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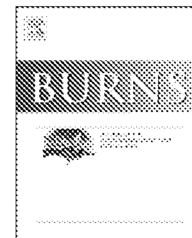

A randomised controlled trial of amniotic membrane in the treatment of a standardised burn injury in the merino lamb

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ABSTRACT

Burn injury is associated with disabling scar formation which impacts on many aspects of the patient's life. Previously we have shown that the fetus heals a deep dermal burn in a scarless fashion. Amniotic membrane (AM) is the outermost fetal tisue and has beeen used as a dressing in thermal injuries, though there is little data to support this use. To assess the efficacy of AM in scar minimisation after deep dermal burn wound, we conducted a randomised controlled study in the 1-month lamb. Lambs were delivered by caesarian section and the amniotic membranes stored after which lambs were returned to their mothers post-operatively. At 1 month, a standardised deep dermal burn was created under general anaesthesia on both flanks of the lamb. One flank was covered with unmatched AM, the other with paraffin gauze. Animals were sequentially euthanased from Day 3–60 after injury and tissue analysed for histopathology and immunohistochemically for α-smooth muscle actin (αSMA) content. AM resulted in reduced scar tissue as assessed histopathologically and reduced αSMA content. This study provides the first laboratory evidence that AM may reduce scar formation after burn injury.

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1. Introduction

Burn is a major cause of morbidity and mortality in both children and adults. The advances in critical care, resuscitation and aggressive early surgery have led to a marked survival in patients with major burns [1]. However, patients surviving these huge injuries, together with those patients with smaller injuries, are surviving with disfiguring hypertrophic scars. Post-burn scar is frequently a more problematic issue in the paediatric population than a corresponding injury in the adult as the rate of constitutive growth of the paediatric patient far outstrips the growth of the scar tissue [1,2]. Scar tissue over flexure surfaces and associated with joints can cause major disruption with normal range of movement and inhibit optimal physical and psychosocial development of the child regardless of therapy [3]. A dressing that is relatively inexpensive, improves epithelialisation rate and reduces scar formation, whilst being relatively non-immunogenic would be an ideal burn dressing.

Amniotic membrane (AM) is the outermost layer of the fetus, and innermost layer of the placenta, and consists of a columnar epithelial monolayer, an avascular stromal matrix

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and a thick basement membrane composed of type IV and V collagen and laminin [4]. The use of AM in burn wounds was first described by Davies in 1910 in the United States as an effective dressing which reduced pain [5]. It is still used frequently in the treatment of corneal injuries and in the developing world as a burn dressing where it is has been described as effective in promoting epithelialisation, reducing hospital length of stay and reducing pain [6,7]. In the developed world, AM has been used occasionally in the treatment of deep dermal burn as a temporary dressing, and as an inexpensive substitute for cadaveric skin, where it has been shown to optimise the wound bed for future skin grafting, by reducing infection and encouraging angiogenesis [8]. However, there has been limited scientific study comparing AM with any standard dressing. As a fetal tissue, AM has a number of properties which may be seen as ideal. Previous work has identified that the early to mid-term fetus heals incisional wounds in a scarless fashion [9]. More recently, our group has shown the fetus can also heal a deep dermal burn injury (DDBI) in a scarless fashion [10]. It has been postulated that some of these beneficial properties shown by AM may be due to its fetal nature.

We hypothesised that AM would confer a fetal-like wound repair process after standardised DDBI. To test this hypothesis, we compared AM versus a neutral burn dressing in 1 month old merino lambs, and assessed the morphological, microscopic and immunohistochemical differences in the wound regeneration.

2. Methods

2.1. Amniotic membrane harvest

Following Animal Ethics Committee approval, merino ewes were time mated, following super ovulation (500 IU Folligon-Intervet International, Worthington, MN) and housed in the animal research centre. At day 145–147 (normal gestation = 150) pregnant ewes were fasted for 24 h prior to induction of anaesthesia with thiopentone 10–15 mg/kg and intubation of the larynx with a size 10 cuffed endotracheal tube (Portex™ Smiths Medical International, UK). Anaesthesia was maintained with halothane in 100% oxygen and continuous pulse oximetry monitoring was used throughout the procedure. Ten milligrams buprenorphine was administered intra-muscularly prior to the initial incision. Intravenous access was achieved, placing a triple lumen central venous catheter (Arrow International, Reading, USA) in the right internal jugular vein through which 1 L of Hartmann's solution was administered through the course of the operation and recovery. Following sterile preparation of the abdomen, a paramedian incision was created, and the uterus delivered to the operating field. The fetus was retrieved through a hysterotomy, the umbilical cord ligated and the lamb was intubated and resuscitated. Once fully awake and spontaneously breathing, the lamb was extubated and placed in a box filled with warmed towels. The uterus was exteriorised, and uterine vessels crushed and ligated. Hysterectomy was then carried out. Following haemostasis, the laparotomy wound was closed in a sterile fashion. The AM was then retrieved from the uterus. The membranes were lavaged with sterile PBS solution containing antibiotics (50 units/mL penicillin, 50 μg/mL streptomycin and 0.125 μg/mL amphotericin B) to minimise the risk of infection. Following lavage, the AM was then placed in a sterile manner onto discs of sterilised paper as a support and stored in 50% Dulbecco's modified Eagle medium (DMEM)/50% glycerol. The ewe was then extubated and recovered in the pen along side the newly born lamb, where analgesia was administered. All lambs were able to suckle from the mother over the first night.

2.2. Creation of scald and treatment

One month later, a standardised deep dermal burn was created as previously described by our group [10]. The lambs were anaesthetised with thiopentone (15 mg/kg) prior to intubation of the larynx with a size 6 cuffed endotracheal tube (Portex™ Smiths Medical International, UK). Anaesthesia was continued with halothane/100% oxygen mixture, and continuous pulse oximetry monitoring was used throughout the procedure. A single intramuscular dose of Flunixin™ (Finadyne) at a dose of 2 mg/kg was administered preoperatively. The lamb was shaved on a standard area of the lower abdomen, initially with sheep clippers then with a safety razor, lukewarm water and shaving cream, until the skin was free of wool. The wound was then gently washed with lukewarm water to remove any soap residue.

Polypropylene tubes of 15 mL volume, with a 1.5 cm diameter were filled with sterile water and were heated in a hot water bath. The temperature of the water was measured using a digital thermometer (N19-Q1436 Dick Smith, Australia, range −50 °C to 100 °C (±0.5%)). We have previously identified a temperature of 82 °C for 10 s consistently creates a deep dermal injury in the lamb at 1-month post-delivery [10]. Once the tubes had reached this temperature, they were removed in a sterile manner from the water bath and promptly inverted onto the flank of the lamb, care being taken to avoid spillage. A total of six identical scalds were created—three on the left and three on the right flank. To aid in identification of wounds at euthanasia, the margins of all wounds were identified by tattooing of the wound with ink with a 25 gauge needle. The discs of stored AM were brought to room temperature, rinsed three times in sterile 0.9% saline, and laid amnion side upwards onto a piece of paraffin impregnated gauze (Jelonet™, Smith and Nephew, Australia). This amnion side was then placed directly onto the wounds on one flank, ensuring coverage of the entire wound area. The wounds on the other flank were covered with Jelonet™ alone. All wounds were then covered with an inert absorbent dressing (Melonin™ Smith and Nephew, Australia) to reduce direct trauma and soiling to the wound, and the whole treatment stitched to the skin (at a minimum 3 cm margin from the wound). A stocking bandage was passed over the entire dressing, to prevent dislodgement of the dressings.

2.3. Tissue retrieval

A total of 21 lambs were scalded in accordance with the model described above. Three animals were randomly selected on each of post-operative days 1, 3, 5, 7, 14, 21 and 60 and

euthanased. Discs of injured skin from the experimental and control areas (i.e. injured skin covered with either amnion or paraffin gauze) were obtained from each animal. Uninjured skin was also obtained from the same animals to allow for direct comparison.

2.4. *Histopathology*

Tissue sections were fixed in 4% buffered formalin and blocked in paraffin. Sections of 4 μm thickness were stained with haematoxylin and eosin. These sections were then examined in a blinded manner by the same histopathologist who assisted in the creation of the burn model. Between three and four sections on each slide were examined. We have previously validated this technique of scoring thermally injured skin [10]. Briefly, to quantify degree of tissue damage/recovery, a scoring system was developed to enable objective assessment. A total of five markers of injury were observed: number of fibroblasts, alteration of interstitial tissue, epidermal thickness, number of hair follicles and alteration in papillary and reticular dermis. These markers were graded from 0 to 3, with 0 = normal and 3 = severe abnormality (for example: 0 = normal number of hair follicles and 3 = very few hair follicles remaining). For each tissue section these scores were then added together to get a total score for each sample. To validate this novel scoring system, the same histopathologist scored the slides on two separate occasions, between 1 and 3 months apart and was blinded to the previous scores of the same slides. Once all slides were assessed and scored on the second occasion, the scores in each category were compared against those from the first assessment and a kappa statistic was determined which gives a measure of concordance between these scores. A kappa value greater than 0.4 is considered to show good concordance.

2.5. *Immunohistochemical analysis of scarring*

Paraffin sections of 4 μm thickness of burn and control skin were immunohistochemically analysed for α-smooth muscle actin (αSMA). Slides were de-waxed, hydrated and washed with tris-buffered saline (TBS). All slides had an endogenous peroxide removal step (0.6% H_2O_2 in TBS, 10 min) followed by blocking in 4% powdered milk in TBS (αSMA). The primary antibody used was monoclonal mouse anti-αSMA (#A2547 Sigma, St Louis, MI)) 1/400 in 1% BSA in TBS for 90 min at room temperature. The secondary antibody used was goat anti-mouse-HRP (#P0447, Dako Cytomation, Zug, Switzerland) 1/100 for 60 min. Development was with DAB (3′-diaminobenzidine) (#2014, Zymed Laboratories, Co. San Francisco, CA) for 2 min followed by a counterstain with Haematoxylin. A negative control was used for each protein with no primary antibody. To enable an objective and quantitative scoring system, slides were then visualised using a Nikon EP600 microscope fitted with a Spot RT slider cooled CCD camera and captured directly as digital images. Up to five fields were captured from each slide and all slides were photographed on the same day to avoid any variability associated with the light source. Image morphometry was analysed using ImagePro Plus® image analysis software (Version 4.1.29, Media Cybernetics, L.P., USA) which can automatically calculate the area (square microns) of stained protein (brown DAB) in each of the sections. Blood vessels and the *erector pili* muscle associated with hair follicles, as well as in myofibroblasts contain αSMA. To ensure only interstitial αSMA was included, counting was performed inside 50 μm square boxes positioned within the interstitial area (avoiding epidermis, hair follicles and blood vessels). The average of six of these boxes was used for each field, with five fields viewed per slide.

3. Results

3.1. *Surgery*

There was one maternal death related to blood loss at time of operation. This lamb was bottle fed until taken by one of the other ewes. All lambs survived the caesarean section, and the subsequent scalding procedure.

3.2. *Histopathology*

3.2.1. *Histology*

Using our previously validated model of scoring [10], the aggregated scores for each burn were calculated as arithmetic average of the specific scores. Both time and treatment (experimental versus control) effects on the histology scores were tested using ANOVA based on these aggregated scores. The composite Cohen's kappa statistics were greater than 0.4 and significant at the 0.1% significance level, for all except two categories in the experimental group (number of hair follicles and alteration in papillary dermis). We therefore excluded these groups from the analysis. The remaining variables were assessed individually using ANOVA. There was a significant difference over time in fibroblasts and epidermal thickness seen in the AM group, but the difference detected in reticular dermal thickness did not attain statistical significance. Analysed in isolation, trends towards improvement were seen in the AM category; however these did not achieve statistical significance. The strongest individual trend was seen in number of fibroblasts with a $p = 0.065$.

The average composite score for each burn wound group was calculated using the scores which we had shown had good concordance between analyses (epidermis, fibroblast count and reticular dermis) and log-transformation was performed prior to ANOVA analysis. As expected, wound disruption improved over time in both groups. When the categories were combined, analysing the scores in combination demonstrated an improved score in the AM group ($p = 0.04$). We analysed Day 60 in isolation, as this was the end point of the study and is the point which most closely reflects "chronic scarring". At day 60, the ratio of combined histology scores of AM versus control group was 0.82 – (CI 0.65–0.98), confirming a small but statistically significant improvement in histology scores in the AM group at the end point of the study.

3.3. *Immunohistochemical analysis of αSMA*

Statistical analysis demonstrated variation in αSMA expression over time and between AM treatment and control. There was a high degree of variability seen between animals. However, when the mean amount of αSMA on the total number of sections was analyzed, there was significantly less

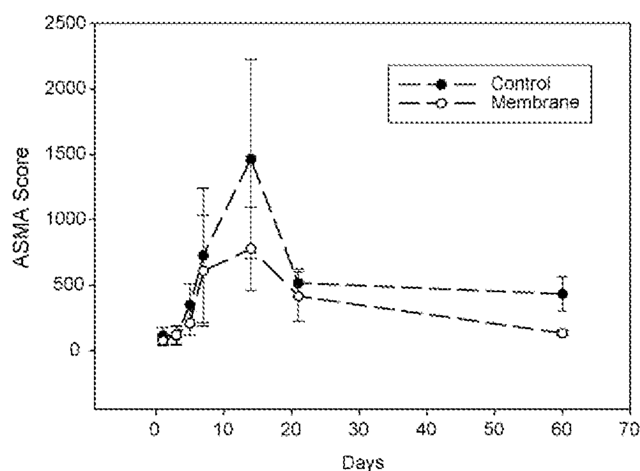

Fig. 1 – The averages of the αSMA scores over time (each points represents composite score acquired from three animals at each time point in each group). The standard error of the estimated treatment means within days is approximately 170 μm^2.

αSMA in animals treated with AM compared to control ($p < 0.001$) (Fig. 1). There was also a trend of lower αSMA scores from day 3 onwards. At the final study point (day 60), the average log-αSMA was 5.988 ± 0.315 (average ± standard error) for the control and 4.873 ± 0.315 for the AM treatment, demonstrating a highly significant difference in the amount of αSMA expressed in AM treated and control tissue at Day 60 (p-value = 0.002).

Macroscopically, all wounds were raised with overlying amnion and/or dried tissue exudate for the first 7–14 days. None of the wounds were macroscopically infected or inflamed. On two occasions (one at day 14 and one at day 21), the AM appeared more adherent than on other occasions. Wool obscured all wounds by day 21, but in general, following wool removal, the AM-covered wounds were less palpable and devoid of the erythematous appearance common on the control-treated wounds (Fig. 2). A scoring system of macroscopic wound appearance was not attempted as wool overgrowth made objective assessment impractical.

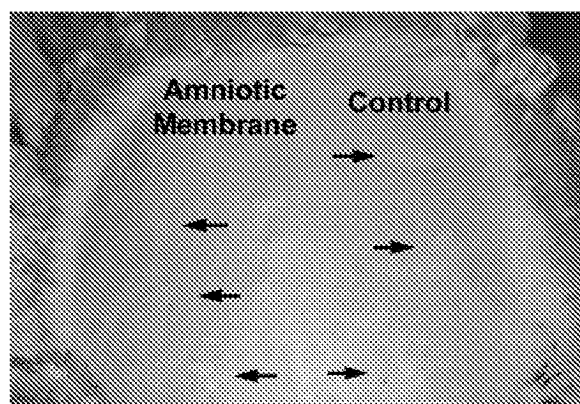

Fig. 2 – The macroscopic appearance of burn scars 60 days post-thermal injury. The AM treated wounds on the left appear to have much less scarring than the control wounds on the right. Arrows point to the wound perimeters which were tattooed for easier identification.

In summary, AM treatment of the DDBI results in reduced αSMA content across the experimental time course in general and at day 60 specifically, and a small but significant difference in histology scores at day 60, with a trend to improvement throughout the study period.

4. Discussion

AM has been considered as being associated with healing properties for centuries. Sailors who had been born with the membranes intact (the "caul") would carry this with them whilst sailing in the belief that this protected them from drowning [11]. Davies reported AM use as a burn dressing in 1910 and its widespread use was adopted for numerous wounds in the ensuing 15 years. AM as a wound dressing was introduced in the field of ophthalmology approximately 30 years later [12]. It then enjoyed a brief period in vogue for treatment of chemical burn to the eye, before falling out of favour, despite apparently good clinical results. More recently, AM has been used successfully in optimising corneal healing post-chemical burn and corneal surface reconstruction [13,14].

In its use in the field of corneal grafting, AM has been associated with reduced scar formation [13]. The beneficial profile of AM has been attributed to the promotion of epithelialisation, the prevention of fibrosis and adhesion. Interest was renewed in AM when Adinolfi et al. reported the relative deficiency in MHC class one antigens in AM [15]. These antigens are the identifier of self/non-self and are the key which stimulates the body to reject a non-self tissue. Relative deficiency in MHC may reduce the rejection response of host to foreign AM and diminish the subsequent inflammatory response. Since these reports, there have been several publications, *in vivo* and *in vitro*, demonstrating the lack of immunogenicity and down regulation of receptors which are associated with scar tissue formation in the post-natal animal [16]. Recently AM cells have been shown to produce potent natural antimicrobials [17]. AM cells do not express HLA-A, B, C or DR antigens of B2 microglobulins, though *in vitro* culture of these cells can induce the production of small quantities [15]. However, AM does produce a number of cytokines *in vitro*, with cultured cells producing large quantities of interleukin 6 and 8 [18,19]. Removal of epithelial layer from AM has resulted in lower levels of growth factor production (such as TGFβ1 and KGF) indicating the importance of epithelium in their production [20].

A number of human studies have demonstrated minimal or no immunological reaction to transplantation of AM to skin or cornea [21]. Despite the *in vitro* production of significant quantities of various pro-inflammatory cytokines, a recent study examining the effect of amniotic epithelial cells on chemotaxis of neutrophils and macrophages, as well as lymphocytic proliferation and apoptosis confirmed a potent immunosuppressive effect on chemotaxis of the neutrophils and macrophages, as well as diminished T and B cell proliferation and inducing apoptosis [22]. Hence, it would appear that AM diminishes both the innate and adaptive

immune system response to a number of stimuli, despite its ability to produce pro-inflammatory cytokines. In any burn, there is a massive up-regulation of both innate and adaptive immune systems, both at a local and systemic level. The beneficial effects of AM may in part be due to these immunosuppressive qualities, as well as the as yet un-defined fetal properties which have been shown to diminish scar formation post-burn [10].

Alpha-SMA is the hallmark of the myofibroblast, the effector of scar formation which is associated with increasing degree of fibrosis in a number of fibro-proliferative conditions, such as Dupuytren contracture [23]. The pattern of αSMA seen in this study in the control group after injury is similar to expression levels seen in our previously reported model [10]. The application of AM is associated with a significant reduction in αSMA most apparent at the end point of the study.

Direct application of AM to the wound may impart a fetal-type wound healing property. As yet it is unclear exactly what this factor/factors are, but further studies by our group are keenly investigating a number of novel proteins which are expressed in the fetus and seem to induce a reduction in scar formation.

This novel model of burn injury provides a useful vehicle upon which to assess the effects of AM on wound healing post-burn injury. The use of a control and experimental dressing on the same animal minimises the inter-animal variation as each animal acts as its own control. The sheep model however has a number of drawbacks—in particular the rapid growth of wool, which removes all dressings from the wound surface. However, the main effect of biological wound dressings is likely to be in the immediate post-application phase, after which time their effect is likely to be minimal. The majority of biological dressings such as Transcyte^{TM} (Advanced Tissue Sciences Inc, La jolia, CA) and Biobrane^{TM} (Bertek Pharmaceuticals Inc., Morgantown) [24] are applied for 1–2 weeks. Hence, the majority of the biological effect will occur well before the wool regeneration has had time to occur. As with any animal model, it is not possible to recreate the same environment as the human patient. Dressings were not replaced on a daily or weekly schedule, though the wounds were well protected from physical trauma, and there was no macroscopic evidence of infection in any of the animals. However, the conditions of animals and humans are different in terms of nursing, cleansing, etc., and these factors should be considered when reviewing the data. The small but significant improvement seen in the AM group may be underestimated by large degree of inter-animal variation, and a relatively small cohort of animals.

5. Conclusions

The improvement in survival rate of children suffering large burns is gratifying, but there has been minimal improvement of quality of life due to the multitude of restraints enforced by severe and unsightly scar tissue development. AM is a readily available dressing with a documented clinical usage in the treatment in burns. It is effective acutely as an antibacterial dressing [4]. Laboratory data supports the safety of AM with respect to relatively minimal immunogenic response [23]. In this controlled animal trial, AM used post-DDBI appears to be safe. It results in a small but statistically significant reduction in the amount of scar tissue generated in response to a standardised burn injury when compared with an inert moist wound dressing. These results support proceeding to a randomised controlled trial in patients with burns.

Conflict of interest

There are no conflicts of interest to report.

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