ORIGINAL ARTICLES

The expression of COX-2 and VEGF in mixed grafting of sheepskin and autoskin in rats

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Abstract

Objective: To investigate the expression of cycloxygenase-2 (COX-2), and vascular endothelial growth factor (VEGF) by using immunohistochemistry method and observe the changes of histological structure between the all skin group and the xenoskin (sheepskin) group, and explore the effect of the xenoskin (sheepskin) which is a kind of covering of autogenous microskin grafting.
Methods: A total of 180 Wistar healthy male rats were randomly divided into 3 groups: the autoskin group (the control group), the alloskin group and the xenoskin group (the sheepskin group), with 60 rats in each group. By using immunohistochemistry method, the expression of COX-2 and VEGF were revealed on 3, 5, 7, 14, 21 and 28 d, with 10 executed rats in each group on each time points, and the structure of histology was observed by light microscope.
Results: There were obvious difference of expression of COX-2 and VEGF on wound in the autoskin group compared with other two groups ($p < .01$); but there were no differences between the alloskin group and xenoskin group ($p > .05$). Observed on light microscope, the paralleled collagen and elastic fiber appeared in the autoskin group on 14 post-transplantation days, but they appeared in the alloskin group and the xenoskin group only on 21 post-transplantation days.
Conclusions: There were no differences of the expression of COX-2 and VEGF in the alloskin group and the xenoskin group, which provided clinical theoretical basis on sheepskin as a substitute for the alloskin to cover autogenous microskin.

Key Words: Sheepskin, Angiogenesis, Cycloxygenase-2, Vascular endothelial growth factor

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Burn wound is the root cause of symptoms such as shock, imbalance of water and electrolyte, and wound infection in patients suffering from extensive deep burn. Therefore, timely and effective sealing of burn wounds is the key to the successful treatment of such patients. The common clinical autogenous skin is proved to be an effective approach for wound closure of small area burned patients, such as large skin grafting, stamp skin grafting, mesh skin grafting, various pedicled and free flaps. The skin supply of autologous donor site for large deep burn patients is seriously insufficient, so autologous skin substitutes must be applied. It will lead to a variety of serious complications, even life-threatening if the wound can not be repaired in time.

Autologous particulate skin combined with alloskin graft is a common method in the treatment of large area deep burn wounds at present. However, the clinical practice is limited due to insufficient source of alloskin and its expensive price.

Thus, the mechanism of alloskin substitutes in the treatment of burn wounds should be further investigated.

Cyclooxyenase-2 (COX-2) is an induced subtype of cycloxyenase, which promotes cell proliferation[1] and inhibits the immune response[2,3] by inducing the activity of epidermal growth factor receptor (EGFR), and boosts the forma-

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tion of new blood vessels by stimulating the expression of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor.[4,5] VEGF is currently known as the most powerful and specific regulator of cell proliferation and migration. It selectively acts on vascular endothelial cells, and then promotes the cells growth and division, finally improves vascular permeability and promotes angiogenesis.[6] Under normal conditions, the concentration of VEGF in the blood is very low. However, the level of VEGF can rise rapidly when acute trauma occurs.[7]

In this study, the expression levels of COX-2 and VEGF were detected at skin wound of replicated traumatic model in rats to analyze their role in the mechanism of wound healing, and to provide a theoretical basis for clinical application of alloskin as a microskin-grafting substitute.

1 Material and methods

1.1 Principal instruments and reagents

Rotary microtome (LEICA), Computer image analysis system (aerial image center), Rabbit anti-Mouse COX-2 Antibody, Rabbit anti-Mouse VEGF-A Polyclonal Antibody.

1.2 Preparation of animal models

180 healthy male Wistar rats (clean grade, 3-4 mo, weight M = 232.25 g, QR = 52.75 g) were obtained from the Experimental Center of Inner Mongolia University. They were treated with complex model diet feeding (provided by the Experimental Center of Inner Mongolia University) with the same feeding conditions (temperature 25-30 °C, humidity 40%-50%, light 12 h/d). Rats were randomly divided into three groups: the autoskin group (control group), the alloskin group (alloskin + autoskin) and the xenoskin group (sheepskin + autoskin), with 60 rats in each group. Each group was observed at 6 time points after 3, 5, 7, 14, 21 and 28 d at the time of molding, with 10 points at each time.

Hair removal at the back of the rats was performed 24 h before the operation, with dip depilatory (10 g barium sulphide, 15 g lime, with distilled water to 100 mL[8]), followed by intraperitoneal injection of anesthesia. The dorsal skin was cut to the deep fascia to form a wound with an traumatic area of 4 cm × 6 cm. The skin was covered with antibiotic saline gauze, and then autoskin, alloskin, xenoskin (sheepskin + autoskin) were performed respectively.

Methods: (1) The autoskin group (control group): the harvested skin (4 cm × 6 cm) was tailored into full skin flap to cover the back wound of the same rat. (2) The alloskin group: the autogenous skin of the rats was peeled into 0.3 mm thick skin, and the skin was cut into a patch by the ratio of 1:10 (area ratio)[9] (the maximum is not more than 1 mm^2). The particles of autoskin were uniformly smeared onto dermalsurface of the rats. The alloskin rat skin with autologous particulate skin was also transplanted onto the wound surface. (3) The xenoskin (sheepskin) group: using the same method to make rat skin into particles. The particles of autoskin were uniformly smeared onto dermalsurface of the rats. Xenoskin (sheepskin) with autoskin particles was also transplanted on the wound surface.

After the transplantion, the skin grafts were sutured, packed and wrapped with pressure. After intraperitoneal injection of 5 mL saline, each rat was placed and fed in a single cage. 1/2-3 d dressings were used to observe the survival and growth of mixed grafts.

1.3 Specimen collection

10 rats in each group were killed and the samples were collected immediately after 3, 5, 7, 14, 21, 28 d. 4% paraformaldehyde fixed, paraffin embedded samples were cut and 5 μm-thick longitudinal section was obtained. HE, VG combined with Verhoeff and Perdrau staining were used and observed their histological morphology under light microscope.

1.4 Method to detect COX-2 and VEGF

After paraffin removal and hydration, paraffin sections were repaired with trypsin (0.025 g/L, PH = 7.8), with 1 drop of monoclonal antibody (anti COX-2 and VEGF) to each slice, and were stored in the refrigerator (4°C) overnight. The PBS solution was used as blank control. Each slice was incubated with 1 drop of biotin labeled second antibody (reagent C) at room temperature. Each slice was added with freshly prepared DAB solution, and was observed under microscope for 3-5 min. Results found: Both COX-2 and VEGF were found to express in the cytoplasm with yellow and pale brown staining. No staining in the cytoplasm was scored as negative. At high magnification, we counted 1,000 cells, dividing them according to the percentage of positive cells, < 10% negative, > 10% positive.[10] Positive cell rate = positive cell number /60 × 100%

1.5 Statistical processing

Data were presented as percentage and case number. Count data between groups were compared using χ^2 test, and analyzed by SPSS 11.5 statistical software package. The test level was set at α = 0.05. $P \leq .05$ was considered statistically significant.

2 Results

2.1 General observation

1 rat (No. 39 in control group) died of excessive force of preoperative hair removal grab. 3 rats (No. 19 and 55

from sheepskin group, No. 54 from alloskin) died during intraperitoneal administered 20% urethane 1 ml/50 g to anesthesia, which were remodeled in follow-up experiments. The rest of the experimental animals were well tolerated with surgery and grafts, and they were free to move after anesthesia anabiosis.

2.2 Histological observation of wound healing

2.2.1 Light microscopy observation

There was no rejection in the autoskin group after extensive skin transplantation, and the wound healing regularity was similar to the general injury. However, moderate inflammatory reactions and the process of tissue cell repair and healing still occurred. New blood capillary hyperplasia and thin layer granulation tissue began to grow on 3 post-transplantion days. After 5-7 days, capillaries were inserted into the skin graft, while the intradermal hair follicle epithelial cells and epidermal cells proliferated slightly. 14 days later, the epidermis continued to proliferate, the layers increased, and became well differentiated. After 3-4 weeks, the epithelium of the graft area was well covered and hair follicles were found at the epithelial surface.

Obvious immunological rejection appeared at the metaphase after the grafting in the alloskin group and the xenoskin (sheepskin) group mixed with autoskin. In addition to inflammatory reaction, there was obvious immunological rejection in wound healing process. On 3 and 5 post-transplantion days, the structure of epidermis cells of alloskin and xenoskin (sheepskin) was basically normal, and the new capillaries grew to the bottom of the dermis of alloskin and xenoskin (sheepskin) with corpuscular hyperplasia. On 7 post-transplantion days, graft rejection was observed in these two groups with necrosis and exudation in different degrees found at epidermal layer. And partial separation of the epidermis and dermis was presented. In the dermis layer, there were some inflammatory cell infiltrations composed of lymphocytes. The dermis layer had more new capillaries, and the skin continued proliferating and growing. After 14 d, the rejection was even more obvious, and a large cavitation bubble in the epidermis was shown. The alloskin and xenoskin (sheepskin) were separated from the dermis, and the microskin was fused into synthetic slices. After 21 d, the granulation tissue tended to mature and the capillary decreased. Autologous particles were fused into each other to repair the wound 28 days later.

2.2.2 Stained with VG + Verhoeff

In the autoskin group (control group), the parallel arrangement of collagen fibers and elastic fibers occurred on 14 post-transplantion days, and the collagen and elastic fibers appeared parallel with 21 d in the alloskin and xenoskin (sheepskin) group after transplantation.

2.2.3 Stained with Perdrau

Reticular fibers appeared in the around of capillaries. There was no difference between the alloskin group, xenoskin (sheepskin) group and autoskin group (control group).

2.3 COX-2 expression

(1) The positive expression rates of COX-2 were 45%, 71.7% and 75%, respectively in the autoskin group (control group), alloskin group and xenoskin (sheepskin) group. There were significant differences between the three groups (see Table 1, $p < .01$). The expressions of COX-2 protein were found as brownish yellow in cytoplasm and nuclear membrane (see Figures 1-3).

(2) The expression of COX-2 in the autoskin group showed significantly difference compared with the other two groups ($p < .01$), while the difference of COX-2 expression between the alloskin group and xenoskin (sheepskin) group was not significant ($p > .05$). The number of positive cases of COX-2 in each phase was shown as follows (see Figure 4). The trends showed a decreased number of positive COX-2 in the autoskin group (control group) with time, while the other two groups showed the highest number of positive points on 14 post-transplantion days, and then declined sharply.

2.4 VEGF expression

(1) The positive expression rates of VEGF in the autoskin group (control group), alloskin group and xenoskin (sheepskin) group were 68.3%, 45.0%, 38.3%, $\chi^2 = 11.913$, $p = .003$, which showed that the differences between the three groups were significant (see Table 2). VEGF protein was expressed in cytoplasm and nuclear membrane as brownish yellow (see Figure 5).

Table 1: The expression of COX-2 in each group (n = 60)

Groups	Number of positive (n)	Number of negative (n)	Positive rate (%)	χ^2 value	*p* value
Autoskin	27	33	45.0	8.777$^{\triangle}$	.003
Alloskin	43	17	71.7	11.250$^{\triangle※}$	.001
Sheepskin	45	15	75.0	0.170$^{※}$	.680

Note. $^{\triangle}$ presents difference between the autoskin and alloskin group, $p < .01$; $^{\triangle※}$ presents difference between the autoskin and sheepskin group, $p < .01$; $^{※}$presents difference between the alloskin and sheepskin group, $p > .05$

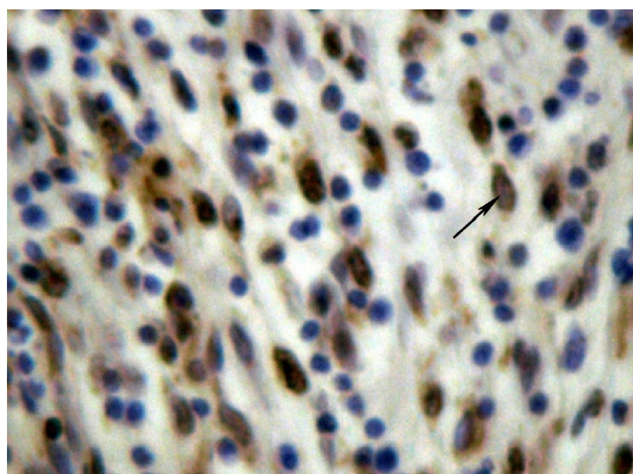

Figure 1: The positive expression of COX-2 in sheepskin (S-P, × 400)

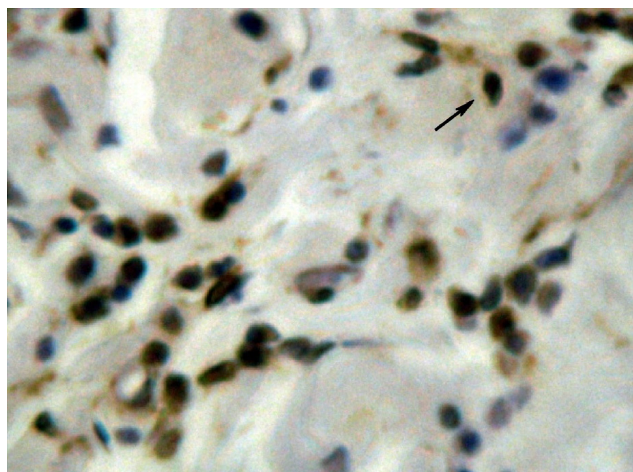

Figure 2: The positive expression of COX-2 in alloskin (S-P, × 400)

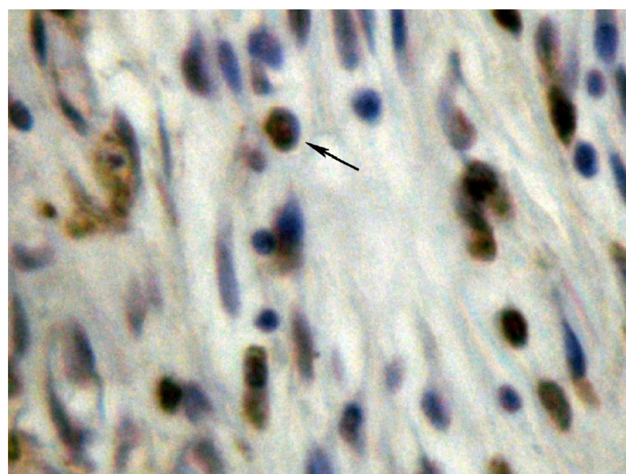

Figure 3: The positive expression of COX-2 in autoskin (S-P, × 400)

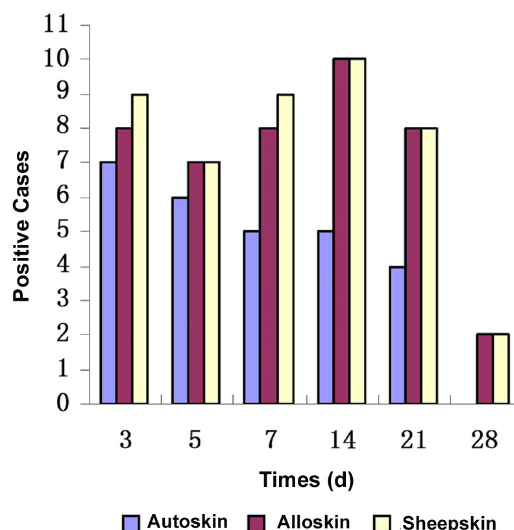

Figure 4: The expression of COX-2 in each group on each time point (S-P, × 400)

Table 2: The expression of VEGF in each group (n = 60)

Groups	Number of positive (n)	Number of negative (n)	Positive rate (%)	χ^2 value	*p* value
Autoskin	41	19	68.3	6.625△	.010
Alloskin	27	33	45.0	10.848△※	.001
Sheepskin	23	37	38.3	0.549※	.459

Note. △ presents difference between the autoskin and alloskin group, $p < .01$; △※ presents difference between the autoskin and sheepskin group, $p < .01$; ※presents difference between the alloskin and sheepskin group, $p > .05$

(2) The positive expression of VEGF in the autoskin group (control group) showed significant differences compared with the other two groups ($p < .01$). While, the difference was not significant between the alloskin group and xenoskin group ($p > .05$). The number of positive cases of VEGF in each phase was shown in Figure 6. The trends showed that the number of positive VEGF in the autoskin group (control group) was the highest on 5 post-transplantion days, and then decreased with time, while the other two groups showed a relatively positive number on 5 and 14 post-

transplantion days, respectively.

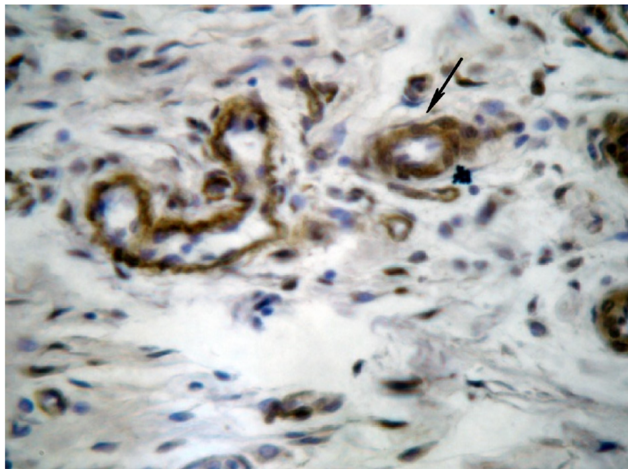

Figure 5: The positive expression of VEGF (S-P, × 400)

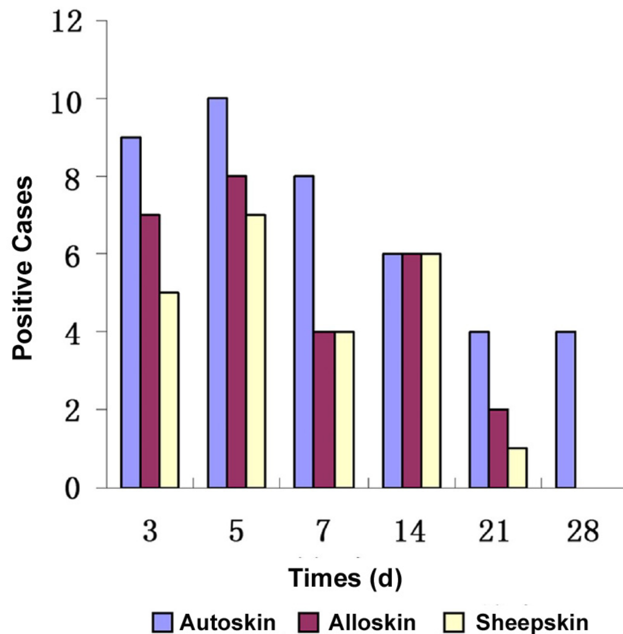

Figure 6: The expression of VEGF in each group on each time point

3 Discussion

Faced with the problem of the shortage of healthy skin, we are continuously seeking for skin substitutes to treat large area deeply burned wounds. The selection of skin substitutes for extensive deep burns has been explored continuously by domestic and overseas searchers. Pigskin and frog skin etc. have been proved as successful substitutes in previous studies. Pigskin is not well applied due to its stiffness and the patients' ethnic and religious beliefs. The clinical application of frog skin is unsatisfactory as well since the skin area is smaller. Artificial biological dressings have been put into use, but they can not be widely used due to expensive price.

The healing of deep burn wounds is not merely a simple process of proliferation, differentiation and migration of epithelial cells. Deep burn wounds have defects in the whole skin and skin appendages. The basic repairing process is the proliferation of vascular endothelial cells and fibroblasts, connective tissue formation, and finally the wound remodeling. Due to obvious necrotic tissue of deep burn wounds, therefore, in the early stage of inflammation in wound healing, inflammatory cells use the release of a variety media or cytokines in the initiation and regulation of fibroblast, vascular endothelial cells and other cells to repair wound. Inflammatory cells also secrete various enzymes to dissolve and separate the necrotic tissue. Combined with the phagocytosis of inflammatory cells, the necrotic cells are removed as well as the necrotic tissue of the wound to form a healthy basal tissue for wound healing. Wound healing in large area deep burn patients is a complex healing process which includes obvious inflammatory reaction and immune rejection reaction.[11]

Our histological observation indicated that inflammatory response was stronger in the alloskin and the xenoskin (sheepskin) group than that in autoskin group (control group). The collagen fibers and elastic fibers in the alloskin and xenoskin (sheepskin) group were regenerated later than those in the autoskin group (control group). This might be the result of the strong inflammatory reaction and the cytokines function induced by the immune rejection in the process of wound healing in the alloskin and xenoskin (sheepskin) group. This result was consistent with previous studies.[12]

The inflammatory factor test showed that the numbers of positive COX-2 in the autoskin group (control group) decreased with time, while the numbers of COX-2 in the alloskin group and xenoskin (sheepskin) group were the highest on 14 post-transplantion days. The results were consistent with the literature which reported that the expression of COX-2 increased markedly when the tissue was inflamed.[13] The accelerated wound healing in the autoskin group may probably be associated with moderate inflammatory response and the repairing process of tissue cells, which has a relationship with COX-2. The strong immune rejection might be caused by marked increase of COX-2 in the alloskin group and xenoskin (sheepskin) group.

The experimental results also showed that the positive expression of VEGF in the autoskin group (control group) was the largest on 5 post-transplantion days, and then decreased with time. The other two groups showed relatively positive numbers on 5 and 14 post-transplantion days respectively. VEGF was proved to have two peaks in mixed grafts,[14] appearing at 3 d and 14 d, respectively. VEGF has been reported to induce the expression of COX-2,[5] whereas COX-2-induced PGE2 carried the function of enhancing VEGF

mRNA expression and promoting its protein synthesis.[15] This may be the results of the relatively higher number of positive points in the alloskin group and xenoskin (sheepskin) group on 5 and 14 post-transplantion days. According to the biological function of VEGF, it can be inferred that the first peak may be related to its promotion of microskin transplantation bed and establish blood supply in the alloskin group and xenoskin group, and the other peak may be relative with the reconstruction of vessels and extracellular matrix of alloskin and xenoskin.

When inflammation occurred, COX-2 showed a high expression.[16] The study found that COX-2 in the alloskin group and xenoskin (sheepskin) group was higher than that in the autograft group (control group) ($p < .01$). Selective effects of VEGF on vascular endothelial cells promoted the growth of split and improved the vascular permeability and angiogenesis. The expression levels of VEGF in the alloskin group and xenoskin (sheepskin) group were lower than that in the autograft group (control group) ($p < .01$), which indicated that the inflammatory reaction caused by autoskin wound repair could quickly promote wound healing. This study also discovered that there was no significant difference between the alloskin group and xenoskin group with regard to the expression levels of COX-2 and VEGF ($p > .05$). This indicated that there was no obvious difference in the inflammatory reaction and wound healing between the alloskin group and xenoskin (sheepskin) group. In addition, sheepskin is the best candidate of skin substitute for extensive burn patients due to its features of thinness, easy folding, easy to dry and great area. Therefore, it provides a theoretical basis for xenoskin (sheepskin) as an autologous particle skin graft cover instead of alloskin.

Sheepskin has many advantages over its counterpart, such as wide source, low price, soft and elastic, and similar human skin structure. Our research has shown encouraging prospects. With the gradual development and in-depth study of cell biology, molecular biology and biological engineering, materials science and medicine, xenoskin (sheepskin) can be used as a particulate skin graft covering for clinical application instead of alloskin. It also brings great benefits to the low-income deep burn patients since sheepskin prices are low.

Conflicts of Interest Disclosure

The authors have no conflict of interest related to this article.

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