ORIGINAL ARTICLE

Study on the mechanism of microporous sheep ADM combined with hUCMSC in promoting wound healing

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ABSTRACT

Objective: To explore the therapeutic mechanism of microporous sheep acellular dermal matrix (ADM) combined with human umbilical cord mesenchymal stem cell (hUCMSC) to promote the healing of full-thickness skin defect.

Methods: hUCMSC was co-cultured on microporous sheep ADM to form composite biological dressings. Seventy-two nude mice were selected to make full-thickness skin injury models and randomly divided into 3 groups (hUCMSC + microporous sheep ADM group, sheep ADM group and iodophor gauze group), with 24 mice in each group. The wound healing rate of each group was detected at 14 d, 21 d and 28 d after operation, qRT-PCR technique was used to detect the expression of Bax and Bcl-2 in the wound tissues, and the immunohistochemical staining technique was used to detect the expression of Collagen I and vascular endothelial growth factor (VEGF). Data were analyzed with one-way ANOVA and t test.

Results: At 14 d after operation, the wound healing rate of the hUCMSC + microporous sheep ADM group was (65.34 $\pm$ 14.72)%, which was significantly higher than that of the iodophor gauze group [(37.54 $\pm$ 10.21)%], and higher than that of the sheep ADM group [(49.08 $\pm$ 11.16)%], the differences were statistically significant (t = 19.52, 14.72; p < .05). With the gradual healing of the wound, at 28 d after operation, the wound healing rate of the hUCMSC + microporous sheep ADM group was (98.63 $\pm$ 15.41)%, which was significantly higher than that of the iodophor gauze group [(81.74 $\pm$ 16.27)%], and higher than that of the sheep ADM group [(63.47 $\pm$ 14.80)%], the differences were statistically significant (t = -16.42, 20.35; p < .05). The expression of Bax in the wound tissues of the hUCMSC + microporous sheep ADM group was significantly reduced, especially at 21 d after operation, the expression level was 0.25 $\pm$ 0.06, which was significantly lower than the iodophor gauze group (0.53 $\pm$ 0.16) and the sheep ADM group (0.41 $\pm$ 0.12), the differences were statistically significant (t = 3.52, -2.83; p < .05). The expression of Bcl-2 in the wound tissues of the hUCMSC + microporous sheep ADM group was significantly higher than those of the other two groups, especially at 21 d after operation, the expression level was 0.63 $\pm$ 0.19, which was significantly higher than the iodophor gauze group (0.34 $\pm$ 0.09) and the sheep ADM group (0.46 $\pm$ 0.13), the differences were statistically significant (t = 5.31, -6.07; p < .05). Immunohistochemical detection showed that the expression of Collagen I and VEGF in the hUCMSC + microporous sheep ADM group was slightly more than that of the sheep ADM group and the iodophor gauze group, but the effect was not remarkable.

Conclusions: hUCMSC + microporous sheep ADM composite dressing can promote the healing of the full-thickness skin injury and reduce the production of apoptotic cells by carrying hUCMSC.

Key Words: Mice, Wounds and injuries, Mesenchymal stem cells, Microporation, Sheep acellular dermal matrix, Wound healing

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1. INTRODUCTION

During the treatment process of severe large-scale deep burn, it is needed to apply various skin substitutes to the wound due to autologous and allogenic skin deficiency. Currently, the biological dressings used clinically primarily comprise pigskin, collagen membrane, artificial skin, amniotic membrane and so on.[1] Taking the special beliefs of the ethnic minorities (Muslim) into consideration, in this study, the research on acellular dermal matrix (ADM) is given top priority by virtue of the abundant resource of sheepskin.[2,3]

Some researches have shown that in the skin wound or the burn wound, MSCs can be induced and transformed into epidermal cells or fibroblasts and so on, in order to fill the defect in the wound tissues, secrete some factors to promote the wound healing and then improve the repair quality.[4] MSCs are remarkably affected by age, i.e., the potential of proliferation and differentiation is gradually decreased with the increase of age. Simultaneously, it is required to conduct bone marrow puncture when collecting stem cells. The disease factors such as infection and poor coagulation can also restrict the application of autologous MSCs. Human umbilical cord mesenchymal stem cell (hUCMSC) can be extracted from the umbilical cord blood. It is characterized by relatively high purity, convenient collection, lower probability of viral/bacterial pollution and oncogenicity than MSCs. In the precedent experiment, it was found that hUCMSC could survive and grow in the micropores and superficial layer of heparinization sheep ADM.[5] Therefore, it was designed to culture hUCMSC + microporous sheep ADM in the in-vitro study in this issue, in order to heal the full-thickness skin defect of the nude rats. Now it was reported as follows.

2. MATERIALS AND METHODS

2.1 Experimental animals, main reagents and instruments

Experimental animals, main reagents and instruments are listed as follows: one healthy adult female sheep originating from Darhan Muminggan Joint Banner, Baotou City of Inner Mongolia, weighing 30 kg; 72 SPF healthy nude rats of either gender (8-week-old, purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd.), weighing (20 $\pm$ 4) g; Collagen I (Boster, 1:400); VEGF antibody (Santa cruz, 1:500); BsMAb SP kits and DAB chromogenic kits (Fuzhou Maixin Biotechnologies Development Company); real-time PCR system (ABI Steponeplus, USA), high-speed refrigerated centrifuge (5424, Eppendorf, Germany), Rotary Microtome (RM2235, Leica, Germany), optical microscope (CH-20, Olympus, Japan), microscope camera system (Olympus, Japan).

2.2 Co-cultivation of hUCMSC and microporous sheep ADM

Healthy age-appropriate sheep were sacrificed and fleeced, with subcutaneous adipose tissue removed. Meanwhile, acellular sheep skin biological dressings (10 cm $\times$ 20 cm in size) were made, aseptically packaged, and preserved at -80 °C for use. The cryopreserved acellular sheep skin was placed in a freeze-dryer and freeze-dried to freeze the water in the interstitial space with the ice crystals directly sublimated, and expected to leave uniform pores.

With the informed consent signed, 3 to 5 cm of neonatal umbilical cord were obtained in order to cultivate hUCMSC and then identify the cultured cells, as previously reported by our group.[5] The previously prepared sterile microporous sheep ADM was trimmed to the size of 3 cm $\times$ 3 cm, placed at the bottom of a sterile petrie dish with the dermis upwards, and then soaked with hUCMSC culture medium for 1 day, with the medium removed. The fourth passage of hUCMSC was seeded and cultured for 6 days, with the culture medium changed every 3 days.

2.3 The establishment of nude mouse full-thickness skin injury models

72 mice were anesthetized by intra-peritoneal injection of 2 ml/100 g chloral hydrate with a mass concentration of 1.8% in order to make a full-thickness injury wound with a diameter of 2 cm on the back of each nude mouse. These nude mice were randomly divided into 3 groups (hUCMSC + microporous sheep ADM group, sheep ADM group and iodophor gauze group), with 24 mice in each group. After iodophor disinfection of the wound surface, the mice in the three groups were covered with the prepared microporous sheep ADM combined with hUCMSC and iodophor gauze respectively. The periphery of the wound was sutured and fixed with 2-0 sutures.

2.4 Wound healing rate

8 nude mice were randomly selected from each group at 14 d, 21 d and 28 d after surgery and then anesthetized by intra-peritoneal injection of chloral hydrate with a mass concentration of 1.8% in order to observe the wound healing status. The unhealed part of the wound was profiled on the cellophane tape in a ratio of 1:1 and the image was then scanned. AutoCAD (Autodesk USA) and CorelDRAW (Corel, Canada) were used to figure out the surface area of the unhealed wound and then calculate the wound healing rate at 14 d, 21 d and 28 d after surgery.

The formula is listed as follows: wound healing rate (%) = (the original area of the wound – the unhealed area of the wound)/the original area of the wound $\times$ 100%.

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2.5 The expression levels of apoptosis-related genes Bax and Bcl-2 detected by use of qRT-PCR

Upon the anesthesia performed in Part IV of the experiment at 14 d, 21 d and 28 d after surgery, these nude mice were sacrificed by cervical dislocation. Half of the wound tissues were preserved in liquid nitrogen and the other half in 4% paraformaldehyde for immunohistochemical staining. The wound tissues cryopreserved in liquid nitrogen were quickly ground into powder with a mortar. 100 mg of powder was taken and put into a centrifugal tube containing 1 ml of Triol solution, mixed well, in order to extra RNA. The targeted cDNA was synthesized through reverse transcription reaction. The primers were designed in accordance with the mRNA sequences of related genes in Genebank through Primer Premier 5.0 software, and synthesized by BGI Technology Co., Ltd. PCR amplifier was used to amplify the primers. The primer sequences are as follows: GAPDH-F: 5'-CGTGCCGCCTGGAGAAACCTG-3', GAPDH-R: 5'-AGAGTGGGAGTTGCTGTTGAAGTCG-3'; Bax-F: 5'-CAG GAT GCGTCCACCAAGAA -3', Bax-R: 5'-CGTGTCCACGTCAGCAATCA-3'; Bcl-2 F: 5'-TCTGTTTGATTTCTCCTGGCTGT-3', Bcl-2 R: 5'-ATTTGT TTG GGGCAGGTTTG-3'. 20 μl reaction system was that: the reaction conditions were 95 °C, 3 min; 95 °C, 30 s, Tm, 30 s, 72 °C, 30 s; 45 cycles; 72 °C, 10 min. The relative mRNA expression of Bax and Bcl-2 was calculated according to the following formula: Relative expression = $2^{-[ct\ (targeted\ gene\ -\ reference\ gene)\ -\ ct\ (control\ gene\ -\ reference\ gene)]}$.

2.6 The expression levels of Collagen I and vascular endothelial growth factor (VEGF) detected by use of immunohistochemical staining

At 14 d, 21 d and 28 d after surgery, the other half of the wound tissues preserved in 4% paraformaldehyde in Part V of the experiment were taken and fixed in 4% paraformaldehyde to prepare paraffin sections, and the expression of Collagen I and VEGF protein in the wound tissues was detected by use of immunohistochemical staining.

2.7 Statistical treatment

SPSS18.0 software was used to make an analysis of the experimental data. The wound healing rate and the mRNA expression of Bax and Bcl-2 detected by qRT-PCR were represented by mean ± standard deviation ($\bar{x}\pm$ s). One-way ANOVA was applied to the intra-group comparison, and t-test was used in the inter-group comparison. The difference ($p < .05$) was of statistical significance.

3. RESULTS

3.1 Wound healing rate

After the full-thickness injury was treated with different dressings, there were statistically significant differences in the wound healing rates of the three groups at 14 d, 21 d and 28 d after surgery ($p < .05$). At 14 d, the wound healing rate in the iodophor gauze group was (37.54 ± 10.21)%, which was obviously lower than that in the sheep ADM group [(49.08 ± 11.16)%], and the difference was of statistical significance (f = 17.65, $p < .05$); the wound healing rate in the hUCMSC + microporous sheep ADM group was (65.34 ± 14.72)%, which was obviously higher than the other two groups (t = 19.52, 14.72, $p < .05$). With the gradual healing of the wound surface, at 28 d, the wound healing rate in the hUCMSC + microporous sheep ADM group was (98.63 ± 15.41)%, which was remarkably higher than the sheep ADM group [(81.74 ± 16.27)%] and the iodophor gauze group [(63.47 ± 14.80)%], and the differences were statistically significant (t = 16.42, 20.35, $p < .05$). See Table 1 for details.

Table 1. The comparison in the wound healing rates at different time points after surgery among different groups of nude mice with full-thickness skin injury (%, $\bar{x}\pm$s)

Group	n	14 d after surgery	21 d after surgery	28 d after surgery
Iodophor Gauze Group	12	37.54 ± 10.21	52.36 ± 13.54	63.47 ± 14.80
Sheep ADM Group	12	49.08 ± 11.16 *	71.53 ± 15.40 *	81.74 ± 16.27 *
hUCMSC + Microporous Sheep ADM Group	12	65.34 ± 14.72 *#	84.53 ± 19.24 *#	98.63 ± 15.41 *#
F Value		167.25	172.43	186.48
p Value		< .05	< .05	< .05

Note. In comparison with the iodophor gauze group, * $p < .05$; in comparison with the sheep ADM group, # $p < .05$; ADM is acellular dermal matrix; hUCMSC stands for human umbilical cord mesenchymal stem cell

3.2 The change in the expression levels of apoptosis-related genes detected by use of qRT-PCR

At 14 d, 21d and 28 d, there were statistically significant differences in the mRNA expression of Bax in the three groups ($p < .05$); at 14 d, the expression level of Bax in the hUCMSC + microporous sheep ADM was obviously lower than the other two groups, and the differences were statistically significant (t = -5.86, 2.14, $p < .05$). At 21 d after

surgery, the expression levels of Bax in the three groups were relatively lowered. However, the expression level of Bax in the hUCMSC + microporous sheep ADM group was still lower than the other two groups, and the differences were of statistical significance (t = 3.52, -2.83, p < .05). At 28 d, the expression levels of Bax in the three groups were somewhat increased. However, the expression level of Bax in the hUCMSC + microporous ADM group was still lower than that in the iodophor gauze group, and the difference was of statistical significance (t = 4.71, p < .05). See Table 2 for details.

Bcl-2 were expressed in the three groups in different degrees. At 14 d, 21d and 28 d, there were statistically significant differences in the mRNA expression of Bcl-2 in the three groups (p < .05); at 14 d, the expression level of Bcl-2 in the hUCMSC + microporous sheep ADM was obviously higher than the other two groups, and the differences were statistically significant (t = -3.19, -4.23, p < .05). At 21 d after surgery, the expression levels of Bcl-2 in the three groups were relatively increased. However, the expression level of Bcl-2 in the hUCMSC + microporous sheep ADM group was still higher than the other two groups, and the differences were of statistical significance (t = 5.31, -6.07, p < .05). At 28 d, the expression levels of Bcl-2 in the three groups were somewhat lowered. However, the expression level of Bcl-2 in the hUCMSC + microporous ADM group was still higher than the other two groups, and the differences were of statistical significance (t = 3.46, -1.63, p < .05). See Table 3 for details.

Table 2. The comparison in the mRNA expression of Bax among different groups of full-thickness wound tissues ($\bar{x}\pm$s)

Group	n	14 d after surgery	21 d after surgery	28 d after surgery
Iodophor Gauze Group	8	0.83 ± 0.19	0.53 ± 0.16	0.74 ± 0.21
Sheep ADM Group	8	0.56 ± 0.13 *	0.41 ± 0.12 *	0.52 ± 0.18 *
hUCMSC + Microporous Sheep ADM Group	8	0.38 ± 0.09 *#	0.25 ± 0.06 *#	0.41 ± 0.15 *#
F Value		9.21	17.58	2.45
p Value		< .05	< .05	< .05

Note. In comparison with the iodophor gauze group, * p < .05; in comparison with the sheep ADM group, # p < .05; ADM is acellular dermal matrix; hUCMSC stands for human umbilical cord mesenchymal stem cell

Table 3. The comparison in the mRNA expression of Bcl-2 among different groups of full-thickness wound tissues ($\bar{x}\pm$s)

Group	n	14 d after surgery	21 d after surgery	28 d after surgery
Iodophor Gauze Group	8	0.25 ± 0.07	0.34 ± 0.09	0.26 ± 0.05
Sheep ADM Group	8	0.39 ± 0.11 *	0.46 ± 0.13 *	0.37 ± 0.10 *
hUCMSC + Microporous Sheep ADM Group	8	0.51 ± 0.16 *#	0.63 ± 0.19 *#	0.54 ± 0.15 *#
F Value		4.57	3.17	6.24
p Value		< .05	< .05	< .05

Note. In comparison with the iodophor gauze group, * p < .05; in comparison with the sheep ADM group, # p < .05; ADM is acellular dermal matrix; hUCMSC stands for human umbilical cord mesenchymal stem cell

3.3 Immunohistochemical detection

VEGF and Collagen I were expressed in the three groups in different degrees at different time points. Positive immunohistochemical staining was expressed by dark brown cells and negative results were expressed by light brown cells. With the gradual healing of the wound surface, the dark brown positive cells of Collagen I and VEGF in each group at 28 d after surgery were expressed darker than those at 14 d and 21 d after surgery. At the same time point, immunohistochemical detection showed that the expression of Collagen I and VEGF in the hUCMSC + microporous sheep ADM group was slightly more than that of the sheep ADM group, but the effect was not remarkable (see Figures 1-2).

4. DISCUSSION

During the treatment of full-thickness injury or burn, the application of ADM can provide a suitable environment for wound healing, induce cell proliferation and angiogenesis, and promote wound healing. Meanwhile, it can also reduce the incidence of some problems such as scar hyperplasia during wound healing.[6] Previous studies by our group have found that sheep ADM can provide a suitable microenvironment for the wound tissues and promote the healing of the wound surface as a natural matrix for tissue regeneration. Since sheep ADM has no antigenic interference, it has a good biocompatibility with wound tissues and makes for epithelial regeneration of the wound surface.[3, 7]

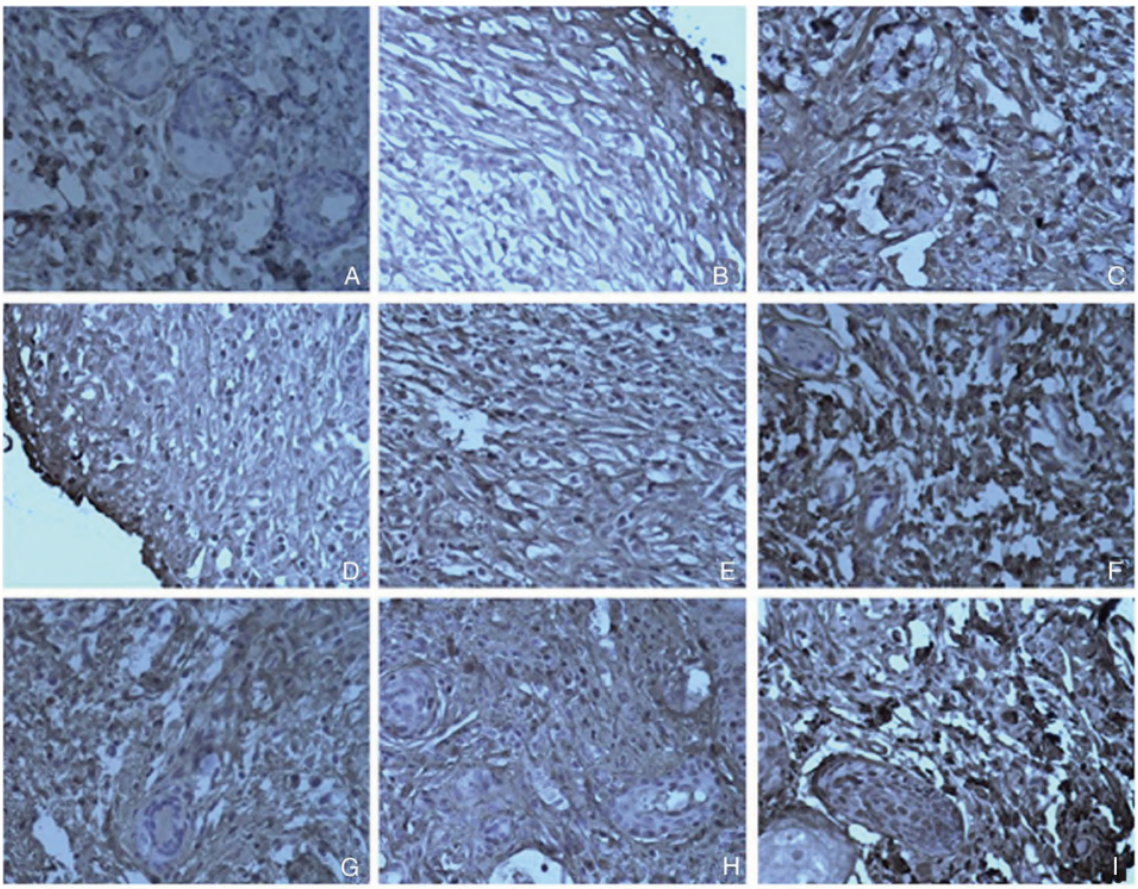

Figure 1. Immunohistochemical staining of Collagen I in different groups of wound tissues (× 400)
A, B and C are the iodophor gauze group, the sheep ADM group and the hUCMSC + microporous sheep ADM group at 14 d after surgery; D, E and F are the iodophor gauze group, the sheep ADM group and the hUCMSC + microporous sheep ADM group at 21 d after surgery; G, H and I are the iodophor gauze group, the sheep ADM group and the hUCMSC + microporous sheep ADM group at 28 d after surgery; the expression of Collagen I was shown in each group, the expression of Collagen I could be seen in the wound interstitium of each group, which was slightly higher in the hUCMSC + microporous sheep ADM group and slightly lower in the iodophor gauze group, but the expression intensity was relatively consistent within each group at the three time points, with no significant change; ADM is acellular dermal matrix; hUCMSC stands for human umbilical cord mesenchymal stem cell

Moreover, hUCMSC has the advantages of convenient sampling, low oncogenicity and relative purity. Some studies have reported that the use of hUCMSC in the treatment of pressure ulcer can improve the blood circulation around the wound, promote granulation tissue growth and accelerate wound healing.[8] Some clinical studies have also found that the use of hUCMSC in the treatment of skin ulcer can greatly improve the healing of ulcer wounds.[9] In vitro studies revealed that hUCMSC can be induced to differentiate into wound repair cells such as fibroblasts, endothelial cells, keratinocytes, sweat gland cells and peripheral parietal cells.[10–12] It has also been reported that hUCMSC can be transplanted to the rat skin defect models, and mesenchymal stem cells can differentiate into peripheral parietal cells, keratinocytes and endothelial cells to different degrees, promote the neovascularization, improve the blood circulation around the wound tissues and accelerate the wound healing.[13,14] In this experiment, hUCMSC were implanted on the microporous sheep ADM biological dressing, which not only exerted the advantage of sheep ADM in protecting the injured wound tissues, but also served as a carrier of hUCMSC to transplant hUCMSC into the injured wound area. It not only filled in the tissue defect, but also played a role in differentiating into functional cells and secreting growth factors, further promoting the healing of the injured wound tissues. By detecting the expression of VEGF and Collagen I, it was also found that the expression in the hUCMSC + microporous sheep ADM group was higher than that in the other two groups, and the wound healing rate in the hUCMSC + microporous sheep ADM group was also higher than that in the sheep ADM group and the iodophor gauze group. The differences were of statistical significance ($p < .05$). It was indicated that hUCMSC has a good promotive effect on the wound healing.

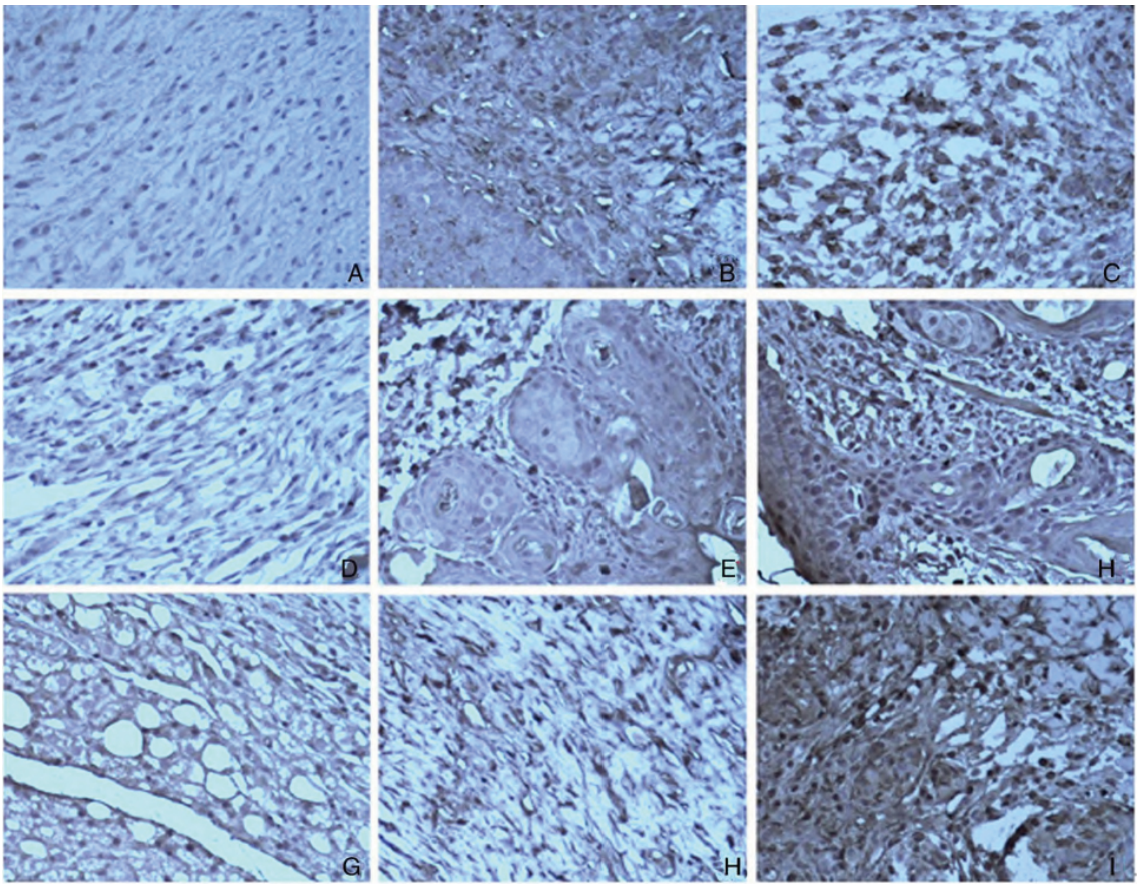

Figure 2. Immunohistochemical staining of VEGF in different groups of wound tissues (× 400)

A, B and C are the iodophor gauze group, the sheep ADM group and the hUCMSC + microporous sheep ADM group at 14 d after surgery, showing VEGF was expressed both in fibroblasts and endothelial cells of the wound tissues, the expression in A was weakest and the expression in C was enhanced; D, E and F are the iodophor gauze group, the sheep ADM group and the hUCMSC + microporous sheep ADM group at 21 d after surgery, showing the expression of VEGF in the three groups were slightly higher than that at 14 d after surgery; G, H and I are the iodophor gauze group, the sheep ADM group and the hUCMSC + microporous sheep ADM group at 28 d after surgery, showing obvious vasculogenesis and a higher expression of VEGF than before; I indicates the strongest expression of VEGF; VEGF represents vascular endothelial growth factor; ADM is acellular dermal matrix; hUCMSC stands for human umbilical cord mesenchymal stem cell

Apoptosis usually occurs at every stage of wound healing, and it is a type of programmed cell death process. Bcl-2 has an effect of inhibiting cell apoptosis, while Bax can promote apoptosis. Bcl-2/Bax can regulate cell apoptosis by forming dimers.[15] Therefore, by detecting the expression changes of Bcl-2/Bax, it is feasible to evaluate the effect of microporous sheep ADM + hUCMSC biological dressing on cell apoptosis during the process of wound healing. It has been found that hUCMSC also have anti-apoptotic effects. It is speculated that it may paracrine some anti-apoptotic cytokines.[16] Because it has been confirmed that mesenchymal stem cells can promote the survival of dangerous cells at the edge of the myocardial infarction area and repair myocardial function.[17] Some other studies have also confirmed that mesenchymal stem cells can significantly reduce the number of apoptotic cells in acute lung injury and acute kidney injury tissues of rats, which further improves the survival rate.[18] In this experiment, it was found that the expression of Bax in the hUCMSC + microporous sheep ADM group was decreased, while the expression of Bcl-2, which inhibited apoptosis, was increased, indicating that microporous sheep ADM + hUCMSC inhibited cell apoptosis in the wound tissues, which was consistent with the above literature reports.

In conclusion, in this study, the use of microporous sheep ADM dressing combined with hUCMSC can greatly promote the healing of full-thickness injuries, providing a theoretical support for clinical treatment.

Conflicts of Interest Disclosure

The authors declare they have no conflicts of interest.

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