



Cultured Equine Autologous Keratinocytes on Collagen Membrane for Limb Wound Healing

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Abstract

Objective- Use of equine autologous keratinocyte on collagen membrane grafts (KCMG) for treatment of wounds in the distal aspect of the horse limb.

Design- Experimental study

Animals- Four horses.

Procedures- Keratinocytes have been separated by enzyme digestion from limb skin sample and proliferated in vitro. Full thickness excision wounds (6.25 cm²) were created on the mid-lateral of both metatars of each horse aseptically. The wounds were classified into two groups, group A and B as control (n=4) and keratinocyte collagen membrane graft (KCMG [n=4]) group respectively.

Acid soluble collagen has been extracted from calf skin and polymerized in vitro, then keratinocytes, cultured on collagen membrane for one day. Cell graft was performed once on the 4-day-old wounds. Photographs were taken twice weekly. Whole wounds excision biopsies were performed on 28-day-old wounds.

Results- Geometrically, there were increase in epithelialization, contraction and total wound healing per day in group B, but it was not significant statistically. Histopathologically both groups had epidermal cells in superficial layer, but the amount of this layer, differentiation and maturation of stratum spinosum cells of epiderm in group B was better than group A. These differences were not significant (P-Value>0.05).

Conclusion and Clinical Relevance- Perhaps use of keratinocyte collagen membrane graft (KCMG) have positive effects and cause better wound healing in derm and specially epiderm of equine lower limb wounds.

For increase of cell treatment effects significantly, it is better to use cell grafts on large wound, also preparation of wound bed and repeated treatments are essential.

Key Words- Keratinocyte, Collagen membrane, Equine, Limb, Wound healing.

Introduction

The progression of wound healing is a complicated but well-known process involving many factors, yet there are few products on the market that enhance and accelerate wound healing. This is particularly problematic in veterinary medicine where multiple

species must be treated and large animals heal slower, often times with complicating factors such as the development of exuberant granulation tissue.⁴⁴ The historical gold standard for replacement of lost skin is the autologous skin graft. However, the horse's lack of redundant donor skin limits the practicality of full-thickness grafting to smaller wounds; moreover, graft failure is relatively common in equine patients as a result of infection, inflammation, fluid accumulation beneath the graft, and motion. Tissue engineering (the science that studies the creation of artificial and semiartificial organs and tissues) has emerged as an interdisciplinary field with the aim to regenerate new biological material for replacing diseased or damaged tissues or organs. For this to be achieved, not only is an appropriate source of cells required, but also a scaffold

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designed from natural or synthetic polymers.³⁴ Cultured keratinocyte cells have been used for human extensive burns and chronic ulcers with different origins since 20 years ago. Transplantation of autologous cultured keratinocytes is one of the most advanced areas of tissue engineering which has clinical application in treatment of wounds. Current tissue-culture techniques enable keratinocytes from a small piece of skin to be grown into sheets of epithelium, or cultured keratinocyte grafts, that are suitable for treating wounds.²² Humans and horses are the only mammals known to naturally develop excessive granulation during wound healing; inflammatory response may contribute to observed differences in the cellular populations, with exuberant granulation tissue (EGT) possessing markedly increased myofibroblasts, small vessels, and acute inflammatory cells compared with keloids.³⁶ Equine lower limb wounds are very closely resemble to human chronic leg ulcers, and wounds of this region are expected to be treated with these methods.

In horses, wounds of the distal portion of the limbs have slower rate of healing and propensity to develop EGT, compared with other species or similar wounds proximal to corpus and tarsus, wounds have a longer preparatory phase of healing owing to greater wounds retraction, slower rate and earlier cessation of wound contraction, and slower rate of epithelialization.^{2,41,42} Cutaneous wound temperature (CWT) varied with anatomic location and throughout healing. CWT of wounds developing EGT was significantly less than that of wounds without EGT.⁷

The relative hypoxia present acutely in limb wounds of horses may promote a feeble yet prolonged inflammatory response, which could interfere with and retard the subsequent phases of healing⁶ but the use of hyperbaric oxygen therapy (HBOT) after full-thickness skin grafting of uncompromised fresh and granulating wounds of horses is not indicated.²¹

In the last few years, progress in the field of tissue engineering has been to the production of numerous biomaterials of particular interest in burn and chronic wound therapy.³⁸

One study demonstrates that upside-down grafts of undifferentiated monolayer of keratinocytes on non-cross-linked bovine type I collagen membranes onto standard nude mice full thickness wounds do lead to an early reconstitution of multilayered squamous epithelium with enhance wound healing compared to the control group.²⁴

The object of this study is creating and use of autologous equine keratinocyte collagen membrane grafts (KCMG) for treatment of wounds of the distal aspect of the horse limb.

Materials and methods

Animals

Four healthy adult horses between 4 to 18 years old and weighing 325-450 kg, with normal findings on physical and hematological examinations were used in this study and rule out pre-existing diseases. Horses were taken anthelmintic drugs and hoof care was performed 2 weeks before the beginning of the study. The experiment ethical protocol had been approved by the research council of faculty of veterinary medicine of University of Tehran. Procaine penicillin G (20000 IU/kg) and streptomycin (20 mg/kg) were administered for 8 days, commencing several hours before wounding. Diet consisted of alfalfa hay, free choice commercial grain was fed as necessary to maintain body condition. Each horse was confined to a box stall.

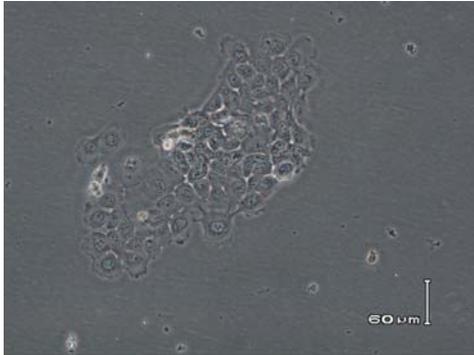
Collagen extraction from calf skin

Collagen membrane was prepared with acid soluble collagen,⁴⁰ containing collagen type I and III. SDS gel electrophoresis demonstrated the typical banding pattern for collagen I and one retarded electrophoresis for collagen III. This solution was lyophilized and stored in -20°C.

Isolation and cultivation of primary equine keratinocyte

Biopsies were aseptically obtained from lower limb epithelium of horses under local anesthesia with 2 % lidocaine HCl, and placed into 10 ml William's medium E(WME + Glutamax I, Gibco, 32551/020 USA) containing gentamycin (50 mg/ml). The tissue then stored at 2 to 8°C until use. Samples were washed with sterile PBS (Phosphate buffer saline) containing gentamycin (50 mg/ml) and the epidermis separated using 10 mg/ml dispase II (Dispase, Gibco, 17105 – 41, Germany) in Hank's balanced salt solution for 24 h (4°C). After trypsinisation (Trypsin 1: 250, sigma, T-4799, Germany) of separated epidermis, the basal cell layer was scraped off. Cells were re-suspended in approximately 5 ml of medium and seeded at cell culture T – 25 cm² flask and grown at 37°C in 5 % CO₂ incubator (Picture 1). William's medium E supplemented with 10 % FCS (Fetal calf serum), 1 ng/ml recombinant epidermal growth factor (EGF, Gibco, 10450 – 2.5 µg, USA), 15µg/ml bovine pituitary extract (BPE, Gibco, 13028 – 25 mg, USA), 0.4 mg/ml hydrocortisone, 5 µg/ml insuline and 50 mg/ml gentamycine. The medium of flasks was changed with fresh complete medium and gassed with 5 % CO₂ every other day. Upon reaching 70-80 % confluency, the medium is removed and the flasks washed once with 10 – 15 ml of sterile PBS. Then one ml of trypsin added to the flask and it was incubated for 5 – 10 minutes at 37°C until cells were dislodged. The trypsin reaction was stopped by adding 5 ml of WME containing 10 % FCS. The cells were spun at 500 rpm for 10 minutes at room temperature and the cell pellet was gently re-suspended in complete WME and seeded into T – 75cm² flasks.

The flasks were gassed with 5 % CO₂ and incubated at 37°C. The medium of flasks were changed every other day until cells reach 70 % - 80 % confluency. Finally these cells (second passage) were transferred to collagen membrane.



Picture 1- Keratinocytes after one day seeded at cell culture (T – 25 cm² flask).

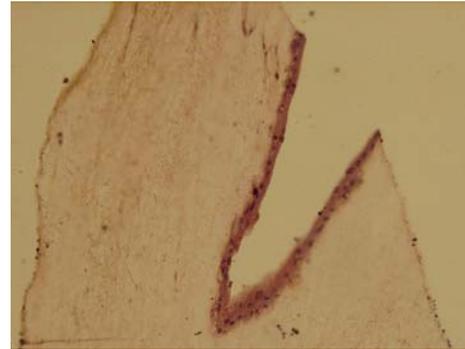
Wound creating

Horses were anesthetized with xylazine (1.1 mg/kg IV) and ketamine (2.2 mg/kg IV). After aseptic preparation of the surgical sites, a standardized full-thickness skin wound was created on the mid-lateral of each metatarsal bone using a sterile 2.5cm×2.5cm template, so each hind limb had a wound (a total of eight wounds). Wound on one limb had no treatment (n = 4) whereas wound on the contralateral limb received KCMG (n = 4). The wounds were covered with sterile nonadhesive pad (Roundpad Dr. Wusthoff GmbH, D – 42929, Germany) and bandaged with gauze – coated cotton wool and an elastic bandage before recovery from anesthesia for preventing of contamination. Bandages were removed and wounds rinsed with isotonic saline solution twice weekly. A ruler was attached to the leg around the wound and photograph was taken before re-bandaging. Wound area was measured twice weekly for 28 days. Wound area and rate of healing of treated and control wounds were compared statistically.

Preparation of keratinocyte collagen membrane graft (KCMG)

Lyophilized collagen was re-dissolved in 0.1 M acetic acid. The final concentration was adjusted to 3 mg/ml (stock solution). For preparation of collagen membrane 2.8 ml of the stock solution of collagen was mixed on ice with 1.7 cc of 3x DMEM (Dulbecco's modified eagle's medium, sigma-Aldrich D7777-1L, Germany) followed by neutralization with 1M NaOH and addition of 0.5 ml FCS. This solution was poured into 60mm diameter dish. Polymerization of the gels occurred at

37°C within 30 minutes. After one day of preparation of collagen membrane keratinocytes were trypsinized and washed with PBS twice. Keratinocytes were inoculated onto membranes at a density of 42×10^3 per cm². Keratinocytes attached and flattened on membrane after one day of culturing on the collagen membrane. Sample of collagen membrane with and without equine keratinocytes was put in 10% formalin and prepared for histopathologic studies (Picture 2).



Picture 2- Histopathologic slide of Keratinocyte collagen membrane graft (KCMG) that had a layer of flattened cells with large nucleus (keratinocytes) on a collagen membrane showed an amount of thin, parallel, pinkish yellow collagen fibers.

Wound treatments

In 4-day-old wounds, proper granulation tissue developed and cell grafts were preformed. Wounds were divided into two groups. A-control group (n=4) just were changed bandage and B- keratinocyte collagen membrane graft (KCMG) (n=4). Each horse had a control and a treatment, one on the left and other on the right limb. Treatment was done once. All 4-day-old wounds rinsed with normal saline, for group B, keratinocyte collagen membrane grafts (KCMG) was transplanted as an upside-down graft onto each horse full-thickness wounds (Picture 3), and then all of wounds were re-bandaged. Bandages were changed and digital photographs were taken twice weekly.



Picture 3- Keratinocyte collagen membrane graft (KCMG) transferring upside down on the wound.

Histopathology

Full-thickness biopsies with 2-3 mm margin of healthy skin for histopathology were taken from all 28-day-old wounds and fixed in 10% formalin and processed to 6 µm paraffin sections and stained with hematoxylin and eosin (H & E), and Masson's trichrom stain (MT) for collagen fibers. Derm and epiderm of treatment and control wounds were compared semiquantitatively with each other in the same horse and then two groups were evaluated. Histopathologic evaluation were taken on score as: +1 (mild), +2 (medium) and +3 (severe) in extension or intensity of changes.

In epiderm, arrangement, differentiation and number of cell layers were evaluated. In derm amount (expansion), arrangement and direction of collagen fiber (parallel to each other and skin), and also number, direction and orifice of vessels (endothelial cells) were studied. Extention of acute inflammation (platelet, fibrin, polymorphonuclear cells [PMN]), and chronic inflammation (mononuclear cell infiltration [MN]) were examined.

Data Analysis

Wound area (cm²) was measured using the digital images and a software package (Scion image for windows beta 4.0.2, Scion Corporation, USA). Variables were analyzed included: total wound healing, wound contraction and epithelialization rate. These variables (macroscopic data) were converted to percentage and analyzed as a repeated measurement analysis of variance. Student "t" test was used for histopathologic variables. Significance was set at P-Value < 0.05.

Results

Cross finding

Four horses were used for this study each hind limb had a wound, a total of eight wounds, 4 wounds as control(A) and 4 wounds as treatment(B). Healing was studied for 28-day-old wounds (24 days after cells grafting). Animals showed no signs of discomfort or significant inflammation.

Differences were seen in mean epithelialization, wound contraction and especially total wound healing per day in two groups, and in group B (KCMG), these are more than group A (control) but no significant difference between two groups was seen(P-Value>0.05) (Fig 1). Significant differences in mean epithelialization percentage, mean wound contraction percentage and mean total wound healing percentage during 28 days between control and treatment wounds were not seen(P-Value>0.05) (Fig 2-4).

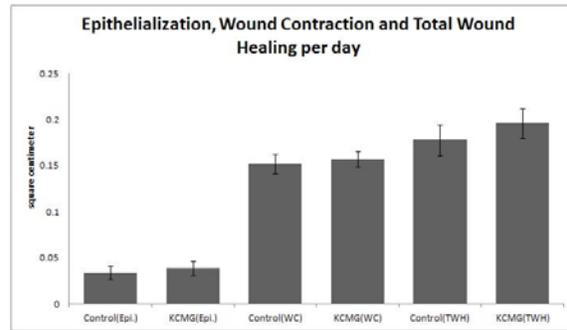


Figure 1- Epithelialization (Epi.), wound contraction (WC) and total wound healing (TWH) per day, in control and KCMG groups. Mean epithelialization, wound contraction and especially total wound healing per day in KCMG group are more than control group, (P-Value>0.05).

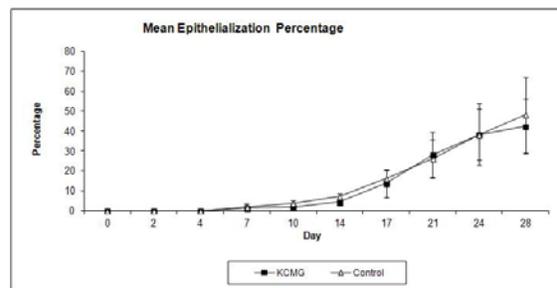


Figure 2- Mean epithelialization percentage during 28 days, Control and Keratinocyte collagen membrane graft (KCMG) groups (P-Value>0.05).

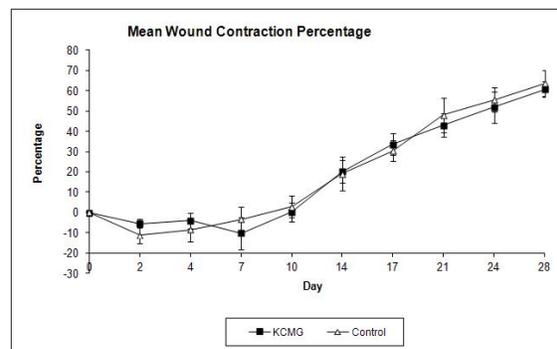


Figure 3- Mean wound contraction percentage during 28 days, Control and Keratinocyte collagen membrane graft (KCMG) groups (P-Value>0.05).

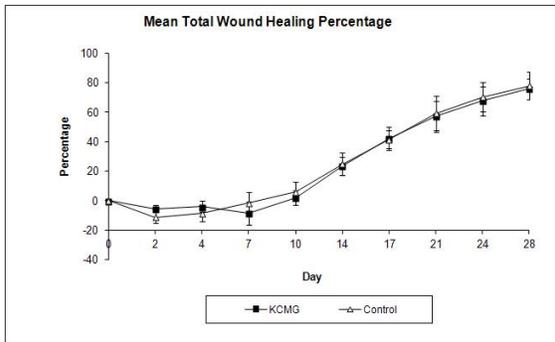


Figure 4- Mean total wound healing percentage during 28 days, Control and Keratinocyte collagen membrane graft (KCMG) groups (P-Value>0.05).

Histopathological findings

Histopathologic slides of collagen membrane without cells, which prepared in vitro, showed an amount of thin, parallel, pinkish yellow collagen fibers without cross linking and bundle. This membrane with cells (KCMG) had a collagen membrane like above and a layer of flattened cells with large nucleus. Cells attached to collagen membrane well after one day of culture. Diameter of this layer was similar to cuboidal cells that covered internal surface of body (like mesothelial cells) and in some portion of this layer, cells were multilayer (2-3 layers) and they were similar to stratum spongiosum cells and have a view like a stratified epithelial tissue on the one side of collagen membrane (Picture 2).

Full-thickness biopsies were taken from 28-day-old wounds, derm and epiderm of treatment and control wounds compared semiquantitatively with each other in the same horse and then two groups were evaluated. Histopathologic evaluation were taken on score as: +1 (mild), +2 (medium) and +3 (severe) in extension or intensity of changes.

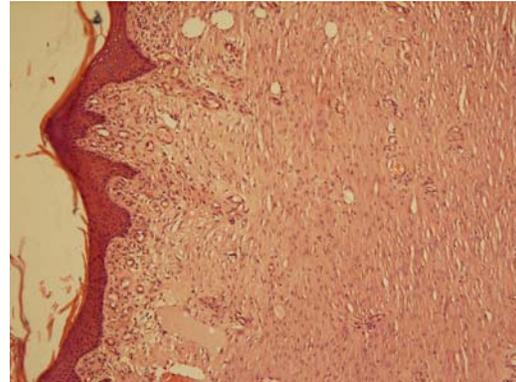
There were some differences between two groups:

1- Both groups had epidermal cells in superficial layer and they often covered total wound bed, but number of epithelial cell layer, in group B was more than group A. Differentiation and maturation especially in stratum spinosum cells of epiderm, in group B was better than group A (Pictures 4,5).

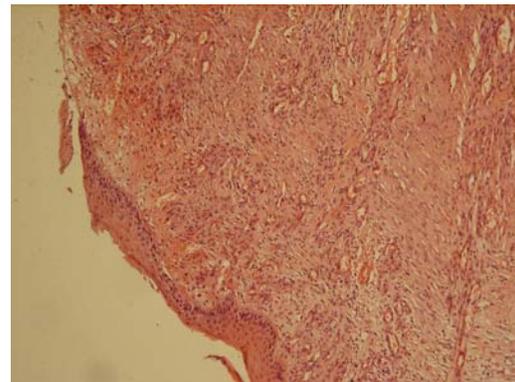
2- Derm of group B was better than group A: because Inflammation, congestion and edema in superficial derm of group B were lesser than group A. Angiogenesis, maturation and organization of blood vessel in group B were better than group A, and infection and infiltration of inflammatory cells in group A were lesser than group A (Pictures 4,5).

Amount (expansion) and arrangement of collagen fibers were more parallel to each other and skin in group B. (Pictures 6,7).

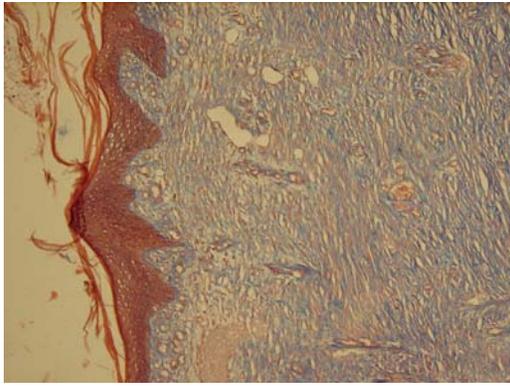
But these histopathological differences were not significant (P-Value>0.05).



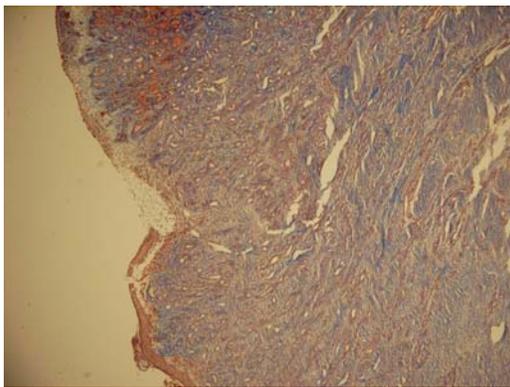
Picture 4- Keratinocyte collagen membrane graft (KCMG [group B]) horse no 1. Basal cells are regular. Number of epithelial cell layer, differentiation and maturation especially in stratum spinosum cells of epiderm, in group B were better than group A (Picture 5). Inflammation, congestion and edema of superficial derm of group B were lesser than group A. Amount and arrangement of collagen fibers in group B was better than group A. Maturation and differentiation of blood vessel in group B were better than group A (H&E $\times 100$). Histopathological differences were not significant (P-Value>0.05).



Picture 5- Control wound (group A) horse no 1 (H&E $\times 100$).



Picture 6- Keratinocyte collagen membrane graft (KCMG [group B] horse no 1. Amount (expansion) and arrangement of collagen fibers in derm were more parallel to each other and skin in group B (Masson's trichrom stain [MT] $\times 100$) (P-Value >0.05).



Picture 7- Control wound (group A) horse no 1 (Masson's trichrom stain [MT] $\times 100$).

Discussion

Engineering of skin substitutes provides a prospective source of advanced therapies for treatment of acute and chronic skin wounds.⁴ Horse chronic limb wounds have a pathology that very closely resembles of human chronic leg ulcers,⁹ and human cultured keratinocyte was used in engineering of skin substitutes for treatment of chronic ulcers.^{23, 38}

During one week after injury, wounds retraction happen especially in distal limb and wounds enlarge.² This fact pulls down the percentage of wound contraction and total wound healing below zero (Figure 3,4).

In one study, preformed collagen gel was topically applied to cutaneous wounds of the equine dorsal fetlock and metatarsal regions to evaluate the effect on exuberant granulation tissue production and wound healing. Significant differences in the production of exuberant granulation tissue, rate of epithelialization, or degree of wound contraction were not detected

between the collagen treated and control wounds.³ In another study, the effect of a porous bovine-derived collagen membrane (PBCM) evaluated on the horse limb wound healing, and in that study, fibrin score, neutrophil score and degree of inflammation were significantly greater in the PBCM treated wounds,⁴⁵ There were not alive cells, on collagen gel and PBCM in two above studies and in our study we had a layer of keratinocytes on the collagen membrane.

For the first time, cultivation of keratinocytes of the stratum medium of the equine hoof wall was preformed for study on pathophysiologic effects of laminitis.¹⁷ Culture and characterization of equine hoof keratinocytes was preformed and keratin and vimentin expression were seen in hoof keratinocytes,⁴³ after that, the isolation and cultivation of primary equine keratinocytes derived from lip epithelium was done, and their maintenance of a high proliferative capacity up to second passage and high cell yields correlated with a cell morphology of cobble-stone shape and small size¹⁵ and we had same results in our keratinocytes cultures.

Keratinocytes can be cultured directly onto a delivery membrane in culture vessels, which is then peeled off when required for use. It is therefore possible to use keratinocytes before the cells achieve confluence.⁸ The membrane is then inverted and placed on the wound. This method eliminates the need for enzymatic release of cells before use. A number of systems^{8,11,14} have been developed, some are based on biological tissue, and others are synthetic polymers.⁸ It has shown collagen type I is a suitable matrix for growth of human keratinocytes in vitro.²⁴ In one study human keratinocytes were transplanted as an upside-down graft on collagen membranes (KCMG) onto standard nude mice full-thickness wounds. Fully differentiated epidermis was found at 21 days after grafting KCMG with persistence of human keratinocytes. They showed that upside-down grafts do lead to an early reconstitution of multilayered squamous epithelium with enhanced wound healing²⁴ and we obtained the same results and our treatment group had more differentiated layer compared to control group but that was not significant.

Canine keratinocyte culture and the use of a cultured epidermal autograft (CEA) in a dog was preformed successfully.¹ They used confluent sheet of keratinocytes and in our study subconfluent monolayer of cultured equine keratinocytes on collagen membrane were used.

In different references, wounds were created with 1 cm²³⁵ to 12 cm²³ templates on different sites of horses' limb, and in this study we used 6.25 cm² template. In some studies more than one wound created on each limb^{5,19} but we had just a wound on each hind limb. A more traumatically induced wound may have been ethically questionable.

Granulation tissue appears after days 4 to 5 and prevents wound infection, promotes wound contraction, provides

scaffold for epithelial cells to cover the skin defect and provides nutrient for subsequent skin grafts² and keratinocyte grafts was done in 4-day-old wounds and cell grafted onto granulation tissue.

The development of new techniques and modification to overcome some of the disadvantages in cultured keratinocyte grafting has been motivated by several well-known drawbacks in the use of cultured epithelial autograft such as long culture periods, lack of adherence, difficulty in handling, lack of dermal substrates, and high costs. Two recent insights have influenced further research on the one hand, It has been shown that the use of undifferentiated proliferative cells in fibrin glue suspension is effective in epithelial reconstitution on the other hand; the enzymatic release of cells from the culture surfaces is a critical step leading to at least temporary distraction on anchoring structures of the cultured cells. In this study, we tried to combine these two aspects in an attempt to modify common modalities of keratinocyte transplantation. To avoid disperse dissolving of the cultured cells, keratinocytes were seeded onto bovine collagen membranes without feeder layers culture conditions subconfluent monolayer of cultured equine keratinocytes were transplanted as an upside-down graft on collagen membranes (keratinocytes collagen membrane grafts [KCMG]) onto horse full-thickness wounds. This study demonstrates that upside down grafts of undifferentiated monolayer of keratinocytes on bovine collagen membranes can lead to an early reconstitution of multilayered squamous epithelium with enhanced wound healing in derm compared to the

control group histopathologically but this is not so much to be significant.

Polymeric membranes have the advantage of being readily available and relatively non toxic and do not carry the inherent risks of disease transmission. Their efficacy, however, must be proved in large clinical series.⁸

If significant levels of expansion are required like our study, however standard culture techniques may need to be used before transferring the proliferated keratinocytes to the delivery system (like collagen membrane)^{20,39} clearly this delivery method only transfers keratinocytes are only part of the solution to wound coverage after full-thickness skin loss in burns patient. It is widely appreciated that the addition of a dermal substitute to such a wound is important for stable wound healing,^{26,29} this may also require the transplantation of fibroblasts to enhance healing further and to improve the mechanical properties of the graft^{12,13} The role of the delivery of pre-confluent keratinocytes in conjunction with methods of dermal delivery should also be assessed.

This study showed 42×10^3 keratinocyte cells per each square centimeter of bovine collagen membrane on small lower limb wounds of horses did not have significant differences grossly and histopathologically. For increase of cell treatment effects significantly, it is better to use cell grafts on large wound, also preparation of wound bed and repeated treatments are essential.

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چکیده

کراتینوسیت‌های خودی کشت داده شده اسب بر روی غشاء کلاژن برای درمان زخمهای اندام حرکتی

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هدف- هدف از مطالعه استفاده از کراتینوسیت‌های کشت داده شده خودی اسب بر روی غشاء کلاژن جهت درمان زخم‌های نواحی پایینی اندام حرکتی می‌باشد.

طرح مطالعه- مطالعه تجربی

حیوانات- چهار راس اسب

روش کار- کراتینوسیت‌ها با روش‌های هضم آنزیمی از نمونه لب هر اسب در آزمایشگاه جدا و تکثیر شدند. زخم‌های تمام ضخامت (۶.۲۵ سانتیمتر مربع) در قسمت میانی و جانبی هر دو متاتارس هر اسب به صورت آسپتیک ایجاد گردید. زخمها به دو گروه چهارتایی شاهد (A) و پیوند کراتینوسیت‌ها بر روی غشاء کلاژن (B) تقسیم شدند. کلاژن محلول در اسید از پوست گوساله جدا شد و در آزمایشگاه پلیمریزه گردید، سپس کراتینوسیت‌ها به مدت یک روز بر روی این غشاء کشت داده شدند. پیوند سلولی تنها یک بار در زخم‌های چهار روزه انجام گردید. عکس برداری دو بار در هفته صورت گرفت. نمونه تمام ضخامت پوست از زخمهای ۲۸ روزه گرفته شد.

نتایج- از نظر ماکروسکوپی میزان تشکیل مجدد بافت پوششی در روز، میزان انقباض زخم در روز و میزان التیام کلی زخم در روز در گروه B افزایشی مشاهده شد، هرچند این افزایش از نظر آماری غیر معنی‌دار بود. از نظر هیستوپاتولوژی در هر دو گروه لایه سطحی پوست (اپیدرم) در بیشتر موارد تمام سطح زخم را پوشانده بود ولی از نظر تعداد لایه‌های اپیدرم و نیز تمایز آنها بخصوص بلوغ و تمایز سلولهای خاردار، زخم‌های گروه B وضعیت بهتری از زخم‌های گروه A داشت. البته این تفاوتها از نظر آماری معنی‌دار نبود.

نتیجه‌گیری و کاربرد بالینی- احتمالاً استفاده از کراتینوسیت‌های کشت داده شده خودی اسب بر روی غشاء کلاژن در التیام زخم‌های ایجاد شده در نواحی پایینی اندام حرکتی تأثیر مثبتی داشته و باعث بهبود و التیام مطلوب‌تر زخم درم و بخصوص اپیدرم می‌شود. برای افزایش اثرات سلول درمانی به صورت معنی‌دار بهتر است پیوند سلولها در زخمهای وسیع صورت گیرد، همچنین ایجاد بستر مناسب و درمان‌های مکرر ضروری می‌باشد.

کلمات کلیدی - کراتینوسیت، غشاء کلاژن، اسب، اندام حرکتی، التیام زخم.

