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The effect of nasal packing and prednisolone on mucosal healing and reciliation in a sheep model*

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SUMMARY

Statement of problems: *To determine whether topical prednisolone affected sinus mucosal healing in a sheep animal model.*

Methods of study: *A standardised sheep model with concurrent Oestrus ovus infection was utilised. Following full thickness nasal mucosal injuries, hyaluronic acid packs soaked with prednisolone were applied to one side of the nasal cavity, with hyaluronic acid packs without prednisolone, to the other. At four weekly intervals, for 16 weeks, mucosal biopsies were taken and analysed for epithelial thickness and length, for cilial height and percentage of ciliated mucosa and mucosal inflammation.*

Results: *Eighteen sheep were utilised, with each acting as its own control. At completion of the study there was no statistical difference between the control or treatment group for the percentage of mucosal epithelialisation (mean: 86.5% vs 92.2%), epithelial thickness (mean: 39.7μm vs 39.3μm or the percentage of epithelium ciliated (mean: 26.5% vs 30.8%). Furthermore prednisolone had no effect on mucosal inflammation with the control arm mean score of 3.44 (SD .022) and treatment arm mean of 3.77 (SD 0.21).*

Conclusion: *Hyaluronic acid nasal packs soaked in prednisolone failed to improve the speed of mucosal healing or re-ciliation in the sheep model of eosinophilic rhinosinusitis.*

Key words: sheep model, prednisolone, mucosal healing

INTRODUCTION

The healing of mucosal injuries following sinus surgery is a complex process. Although there is a good understanding of the physiology of wound healing, there is still a significant gap between this understanding and the practicalities of positively modifying mucosal wound healing. Post-operative wound healing is characterised histologically by four different phases (Watelet et al., 2002). Following sinus surgery the first coagulation phase lasts between 7 and 12 days and clinically is identified by blood crusting over the area of mucosal injury (Watelet et al., 2002). The second inflammatory phase is characterised by the formation of granulation tissue which lasts two to four weeks (Steed, 1997). The third phase of tissue remodelling is characterised by angiogenesis, reepithelialisation and fibroblast cell migration is maintained by cytokines secreted by macrophages within the lamina propria (Moriyama et al., 1996). This phase lasts up to 16 weeks (Rajapaksa et al., 2004). The last phase of tissue remodelling is characterised by the restoration of normal mucosa and lasts up to 6 months. These phases of wound healing are controlled by a variety of growth factors, including TGF-β (Shah et al., 1995), PDGF, Epidermal growth factors such as TGFα and FGF, which work in a coordinated way largely due to variations in their tissue concentrations. These polypeptides control the growth, metabolism and differentiation of cells is involved in the stimulation of fibronectin, elastin and collagen synthesis.

Work at our institution has demonstrated that the presence of mucosal inflammation impairs healing after endoscopic sinus surgery (ESS) (Rajapaksa et al., 2004). In the sheep model we demonstrated a significant reduction in mucosal re-epithelialisation in the presence of eosinophilic sinusitis. Other authors have demonstrated the presence of persistent inflammation due to incomplete removal of diseased tissue, may lead to recurrence of the primary pathology (Hinni et al., 1992). The use of steroids in reducing postoperative inflammation shows some promise. Steroids have anti-inflammatory effects which act due to the inhibition of mRNA synthesis. As such they inhibit the production of leukotrienes, histamines, cytokines like IL-4 and IL-13 as well as regulating IgE production. Studies show that steroids aid in the acceleration of healing, reduces oedema which is characteristically seen in the third phase of wound healing, reduces the formation of granulation

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tissue and accelerates the late phases of epithelial wound closure (Lavigne et al., 2002; Watelet et al., 2002).
The aim of this study was to use a sheep model with diseased nasal mucosa similar to that found in humans with rhinosinusitis, and evaluate the effect of Hyaluronic acid impregnated with prednisolone on the healing process. Our research group has developed a sheep model for the investigation of ESS (Shaw et al., 2001). We have demonstrated that this model is suitable for the investigation of mucosal healing in the presence of concurrent inflammation (Rajapaksa et al., 2004).

In order to apply the steroid to the wound a dissolvable substance (hyaluronic acid ester) was used that was soaked in prednisolone. The hyaluronic acid nasal pack (Merogel, Medtronic Xomed®, Jacksonsville, USA) is a biomaterial and represents an esterified version of Hyaluronic acid. Hyaluronic acid has been implicated in several biological situations from the regulation of embryonic development to wound healing and effects on cellular structural integrity (Longaker et al., 1991; Jacob et al., 2002). Its use in the post-ESS situation is of interest because of its potential benefit on reducing postoperative scarring and thus improving the surgical outcomes. Jacobs demonstrated in the rabbit model that merogel encourages new bone formation in the sinonasal cavity (Jacob et al., 2002). However their experiment utilised a large amount of merogel in an unsoaked form, which was tightly packed onto a sinus denuded of tissue. Work in our department showed in a healthy sheep model that merogel significantly improved mucosal healing when compared to controls without any evidence of new bone formation (McIntosh et al., 2002). However a similar study using the same sheep model but with active parasitic infection failed to demonstrate a treatment benefit with Merogel (Rajapaksa et al., 2004). Previous human trials have utilised both Hyaluronic acid as a cream (Rhinogen®) and pack (Supragel Sinus-Hylan B®) after ESS, and have demonstrated significant reductions in nasal crusting, improved mucosal healing and reduced synechia (Soldati et al., 1999; Kimmelman et al., 2001). Merogel was selected as the transport medium for the prednisolone principally because our department had documented its affects on wound healing, thus allowing us to determine any additional effects the prednisolone would have on mucosal regeneration.

METHODS

The Animal Ethics Committees of the University of Adelaide granted approval for the study. Eighteen sheep that had not been treated for the parasite *Oestrus ovus* were utilised. The study proceeded in two-phases. Standardisation of the nasal cavity followed our previously published protocol (Shaw et al., 2001).

Surgical procedure

Under general anaesthesia, two full thickness nasal mucosal injuries were performed. Lateral nasal wall mucosal biopsies were taken from both sides. These were designated Day 0 samples. Next a 4mm microdebrider (Medtronic Xomed®) was used to create a 40x20mm full thickness mucosal injury on the lateral nasal wall of each nasal cavity. A 40x20mm piece of Merogel (Medtronic Xomed®) was placed on each mucosal injury. The sheep were used as their own controls, and were randomised to receive the treatment to one side or the other. The treatment involved soaking one of the merogel packs with 5mls of 0.5% prednisolone. On the other side (control side) plain merogel was applied. Three days after the treatment the sheep were dipped to eradicate the *Oestrus ovus*. At days 28, 56, 84 and 112 after the mucosal injuries, further biopsies were taken. All biopsies were harvested under sedation using Xylazine 20mg/ml, with topical anaesthesia. Four different areas of the original mucosal injury were harvested on each occasion.

Tissue preparation

Biopsies were fixed in formalin for 12 hours then transferred to 70% ethanol before being embedded in paraffin wax. Sections of 4μm thickness were cut on Leica Biocut 2035 microtome and baked in a 55°C oven for 24 hours. All sections were stained in haematoxylin and eosin (H&E). Briefly, samples were dewaxed in xylene and brought to water through graduated ethanol baths. They were then stained in Meyer's haematoxylin (Merck KGaA, Darmstadt, Germany), for 5 minutes, washed in water and then rinsed briefly (10 sec) in $LiCO_3$ (saturated solution diluted 1:5), to "blue" the sections and washed again in water. The sections were then counter-stained in Eosin B (Sigma-Aldrich, St. Louis, MI, USA), for 90 seconds, washed in water then dehydrated through graduated ethanol baths to xylene. Slides were mounted using DePeX mounting medium (BDH Laboratory Supplies, Poole, England).

Specimens were viewed with a Sony SSC-DC50P digital camera attached to an Olympus BH-2 bright-field microscope utilising the Image-Pro Plus software (Media Cybernetics, Maryland, USA). Epithelial length and thickness were measured under 10x magnification. Epithelial length and epithelialisation was measured by drawing a line along the surface of the keratinocytes and calculated as a percentage of the entire length of the specimen (surface length). Epithelial thickness was measured between the line drawn on the surface of the keratinocytes and another line drawn along the basement membrane. It was calculated using the software package and expressed as an average height for the length of epithelium. The length of ciliation was measured under 20x magnification and is expressed as a percentage of total epithelial length. (not total specimen length). The epithelia inflammation was graded from H&E slides. The level of polymorphonuclear lymphocyte infiltration was graded from 1 to 5 (one being the least inflamed). All data was assessed in triplicate.

All data is presented as mean values (+/- standard error of the

mean (SEM)). Significance was determined using a two-tailed, unequal variance, Students' t-test. A significant result is viewed as $p < 0.05$.

RESULTS

Eighteen sheep infected with the parasite *Oestrus ovus* underwent the study protocol.

Effect of prednisolone on the percentage of mucosal epithelialisation

On day zero there was 83.2% (control) vs 78.4% (treatment) epithelialisation. In the process of harvesting the mucosa we expected a 15% loss in epithelialisation, due to trauma of through-biting forceps around the edges of the specimen. Therefore day zero figures represented complete epithelisation. There was no statistical difference in the epithelisation between treatment arms at days 28, 56, 84 or 112 (Table 1). The percentage epithelialisation returned to day zero levels by day 84.

Effect of prednisolone on epithelial thickness

The thickness of mucosal epithelium at day zero was 29.2μm (control) vs 34.9μm (treatment) arms. There was a substantial increase in mucosal thickness at day 28 after mucosal injury. This represents tissue oedema during the first two phases of wound healing following mucosal injury. There was no statistical difference between the two treatment arms at days 28, 56, 84 or 112 (Table 1). Epithelial thickness reduced over the healing period and approached but did not achieve day zero levels.

Effect of prednisolone on the percentage of epithelium ciliated

At day 28 after the mucosal injuries there was almost complete absence of cilia (Table 1). The restoration of normal mucosal ciliation improved with each sequential biopsy but had still not returned to normal by day 112.There was no treatment effect at days 28, 56, 84 or 112 for the percentage of epithelium ciliated.

Table 1. Results of mucosal biopsies at Days 28-112, comparing the control (merogel) with treatment (merogel-prednisolone) arms expressed as means for percentage of mucosa fully epitheliated, epithelial thickness and the percentage of epithelium ciliated.

		Percentage of mucosal biopsy fully epitheliated		**Epithelial thickness (μm)**		**Percentage of epithelium Ciliated**	
Day 28	Control	47.2	CI:8.6	59.1	CI:9.2	0.3	CI:.2
	Treatment	32.4	CI:7.2	49.9	CI:6.9	0.4	CI:0.2
	p-value	**0.18**		**0.43**		**0.79**	
Day 56	Control	74.5	CI:7.1	50.1	CI:6.5	17.2	CI:4.0
	Treatment	63.6	CI:7.9	40.3	CI:4.9	19.3	CI:3.1
	p-value	**0.31**		**0.23**		**0.70**	
Day 84	Control	77.9	CI:5.6	52.3	CI:3.2	24.8	CI:4.4
	Treatment	83.4	CI:5.1	59.1	CI:2.3	24.0	CI:4.3
	p-value	**0.47**		**0.095**		**0.92**	
Day 112	Control	86.5	CI:3.5	39.7	CI:3.2	26.5	CI:4.7
	Treatment	92.2	CI:2.2	39.3	CI:2.8	30.8	CI:3.6
	p-value	**0.15**		**0.91**		**0.73**	

(CI=95% Confidence Intervals)

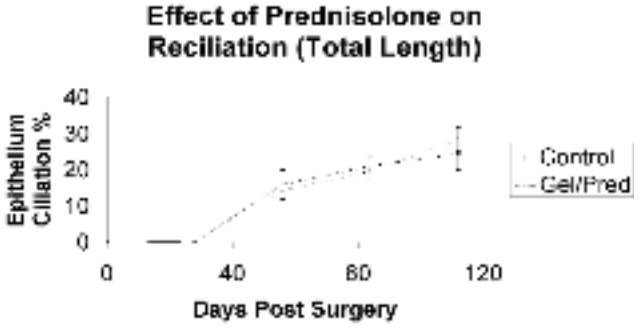

Figure 1. Effect of prednisolone on reciliation (total length).

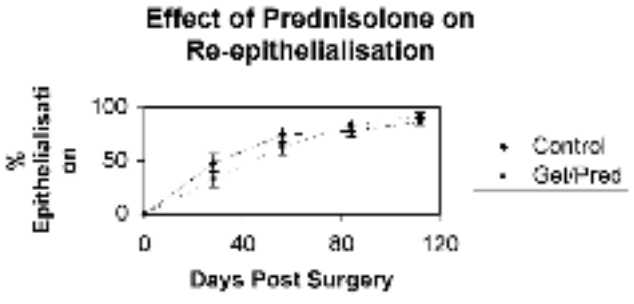

Figure 2. Effect of prednisolone on re-epitheliasation (total length).

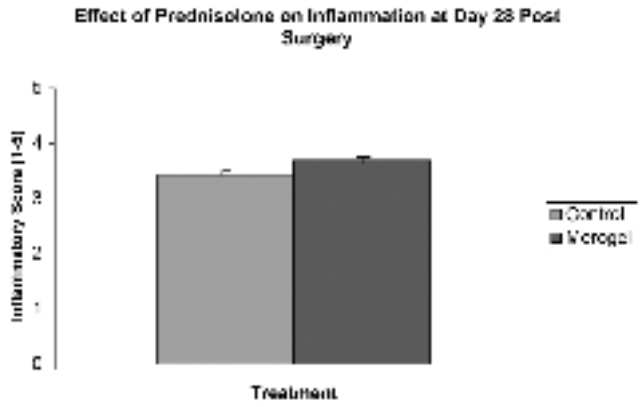

Figure 3. Effect of prednisolone on inflammation at day 28 post surgery.

Effect of prednisolone on mucosal inflammation

There was no treatment effect with prednisolone on mucosal inflammation at day 28, with a score of 3.48 (control) vs 3.77 (treatment) (p=0.38). Analysis was not performed at days 56, 84 or 112 as mucosal inflammation is limited to the first two to four weeks following mucosal injury.

DISCUSSION

Several factors influence the outcomes of patients undergoing ESS. These include patient factors (including previous sinonasal surgery, age, smoking, nasal allergy), disease factors (including the presence of active infection, disease type) and technical aspects relating to the surgeons skill and extent of surgery (Senior et al., 1998). We were interested in identifying factors which could be favourably modified and would improve the healing of sinonasal mucosa after ESS. Our sheep model demonstrated that when prednisolone soaked Merogel was placed over full thickness mucosal injuries as opposed to Merogel alone, there was no difference in healing as measured by epithelial thickness, percentage epithelialisation or the percentage of mucosa that had reciliated. However, the Merogel soaked in prednisolone did not detrimentally affect mucosal healing.

Our department has developed a sheep model for rhinosinusitis (Shaw et al., 2001). We used the sheep model to investigate the use of nasal packing, and especially its removal can hamper the healing of the nasal mucosa, as measured by the return of normal ciliary numbers post operatively (Shaw et al., 2000). Further work has investigated the used of 2 new forms of dissolvable packing, namely Merogel and Merogel impregnated with IGF-1 (McIntosh et al., 2002). This has been done in both healthy sheep, as well as sheep infected with *Oestrous ovi*, a nasal parasite which causes an eosinophilic sinusitis. Initial analysis of results of this work suggests that neither Merogel nor Merogel impregnated with IGF-1 seems to have significant effect on the healing process in sheep with sinusitis. The findings in this study, along with the use of topical IGF-1, indicate that the topical application of growth factors to modify mucosal healing do not appear to be successful.

Sinonasal mucosa is composed of pseudo-stratified epithelium (ciliated and non-ciliated cells, goblet and basal cells) which lies on a basement membrane, underneath of which is the lamina propria. After mucosal injury the speed and completeness of healing of this mucosa is dependent on several variables including the thickness of mucosal injury and the presence or absence of infection. The migration of new respiratory epithelium into a wound will begin within a few hours of the injury at a rate of 0.04mm/hour (Hosemann et al., 1990). Moriyama et al. (1996) investigated variation in the healing of sinus mucosa after superficial (superficial epithelium removed) and full thickness (bone exposed) injuries (Moriyama et al., 1996). At 6 months following injury normal epithelium was present in the superficial group, whereas there were scattered scabs and scar tissue along with incomplete epithelial regeneration in the full thickness group. At 12 to 18 months after injury there were still only scattered ciliated cells in the bone exposed group. In our sheep model we formed a full thickness mucosal injury. Epithelial thickness did not return fully to its pre-injury state, though there was a steady progression with time towards this. The oedematous phase, as described above, is said to regress faster with the application of steroids (Watelet et al., 2002). This was not the case in our study. Reciliation was identical for the two treatment groups. It has been shown that sinonasal mucosa will regenerate successfully after ESS with improvements in mucosal architecture and ciliation (Keles et al., 2001).

Our study failed to show an improvement in sinonasal mucosal healing with the topical application of prednisolone. Possible reasons for this lack of effectiveness may be the low level of parasitic prevalence in this cohort of sheep. Infection of sheep with *Oestrus ovus* in South Australia is endemic. In our past studies larva are usually seen to migrate from the sinuses into the nasal cavity when endoscopy is performed. It was noted at the time of this study that few *Oestrus ovus* larva were seen. A low level of *Oestrus ovus* infection would lessen the generation of an eosinophilic inflammation of the sinonasal mucosa. If the inflammation present was mild, the potential benefit of prednisolone would be less than that seen with an overt eosinophilic inflammation. Secondly, merogel may not provide sufficient prednisolone at the wound site in the post-operative period. This because as Merogel absorbs a significant amount of fluid, the Merogel may have been partially saturated with transudate and blood prior to soaking with prednisolone.

CONCLUSION

Hyaluronic acid nasal packs soaked in prednisolone failed to improve the speed of re-epithelialisation or re-ciliation in the sheep model of eosinophilic rhinosinusitis.

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