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Proof of concept of a new autologous skin substitute for the treatment of deep wounds in dogs



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ABSTRACT

Autologous skin grafts are effective for the repair of large skin wounds, but the availability of large amounts of skin is often limited. Through bioengineering, several autologous skin substitutes have been developed for use in human clinical practice. However, few skin substitutes are available for use in animals. The aim of this study was to develop and assess an engineered autologous skin substitute for the treatment of deep wounds in veterinary medicine. Canine keratinocytes and fibroblasts were isolated after double enzyme digestion from 8 mm punch biopsies from four healthy Beagle dogs. Skin substitutes were constructed on a fibrin-based matrix and grafting capacity was assessed by xenografting in six athymic mice. Bioengineered autologous skin was assessed clinically in two dogs with large deep skin wounds. The canine skin construct was ready for use within 12–14 days after the initial biopsy specimens were obtained. Grafting capacity in this model was confirmed by successful grafting of the construct in athymic mice. In both dogs, grafts were established and permanent epithelialisation occurred. Histological studies confirmed successful grafting. This full thickness skin substitute developed for the management of large skin defects in dogs appears to be a safe and useful tool for clinical veterinary practice. Further studies are needed to validate its efficacy for the treatment of deep wounds.

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Introduction

Llames et al. (2006) developed an autologous bioengineered skin graft which proved effective for treating human patients with severe skin injuries. In dogs, biological wound dressings, such as peritoneum, amnion, omentum, collagen dressings and extracellular matrix (ECM) products, have been used with varying success in treating skin defects caused by trauma or tumour excision (Schallberger et al., 2008). Skin autografts, allografts and xenografts are options for treating large skin defects, but complications, such as donor site morbidity, infection, rejection or transmission of infectious agents, have been reported (Schallberger et al., 2008). Autologous techniques in veterinary medicine include the use of local skin flaps, axial pattern flaps and free meshed skin grafts (Aper and Smeak, 2005; Tong and Simpson, 2012; Stanley et al., 2013). However, using autologous skin to repair massive defects, such as burns or trauma wounds, is limited by the

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https://doi.org/10.1016/j.tvjl.2017.11.003 1090-0233/© 2017 Elsevier Ltd. All rights reserved. availability of viable tissue, and optimal wound treatment is difficult (Wells and Gottfried, 2010). Furthermore, free skin grafts have a higher risk of failure than local skin flaps and axial pattern flaps, since insufficient blood flow to the graft can result in infection or graft displacement (Thilagar et al., 2009; Liptak, 2012).

Developments in bioengineering include biosynthetic skin, consisting of keratinocytes and fibroblasts cultured on biomaterial scaffolds. These skin substitutes have effectively treated wounds with delayed healing (Kim et al., 2013). Reconstructing skin using the patient's own cells offers the best approach to repairing large, deep skin wounds (Blais et al., 2013). Culture-based techniques amplify the number of cells available to treat large skin lesions. To date, most animal studies have acted as models for wound healing in human beings. Reconstructed human skin, grafted onto athymic mice, has proved effective as a permanent dressing for burns (Pouliot et al., 2002; Martínez-Santamaría et al., 2012). Spraving cultured autologous keratinocytes onto a skin defect in a pig model improved epidermal thickness, confluence and blood vessel formation in the graft (Reid et al., 2007). An engineered skin substitute has been developed for use in dogs, but its grafting capacity and usefulness in clinical practice remains unclear (Serra et al., 2007; Cerrato et al., 2013). Autologous epidermal sheets generated using genetically modified epidermal keratinocytes have been successfully grafted in dogs to treat dystrophic epidermolysis bullosa (Gache et al., 2011). In this study, we describe an autologous canine skin construct designed to treat large, deep or delayed-healing wounds in veterinary practice.

Materials and methods

Engineered canine skin

The study protocol was approved by the Committee on Animal Care and Use of the Universitat Autónoma de Barcelona, Spain (approval numbers CEEAH 1990 and CEEAH 1624). Canine keratinocytes and fibroblasts were isolated from fresh 8 mm diameter abdominal skin punch biopsies from four healthy Beagle dogs (two male, two female) by double-enzyme digestion with collagenase type I solution and trypsin, as described by Meana et al. (1998). Primary keratinocytes and fibroblasts were cultured and grown as described by Cerrato et al. (2013).

Skin constructs were prepared following the method of Llames et al. (2006). Cultured dermal fibroblasts (4×10^4 cells/mL) were suspended in canine plasma diluted in saline (1:1 V/V) containing tranexamic acid (0.4 mg/mL). The mixture was placed in 9.5 cm² six-well culture plates, then 1:10 V/V of CaCl₂ (1% in saline) was added to promote plasma clotting and the plates were incubated at 3° c for 30 min. The resultant matrices were covered with culture medium and incubated for a further 24 h. Following incubation, keratinocytes (1×10^4 cells/cm²) were seeded onto plasma matrices and cultured until they covered the matrix surface. The skin constructs were then detached manually from the wells for grafting.

Grafting engineered canine skin in nude mice

The engineered canine skin was assessed in terms of its viability and grafting capacity by xenografting in nude mice. Six-week old female NMRI-*Foxn1nu* mice (n = 6; Harlan Laboratories), were housed in pathogen-free conditions during the experiments. The mice were anaesthetised with 125 mg/kg ketamine (Merial) and 1 mg/kg medetomidine (Esteve), and placed in a sterile field warmed with a heating pad.

Full-thickness, 12 mm diameter, circular wounds were created on the backs of the mice and skin constructs of similar size were orthotopically grafted onto the wounds. The removed circles of mouse skin were devitalised by three repeated freeze/thaw cycles and then sutured over the grafts to act as a 'biological bandage' (Del Rio et al., 2002). Anaesthesia was reversed with 2.5 mg/kg atipamezole hydrochloride intraperitoneally; 10 mg/kg ciprofloxacin was administered intraperitoneally as antibiotic prophylaxis. Over the next 5 days, meloxicam was administered at 0.3 mg/kg/day for pain relief. Grafted skin samples were harvested 2, 3, 6 and 14 weeks after grafting and fixed in 10% neutral buffered formalin. Each animal was individually sampled at a single time point and then euthanased using an overdose of isoflurane.

Grafting engineered canine skin in dogs

Two dogs with large, full-thickness skin wounds were recruited for this study once written informed consent was obtained from the owners: (1) a 1-year-old male crossbreed (beagle × pointer) dog with a wound on the lower back (case 1) and a 7-month-old, male golden retriever with a lesion on the limb (case 2). Fresh blood (10 mL) collected into sodium citrate-coated tubes (Vacutainer) and an 8 mm punch biopsy were obtained from each dog. Skin biopsies were placed in 50 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 IU/mL penicillin, 100 μ g/mL streptomycin and 2.5 μ g/mL amphotericin B. During the preparation of the autologous skin constructs (10–14 days) granulation tissue was induced in the skin wounds using honey-based creams to obtain a suitable bed for skin grafting (Bischofberger et al., 2016).

The skin constructs were prepared using autologous fibroblasts and keratinocytes from each dog prepared in 75 cm² culture flasks, as described above, and fixed to non-petroleum gauzes using an inorganic polymer glue (Meana et al., 1997). The autologous skin constructs were detached manually from the culture flasks, rolled up and introduced into a 50 mL tube containing DMEM supplemented with 100 IU/ mL penicillin and 100 μ g/mL streptomycin. The constructs were then sent to the corresponding veterinary clinic.

Prior to the grafting procedure, the two dogs were sedated using 0.05 mg/kg acepromazine intramuscularly and 0.47 mg/kg 2% morphine intravenously. Anaesthesia was induced and maintained with 4 mg/kg 1% propofol intravenously and 1.5-2% isoflurane by inhalation. The canine skin constructs were unrolled and grafted with the dermal side facing the wound bed using loose sutures or staples at the margins to fix the grafts.

Canine plasma was placed in a Petri dish and 1% CaCl₂ was added. This plasma formed a coagulated sheet that was placed over the graft to maintain humidity and supply nutrients. A Robert Jones-like bandage was also used to hold the graft in place and to provide protection. The dogs were sedated for 3 days after surgery with

Results

Engineered canine skin

Sufficient numbers of cells for the skin constructs (at least 1×10^6 each of fibroblasts and keratinocytes) were obtained after 10–12 days of culture from each 8 mm punch biopsy specimen taken. The addition of calcium chloride induced plasma coagulation and gave rise to a thick, resistant and easy to handle plasmabased skin substitute. Tranexamic acid prevented plasma fibrinolysis. Embedded fibroblasts grew and expanded in the fibrin matrix. Keratinocytes also grew and reached confluence 48 h after seeding on the fibroblast-matrix surface (Fig. 1). The canine skin constructs were ready for use 11–15 days after the skin biopsy specimens were collected.

Skin grafts in nude mice

The engineered canine skin was successfully grafted onto the wounds created in the nude mice (Fig. 2). At week 3, the devitalised skin 'biological bandage' sloughed off, exposing the underlying graft (Fig. 2). On histological examination, melanin was observed in the grafted constructs on day 14, indicating the presence of cells of canine origin (Fig. 3); however, on post-grafting day 21, no traces of melanin could be seen.

Skin grafts in dogs

The autologous skin grafts took well and we observed permanent epithelialisation in both dogs (Fig. 4). We did not observe any epithelial loss or blistering. The dog with the lower back wound (case 1) exhibited full wound closure after 25 days, while the dog with the leg wound (case 2) exhibited full wound closure after 40 days. We considered outcomes satisfactory in cosmetic and functional terms. However, case 1 experienced pruritus, requiring the use of emollients and moisturisers during the healing process.

We obtained biopsies from the wound centres and margins on day 25 (case 1) and day 50 (case 2) when the wounds had healed



Fig. 1. Histology of the canine engineered skin graft obtained in 12–14 days. Keratinocytes were seeded on the top of the fibrin-based matrix (black arrow) where fibroblast were embedded (asterisks). Scale bar=50 μ m. Stained with haematoxylin and eosin.



Fig. 2. Grafting the engineered canine skin construct in nude mice. (a) Skin removed from the mouse was devitalised and used as a bandage. (b) Three weeks after grafting, the dead mouse skin had sloughed off, exposing the engineered canine graft. (c) Nude mice with canine skin constructs at 6 weeks post-grafting.

completely. Histologically, all biopsies showed complete differentiation and maturation of the epidermis, and mature fibroblasts were embedded in a collagen matrix in the dermis. However, no skin adnexal structures were observed in the grafted areas (Fig. 5).

Discussion

In this proof of concept study, we developed a full-thickness skin substitute (dermis and epidermis) for the management of large skin defects in dogs. The canine skin construct remained viable in nude mice with dermal wounds and appeared to perform as expected in two dogs with skin defects. These encouraging results warrant further investigation of cultured skin substitutes in treatment of large canine skin defects.

The use of engineered grafts resolves two of the most important problems of skin transplantation: the availability of mobile or free skin to cover the wound and the process of graft preparation. The removal of subcutaneous fat during graft preparation is essential to ensure graft viability, but can be time consuming, tedious and incomplete (Townsend et al., 2012). In small animal surgery, a dog or cat undergoing a 90 min procedure has around twice the risk of post-operative infection compared to one undergoing a 60 min procedure (Brown et al., 1997). Therefore, engineered skin, rather than real skin, for grafting could provide a faster and, consequently, a safer way to repair large skin defects, since it reduces surgery and anaesthesia times, and no graft preparation is necessary.

The prototype artificial skin construct we developed has several benefits. It was derived from an 8 mm diameter skin biopsy and was ready for use within 12–14 days. This preparation time is a week shorter than for the human skin model described by Llames et al. (2006). This is a huge benefit in critical cases, in which a short procurement time is critical for the recovery of skin function.

Moreover, the minimal size of the source biopsy is a further benefit when managing large wounds, for which the availability of the patient's own skin is compromised. Another advantage of our model is that no feeder layers were used in keratinocyte culture, thus avoiding possible graft immunogenicity. Graft destruction can be immunologically mediated and initiated by foreign fibroblasts used as a feeder layer to optimise keratinocyte growth during culture (Svensjö et al., 2001). In our model, the artificial dermis was composed of autologous fibroblasts embedded in a scaffold of coagulated plasma. The presence of autologous fibroblasts could also help to repair the damaged dermis, since fibroblasts have been observed to persist in the wound bed, improving the grafting process (Wisser and Steffes, 2003; Llames et al., 2004).

We assessed the viability and grafting capacity of our engineered skin by xenografting the skin constructs in nude mice. Nude mice are widely used in xenografting studies, since they are immunodeficient, athymic and have melanin-free skin (Martínez-Santamaría et al., 2012; Yohan et al., 2014); because of this lack of melanin, the detection of melanin in a re-epithelialised wound confirms the integration of melanocyte-containing canine engineered skin into the mouse skin. However, we observed a lack of melanin on day 21, which is likely to be due to the effects of murine fibroblasts. According to the findings of experiments in human pigmented epidermal reconstructs, fibroblasts might influence the pigmentation of grafted skin (Hedley et al., 2002; Casoli et al., 2004). This hypothesis predicts that in our model, colonisation of the grafted dermis by murine fibroblasts impairs melanocyte survival or melanin synthesis, leading to the elimination of melanin from the canine graft.

The success of a skin graft is closely related to formation of granulation tissue in the wound. Optimal graft taking is dependent on formation of healthy, active, well-organised, new granulation



Fig. 3. Histology of the canine engineered graft in the nude mice at day 14. (a) Canine engineered (left) and murine regenerated skin (right). Scale bar = 50 μ m. (b) Presence of melanin (black arrow) in the epidermal layers of the canine engineered skin. Scale bar = 25 μ m. Haematoxylin and eosin.



Fig. 4. Grafting the canine skin constructs in two dogs. (a–d) Case 1. (e–h) Case 2. Skin defects before transplantation (a and e) and at day 0 showing the engineered skin covering the wound (b and f). After 10 days (c and g), note the epithelialised area separated from the wound margins (black arrowhead). On day 25 (d) and day 40 (h), the wounds had fully healed.

tissue in the first 48 h of transfer. As a free graft, artificial autologous skin survives by absorbing tissue fluid from the host bed through plasma imbibition (Swaim, 2003). At approximately 72 h, fragile capillary buds emerge from the host bed and start to vascularise the graft (Swaim, 2003). A well applied, immobile graft is vital to the final outcome, since dressing displacement caused by animal rubbing has been described as one of the most frequent



Fig. 5. Histology of the engineered skin graft after complete wound healing in a canine clinical case (case 1, day 25). Complete and 8–10 layered epidermis (black arrow), with mature fibroblasts (asterisks) in the dermis were observed after grafting. Scale bar = $100 \,\mu$ m. Haematoxylin and eosin.

causes of graft failure (Stanley et al., 2013). In our proof of concept trial, both dogs were sedated for 3 days after transplantation to avoid excessive movement.

Excessive wound contraction complicates deep wound healing, limiting and reducing the functionality and movement of the healed wound, and occasionally producing pruritus (Reid et al., 2007). However, when wound repair takes place within 3 weeks, this phenomenon is markedly reduced (Reid et al., 2007). Our canine skin construct can be prepared and transplanted in under 3 weeks. Thus, given the earlier recovery of epidermal function, wound contraction might be reduced, and functionality loss and pruritus should be diminished. Notwithstanding, one of the dogs experienced pruritus 35 days after grafting the autologous skin construct. Dry skin is common during wound healing and particularly affects wound sites with grafted skin, possibly via mechanisms of transepidermal water loss or sebaceous gland destruction, leading to impaired barrier function and, consequently, pruritus (Kuipers et al., 2015). Emollients help to restore barrier function and therefore might provide relief from pruritus; in our clinical case, conventional emollients and moisturisers resolved the problem.

One of the limitations of our engineered canine skin was that this prototype did not contain hair follicles or annexed glands. In one artificial model, de novo hair follicle formation in vivo was possible using epithelial and mesenchymal cells from embryos, as well as from newborn mice or stem cells from the adult follicle bulge area (Zheng et al., 2010). It therefore seems that incorporating hair follicles derived from autologous stem cells into tissueengineered skin, besides providing a niche for cells involved in hair renewal, also offers a useful stem cell reservoir for wound healing (Blais et al., 2013). For the next generation of tissue-engineered skin, this approach should help recover the sense of touch and improve the cosmetic appearance of the skin (Blais et al., 2013).

The ideal engineered skin substitute should be safe, clinically efficacious, simple to produce, and easy to handle and administer (Sun et al., 2014). Our canine skin model consists of autologous keratinocytes, fibroblasts and plasma, Plasma makes an excellent three-dimensional (3D), fibrin-based scaffold, which is rich in growth factors and cytokines, and allows for the rapid growth of both keratinocytes and fibroblasts (Llames et al., 2006). Plasma acts as a reservoir of different growth factors, reproduces the normal healing process in vitro, and also provides an appropriate 3D scaffold that promotes the migration, proliferation and differentiation of cells in the wound bed (Martínez-Santamaría et al., 2012). The use of cells and plasma from the same animal makes this prototype safer, avoiding rejection and virus transmission. Furthermore, canine plasma could be obtained from blood banks, making the procedure less costly. Plasma can be stored frozen and quarantined to avoid the risk of virus transmission. Our model, based on creating a thick, resistant and easy to manipulate fibrin 3D scaffold, appears to be a useful tool for the treatment of large wounds in veterinary medicine, fulfilling most of the requirements of an ideal skin substitute.

Conclusions

The proposed autologous, engineered skin construct can be prepared from a small biopsy specimen in 2 weeks. The artificial skin was easy to manage and transport, and no special techniques or instruments were required for its preparation and use. The engineered skin proposed showed a good grafting capacity and achieved complete permanent reepithelialisation of the wound site with no signs of rejection. These observations warrant further investigation in larger and controlled trials.

Conflict of interest statement

None of the authors of this paper have a financial or personal relationship that could inappropriately influence or bias the content of the paper.

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