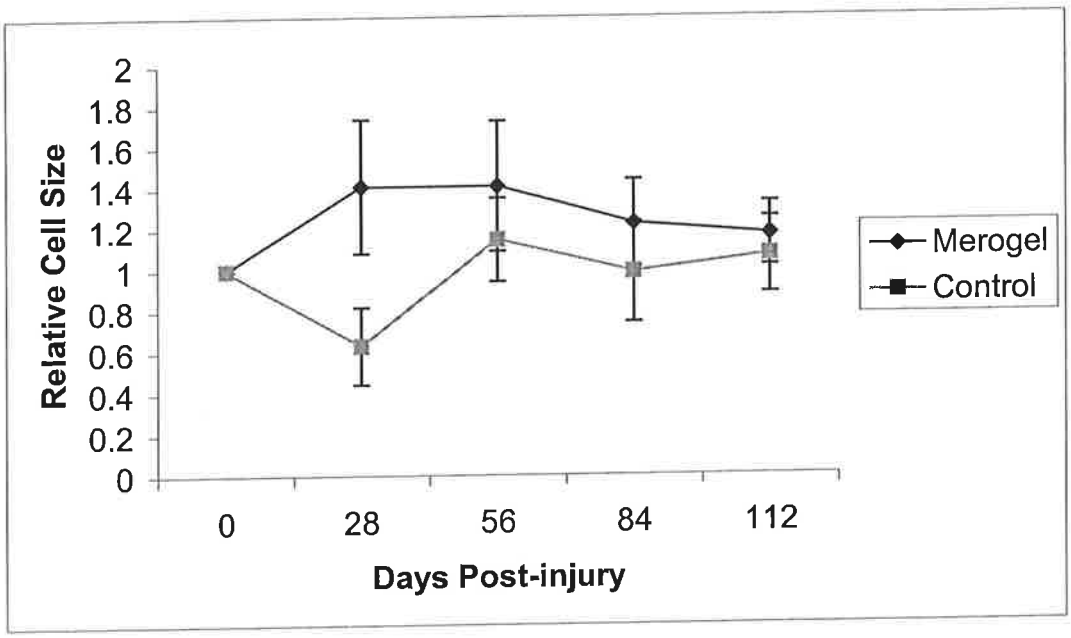

Figure 14-24. Graph of relative average cell size over time. Comparison of Merogel packed wounds to control wounds. Average cell size related to baseline (designated to equal 1). Error bars indicate standard error of mean.

Immunostaining

Immunostaining for collagens I and III was performed. The integrated optical density was calculated and expressed as a ratio of collagen I:collagen III. These ratios were compared to the baseline ratio (relative to a baseline of 100). An increase in the relative measures would be representative of early predominance of collagen I compared to collagen III, which would be indicative of increased submucosal healing. The result is demonstrated in Figure 15-25 (see also Appendix to chapter 15). There was no significant difference between packed and unpacked wounds. At day 28 the relative ratio was 114.01 for packed wounds (standard error of mean 52.15), compared to 98.93 for control wounds (standard error of mean 73.17). At day 112, it was a 146.61 for packed wounds (standard error of mean 71.78) and 74.96 for control wounds (standard error of mean 51.47).

SEM

Scanning electron microscopic analysis of cilia present on the epithelium was performed at days 56 and 112 post-wounding at 500x and 2000x magnification. A representative scanning electron microscopic picture of cilia taken at 2000X magnification in control and packed wounds at day 112 is shown (Figure 15-26). Image analysis software was used to assess cilia regeneration at 500X magnification (Figure 15-27) (see also Appendix to chapter 15). There was no significant difference in cilia regeneration between packed and unpacked wounds (74% control versus 71% Merogel at day 112 using 500x magnification and image analysis). An assessment of the maturity of the cilia was performed at 2000X magnification. Approximately 55% of the cilia seen were mature in both the treatment and control groups at day 112 (Figure 15-28).

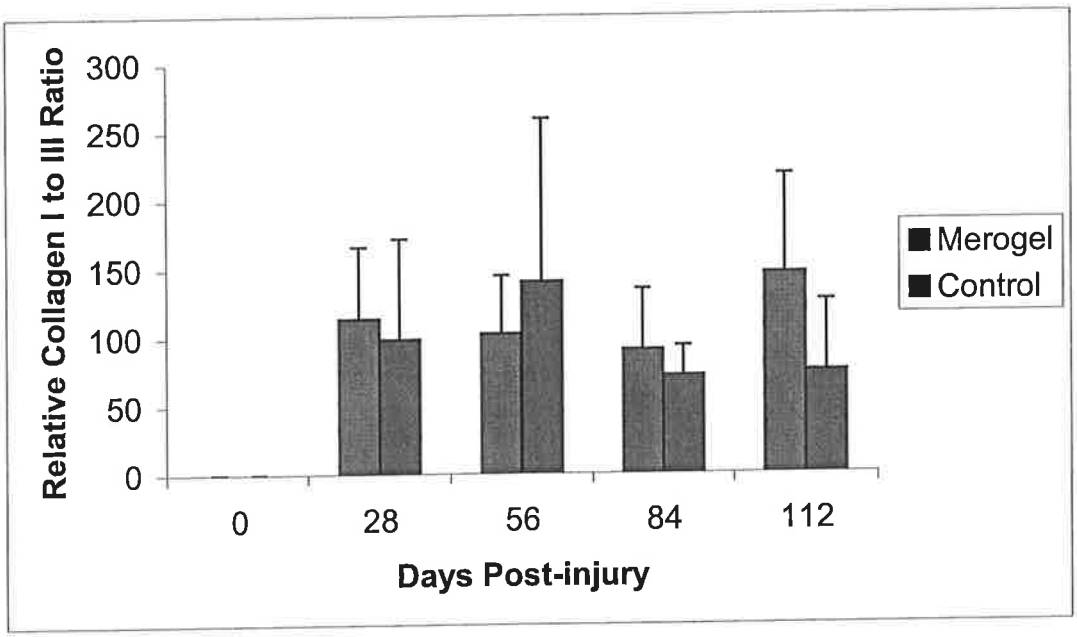

Figure 14-25. Graph of relative collagen I to collagen III ratio over time. Comparison of Merogel packed wounds to control wounds. Collagen I to III ratio related to baseline (designated to equal 100). Error bars indicate standard error of mean.

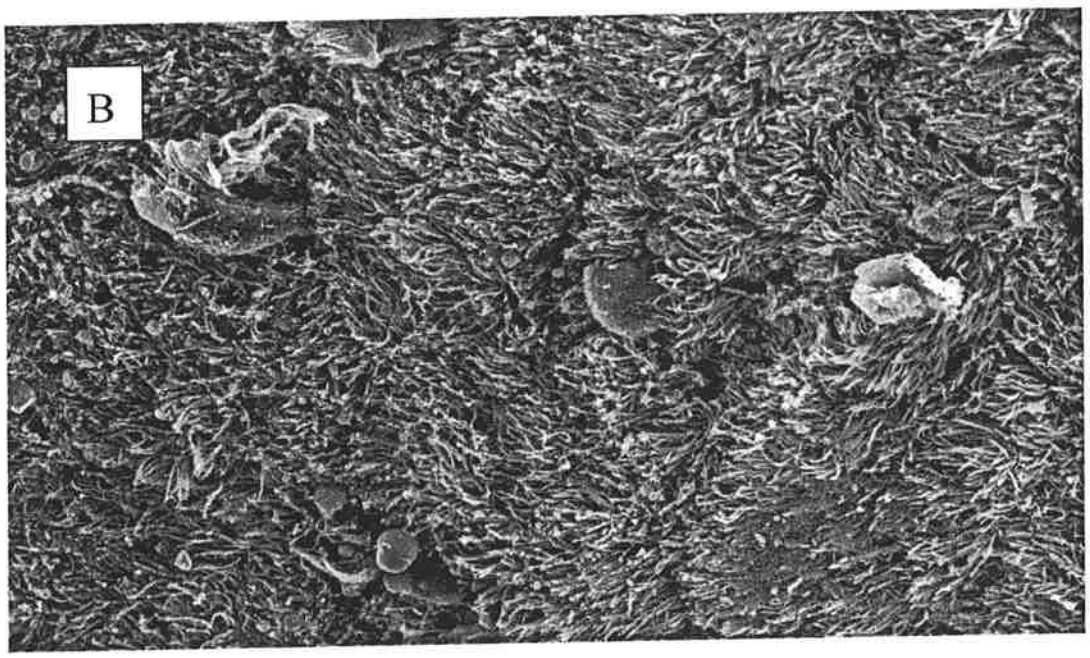

Figure 14-26. Surface electron microscopy images of Merogel packed and control wounds at day 112 at 2000x magnification. A) Control wound, B) Merogel wound.

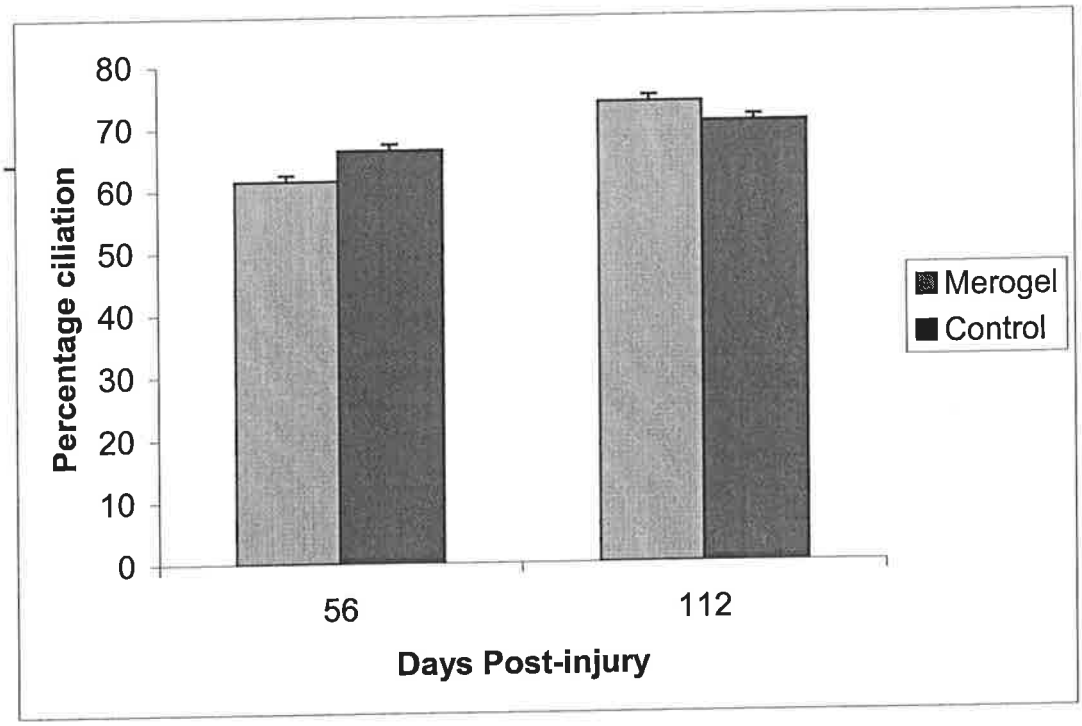

Figure 14-27. Graph of cilia regeneration over time.
Comparison of Merogel packed wounds to control wounds. Error bars indicate standard error of mean.

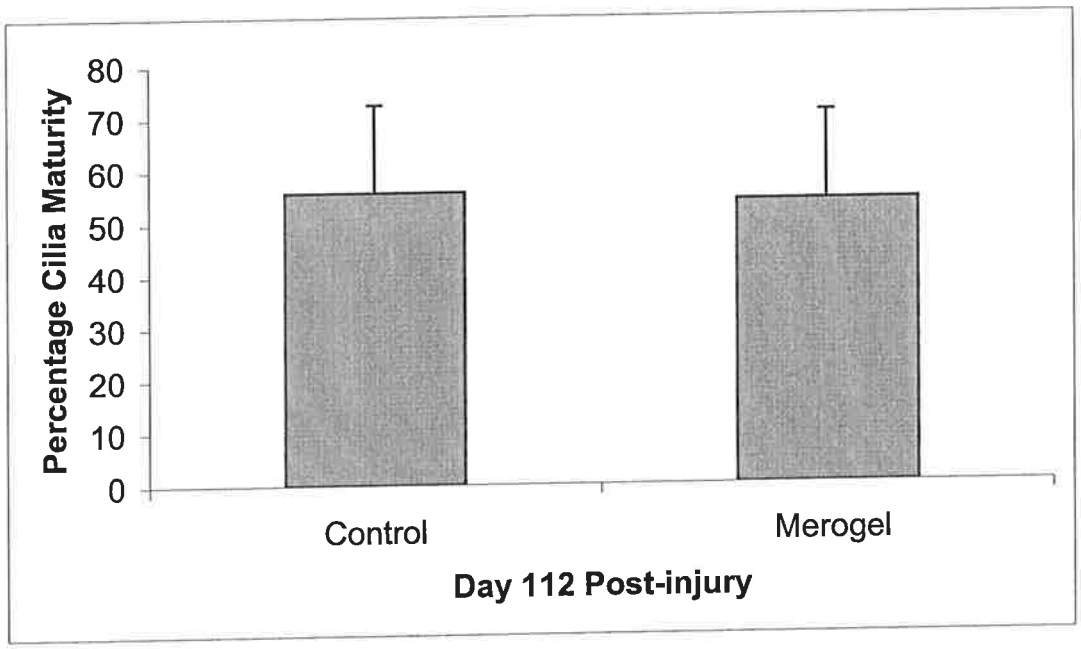

Figure 14-28. Graph of cilia maturity at day 112.
Comparison of Merogel packed wounds to control wounds. Error bars indicate standard error of mean.

3. Merogel and IGF-I nasal packing versus control

There were 12 sheep in this arm of the trial. Measurements of re-epithelialisation, epithelial height, average cell volume index and ratios were performed for all animals in suitable specimens at all time points. Reciliation was recorded using SEM at days 56 and 112. Collagen I and III measurements were performed in 4 sheep that had suitable H&E sections at all time points. This number was chosen due to the high labour intensity associated with this technique. There was no bias in the selection of these 4 sheep other than prior determination of their suitable H&E stained sections.

Light microscopy

The effect of IGF-I compared to control in rates of re-epithelialisation and relative epithelial height are demonstrated in Figures 15-29 and 15-30 respectively (see also Appendix to chapter 15). There was a statistically significant ($p<0.05$) improvement in re-epithelialisation at day 28 for IGF-I packed wounds (88.77%, standard error of mean 7.11%) when compared to the unpacked control wounds (44.15%, standard error of mean 16.50%). The re-epithelialisation at other time points measured after this was similar, with no statistically significant differences present (see Figure 15-29).

Epithelial height was measured as a marker of epithelial maturation. The height of the epithelium was measured at all time points (Figure 15-30) (see also Appendix to chapter 15). The epithelial height is expressed as a relative value of the original baseline height. There was no significant difference between the relative epithelial heights of IGF-I treated and control wounds. At day 112, the relative height of

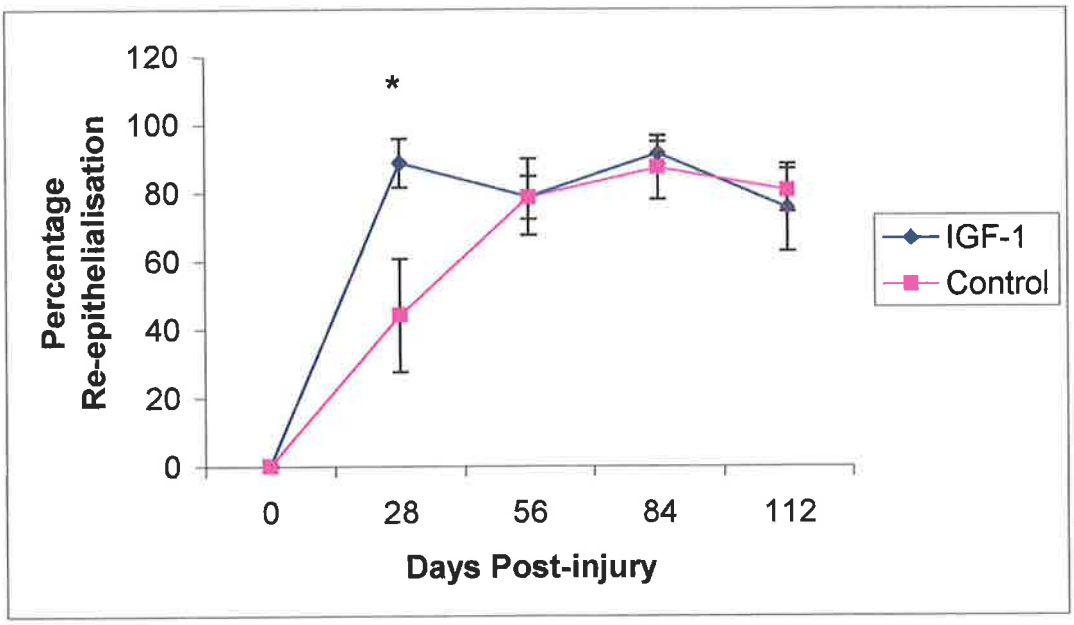

Figure 14-29. Graph of epithelial regeneration over time. Comparison of Merogel-IGF-I packed wounds to control wounds. Error bars indicate standard error of mean. *=p<0.05.

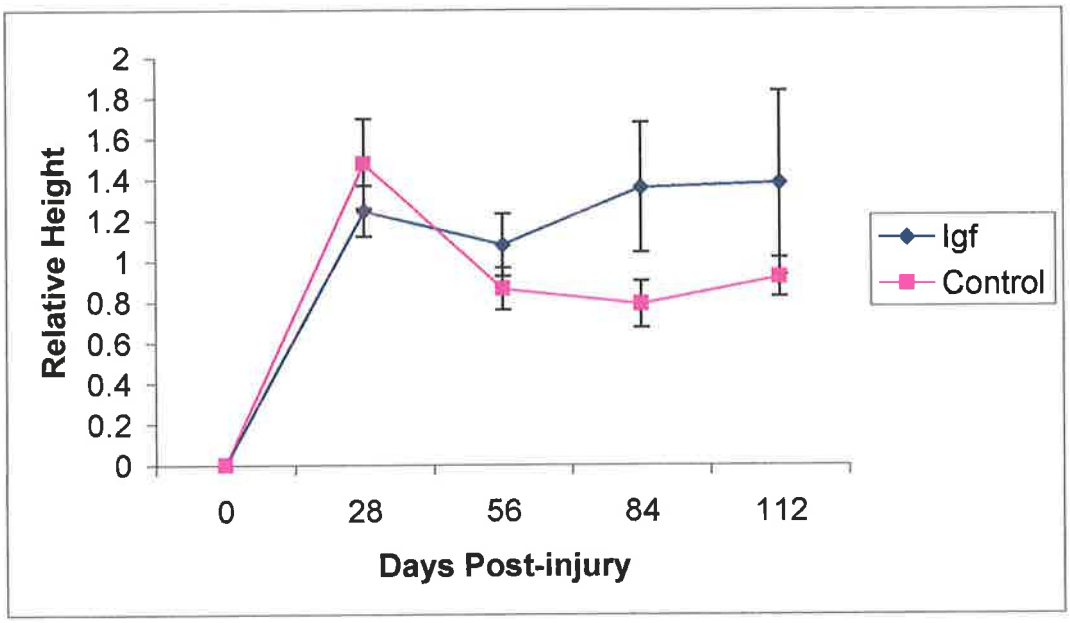

Figure 14-30. Graph of relative epithelial height over time. Comparison of Merogel-IGF-I packed wounds to control wounds. Epithelial hight related to baseline (designated to equal 1). Error bars indicate standard error of mean.

control wounds was 0.92 (standard error of mean 0.10) and for packed wounds was 1.38 (standard error of mean 0.45)

Average cell size

Measurement of epithelial height and cell density allows the calculation of an index of cell size. By comparing this index at each time point to the baseline it is possible to determine if there is a change in the cell numbers in the healing wound. The result for this measurement is demonstrated in Figure 15-31 (see also Appendix to chapter 15). There was no significant difference between packed and unpacked wounds. At day 28 the relative cell size was 1.38 for packed wounds (standard error of mean 0.26), compared to 1.63 for control wounds (standard error of mean 0.35). At day 112, it was a 1.13 for packed wounds (standard error of mean 0.21) and 1.16 for control wounds (standard error of mean 0.17).

Collagen I: Collagen III as a marker of epithelial maturation

Immunostaining for collagens I and III was performed. The integrated optical density was calculated and expressed as a ratio of collagen I:collagen III. These ratios were compared to the baseline ratio (relative to a baseline of 100). An increase in the relative measures would be representative of early predominance of collagen I compared to collagen III, which would be indicative of increased submucosal healing. The result is demonstrated in Figure 15-32 (see also Appendix to chapter 15). There was no significant difference between packed and unpacked wounds. At day 28 the relative ratio was 204.74 for packed wounds (standard error of mean 115.00), compared to 132.28 for control wounds (standard error of mean

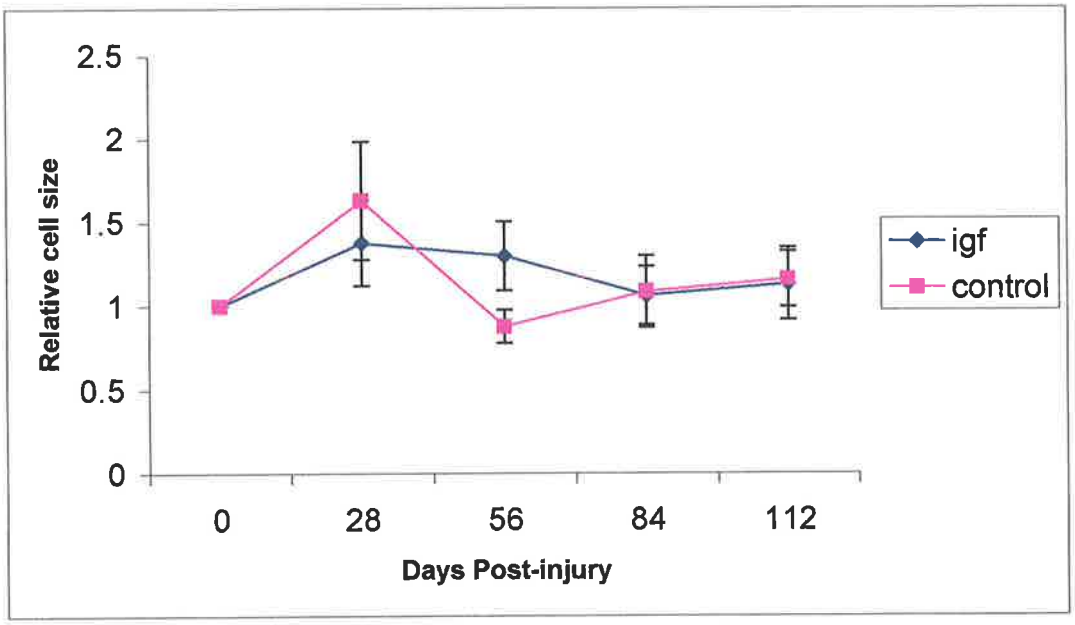

Figure 14-31. Graph of relative average cell size over time. Comparison of Merogel-IGF-I packed wounds to control wounds. Average cell size related to baseline (designated to equal 1). Error bars indicate standard error of mean.

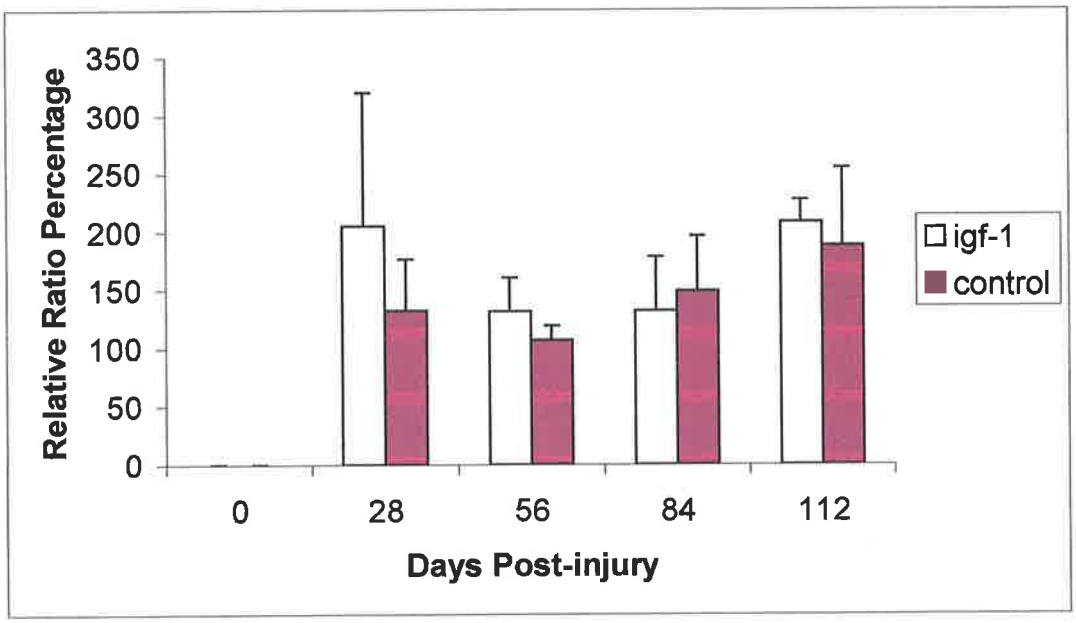

Figure 14-32. Graph of relative collagen I to collagen III ratio over time. Comparison of Merogel-IGF-I packed wounds to control wounds. Collagen I to collagen III related to baseline (designated to equal 100). Error bars indicate standard error of mean.

43.85). At day 112, it was a 208.24 for packed wounds (standard error of mean 18.98) and 187.70 for control wounds (standard error of mean 67.08).

Electron microscopy

Reciliation and cilia maturity were assessed. These are summarised in Figures 15-33 and 15-34 (see also Appendix to chapter 15) respectively. There was no statistical difference between reciliation at days 56 and 112. At day 112 ciliation was 73% for the IGF-I wounds compared to 70% for controls. The proportion on the surface covered by mature cilia was 41% in the packed wounds compared to 57% in the control wounds at day 112. This difference was not statistically significant.

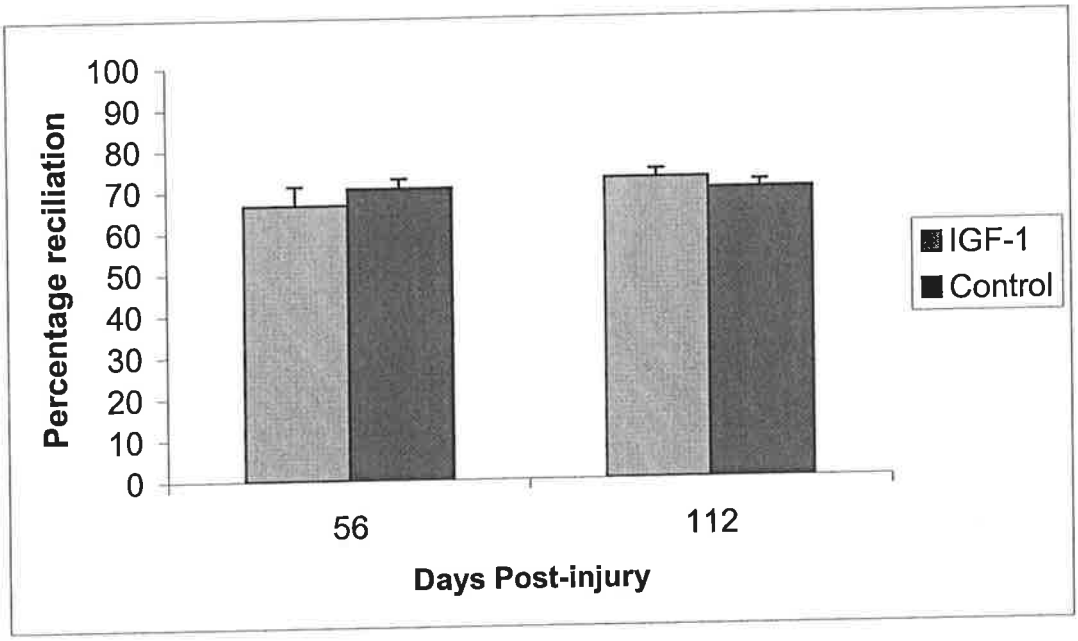

Figure 14-33. Graph of reciliation over time. Comparison of Merogel-IGF-I packed wounds to control wounds. Error bars indicate standard error of mean.

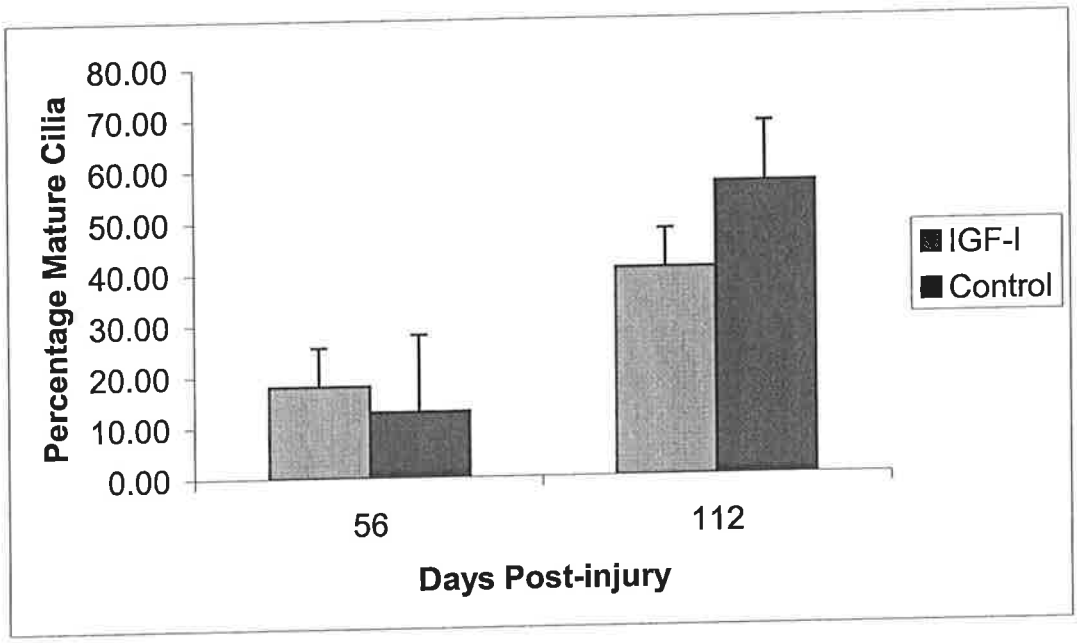

Figure 14-34. Graph of cilia maturity over time. Comparison of Merogel-IGF-I packed wounds to control wounds. Error bars indicate standard error of mean.

Discussion

Re-epithelialisation

Merocel is the most commonly available commercial nasal packing material used. When this pack is removed, concern has existed as to its effect upon the wound healing process. This research has demonstrated that the use of Merocel as a post-operative packing material has no significant detrimental or beneficial effect on re-epithelialization. The concern that pack removal may re-open the wound and have a deleterious effect on the healing process was not supported by the findings. However, it is possible that the pressure exerted by the pack on the wound was different in this study compared to that, which may be seen in humans after ESS. This is because the space in the middle meatus is narrower than the nasal cavity of the sheep. If this is the case, then its removal may induce more tissue damage in humans than what occurred in this study.

Studies on hyaluronic acid, demonstrate that it promotes cell migration and differentiation during non-nasal epithelial wound repair. A statistically significant difference ($p<0.05$) was found between the rates of measurement in re-epithelialisation between each side at day 84. This result confirms published data where the same measurements were performed by a co-author (D. Adams) [235]. The improvement in epithelial covering is at a late time point in the healing process. The clinical significance of this improvement at such a late stage is probably limited.

This most influential factor on re-epithelialisation demonstrated in this research is the beneficial effect of incorporating IGF-I with hyaluronic acid ester. The *in vivo*

use of IGF-I is supported by *in vitro* studies of scratch wound assays using respiratory epithelial cells. In these experiments, IGF-I increased wound closure rates. In the sheep model, full thickness nasal wounds demonstrated increased rates of healing. As early as day 28 post-injury, the treated wounds were re-epithelialised by 88.77%, compared to 44.15% in controls.

Epithelial height

When the height of the regenerating epithelium in both the Merocel packed and control wounds was compared to the normal mucosa taken at the time of wounding, it was found to be higher. There was a trend for the growth of the regenerated mucosa of the packed wounds to be higher than controls. The relative measures for the packed wounds were approximately twice the original starting height compared to 1.5 times the baseline demonstrated in the unpacked wounds. The epithelial height is a marker of epithelial mass, which is the product of total cell numbers and the average size of these cells. These changes from baseline represent either a change in the cell density or average cell size or both. Since the epithelial height is dependant on both cell size and numbers, it can be inferred that a change in epithelial height comes about through an aberration of the control mechanisms of the cell size and/or growth. This concept is well appreciated in the assessment of squamous cell carcinoma, especially with regard to carcinoma in situ. This raises the possibility that the behaviour of regenerating cells of the polyvinyl acetate packed wounds is different to the unpacked wounds. The reason for this is difficult to understand though one possibility is that the packs may act in a similar way to an occlusive dressing, which promotes the retention of growth factors in the local vicinity of the wound [97]. This could have the effect of stimulating cellular

mechanisms involved in growth and division for a longer time than would be expected.

There were significantly greater relative epithelial heights in the hyaluronic acid ester treated wounds compared to controls ($p<0.05$) at day 28. This would infer that the hyaluronic acid ester packs did in fact have an effect upon cell behaviour but that this influence did not translate into improvements in re-epithelialization.

In the IGF-I treated wounds, the relative epithelial height at all time points was similar to control wounds. These measurements were comparable to the baseline.

It has been reported that the nasal epithelium is initially flat or squamous when it regrows [97,122]. The results from this study do not support this statement, with the relative heights of unpacked wounds approaching baseline at day 56. Before this time point, at day 28, most measurements exceeded the baseline height.

Cell Size

The cell size index is used as an indicator of cell growth control. A change in the cell size index would be reflective of altered cell growth characteristics. In the Merocel treated wounds, there was no difference in the index when compared to control wounds. In the Merogel trial, there appeared to be a difference at day 28 but this was not significantly different ($p>0.05$). In the IGF-I treated wounds, there was also no significant difference to control wounds. At day 28, all measurements reveal that the average cell size at this stage was approximately 1.5 times normal regardless of the pack used. At day 56, the Merocel packed wounds were

approximately twice the normal size whilst the results for the other two packs had changed little in their size from day 28. The IGF-I treated wounds showed the earliest trend towards baseline size, which is evident at day 84. These results imply potential favourable influence on cell growth and regulation by both the Merogel and IGF-I impregnated packs. Given the similarity in the two results, this effect may potentially be due to the influence of the hyaluronic acid ester packing material alone.

Immunofluorescence

Research of cutaneous wounds has shown that there is an alteration in the amounts of collagen I and III as wound healing progresses. Immunofluorescence successfully demonstrated collagen I and III in the sheep nasal mucosa. The measurement of inter-optical density were used to calculate the collagen I to III ratio. These ratios were compared to the baseline measurements. Initially, the proportion of collagen III is greater than normal wounds. This results in a corresponding decrease in the amount of collagen I. As the wound matures, the proportion of collagen I increases, and collagen III decreases. The increase in collagen I, and /or decrease in collagen III, will be reflected in the collagen I to collagen III ratio. The greater the ratio, the more mature the wound. Merocel packed wounds demonstrated a trend towards lesser collagen maturation compared to controls. Merogel packed wounds demonstrated no discernable difference or trend when compared to controls. However, IGF-I packed wounds demonstrated a trend towards improved collagen maturation at day 28. The lack of statistical significance is belayed by the small numbers used (n=4).

Re-ciliation

The mucosa of the nose and sinuses rids itself of allergens, bacteria and other inhaled materials by the action of ciliary motion. Hence, the return of cilia after an injury is important. Re-ciliation is one of the fundamental end-points in nasal wound healing as failure of re-ciliation leads to mucus stasis, with potential secondary infection, and a return to unhealthy mucosa. The findings in this study are consistent with those of Moriyama [111] and confirm that the re-ciliation in full-thickness wounds is a slow process and is incomplete at day 112 post-wounding. This is regardless of the type of nasal packing material used. The proportion of cilia regenerated at day 112 was 71% for Merocel, 74% for Merogel and 73% for IGF-I. This is compared to similar values for unpacked wounds. With regards to cilia maturity, in the packed wounds, the results were 33% for Merocel, 54% for Merogel, and 41% IGF-I. Furthermore, even at day 112 only approximately 55-60% of the cilia were mature in the control wounds.

Reciliation is an important endpoint in cell differentiation and maturation. The cellular mechanisms involved are influenced by factors in the external environment that activate different cell-signalling pathways. These results suggest that the IGF-I has no influence upon cilia maturity based upon the above results of 41% maturity at day 112. An alternative, or potentially associated explanation, is that the high metabolic demand placed upon the cell in achieving increased re-epithelialisation, is to the detriment of other cell processes, such as reciliation. The exclusion of unsuitable samples also limits statistical assessment.

General Overview

The assessment of the healing process of nasal respiratory epithelium is difficult because of the lack of suitably defined endpoints. In this study, re-epithelialisation and reciliation are probably the most important. The IGF-I impregnated hyaluronic acid ester pack showed the greatest promise in improving re-epithelialisation. Additional research is necessary to further clarify the benefit of IGF-I on nasal epithelial wound healing. For example, it is common for inflammation to be present at the time of endoscopic sinus surgery. An important study would be to assess the effect of inflammation on nasal epithelial wound healing and to then determine the effect of IGF-I on this process. This is a necessary study because prolonged inflammation is detrimental to wound healing in other tissues. Furthermore, the degradative enzymes released in the inflammatory response may neutralise the IGF-I. This study could be performed in an inflammatory sinusitis model. This model is described in the following chapter relating to adhesions.

Chronic rhinosinusitis patients are frequently treated with topical, and occasionally systemic, steroids. The effect of these on nasal mucosa wound healing is unknown. The use of IGF-I for two weeks in patients on corticosteroid treatment has been shown to improve wound-healing outcomes in skin wounds [189]. The hyaluronic acid ester pack used in this research dissolves over a two-week period. Hence, in theory, there is a local sustained released of IGF-I during this time. Therefore, the use of this type of packing material may be of great benefit to this population of patients. Further research would be necessary to investigate this theory.

The clinical significance of the promotion of wound healing in nasal epithelium is potentially high. The commonest complication of endoscopic sinus surgery is the development of adhesions [88,135,136,196,200,207-209,236]. These adhesions are the commonest cause for failure of ESS and may require further surgery to be divided [196,200]. The positive influence of IGF-I on wound healing may reduce adhesion formation. Hence, the use of the hyaluronic acid ester pack, with and without IGF-I, was thought to be the next important step in the assessment of these packs. This is presented in the next section.

XV. Aim: To Determine the Effect of Nasal Packing on the Development of Adhesions in Sheep After Endoscopic Surgery.

Attempts to develop an adhesion model.

The most common site for the formation of intranasal adhesions in humans is between the lateral nasal wall and middle turbinate [237]. This is demonstrated in Figure 16-1. In the sheep, the ethmoid turbinate and lateral nasal wall are related in an analogous manner to the lateral nasal wall and middle turbinate of humans Figure 16-2. This analogy is similar with respect to their size, structure, and distance between them. Hence, the sheep was thought to be suitable for development as a model of intra-nasal adhesion formation.

Adhesion model development

Sheep used in this study were first standardised by middle turbinectomy, as described previously. After the passage of several weeks, an injury was made under general anaesthesia to the lateral nasal wall that lies adjacent to the lateral surface of the most anterior ethmoid turbinate (Figure 16-3). The corresponding mucosa on the surface of this ethmoid turbinate was also injured so as to create the situation of adjacent injured mucosa. This is thought to promote the formation of adhesions (Figure 16-3). Assessments for adhesion formation were made at weekly intervals for a period of four weeks. The injuries were made bilaterally, with one side (computer randomised) packed with the hyaluronic acid packing, and the other side left as control. The scoring of adhesions was done qualitatively as either present or absent. It was anticipated that they could as be scored based upon the surface area involved in adhesion attachment relative to total wound size. However, all but a

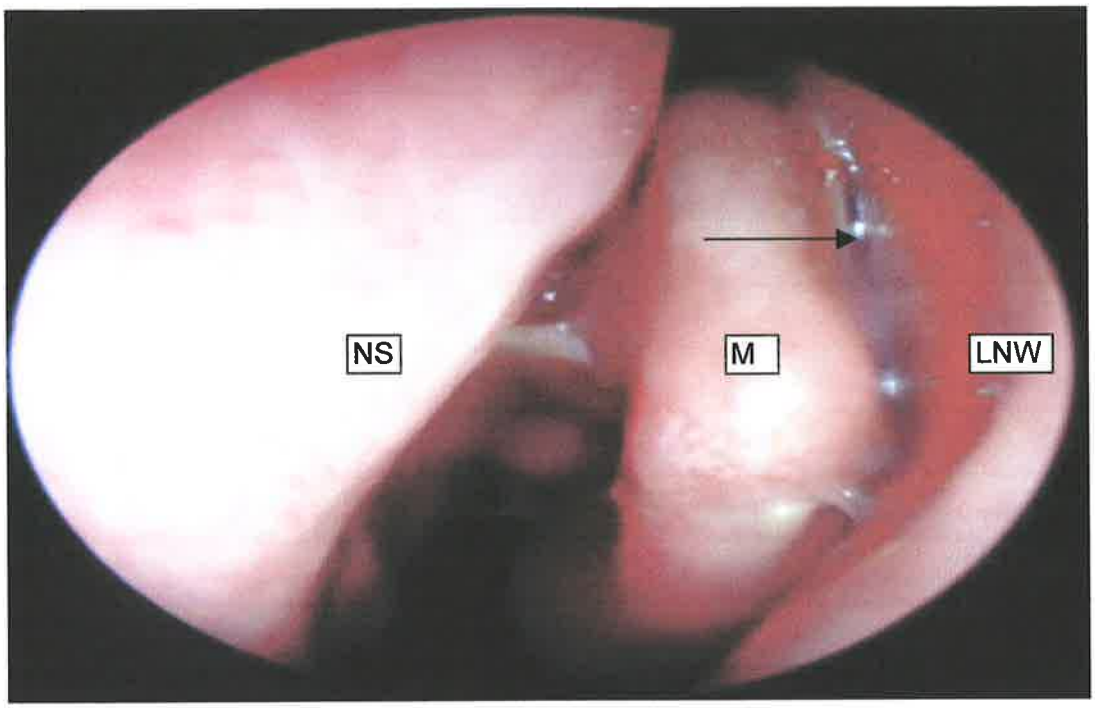

Figure 15-1. Adhesion between middle turbinate and lateral nasal wall. Endoscopic view of left nasal cavity. Arrow indicates adhesion between middle turbinate and lateral nasal wall. NS=nasal septum. MT=middle turbinate. LNW=lateral nasal wall.

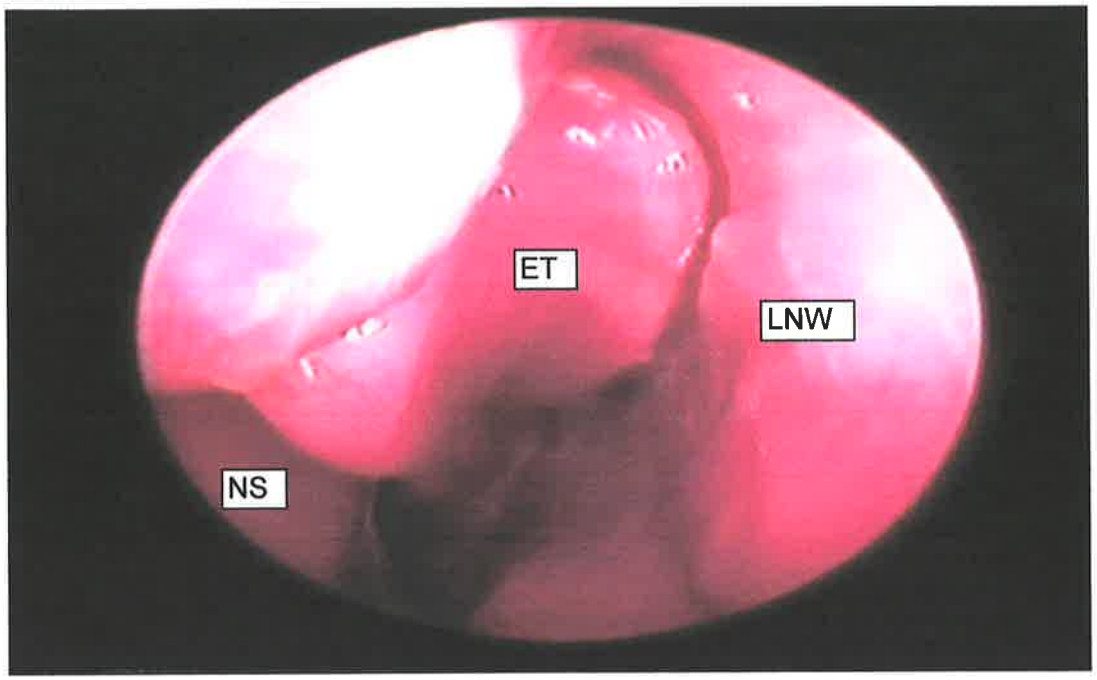

Figure 15-2. Endoscopic view of left nasal cavity of the sheep after middle turbinate removal. NS=nasal septum. ET=ethmoid turbinate. LNW=lateral nasal wall.

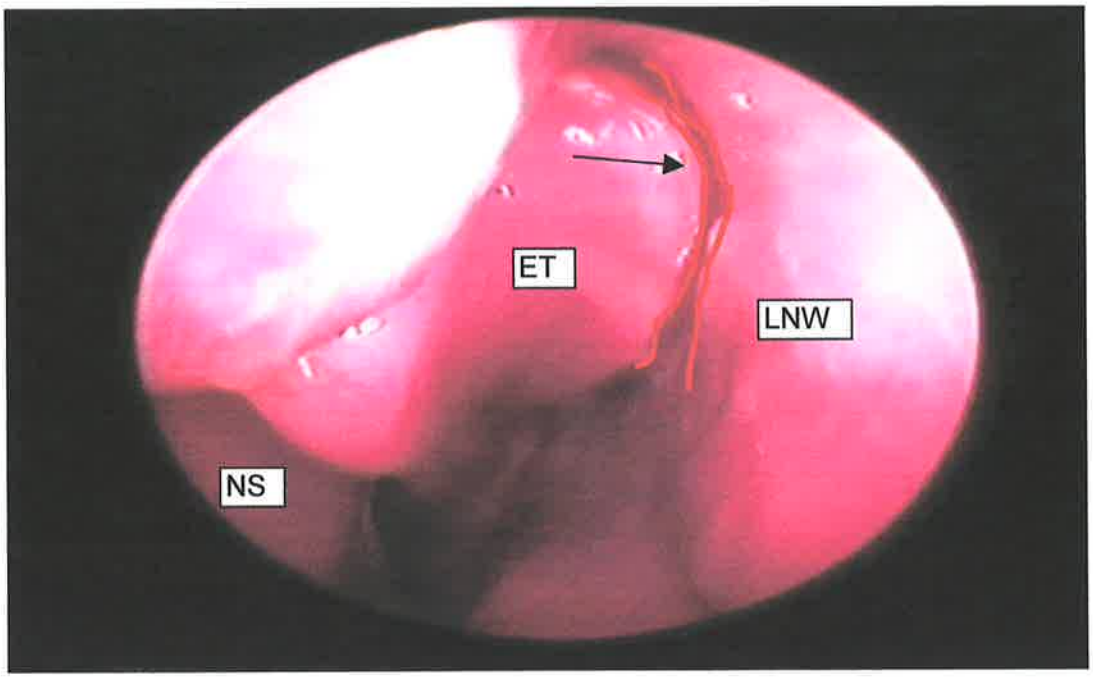

Figure 15-3. Endoscopic view of left nasal cavity of the sheep after middle turbinate removal with computer generated demonstration of areas of injury for adhesion formation. Surface injured indicated by arrow pointing to red lines. NS=nasal septum. ET=ethmoid turbinate. LNW=lateral nasal wall.

small number of adhesions were less than 25% of the wound surface area, and most were less that 50% (Figure 16-4). Hence, a grading system of their size was not performed.

Method 1

A trial of different methods of creating the mucosal injury was attempted. In the first instance, a 4-millimetre diameter Straight-Shot tissue debrider was used (4 sheep). This method was considered to be inappropriate to continue with because the space that the end of the tissue debrider was inserted into was smaller than the tissue debrider diameter. The insertion into the space resulted in fracturing of the superior attachment of the ethmoid turbinate. This resulted in medial displacement of the ethmoid turbinate away from the lateral nasal wall. There was also minimal bleeding caused by the tissue debrider. These combined factors were thought to reduce the likelihood of reliable adhesion formation, which subsequently proved to be correct when the sheep were brought back for assessment. There was no adhesion formation on either side.

Method 2

The next method trialed was the use of a sickle knife, which was passed along the mucosa so as to create multiple full thickness mucosal lacerations that were aligned in both a longitudinal and axial direction. The blood clot that formed within the space was not removed. Five sheep were used in this trial. The outcome of this trial was the formation of small adhesions. These occurred in both packed and unpacked wounds. The results are summarised in Table 16-1. As can be seen, at week 1 unpacked wounds had an adhesion formation rate of 80% (4/5) compared to 40%

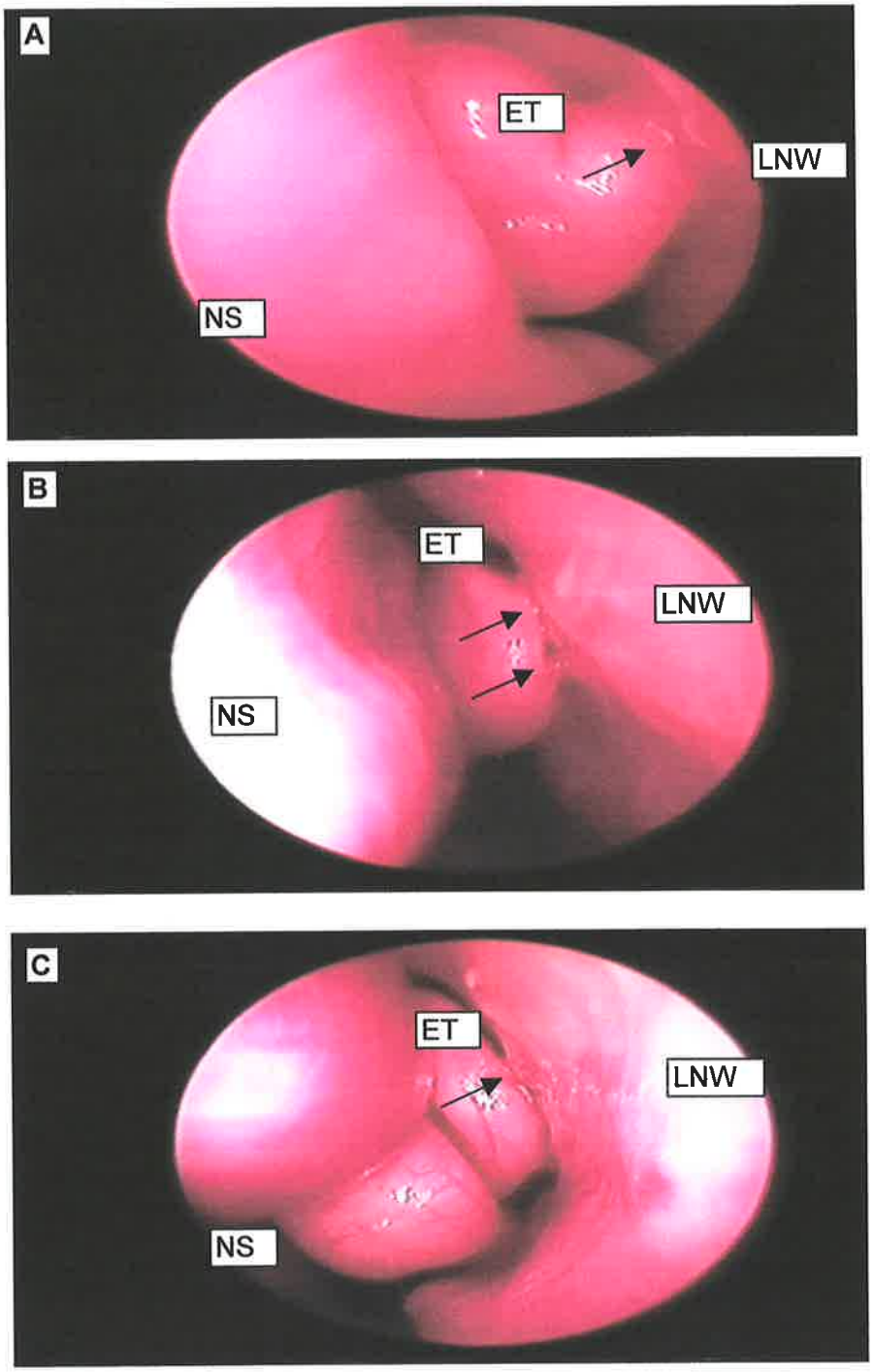

Figure 15-4. Endoscopic view of left nasal cavity of three sheep one week after mucosal injury. Adhesion injury indicated by arrows. Images relate to sheep using Method 2 (see text for details). A) Sheep 1, B) Sheep 3, C) Sheep 5 (see table 1 and text for details). NS=nasal septum. ET=ethmoid turbinate. LNW=lateral nasal wall.

	Packed					Control			
	7	14	21	28		7	14	21	28
1	No	No	No	No		Yes	Yes	Yes	Yes
2	Yes	Yes	Yes	Yes		Yes	Yes	No	No
3	Yes	Yes	No	No		Yes	Yes	No	No
4	No	No	No	No		No	No	No	No
5	No	No	No	No		Yes	Yes	No	No
Adhesion	2	2	1	0		4	4	2	1

Table 15-1. Quantantaive assessment of adhesion formation over time using Method 2. (See text for details).

(2/5) in the packed wounds. At week 2, these were unchanged. However, at week 3, two of the unpacked wound's adhesions had spontaneously resolved, and at the 4-week mark only one unpacked wound demonstrated an adhesion compared to no adhesions in the packed wounds. The size of all the adhesions reduced each week (Figure 16-5). Hence, despite the promising start evident at weeks 1 and 2 suggesting that this model was successful for firstly creating adhesions, and secondly for the packing reducing the incidence of their formation, the later time points demonstrated that these adhesions spontaneously resolved. This was considered to be unacceptable for making scientific comparisons because true adhesions should, by week two, have a collagen matrix that maintains the integrity of the adhesion structure. Furthermore, the persistence of only one adhesion at week 4 may simply have been due to chance rather than a reflection of the utility of the technique.

Method 3

The reason for this poor adhesion formation was thought to be due to inadequate wound creation. Hence, the above technique was modified in an effort to result in larger, and more permanent adhesions by ensuring adequate wound creation. The modification of the above technique was to remove by suctioning the blood clot that formed within the space after the first injury creation with the sickle knife. The created wound could then be visualised and the sickle knife was used to ensure that the injury involved the full extent of both apposing surfaces. Subsequent blood clot formation after this was not removed by suction. Again, one side was packed, the other unpacked as the control. Four sheep were initially involved in this modification but one was excluded due to a larger than desired distance existing

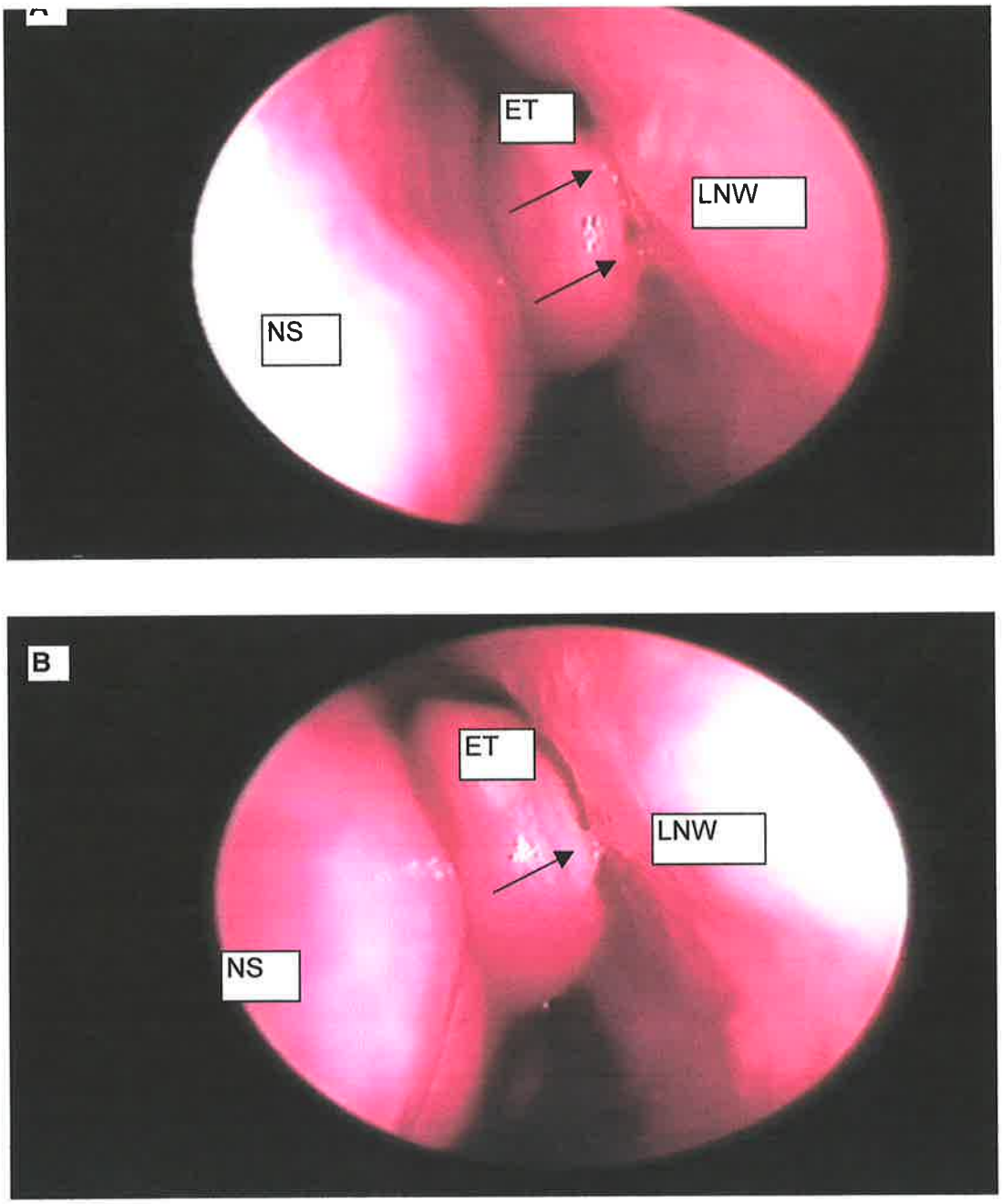

Figure 15-5. Endoscopic view of left (control) nasal cavity of sheep number three from Method 2. Adhesions in A) and B) are indicated by arrows. A) Week 1, B) Week 2 (see table 1 and text for details). NS=nasal septum. ET=ethmoid turbinate. LNW=lateral nasal wall.

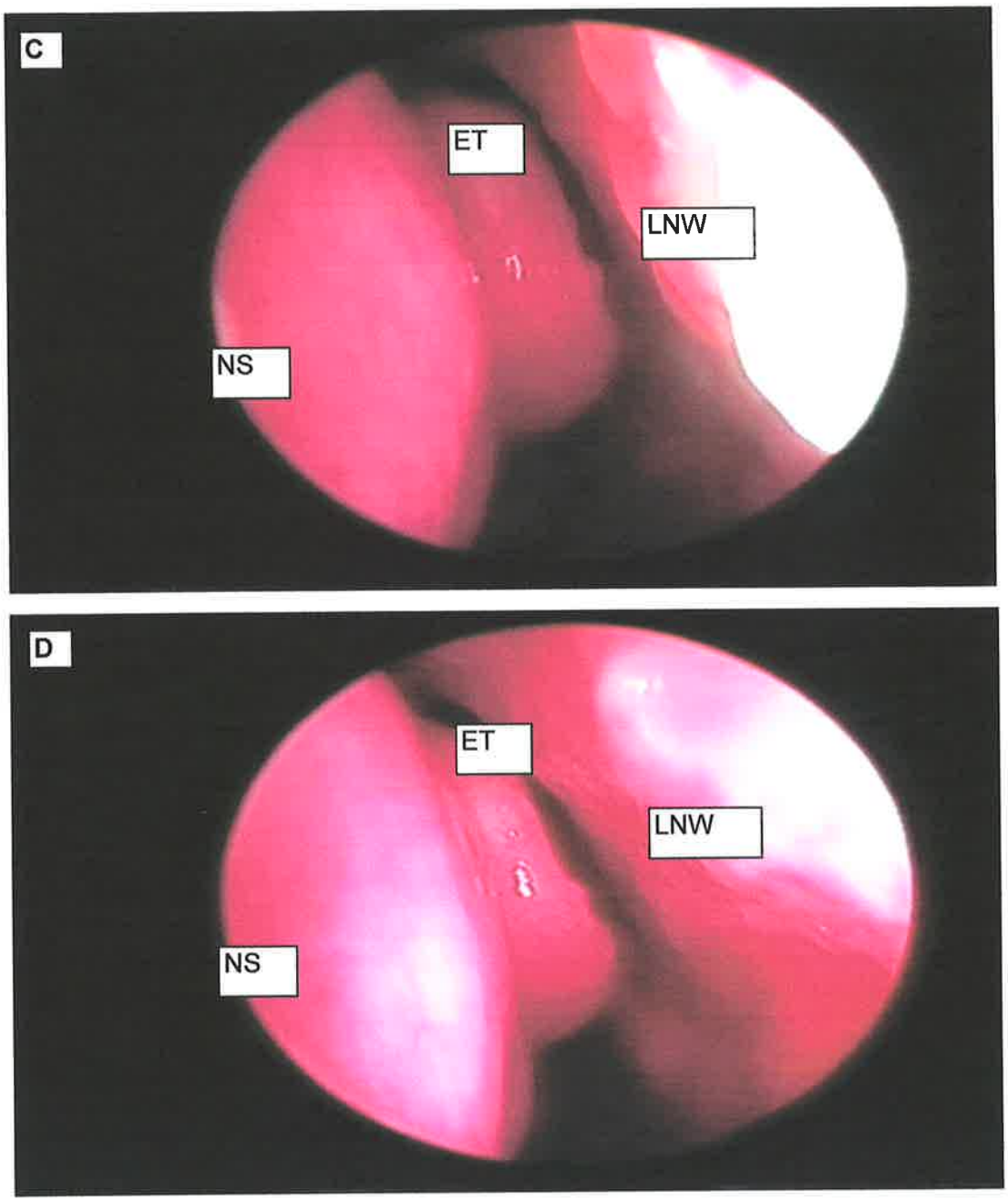

Figure 15-5. Endoscopic view of left (control) nasal cavity of sheep number three from Method 2. C) Week 3, D) Week 4 (see table 1 and text for details). NS=nasal septum. ET=ethmoid turbinate. LNW=lateral nasal wall.

between the lateral nasal wall and the lateral surface of the ethmoid turbinate. The results of this trial are summarised in Table 16-2. Adhesions were present in all sheep in the wounds at week 1, regardless of the presence of packing. However, these seemed to be smaller, overall, than the adhesions seen at week 1 in method 2, described above. At week 2, adhesions, which were now smaller in size, were only present in one of the packed and one of the unpacked wounds (same sheep) and by week 3 there were again no adhesions. Whilst showing more promise initially, based on the 100% adhesion formation at week 1, the later time points were disappointing. The smaller sized adhesions were surprising given the theoretically increased wounding area. The reason that this was thought to have occurred was that the removal of the first blood clot may have removed the important coagulation factors instrumental in adhesion creation.

Method 4

With the 100% rate of adhesion formation at week 1, method 3 was thought to be suggestive of the benefit of ensuring adequate wound creation. However, it also highlighted the probable importance of the initial blood clot in promoting adhesion formation. Hence, method 2 was reverted back to but greater time was spent in abrading the intended wound area. This was done blindly through the blood that came from these wounds. The results in the packed and unpacked wounds is summarised in Table 16-3. There was a 75% adhesion formation rate in both the packed and unpacked wounds at week 1. The adhesions persisted in the packed wounds (although smaller in size) at week 2, and were absent by week 3. In the unpacked wounds, two of the adhesions persisted at week 2, but only 1 remained by

	Packed					Control			
	7	14	21	28		7	14	21	28
1	Yes	No	No	No		Yes	No	No	No
2	Yes	No	No	No		Yes	No	No	No
3	Yes	Yes	No	No		Yes	Yes	No	No
Adhesion	3	1	0	0		3	1	0	0

Table 15-2. Quantantaive assessment of adhesion formation over time using Method 3. (See text for details).

	Packed					Control			
	7	14	21	28		7	14	21	28
1	Yes	Yes	No	No		Yes	Yes	Yes	Yes
2	Yes	Yes	No	No		Yes	Yes	No	No
3	No	No	No	No		No	No	No	No
4	Yes	Yes	No	No		Yes	No	No	No
Adhesion	3	3	0	0		3	2	1	1

Table 15-3. Quantantaive assessment of adhesion formation over time using Method 4. (See text for details).

week 4. These results were considered to still be inadequate due to the inability to produce permanent adhesions.

Method 5

The next trial involved the use a paediatric sized (2 millimetre diameter) Straight Shot cutting blade. It was hope that this blade would consistently produce wounds of adequate size and consistency without fracturing the ethmoid turbinate attachment. Unfortunately, even with the smaller blade, disruption of the ethmoid turbinate attachment did occur with subsequent medial displacement away from the lateral nasal wall. At week 1, 75% (3/4) packed wounds had adhesions, compared to 25% (1/4) unpacked wounds. At week 2, all the adhesions had been dissolved. The failure of this technique was thought to be related to the displacement of the ethmoid turbinate surface away from the lateral nasal wall and possibly the suctioning of blood clot from the wounded areas, which occurs with the use of this type of microdebrider.

Method 6

Hence, at this stage, it was decided that the most important factors in creating adhesions were comprehensive wound creation, apposition of wounded surfaces, and maintenance of the initial blood clot. The sickle knife was again used in this trial but the modification of technique in this instance was to firstly out-fracture the ethmoid turbinate towards the lateral nasal wall, then to medially displace it, create the injuries with the sickle knife and then laterally displace the ethmoid again. The presumed benefit of this was improved apposition of the wounds. The results are summarised in Table 16-4. The adhesion formation rate was poor at week 1 in both

	Packed					Control			
	7	14	21	28		7	14	21	28
1	Yes	No	No	No		No	No	No	No
2	No	No	No	No		No	No	No	No
3	Yes	Yes	No	No		No	No	No	No
4	No	No	No	No		Yes	No	No	No
Adhesion	2	1	0	0		1	0	0	0

Table 15-4. Quantantaive assessment of adhesion formation over time using Method 6. (See text for details).

packed (50%) and unpacked (25%) wounds, with no adhesions present by week 3. Hence, this technique was abandoned.

Method 7

As a last effort to develop the adhesion model, the method of wound injury was changed. Instead of a sickle knife, a curette was used to abrade the mucosa. The results are summarised in Table 16-5. In the unpacked wounds, adhesions were present in all sheep at week 1 and persisted until week 4 in all but 1. In the packed wounds, adhesions formed in two sheep that persisted for 4 weeks. However, all of these sheep developed acute suppuration at the site of injury in at least one of the sides (1 sheep both sides). This was present at week 1 and had resolved with conservative management by week 2. In the total of 4 persistent adhesions, 3 were involved in acute suppuration. It is unknown whether the technique employed was an aetiological factor in this occurring, but in comparison with the other 21 sheep utilised before this particular trial, only 2 had also suffered from acute suppuration (1 from the sickle knife method not involving suction, and the other from the group where the ethmoid turbinate was initially out-fractured). Due to ethical considerations, it was decided not to persist with this technique.

When all of the results from the different methods utilised (excluding methods 1 and 5) are collated (Tables 1-5), the rate of adhesion formation at week one in packed wounds is 12/19 or 63%. This compares to 14/19 or 73% in the control wounds. At week four, there were 2/19 or 11% adhesions persisting in the packed wounds, compared to 4/19 or 23% in the control wounds.

	Packed					Control			
	7	14	21	28		7	14	21	28
1	No	No	No	No		Yes	No	No	No
2	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes
3	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes
Adhesion	2	2	2	2		3	2	2	2

Table 15-5. Quantantaive assessment of adhesion formation over time using Method 7. (See text for details).

Discussion

It is unfortunate that the method that showed the greatest promise for creating adhesions resulted in a suppurative complication (curettage, method 7). This is a confounding variable in terms of the effect the acute inflammation may have had in promoting wound adhesion formation. As highlighted in the adhesion chapter, the presences of either pre-existing inflammation or subsequent wound infection are aetiological factors implicated in promoting adhesion formation.

Sheep develop an inflammatory rhinosinusitis in response to the presence of the *Oestrus ovus* parasite [238,239]. Infestation with this parasite incites a local hypersensitivity reaction, with the recruitment of numerous mast cells and eosinophils to the affected mucosa [240]. Slaughter house surveys have demonstrated a high incidence of infestation in animals not treated with preventative medication [241] Whilst the animals used in this trial had had the prophylactic treatment, there were 3 sheep that were infested with this parasite at the time of injury. There were 2 in the trial where out-fracture of the ethmoid turbinate was performed (method 6) and 1 from the curettage group (method 7). In theory, the inflammation in response to this parasite should favour adhesion formation. In the out-fracture group the two sheep had no adhesion formation on the packed side, and only one adhesion formed in the unpacked side at week 1, which had dissolved by week 2. In the curettage group, this sheep had persistent adhesions on both sides.

The poor rate of adhesion formation in the three sheep with *Oestrus ovus* infestation does not necessarily imply that inflammation is not an important aetiological factor in adhesion formation. In these three sheep the larval burden was low and the larvae

were in an early stage of their life cycle. The degree of inflammation induced by this parasite is related to the larval burden [242]. The number of mast cells in infected sheep is twice the number present in parasite free animals [243]. Also, the number of eosinophils distributed throughout the sino-nasal complex in infected animals, compared to non-infected animals, is increased by a factor of 17 for the septum, 29 for the turbinates and 58 for the sinuses [240,243]. Hence, given the juvenile stage of development, and the low number of larvae in each of the three sheep, it is possible that the inflammatory response was minimal.

Overall, despite the manipulation, the incidence of adhesion formation at week 1 was similar in packed and unpacked wounds (63% versus 73% respectively). At week 4, only 2/19 packed wounds compared to 4/19 unpacked wounds demonstrated persisting adhesions. Both of the packed wounds, and 2 of the 4 unpacked wounds that had persistent adhesions at this time point were in the curettage group. The development of an inflammatory sheep model may be more successful in the creation of an adhesion model. Due to the lack of success in this research, no comment can be made regarding the influence of IGF-I on adhesion prevention.

XVI. Summary

Although ESS is a commonly performed operation little is known about its effect on the healing of the nasal mucosa or the effect of nasal packing on the healing process. This research has confirmed that the nasal epithelium is slow to heal after full thickness injury. Reciliation is also a slow process. This research has also demonstrated the influence of different types of nasal packing on this healing process, including a novel combination of hyaluronic acid ester packing with insulin-like growth factor I.

Polyvinyl acetate sponges are the most commonly used type of nasal packing utilised at the present time. There was no demonstrated beneficial or detrimental effect of the use of this type of packing on the wound healing process. At day 112, the re-epithelialisation of packed wounds was 88%, compared to 86% for unpacked wounds. A measure of relative epithelial height and relative average cell size over all time points also showed no significant difference between packed and unpacked wounds. Immunostaining for collagen I and III showed that there was no difference in the relative ratio of the expression of these collagens over time in the packed wounds when compared to unpacked wounds. There was also no difference in the percentage covering of cilia as assessed by electron microscopy (71% packed, 73% unpacked at day 112). These findings would suggest that the notion of pack removal is detrimental to wound healing are erroneous. The caveat to this statement is that the nasal cavity of the sheep is wider than humans, and hence there is less force applied to the nasal mucosa of the sheep by this packing material.

A newly developed dissolvable nasal packing material composed of a hyaluronic acid ester has been assessed. Histological assessment revealed a statistically significant improvement in re-epithelialistation at day 84 in packed wounds (94.33%) compared to unpacked wounds (83.39%) ($p<0.05$). The clinical significance of this difference at this late time point is unlikely to be important. There was a significant increase ($p<0.05$) in the relative epithelial height at day 28 in the packed wounds (1.75) compared to unpacked wounds (0.81). The reasons for this are speculative, but may be a reflection of increased cellular activity. There was no significant difference in the relative average cell size at any time point. There was no significant difference in the measurement of the relative ratios of collagen I and III expression measured using immunofluorescence. Electron microscopic assessment of cilia regeneration showed no differences in packed and unpacked wounds at days 56 (packed 62%, unpacked 66%) and 112 (packed 74%, unpacked 71%). These findings suggest that this type of nasal packing material does not confer the clinical benefits proposed by the manufacturer. The qualification of this statement is that species differences in cellular hyaluronic acid receptors and hyaluronidase activity may alter the biological effect of the ester used.

The manipulation of the wound healing process, with the intention of improving the rate of healing, was an important aim of this thesis. The use of a stimulating growth factor was intended as the biological agent with which to try and achieve this. IGF-I was selected as the most appropriate growth factor for this thesis. This was initially indicated by a thorough literature review. *In vitro* scratch wound assays demonstrated a significant improvement ($p<0.05$) in wound closure of immortalised cell colonies treated with an IGF-I containing medium (87% closure) when

compared to controls (68% closure) after 24 hours. The presence of IGF-I receptors in the sheep nasal mucosa was demonstrated using immunofluorescence. A measure of relative epithelial height and relative average cell size over all time points showed no significant difference between packed and unpacked wounds. Immunostaining for collagen I and III showed that there was no difference in the relative ratio of the expression of these collagens over time in the packed wounds when compared to unpacked wounds. There was, however, a trend for an improved relative ratio of collagen I to III at day 28 in the packed wounds (205%) compared to control wounds (132%). At day 112, there was no difference in the electron microscopic assessment of cilia regeneration in the packed wounds (73%) compared to the control wounds (70%).

The biological effect of the IGF-I on the promotion of re-epithelialisation was statistically significant at day 28. Despite this benefit in faster re-epithelialisation, the functional differentiation of the epithelium represented by ciliogenesis was not promoted by the addition of IGF-I. The process of ciliogenesis is of clinical importance but is poorly understood. Further research could be aimed at identifying growth factors that promote ciliogenesis as well as determining the ideal dose of IGF-I to be impregnated in the hyaluronic acid ester nasal pack. It is possible that a combination of growth factors provides the optimum environment for the promotion of wound healing and cell differentiation.

The area of clinical practice that improved wound healing is likely to have an impact upon is the reduction in the formation of adhesions. These adhesions are detrimental to the mucociliary function of the nasal respiratory mucosa and are

related to relapse of disease. The most common location for the formation of these adhesions is near the middle meatus. Attempts at developing an animal adhesion model were unsuccessful. Unfortunately the technique that showed the greatest promise also caused unacceptable morbidity, and hence was appropriately abandoned. Reflecting upon the pathology of chronic rhinosinusitis, it is possible that pre-existing mucosal inflammation is a pre-requisite for such adhesions to be successfully created. The sheep model shows great promise in helping to assess this because of the naturally occurring inflammatory model associated with *Oestrus ovis* infestation. Should it prove possible to develop a reproducible method for consistently creating adhesions in the *Oestrus ovis* infested sheep model, then the next important step would be to assess the effect of the growth factor impregnated pack on reducing the occurrence of these fibrous bands.

XVII. Conclusions

1. Nasal epithelium of the sheep is slow to heal after full thickness mucosal injury.
2. Complete reciliation is also slow to occur, with incomplete reciliation evident 16 weeks after full thickness mucosal injury.
3. The use of polyvinyl acetate based packing does not improve or impair epithelial return.
4. The use of hyaluronic acid based packing is unlikely to improve epithelial return in a clinically significant period of time.
5. The use of hyaluronic acid based packing impregnated with IGF-I significantly improves epithelial return.
6. The potential benefit of the use of merogel packing impregnated with IGF-I in is supported by this work and development of human trials are recommended.
7. The development of an adhesion animal model may require pre-existing mucosal inflammation to be present to be successful.

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Appendix to Chapter 11.
Screening for Potential Growth Factors that Stimulate Respiratory Epithelial Growth
Cell culture results

Data for Figure 11-1 Graph of IGF-I cell culture results

IGF-I	0	3	6	9	12	15	18	24
Treatment	100	89.65	87.80	69.82	73.16	83.42	60.86	48.14
	100	76.09	64.20	51.87	49.14	44.33	30.73	20.11
	100	107.36	94.67	74.32	78.37	75.60	65.13	52.98
	100	71.69	58.36	47.81	47.87	36.16	39.59	26.30
	100	110.71	102.19	75.06	75.19	60.31	52.35	30.84
	100	68.63	67.80	59.09	66.78	55.58	44.34	33.46
	100	74.86	67.93	46.04	46.12	43.50	35.14	21.20
	100	87.02	74.21	58.19	60.32	63.23	44.50	31.06
	100	109.68	112.15	63.33	67.33	50.83	41.19	26.84
Control	100	82.98	60.64	46.15	45.47	42.99	32.79	28.69
	100	77.20	57.91	32.85	33.85	23.43	13.90	6.59
	100	70.65	49.39	28.06	30.74	17.25	11.32	7.04
	100	76.24	63.66	53.60	53.97	45.38	36.44	29.44
	100	78.30	54.87	49.81	47.02	45.32	34.90	27.13
	100	77.76	58.32	37.86	40.11	30.25	25.68	11.71
	100	75.51	61.46	40.61	42.17	33.86	25.00	10.32
	100	75.56	56.15	55.91	44.27	28.08	23.29	0.00
	100	78.78	57.79	50.15	36.02	33.32	23.25	8.88
	100	76.85	56.56	46.27	38.79	32.86	21.28	10.84
	100	88.67	59.71	56.52	42.47	35.22	29.22	19.61
	100	87.49	45.67	63.18	51.25	41.50	32.46	18.16

Data for Figure 11-3 Graph of IGF-II cell culture results

IGF-II	0	3	6	9	12	15	18	24
Treatment	100	73.47	40.41	32.88	15.75	7.65	0.00	0.00
	100	78.84	43.96	20.59	2.97	0.00	0.00	0.00
	100	87.27	47.52	26.20	7.48	0.00	0.00	0.00
	100	132.33	46.03	40.37	26.35	18.98	9.29	11.38
	100	77.70	41.47	28.30	0.00	0.00	0.00	0.00
	100	86.87	69.67	54.84	40.69	25.41	14.63	0.00
	100	90.00	61.02	41.10	30.87	18.63	14.65	2.65
	100	136.91	86.50	63.64	40.36	12.06	4.70	0.00
	100	77.00	60.60	43.06	33.61	19.95	10.92	0.00
Control	100	82.62	63.44	43.79	35.70	26.53	20.50	5.18
	100	84.50	60.90	40.78	28.26	21.30	15.08	2.50
	100		69.29	56.53	38.73	33.70	26.19	8.40
	100	75.35	68.82	56.98	46.69	39.17	32.52	22.15
	100	80.14	59.00	47.28	37.32	25.62	17.39	4.01
	100	91.43	71.77	57.80	39.30	31.69	25.85	10.70
	100	77.62	62.52	45.83	33.21	20.42	13.80	0.00
	100	84.07	62.31	48.32	30.65	21.47	11.75	0.00

Data for Figure 11-4 Graph of EGF cell culture results

EGF	0	3	6	9	12	15	18	24
Treatment	100	70.05	31.20	8.09	0.00	0.00	0.00	0.00
	100	83.42	34.58	14.49	0.00	0.00	0.00	0.00
	100	65.58	28.48	3.50	26.30	0.00	0.00	0.00
	100	85.75	65.47	32.97	32.83	9.94	3.89	0.00
	100	88.75	70.11	49.53	37.76	25.99	20.20	6.90
	100	93.46	56.81	16.54	39.85	1.70	0.00	0.00
	100	83.73	55.26	33.59	12.89	7.44	1.42	0.00
	100	95.49	64.80	36.44	17.91	6.41	1.43	0.00
	100	91.80	63.74	45.38	22.39	13.71	4.06	2.34
Control	100	102.16	80.81	63.92	0.00	0.00	0.00	0.00
	100	104.24	83.67	44.69	0.00	0.00	0.00	0.00
	100	74.88	18.14	7.86	0.00	0.00	0.00	0.00
	100	101.52	79.49	56.30	43.85	30.87	23.40	12.71
	100	76.23	61.87	46.94	27.47	17.98	10.72	4.25
	100	85.40	61.48	45.00	30.78	19.59	14.69	6.27
	100	93.00	68.99	48.25	30.66	18.28	10.02	6.28
	100	98.72	89.75	71.67	57.41	44.93	36.35	0.00
	100	95.15	76.40	64.22	47.94	35.76	25.72	0.00

Appendix to Chapter 14.
The Assessment of the Effect of Nasal Packing on the Healing of the Nasal Mucosa of Sheep After Endoscopic Surgery.
Re-epithelialisation data.

Merocel (Figure 14-14).	Treatment					Control				
Days post-injury	0	28	56	84	112	0	28	56	84	112
1	0	Unsuitable	54.34	Unsuitable	Unsuitable	0	5.74	49.42	Unsuitable	88.08
2	0	Unsuitable	35.21	81.72	100.00	0	7.12	60.49	82.02	52.64
3	0	84.14	88.30	100.00	100.00	0	61.57	100.00	Unsuitable	75.99
4	0	35.22	Unsuitable	Unsuitable	100.00	0	30.76	Unsuitable	Unsuitable	100.00
5	0	51.90	Unsuitable	89.47	100.00	0	Unsuitable	95.00	Unsuitable	75.41
6	0	44.73	Unsuitable	100.00	95.08	0	Unsuitable	Unsuitable	100.00	100.00
7	0	95.15	90.18	70.20	86.54	0	51.85	100.00	Unsuitable	91.38
8	0	82.70	100.00	100.00	76.63	0	80.34	Unsuitable	100.00	100.00
9	0	0.00	42.55	100.00	82.16	0	Unsuitable	Unsuitable	100.00	88.41
10	0	52.27	Unsuitable	100.00	43.19	0	100.00	11.74	83.59	Unsuitable
11	0	77.63	100.00	Unsuitable	100.00	0	23.39	46.45	84.78	Unsuitable
Mean	0	58.19	72.94	92.67	88.36	0	45.10	66.16	91.73	85.77
Standard error of mean	0	10.60	11.41	4.29	6.03	0	12.97	13.73	4.07	5.52

Merogel (Figure 14-22).	Treatment					Control				
Days post-injury	0	28	56	84	112	0	28	56	84	112
1	0	0.00	100.00	81.63	96.32	0	9.92	63.31	67.98	75.41
2	0	27.00	Unsuitable	96.53	100.00	0	41.66	66.57	78.69	100.00
3	0	13.70	Unsuitable	100.00	92.22	0	35.95	Unsuitable	71.27	100.00
4	0	79.66	92.68	100.00	88.67	0	Unsuitable	100.00	Unsuitable	100.00
5	0	75.03	74.79	83.04	100.00	0	68.83	12.08	76.45	93.48
6	0	16.68	Unsuitable	Unsuitable	100.00	0	29.78	93.76	100.00	100.00
7	0	24.86	100.00	90.75	100.00	0	73.57	87.60	93.42	100.00
8	0	75.49	100.00	100.00	0.00	0	Unsuitable	49.61	66.73	78.88
9	0	Unsuitable	85.70	95.51	100.00	0	9.34	80.38	89.22	100.00
10	0	100.00	65.76	84.45	100.00	0	15.58	52.96	73.58	100.00
11	0	25.66	100.00	100.00	100.00	0	31.60	100.00	unsuitable	100.00
12	0	30.84	0.00	100.00	100.00	0	86.79	100.00	100.00	Unsuitable
13	0	100.00	55.05	100.00	59.30	0	Unsuitable	100.00	100.00	88.34
Mean	0	47.41	77.40	94.33	87.42	0	40.30	75.52	83.39	94.68
Standard error of mean	0	10.80	10.53	2.22	8.25	0	9.15	8.30	4.23	2.71

IGF-I (Figure 14-30).	Treatment					Control				
Days post-injury	0	28	56	84	112	0	28	56	84	112
1	0	100.00	77.27	80.41	0.00	0	21.26	100.00	94.51	100.00
2	0	91.03	Unsuitable	91.87	50.45	0	Unsuitable	74.97	91.82	57.20
3	0	100.00	67.93	100.00	100.00	0	12.98	72.73	100.00	67.72
4	0	100.00	85.65	100.00	Unsuitable	0	Unsuitable	100.00	0.00	69.01
5	0	Unsuitable	0.00	Unsuitable	45.25	0	13.54	84.25	89.46	55.00
6	0	Unsuitable	Unsuitable	100.00	100.00	0	Unsuitable	Unsuitable	100.00	Unsuitable
7	0	48.98	100.00	100.00	100.00	0	0.00	57.79	100.00	100.00
8	0	58.88	92.21	100.00	Unsuitable	0	16.84	81.56	Unsuitable	100.00
9	0	Unsuitable	Unsuitable	100.00	Unsuitable	0	Unsuitable	Unsuitable	100.00	Unsuitable
10	0	100.00	100.00	84.75	100.00	0	100.00	56.81	84.66	100.00
11	0	100.00	85.73	75.35	100.00	0	100.00	Unsuitable	100.00	69.52
12	0	100.00	100.00	74.90	84.58	0	88.55	Unsuitable	100.00	87.88
Mean	0	88.77	78.75	91.57	75.59	0	44.15	78.51	87.31	80.63
Standard error of mean	0	7.11	11.15	3.37	12.70	0	16.50	6.25	9.31	6.27

Appendix to Chapter 14.
The Assessment of the Effect of Nasal Packing on the Healing of the Nasal Mucosa of Sheep After Endoscopic Surgery.
Relative epithelial height data.

Merocel (Figure 14-15).	Treatment					Control				
Days post-injury	0	28	56	84	112	0	28	56	84	112
1	1	2.88	1.99	3.22	4.11	1	Unsuitable	0.97	Unsuitable	1.35
2	1	Unsuitable	0.46	1.56	1.06	1	Unsuitable	0.60	1.27	0.95
3	1	0.98	3.24	0.82	0.79	1	0.95	0.60	Unsuitable	Unsuitable
4	1	1.23	1.13	1.17	1.10	1	0.80	1.48	Unsuitable	1.70
5	1	1.27	1.72	0.58	0.53	1	Unsuitable	Unsuitable	Unsuitable	Unsuitable
6	1	0.74	Unsuitable	0.80	0.54	1	Unsuitable	0.52	0.83	Unsuitable
7	1	8.28	6.16	8.04	3.50	1	2.04	4.09	Unsuitable	4.27
8	1	3.49	2.91	2.23	3.07	1	1.78	1.77	2.41	1.72
9	1	Unsuitable	1.23	1.08	1.44	1	Unsuitable	0.09	0.88	0.69
10	1	1.03	1.21	0.86	0.82	1	2.01	2.63	1.02	1.25
11	1	0.81	1.15	1.23	1.12	1	1.11	1.45	1.19	1.48
Mean		2.30	2.12	1.96	1.64		1.45	1.42	1.27	1.67
Standard error of mean		0.86	0.55	0.68	0.40		0.25	0.40	0.26	0.42

Merogel (Figure 14-23).	Treatment					Control				
Days post-injury	0	28	56	84	112	0	28	56	84	112
1	1	Unsuitable	2.55	1.14	1.36	1	0.24	1.30	1.01	0.42
2	1	4.23	4.91	5.60	2.26	1	1.28	1.21	1.09	0.96
3	1	0.76	0.83	1.01	1.48	1	1.30	1.43	0.79	1.29
4	1	0.96	1.01	0.77	0.63	1	Unsuitable	0.29	Unsuitable	0.85
5	1	2.17	0.66	1.06	0.62	1	1.01	0.71	0.74	0.81
6	1	1.38	Unsuitable	1.02	0.95	1	0.45	0.63	Unsuitable	0.77
7	1	1.17	1.33	2.16	1.59	1	0.93	1.06	1.23	1.75
8	1	4.22	2.86	3.02	Unsuitable	1	0.86	0.52	0.56	1.22
9	1	1.88	1.00	1.71	2.07	1	Unsuitable	1.03	1.96	1.35
10	1	1.82	0.95	1.24	0.83	1	1.04	0.83	0.73	0.64
11	1	0.49	0.59	0.67	0.74	1	Unsuitable	0.96	0.55	0.77
12	1	1.00	0.00	0.95	0.79	1	0.85	0.68	0.67	0.37
13	1	0.96	0.24	0.78	0.56	1	0.10	1.00	1.07	0.92
Mean		1.75	1.41	1.63	1.16		0.81	0.90	0.95	0.93
Standard error of mean		0.38	0.42	0.39	0.18		0.14	0.09	0.13	0.11

IGF-I (Figure 14-31).	Treatment					Control				
Days post-injury	0	28	56	84	112	0	28	56	84	112
1	1	1.36	1.18	1.03	Unsuitable	1	2.02	0.83	0.93	1.28
2	1	1.08	0.24	1.13	0.64	1	2.05	1.16	0.39	Unsuitable
3	1	1.50	2.09	1.94	1.94	1	3.07	1.30	1.49	Unsuitable
4	1	1.73	1.40	1.22	0.99	1	1.39	1.14	Unsuitable	1.04
5	1	1.21	Unsuitable	0.64	5.04	1	1.12	1.09	0.86	0.95
6	1	0.63	0.83	0.66	0.55	1	0.63	0.45	0.19	0.50
7	1	1.52	0.99	1.30	1.29	1	Unsuitable	0.68	0.73	1.12
8	1	0.95	0.92	0.98	0.88	1	1.10	0.93	Unsuitable	0.89
9	1	1.78	1.15	0.57	Unsuitable	1	0.82	0.59	0.81	Unsuitable
10	1	0.64	1.18	0.74	0.75	1	1.35	0.45	0.91	1.15
11	1	Unsuitable	0.49	4.44	0.55	1	1.68	Unsuitable	0.84	0.81
12	1	1.32	1.39	1.66	1.19	1	1.03	Unsuitable	0.72	0.54
Mean		1.25	1.08	1.36	1.38		1.48	0.86	0.79	0.92
Standard error of mean		0.12	0.15	0.32	0.45		0.22	0.10	0.11	0.10

Appendix to Chapter 14.
The Assessment of the Effect of Nasal Packing on the Healing of the Nasal Mucosa of Sheep After Endoscopic Surgery.
Average relative cell size index data.

Merocel (Figure 14-17).	Treatment					Control				
Days post-injury	0	28	56	84	112	0	28	56	84	112
1	1	Unsuitable	1.09	0.93	0.88	1	Unsuitable	0.94	Unsuitable	1.18
2	1	Unsuitable	0.69	0.86	0.46	1	Unsuitable	0.28	0.69	1.30
3	1	1.39	4.36	1.09	1.61	1	1.12	0.73	Unsuitable	Unsuitable
4	1	1.25	Unsuitable	Unsuitable	1.54	1	0.74	1.89	Unsuitable	1.97
5	1	0.97	2.58	Unsuitable	0.68	1	Unsuitable	Unsuitable	Unsuitable	Unsuitable
6	1	0.58	Unsuitable	0.40	0.35	1	Unsuitable	Unsuitable	0.63	Unsuitable
7	1	3.14	2.69	2.78	2.18	1	Unsuitable	3.95	Unsuitable	4.14
8	1	2.81	3.17	1.93	4.14	1	Unsuitable	Unsuitable	Unsuitable	Unsuitable
9	1	Unsuitable	1.87	1.67	1.83	1	Unsuitable	Unsuitable	0.73	0.66
10	1	0.82	1.07	0.49	0.63	1	2.47	1.55	1.19	Unsuitable
11	1	0.59	1.17	Unsuitable	0.67	1	1.47	1.20	1.29	0.75
Mean		1.44	2.08	1.27	1.36		1.45	1.51	0.90	1.66
Standard error of mean		0.37	0.43	0.30	0.35		0.43	0.49	0.15	0.58

Merogel (Figure 14-25).	Treatment					Control				
Days post-injury	0	28	56	84	112	0	28	56	84	112
1	1	Unsuitable	3.35	0.95	0.71	1	0.14	0.90	0.37	0.35
2	1	1.85	2.84	2.64	1.70	1	1.31	1.03	0.91	1.08
3	1	0.31	0.59	0.45	0.88	1	0.78	Unsuitable	0.37	0.77
4	1	0.91	0.87	0.52	0.71	1	Unsuitable	0.43	Unsuitable	0.77
5	1	3.32	1.00	0.88	0.88	1	0.87	0.35	0.60	0.61
6	1	0.77	Unsuitable	Unsuitable	0.67	1	0.26	0.69	Unsuitable	0.67
7	1	1.52	1.17	2.17	1.23	1	Unsuitable	2.05	1.83	2.36
8	1	Unsuitable	Unsuitable	Unsuitable	Unsuitable	1	Unsuitable	0.63	0.49	1.04
9	1	Unsuitable	1.54	1.41	1.94	1	Unsuitable	2.52	2.97	2.17
10	1	2.07	1.40	1.28	1.25	1	Unsuitable	1.23	0.83	0.51
11	1	Unsuitable	0.98	0.79	1.35	1	Unsuitable	1.83	0.75	1.38
12	1	1.40	Unsuitable	1.53	2.14	1	0.92	0.74	0.62	0.52
13	1	0.54	0.38	0.89	0.62	1	0.13	1.38	1.14	1.67
Mean		1.41	1.41	1.23	1.17		0.63	1.15	0.99	1.07
Standard error of mean		0.33	0.32	0.22	0.16		0.19	0.21	0.25	0.19

IGF-I (Figure 14-32).	Treatment					Control				
Days post-injury	0	28	56	84	112	0	28	56	84	112
1	1	1.35	1.30	0.66	Unsuitable	1	3.47	1.45	1.07	1.27
2	1	1.18	0.21	1.24	1.17	1	1.61	1.00	0.51	Unsuitable
3	1	2.73	2.68	2.44	2.75	1	Unsuitable	0.92	2.32	Unsuitable
4	1	0.93	1.35	1.64	1.16	1	0.60	0.87	Unsuitable	1.12
5	1	Unsuitable	Unsuitable	0.60	0.50	1	0.70	0.88	0.54	1.04
6	1	Unsuitable	0.96	0.69	0.69	1	Unsuitable	0.61	Unsuitable	0.66
7	1	2.38	1.20	1.41	1.56	1	Unsuitable	0.85	0.81	1.23
8	1	0.76	1.13	0.70	0.94	1	1.13	1.15	Unsuitable	1.03
9	1	1.51	Unsuitable	0.84	Unsuitable	1	1.22	0.37	1.22	Unsuitable
10	1	0.65	1.60	0.60	0.93	1	1.95	0.66	1.49	2.21
11	1	Unsuitable	1.04	0.54	0.64	1	2.98	Unsuitable	1.38	1.28
12	1	0.90	1.51	1.37	0.97	1	1.01	Unsuitable	0.41	0.58
Mean		1.38	1.30	1.06	1.13		1.63	0.88	1.08	1.16
Standard error of mean		0.26	0.21	0.17	0.21		0.35	0.10	0.21	0.17

Appendix to Chapter 14.
The Assessment of the Effect of Nasal Packing on the Healing of the Nasal Mucosa of Sheep After Endoscopic Surgery.
Relative collagen I to III ratio.

Merocel (Figure 14-18).	Treatment					Control				
Days post-injury	0	28	56	84	112	0	28	56	84	112
1	100	80.50	100.65	79.68	66.12	100	194.46	86.02	139.82	54.01
2	100	119.61	Unsuitable	130.84	52.54	100	104.64	157.31	75.53	58.37
3	100	132.10	Unsuitable	90.47	41.76	100	298.52	96.86	306.66	133.77
4	100	39.25	77.40	144.95	114.07	100	96.51	162.61	65.15	59.86
Mean		92.87	89.03	111.49	68.62		173.53	125.70	146.79	76.50
Standard error of mean		24.23	16.44	18.10	18.41		54.51	23.02	64.42	22.09

Merogel (Figure 14-25).	Treatment					Control				
Days post-injury	0	28	56	84	112	0	28	56	84	112
1	100	43.09	42.30	27.64	34.32	100	52.41	56.60	51.13	52.72
2	100	115.93	162.32	175.88	235.11	100	Unsuitable	Unsuitable	Unsuitable	Unsuitable
3	100	56.15	Unsuitable	23.87	45.26	100	26.88	Unsuitable	56.59	15.88
4	100	240.86	103.90	136.01	271.76	100	217.51	224.86	107.52	156.27
Mean		114.01	102.84	90.85	146.61		98.93	140.73	71.75	74.96
Standard error of mean		52.15	42.44	44.41	71.78		73.17	118.97	21.99	51.47

IGF-I (Figure 14-32).	Treatment					Control				
Days post-injury	0	28	56	84	112	0	28	56	84	112
1	100	57.03	84.76	81.22	170.58	100	96.34	95.25	145.42	36.05
2	100	489.15	189.56	235.10	247.19	100	239.00	85.68	254.33	178.07
3	100	77.32	95.75	56.59	195.23	100	64.31	114.14	54.55	315.28
4	100	195.47	156.88	155.13	219.96	100	129.47	132.68	141.19	221.39
Mean		204.74	131.74	132.01	208.24		132.28	106.94	148.87	187.70
Standard error of mean		115.00	28.82	46.46	18.98		43.85	12.03	47.25	67.08

Appendix to Chapter 14.
The Assessment of the Effect of Nasal Packing on the Healing of the Nasal Mucosa of Sheep After Endoscopic Surgery.
Cilial regeneration measured by SEM and image analysis.
Validation of the technique by two blinded observers on two occasions.

Summary of SEM assessment of cilial regeneration evaluated by observer, LS

Specimen	1st Measurement	2nd Measurement	Difference	Average
1	69.31	68.82	0.49	69.07
2	66.99	30.85	36.14	48.92
3	76.61	76.44	0.17	76.53
4	78.22	78.95	0.73	78.59
5	81.83	75.24	6.59	78.54
6	48.38	71.15	22.77	59.76
7	64.28	63.13	1.15	63.71
8	80.65	79.13	2.67	79.89
9	70.56	66.67	3.89	68.62
10	88.75	90.14	1.39	89.45
11	82.9	86.61	3.71	84.75
12	78.63	78.41	0.22	78.52
13	80.54	79.48	1.14	80.01
14	72.11	77.76	5.65	74.94
15	71.12	75.99	4.87	73.55
16	70.77	78.23	7.46	74.5
17	81.1	83.88	2.78	82.49
18	65.75	72.29	6.54	69.02

Summary of SEM assessment of cilial regeneration evaluated by observer, DM

Specimen	1st Measurement	2nd Measurement	Difference	Average
1	64.67	69.5	4.83	67.08
2	48.6	60.06	11.39	54.37
3	67.3	68.3	1	67.8
4	69.67	61.3	8.37	65.5
5	66.25	67.5	1.25	66.88
6	72.5	56	16.5	64.25
7	67.6	65.6	1	66.6
8	64.33	59	5.33	61.67
9	59	69.5	10.5	64.25
10	64	74.5	10.5	69.25
11	72.73	69.3	3.43	71.02
12	85.64	85.52	0.12	85.58
13	78.73	76.1	2.63	77.42
14	70.69	84.05	13.36	77.37
15	79.65	82.07	2.42	80.86
16	74.62	71.39	3.23	73.01
17	81.81	73.57	8.24	77.69
18	67.33	78.58	11.25	72.95

Comparison between LS and DM

Specimen	Mean LS	Mean DM	Difference	Average
1	69.07	67.08	1.99	68.075
2	48.92	54.37	5.45	51.645
3	76.53	67.8	8.73	72.165
4	78.59	65.5	13.09	72.045
5	78.54	66.88	11.66	72.71
6	59.76	64.25	4.49	62.005
7	63.71	66.6	2.89	65.155
8	79.89	61.67	18.22	70.78
9	68.62	64.25	4.37	66.435
10	89.45	69.25	20.2	79.35
11	84.75	71.02	13.73	77.885
12	78.52	85.58	7.06	82.05
13	80.01	77.42	2.59	78.715
14	74.94	77.37	2.43	76.155
15	73.55	80.86	7.31	77.205
16	74.5	73.01	1.49	73.755
17	82.49	77.69	4.8	80.09
18	69.02	72.95	3.93	70.985

Appendix to Chapter 14.
The Assessment of the Effect of Nasal Packing on the Healing of the Nasal Mucosa of Sheep After Endoscopic Surgery.
Cilia regeneration measured with electron microscopy.

Merocel (Figure 14-17).	Treatment		Control	
Days post-injury	56	112	56	112
1	62.95	81.07	59.62	77.25
2	Unsuitable	79.80	Unsuitable	83.44
3	70.29	77.55	71.13	75.09
4	64.96	66.64	68.68	74.08
5	71.75	66.98	78.59	70.48
6	68.10	72.81	74.49	Unsuitable
7	67.21	69.19	Unsuitable	71.57
8		64.94	62.97	79.18
9	73.43	72.98	72.18	60.30
10	68.32	Unsuitable	Unsuitable	67.33
11	Unsuitable	60.62	67.59	Unsuitable
Mean	68.38	71.26	69.41	73.19
Standard error of mean	1.30	2.25	2.31	2.41

Merogel (Figure 14-28).	Treatment		Control	
Days post-injury	56	112	56	112
1	66.20	80.51	50.04	72.34
2	69.00	Unsuitable	72.48	Unsuitable
3	Unsuitable	81.07	Unsuitable	77.25
4	Unsuitable	Unsuitable	71.65	56.84
5	21.87	81.02	59.36	Unsuitable
6	60.83	66.64	71.30	74.08
7	60.43	76.61	72.82	76.41
8	66.63	68.86	75.66	68.67
9	62.65	69.19	57.75	71.57
10	73.38	80.56	68.35	Unsuitable
11	72.57	Unsuitable	Unsuitable	67.48
12	66.27	68.28	63.49	65.67
13	56.65	66.74	67.31	77.18
Mean	61.50	73.95	66.38	70.75
Standard error of mean	4.46	2.17	2.49	2.12

IGF-I (Figure 14-34).	Treatment		Control	
Days post-injury	56	112	56	112
1	Unsuitable	62.38	Unsuitable	72.50
2	40.47	74.41	75.18	67.99
3	61.81	73.08	76.37	67.81
4	68.03	73.74	75.29	68.86
5	71.86	69.38	69.29	68.24
6	75.05	82.12	73.82	82.20
7	Unsuitable	72.83	74.58	Unsuitable
8	82.32	Unsuitable	69.23	70.25
9	64.38	Unsuitable	70.74	Unsuitable
10	Unsuitable	68.28	Unsuitable	65.67
11	Unsuitable	Unsuitable	69.09	Unsuitable
12	69.06	77.98	52.72	66.47
Mean	66.62	72.69	70.63	70.00
Standard error of mean	4.66	2.01	2.30	1.77

Appendix to Chapter 14.
The Assessment of the Effect of Nasal Packing on the Healing of the Nasal Mucosa of Sheep After Endoscopic Surgery.
Cilia maturity measured with electron microscopy.

Merocel (Figure 14-21).	Treatment		Control	
Days post-injury	56	112	56	112
1	0.00	76.92	0.00	72.73
2	Unsuitable	0.00	Unsuitable	0.00
3	0.00	100.00	Unsuitable	14.29
4	0.00	11.11	0.00	91.67
5	Unsuitable	55.56	0.00	100.00
6	0.00	0.00	Unsuitable	Unsuitable
7	Unsuitable	0.00	Unsuitable	Unsuitable
8	Unsuitable	0.00	0.00	100.00
9	Unsuitable	86.67	20.00	80.00
10	0.00	Unsuitable	Unsuitable	0.00
11	0.00	0.00	0.00	Unsuitable
Mean	0.00	33.03	3.33	57.33
Standard error of mean	0.00	13.94	3.65	16.90

Merogel (Figure 14-29).	Treatment		Control	
Days post-injury	56	112	56	112
1	27.27	100.00	Unsuitable	0.00
2	0.00	27.27	0.00	57.14
3	Unsuitable	100.00	Unsuitable	Unsuitable
4	Unsuitable	0.00	0.00	Unsuitable
5	0.00	Unsuitable	Unsuitable	Unsuitable
6	Unsuitable	100.00	0.00	10.00
7	0.00	0.00	50.00	40.00
8	Unsuitable	Unsuitable	50.00	Unsuitable
9	Unsuitable	100.00	22.22	92.31
10	0.00	Unsuitable	0.00	Unsuitable
11	0.00	0.00	20.00	100.00
12	0.00	60.00	0.00	Unsuitable
13	0.00	Unsuitable	50.00	91.67
Mean	3.41	54.14	19.22	55.87
Standard error of mean	3.64	16.75	7.62	16.70

IGF-I (Figure 14-35).	Treatment		Control	
Days post-injury	56	112	56	112
1	Unsuitable	55.56	0.00	57.14
2	Unsuitable	36.36	33.33	87.50
3	0.00	37.50	71.43	66.67
4	Unsuitable	30.77	0.00	0.00
5	18.75	0.00	0.00	Unsuitable
6	0.00	90.00	0.00	Unsuitable
7	0.00	0.00	0.00	100.00
8	100.00	Unsuitable	0.00	Unsuitable
9	0.00	25.00	Unsuitable	0.00
10	Unsuitable	Unsuitable	0.00	Unsuitable
11	Unsuitable	90.00	25.00	88.89
12	7.69	Unsuitable	Unsuitable	Unsuitable
Mean	18.06	40.58	12.98	57.17
Standard error of mean	15.02	11.71	7.98	16.99