Enhanced cryoprecipitate for skin graft and donor site wound healing in pigs

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Key words
Enhanced cryoprecipitate; Pig model; Skin graft and donor site; Wound healing

Abstract
Due to similarities in skin characteristics, the authors hypothesise that a pig model would most accurately show the ability of autologous, enhanced cryoprecipitate (eCryo) to improve the wound healing of split-thickness skin grafts (STSGs) and corresponding donor sites. Fifty-two STSGs (5 × 5 cm) were fashioned and treated according to a randomised protocol with an autologous eCryo-treated and a control group. Macroscopic assessment, histological evaluation and cellular composition were completed at days 7, 14, 21 and 28. Thirty-two donor sites were also created and assessed in a similar manner. Histologic analysis showed enhancement of healing over all time points for eCryo-treated donor sites. All other results showed no statistically significant improvement with the use of eCryo. Autologous cryoprecipitate appears to be a safe, inexpensive and easy-to-use alternative to fibrin glue, which carries risks and is, in many cases, prohibitively expensive. Further studies are necessary to evaluate the full potential of eCryo. Interestingly, eCryo application may improve donor site aesthetic appearance. We believe that a pig model most reliably simulates eCryo’s behaviour in humans to accurately reflect its future clinical applicability.

Key Messages
- burn injuries are notoriously resource intensive and require prolonged hospital stays
- reestablishment of an intact skin barrier often requires autologous skin transplantation
- in this setting, maximising skin graft ‘take’ is paramount
- movement of the graft, infection and bleeding all contribute to the difficulty of applying STSGs to burn wounds in areas such as the axilla, lateral aspects of the trunk, perineum and the gluteal folds
- in this study, the authors investigated eCryo’s enhancement of STSG and donor site healing in an autologous in vivo pig model
- eCryo’s effect on STSG (52 grafts) and donor site (40 sites) healing on a total of nine pigs was evaluated
- due to postoperative agitation, 7 of the 52 (3eCryo treated) STSGs were acutely damaged and subsequently excluded from this study

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• all subsequent discussions will refer to the remaining 45 STSGs
• all 32 donor sites healed appropriately
• wound fibrin and fibronectin concentration is positively correlated with fibroblast mobilisation
• our results are consistent with this hypothesis as we observed a slight increase in the fibroblast concentration throughout the zone of healing through the first week following injury
• in our study, grafts were applied to the pig’s back, presumably over mobile muscle groups in order to effectively evaluate eCryo’s benefit in difficult to graft areas
• our results showed neither a clear benefit nor a detriment associated with adjunctive eCryo use
• the possibility exists that our site selection was overly conservative in order to appreciate a significant improvement in wound healing with eCryo use
• more challenging site selection in the future may serve to elucidate eCryo’s benefit on STSG take
• our application method is less evolved and may result in excessive application, reducing the hypothesised benefits of the solution but could be modified going forward
• the autologous nature of our cryo product eliminates the possibility of foreign body reaction to the solution
• furthermore, the soluble nature of the solution currently makes even application difficult as an unpredictable amount escapes the wound bed as the skin graft is secured and bolstered in place
• this may also serve to explain the lack of observed benefit
• alternate delivery systems are desired and should be investigated
• it is possible that the harvesting process introduced a compromising variable that obscured the observed benefits of eCryo application
• autologous cryoprecipitate appears to be a safe, inexpensive and easy to use alternative to fibrin glue, which carries significant risks and is in many cases prohibitively expensive
• further studies are necessary in order to further refine the use of autologous cryoprecipitate to achieve the hypothesised benefits

Cryoprecipitate is a concentrate of high molecular weight plasma proteins (factor VIII, von Willebrand’s factor, factor XIII, fibronectin, fibrinogen, etc) (16) and has been investigated as a safer alternative to commercially available fibrin glues. Autologous cryoprecipitate is derived from the slow thawing of fresh frozen plasma, resulting in a precipitate that can be resuspended in plasma and stored for prolonged periods. Several protocols have attempted to improve the yield of factor VIII and fibrinogen to achieve parity with commercially available fibrin glues (17–19). Shanbrom and Owens described a ‘super-cryoprecipitate’, or ‘enhanced cryoprecipitate’ (eCryo), derived using high concentrations of sodium citrate to increase the yield of factor VIII and fibrinogen to 97% while simultaneously decreasing the risk of disease transmission (20).

Previous studies carried out in a rat model failed to show a significant benefit to using eCryo as an STSG adhesive substitute (15). Given the histological compatibility between human and porcine skin, the authors believe that a pig model would be the most appropriate setting to evaluate eCryo’s clinical applicability and effect on wound healing. In this study, the authors investigated eCryo’s enhancement of STSG and donor site healing in an autologous in vivo pig model.

**Methods**
eCryo’s effect on STSG (52 grafts) and donor site (40 sites) healing on a total of 9 pigs was evaluated.

**Preparation for blood collection and surgery**
The animals were anaesthetised using an intramuscular injection of ketamine and xylazine followed by intubation and general anaesthesia with isoflurane under IACUC protocol #2007-2734 in conjunction with veterinary services at the University of California Irvine (UCI). The animals were placed in the prone position and subsequently prepped and draped in a sterile manner.

**Blood collection**
One week prior to surgery, approximately 200 ml of blood was obtained from each pig by venotomy of the vena jugularis externa. Following blood collection, the vein was ligated and the straight-line incision closed using deep sutures and skin staples. Bacitracin was applied to the wound site and the pigs were transferred back to their pens for recovery.

**Preparation of the eCryo**
A 0.25 ml 50% weight to volume sodium citrate was added to the extracted plasma to reach a final concentration of 12.5% (w/v). After 24 hours of refrigeration at 4°C, the treated plasma was centrifuged at 2000 g for 30 minutes at 4°C in order to separate the precipitate from the supernatant. The precipitate was further washed to remove excess citrate by

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first adding an equal volume of cold (4°C) normal saline. The suspended precipitate was subsequently ultrafiltered at 4°C through a 10,000 molecular weight (MW) cutoff membrane (Millipore, Billerica, MA). The precipitate was resuspended to create the eCryo used in this experiment. This preparation followed the known successful protocol of Owens and Shanbrom (20).

**Surgeries**

A total of nine Yorkshire pigs were obtained for the study. The STSG harvest sites were outlined using a sterile felt-tipped pen.

Eight to twelve 5 × 5 cm STSGs were harvested from the back of each animal using a dermatome (Aesculap, Tuttingen, Germany), with an intended thickness of 0.01 inches (0.245 mm). To evaluate eCryo on STSGs, 52 harvest sites were randomised such that half received eCryo followed by graft replacement and the remaining simply graft replacement.

The eCryo was applied (Figure 1) and equally distributed as a thin layer between the harvest site and the STSG. In all cases, the unmeshed STSGs were sutured in place and covered with a bolster dressing fashioned from a sterile surgical scrub sponge. To evaluate donor site healing, 32 harvest sites were similarly randomised to receive either eCryo and a sterile bolster dressing or simply a sterile bolster dressing.

The eCryo was applied (Figure 1) and equally distributed as a thin layer between the harvest site and the STSG. In all cases, the unmeshed STSGs were sutured in place and covered with a bolster dressing fashioned from a sterile surgical scrub sponge. To evaluate donor site healing, 32 harvest sites were similarly randomised to receive either eCryo and a sterile bolster dressing or simply a sterile bolster dressing. The eCryo was applied and equally distributed as a thin layer to cover the 16 designated donor sites. Postoperatively, all animals received buprenorphine 0.01 mg/kg intramuscularly for perioperative pain control.

After 7 days, the dressings were removed to assess the healing of all harvest sites. At postoperative days 7, 14, 21 and 28, one 4 mm punch biopsy was obtained from each site in a blinded manner. The biopsy sites were selected from within all grafted and donor site areas and did not contain normal, unharvested skin. The tissue was immediately placed in 4% paraformaldehyde for further pathological evaluation.

The animals were housed in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with the current US Department of Agriculture, Department of Health and Human Services and Natural Institutes of Health Regulations standards. All procedures and subsequent animal care were also performed in accordance with the animal care guidelines of the UCI.

**Macroscopic evaluation**

At postoperative days 7, 14, 21 and 28, two blinded, experienced investigators independently evaluated the macroscopic characteristics of all the wound sites using both visual and tactile sensation. Throughout this evaluative process, care was taken to avoid disruption of skin graft and donor site healing.

A predetermined grading scale was used to evaluate STSGs for adherence, colour and pliability (Table 1). An analogous grading scale specific to donor site healing was adapted by experienced dermatopathologists at the University of California, Irvine Medical Center (UCIMC) and was used to similarly assess the donor site’s macroscopic features.

**Histological assessment**

Harvest site biopsy tissue was fixed in 4% paraformaldehyde and subsequently embedded in paraffin in accordance with standardised protocols used by the department of pathology at UCIMC. Cell populations and tissue architecture were visualised using a hematoxylin and eosin stain. A pathologist at UCIMC using a histomorphological grading scale performed histological evaluation in a blinded manner. The grading system used for the STSG biopsy tissue has been previously described in the quantification of cutaneous scars in burns (21). It has been slightly modified from its original version to fit the parameters of this study. Table 2 shows the evaluated parameters that were scored in order to compare the histological wound healing between the treatment and non-treatment groups. As shown in the table, a score of 14 is the maximum attainable and represents the best possible outcome. A score of 0 denotes the worst outcome. All STSG sites were further evaluated for cellular composition and distribution within the underlying zone of adherence in the wound bed (Figure 2). Specifically, in a blinded manner, a total of 100 cells were selected and identified as either polymorphonuclear (PMN), multinucleated giant cells or fibroblasts and macrophages.

The donor site biopsy tissue was prepared and examined in a similar manner. Experienced dermatopathologists at the UCIMC modified the previously described histomorphological grading scale to more accurately represent the major parameters of donor site healing. The cellular composition of the biopsied tissue was evaluated in a blinded manner using 100 randomly selected cells from the healing wound bed.

**Table 1** Macroscopic split-thickness skin graft evaluation

<table>
<thead>
<tr>
<th>Score</th>
<th>Adherence</th>
<th>Colour</th>
<th>Pliability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Separated from</td>
<td>Black</td>
<td>Very rough</td>
</tr>
<tr>
<td></td>
<td>the wound bed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Loosely</td>
<td>Grey</td>
<td>Hardened</td>
</tr>
<tr>
<td></td>
<td>adherent</td>
<td>or</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Tightly</td>
<td>Pink</td>
<td>Smooth</td>
</tr>
<tr>
<td></td>
<td>adherent</td>
<td>viable</td>
<td>pliable</td>
</tr>
</tbody>
</table>

**Figure 1** Application of autologous cryoprecipitate to the wound bed prior to skin graft replacement.
Table 2  Histomorphological grading scale for split-thickness skin grafts

<table>
<thead>
<tr>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reepithelialisation</td>
<td>Not complete</td>
<td>Complete</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Epidermal–dermal separation</td>
<td>Severe</td>
<td>Moderate</td>
<td>Mild</td>
<td>None</td>
</tr>
<tr>
<td>Fibroplasia</td>
<td>Present</td>
<td>Absent</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Graft adherence</td>
<td>&lt;25%</td>
<td>25–75%</td>
<td>&gt;75%</td>
<td>–</td>
</tr>
<tr>
<td>Inflammatory cells</td>
<td>Present – brisk</td>
<td>Present – non-brisk</td>
<td>Absent</td>
<td>–</td>
</tr>
<tr>
<td>Hair follicles</td>
<td>Absent</td>
<td>Present</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Collagen orientation</td>
<td>Abnormal lower reticular</td>
<td>Abnormal upper reticular</td>
<td>Abnormal papillary</td>
<td>Normal</td>
</tr>
<tr>
<td>Epidermal hyperplasia</td>
<td>Present</td>
<td>Absent</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Figure 2  Tissue architecture in an eCryo-treated split-thickness skin graft site. The arrow denotes the zone of healing. The ‘*’ denotes vacuolisation within this area and indicates an area of graft malunion with the underlying wound bed.

Statistical analysis

Differences between the treatment and non-treatment groups were statistically analysed using a one-way analysis of variance. A \( P \leq 0.05 \) was considered to be significant.

Results

Due to postoperative agitation, 7 of the 52 (3 eCryo treated) STSGs were acutely damaged and subsequently excluded from this study. All subsequent discussions will refer to the remaining 45 STSGs. All 32 donor sites healed appropriately.

Macroscopic wound appearance

All 45 STSGs healed within normal limits over time, with no complications that would serve as barriers to wound healing such as seroma, haematoma or infection. The macroscopic evaluation of the STSGs on postoperative days 7, 14, 21 and 28 showed no statistically significant difference in graft adherence, colour and pliability between eCryo-treated and eCryo-untreated sites (Figure 3).

Similarly, all donor sites healed appropriately without signs of seroma, haematoma or infection. Macroscopic evaluation of donor site healing showed a significant improvement in the eCryo-treated group compared with the untreated group at day 14 (\( P = 0.04 \)) (Figure 4). The remainder of the observations at weeks 7, 21 and 28 showed no statistically significant difference in macroscopic appearance. Interestingly, at all time points, the eCryo-treated donor sites showed improved colouration in the sense that they were less red (Figure 5).

Histological assessment and grading

Through the first week of healing, histological evaluation of the biopsied graft sites showed no statistically significant difference between the treated and untreated STSG sites (Figure 6). During the second week of healing, the untreated graft sites healed significantly better than the eCryo-treated sites (\( P = 0.04 \)). There was no statistically significant difference between the treated and untreated graft sites through the remaining 2 weeks of healing. In addition, there was no statistically significant difference in the graft healing between observation periods.

The tissue architecture within the zone of adherence between the STSG and underlying wound bed was dominated by fibroplasia, epidermal hyperplasia and abnormal collagen...
orientation. Vacuole formation (Figure 2) in this area was noted to be more prominent in the eCryo-treated group. The vacuoles were identified to be collections of multinuclear giant cells, resulting in small areas of graft mal-adherence, but not non-adherence.

The biopsied donor site eCryo-treated tissue showed significantly better healing across all time periods compared with the non-treated group (Figure 7). Biopsies from day 7 post-injury showed a statistically significant difference between the treated and the untreated group ($P = 0.007$). Likewise, during the second week, the healing of the eCryo-treated donor sites was also significantly better compared with the control group ($P = 0.003$). This was unchanged throughout the remaining 2 weeks of observation, with enhanced healing of the eCryo groups ($P = 0.01$) and ($P = 0.0000005$) in the third and fourth weeks, respectively.

**Cellular composition**

Examination of the cellular population within the STSG zone of adherence consistently showed PMN predominance at all healing stages (Figure 8). Furthermore, there was no statistically significant difference in the cellular composition within the treated and untreated graft sites. There was a slight decrease in the number of PMNs and an increase in the number of fibroblasts through the first 2 weeks of healing in the non-treated group (Figures 8 and 9). The remaining
2 weeks showed a reversal of this trend, with an increasing number of PMNs and decreasing fibroblasts (Figures 8 and 9). The eCryo-treated graft sites showed minimal week-to-week fluctuations in the concentration of PMNs and fibroblasts (Figures 8 and 9). A minimal amount of giant cells were observed in the zone of healing throughout all periods of healing (Figure 10). A non-significant increase in giant cells was noticed in the eCryo-treated group at weeks 1 and 4 (Figure 10).

Analysis of the eCryo-treated donor site cellular composition showed a persistent decline in the PMN infiltrate over time compared with a relatively constant presence in the control sites (Figure 8). The difference between these observed trends was statistically significant ($P = 0.004$). Throughout the observed healing period, there was an increase in the number of fibroblasts and macrophages through the first 2 weeks in both groups followed by a slight decrease in weeks 3 and 4 (Figure 9). The subtle difference between the treated and untreated donor sites was not statistically significant. The giant cell presence was negligible in the donor site groups (Figure 10).

**Discussion**

The use of cryoprecipitate to augment wound healing and STSG take has been previously evaluated in rats (15). Due to the dissimilarities between humans and small mammals such as rats, this study had limited clinical applicability. The use of rats in wound healing studies is abundant, as they are easy to handle and inexpensive. However, these mammals have a dense layer of body hair, a thin dermis and epidermis and heal primarily through wound contraction instead of reepithelialisation (22). Porcine skin, on the other hand, is anatomically and physically more similar to human skin with a comparable dermal–epidermal thickness ratio, a measure that accounts for differences in body size (22). Furthermore, both pigs and humans have sparse body hair with follicles that cycle similarly (23), which is important as adnexal structures, including hair follicles, are important in reepithelialisation (22). Not surprisingly, man and pig have a similar epidermal turnover time (24). Finally, porcine dermal collagen and dermal elastic content is similar to that of humans in biochemistry and content, respectively. For these reasons, we believe that a pig model most reliably simulates eCryo’s behaviour in humans to accurately reflect its future clinical applicability.

Fibrin glue has a well-established use as an adhesive in plastic and reconstructive surgery. It is typically formed by the coagulation of cryoprecipitate with the subsequent addition of bovine thrombin (16). During the natural progression
of wound healing, fibrin and fibronectin are deposited into the wound bed from nearby circulation closely following injury (10). Fibroblasts use the fibrin and fibronectin framework to migrate into the wound to deposit type I collagen. Logically, wound fibrin and fibronectin concentration is positively correlated with fibroblast mobilisation. Our results are consistent with this hypothesis as we observed a slight increase in the fibroblast concentration throughout the zone of healing (Figure 9) through the first week following injury. Interestingly, fibronectin has been shown to increase the rate of keratinocyte migration and replication (25), which may aid in earlier wound healing. Fibrin glue emulates the exudative phase of wound healing by creating a stable fibrin polymer framework to facilitate the growth of collagen-producing fibroblasts. Increasing the fibrinogen content has showed benefits on wound healing and shear adhesive strength (26,27).

To achieve adequate fibrinogen concentrations, donor fibrinogen is pooled during the production of many commercially available fibrin glues. This obviously carries the risk of disease transmission, most notably hepatitis B and HIV (10). Although donor screening and product treatment protocols have increased the safety of these products, the risk still remains (28). In addition, some commercial products contain bovine aprotinin, a plasmin inhibitor, and thrombin creating a theoretical risk of Creutzfeldt–Jakob disease transmission (10). Autologous blood products eliminate the aforementioned safety concerns. Several cryoprecipitation protocols have been described in the literature and individually claim an easy and inexpensive method for the concentration of autologous fibrinogen (26,29,30). However, these methods failed to achieve parity with the fibrinogen concentrations found in commercially available fibrin glue (10). Shanbrom and Owens detail their process of autologous cryoprecipitate preparation, which uses high concentrations of sodium citrate to increase the yield of factor VIII and fibrinogen to 97%, establishing this technique as a safer and easier method of fibrinogen delivery to a fresh wound bed for the augmentation of skin graft healing.

Fibrin glue’s role in skin grafting is several-fold. Numerous studies have investigated its role in haemostasis, graft adherence and take (7,31–33). In addition, it has been shown to have clear antibacterial activity (34). Fibrin glue’s adjunctive use in STSG take is most significant in areas of historic difficulty, such as over mobile muscle and close to skin folds (6,8,35–38). In our study, grafts were applied to the pig’s back, presumably over mobile muscle groups in order to effectively evaluate eCryo’s benefit in difficult-to-graft areas. Our results showed neither a clear benefit nor a detriment associated with adjunctive eCryo use. This is similar to studies showing only minimal improvement in STSG take using fibrin glue in easy-to-graft areas (7). The possibility exists that our site selection was overly conservative in order to appreciate a significant improvement in wound healing with eCryo use. More challenging site selection in the future may serve to elucidate eCryo’s benefit on STSG take.

The application of fibrin glue has evolved extensively since it was first used in 1944 by Tidrick and Warner to secure skin grafts (36). Today, many commercially available glues are delivered as an aerosolised compound, which ensures rapid, even application to the wound bed (9). Excessive glue is problematic as it may reduce nutrient diffusion to the graft, causing graft failure (10,13). Our application method is less evolved and may result in excessive application, reducing the hypothesised benefits of the solution (Figure 1), but could be modified going forward. While fibrin glue has been shown to be present in a wound bed for up to 14 days (26,37), the absorption of our autologous cryoprecipitate is yet to be determined. Interestingly, we noted an increase in microscopic vacuolisation (Figure 2) within the zones of healing in wounds treated with eCryo and STSG. This would suggest the potential presence of a barrier to graft adherence, possibly resulting from regions of excessive eCryo deposition and/or delayed absorption. On the other hand, no decrease in graft adherence was noted. The autologous nature of our cryo product eliminates the possibility of foreign body reaction to the solution. Furthermore, the soluble nature of the solution currently makes even application difficult, because an unpredictable amount escapes the wound bed as the skin graft is secured and bolstered in place. This may also serve to explain the lack of observed benefit. Alternate delivery systems are desired and should be investigated.

Consistent with the previous study in rats by Scholz et al., macroscopic and microscopic evaluation of the STSG and donor sites showed no complications that would serve as barriers to wound healing such as seroma, haematoma or infection. Donor sites treated with the eCryo healed significantly better than donor sites without eCryo application. In addition, at any given time point, the donor sites were noted to be less red compared with the control group. However, macroscopic evaluation was only found to be statistically significant 2 weeks following injury.

As mentioned previously, we used a bolster dressing to cover the STSG and donor sites. Occlusive and polyurethane dressings have been shown to improve reepithelialisation rates. Our choice of dressing did not appear to have any observable effect on wound healing. However, postoperative agitation in the pigs may have been partially related to the nature of the dressing as we used staples to secure the bolster dressing to the wound site. In two pigs, relief was achieved by scratching against the metal cage walls, which acutely damaged seven of the STSG sites. The remaining grafts were macroscopically and histologically similar to the graft sites on the other three pigs receiving STSG replacement.

The pig is clearly the most clinically applicable wound healing model. Animal studies in general are challenging to perform and introduce a host of variables that may confound investigative outcomes. For example, human variability in the skin grafting technique combined with an uneven anatomical landscape may result in an inconsistent graft thickness. Autologous skin graft thickness has been shown to be inversely proportional to subsequent healing (38). It is therefore possible that the harvesting process unintentionally introduced a compromising variable that obscured the observed benefits of eCryo application. The variability in graft thickness is unfortunately inherent to skin graft survival studies in animals. Due to the relative similarity between human and pig skin, the effect of this variable is minimised compared with previous studies carried out in rodent models.
Autologous cryoprecipitate appears to be a safe, inexpensive and easy-to-use alternative to fibrin glue, which carries significant risks and is, in many cases, prohibitively expensive. Further studies are necessary in order to further refine the use of autologous cryoprecipitate to achieve the hypothesised benefits.

References