

Animal experimental research on preliminary repairing of full-thickness skin deficiency with two different materials

ZHU Ya-bin¹, FANG Jia-xi¹, ZHU Cheng-tao¹, SHEN Mei-ting¹,
ZHOU Ai-na¹, ZHOU Mi¹, HOU Rui-xia¹, GE Fen-fen²

(1. Medical School, Ningbo University, Ningbo 315211, China;

2. Ningbo Medical Center Lihuili Eastern Hospital, Ningbo 315000, China)

Abstract: Eggshell membrane (EGM) is a natural biomaterial that has been used as an alternative natural bandage on burned or injured skin. Our previous research verified that human amniotic membrane (HAM) possessed good capability to promote skin regeneration after the membrane was decellularized. Considering the similarity between amniotic membrane and the eggshell membrane, these two membranes were detected and compared in wound healing in this work. Using Sprague-Dawley male rats as the experimental animals, the wound appearance and the White Blood Cell (WBC) level were investigated after the animals were made full-thickness skin deficiency. The results showed that the wound healed faster of both EGM and dHAM treatments than that of the control animal. And, both WBC values of dHAM and EGM group were lower than that of control animal group all the time. That is, the wound healing of animals treated with EGM and dHAM separately during 21-day testing time was significantly better and faster than that of the control animals, though the efficiency between EGM and dHAM treatment exhibited little difference. This information is valuable for us to further conduct skin regeneration research.

Key words: eggshell membrane; decellularized Human Amniotic Membrane (dHAM); wound healing; biological materials; skin regeneration

CLC number: G237.5

Document code: A

Article ID: 1001-5132 (2019) 02-0048-06

As the largest protective organ, skin plays an important role in defending human bodies against harms from external environment^[1]. Skin is also the first safeguard of human body in providing the functions of sensation and water equilibrium^[2-3]. Skin deficiency is a very common disease. Large area skin defects and refractory skin ulcer induced by burning, acid/base corrosion or other diseases are very serious and difficult to be cured in clinic^[4]. Some replacements have been tried to repair serious skin deficiency, for example,

autologous skin graft, allogeneic skin transplantation, xenogeneic graft and artificial synthetic skin substitutes etc^[5-6]. However, the application of these materials in clinic is still limited due to the donor shortage, immunological rejection and large scar formation. Therefore, it is of great clinical significance and application prospect to find a good substrate with excellent biological properties and rich sources^[7].

Thanks to the emerging and developing of the interdisciplinary of tissue engineering and regeneration

收稿日期: 2018-08-07.

宁波大学学报(理工版)网址: <http://journal.lg.nbu.edu.cn/>

基金项目: 国家自然科学基金(81471797); 宁波市自然科学基金(2017A610259, 2017A610165).

通信作者: 竺亚斌(1967-), 女, 浙江宁波人, 教授, 主要研究方向: 生物材料与再生医学. E-mail: zhuyabin@nbu.edu.cn

medicine, skin repairing has made large progress in dressing materials and scaffolds, which brings great hope to patients in clinical treatments.

The membrane of egg shell, known as Phoenix dress, is called a collagen tissue, because there are 90% (by weight) collagen contained in the membrane^[8]. The main types are collagen I, IV, and V. Other components include albumin, uronic acid, and sialic acid. The egg white contains lysozyme, avidin (a biotin binder), ovotransferrin (an iron binder), collagenases, and chondroitin sulfate. When used as skin dressing, the membrane together with little egg white will promote adhesion to assist wound closure and maintain gas permeability. The rich protein in the membrane, on the other hand, would provide nutrition for the growth of epithelial cells in the membrane epithelium, and reduce the edema and exudation. Whilst, the lysozyme in egg white has a function of anti-bacterial and anti-inflammatory, which can accelerate the proliferation of granulation tissue and finally promote wound healing^[9-10].

Human Amniotic Membrane (HAM) is the innermost membrane tissue of the placenta. It consists of a thick basement membrane and an avascular stromal matrix. Amniotic membrane acts as a scaffold for cells' proliferation and differentiation because of its protein components like fibronectin, collagen (type I, III, IV, V, and VI), elastin, nidogen, hyaluronic acid, and a variety of growth factors such as epidermal growth factor, hepatocyte growth factor, fibroblast growth factor, transforming growth factor- β 1, and so on^[11]. These components help HAM possess the properties of anti-bacterial, anti-inflammatory, anti-angiogenesis and anti-fibrosis, which make amniotic membrane an ideal therapeutic dress for skin wound healing.

In our previous work, HAM was decellularized (dHAM) in order to eliminate the allogeneic immunization but maintain its bio-function. It has been investigated to possess the capability of promoting wound healing with rabbit as the model animal^[12]. In this work, both dHAM and egg shell membrane (EGM) were used to do *in vivo* test and to compare the

efficiency in full-thickness skin repairing. With the Sprague-Dawley (SD) male rats as the experimental animals, full-thickness skin defects were made on the back. dHAM and EGM were separately covered on the wound. The wound healing progress was followed in 21 d. The results would be important and worthwhile in providing information about their application in clinic treatment for wound repairing in future.

1 Materials and methods

1.1 Decellularization of HAM

HAM was peeled from placenta by mechanical treatment after blood on the fresh placenta was washed away using phosphate buffered saline (PBS). The clean HAM was washed by 70% alcohol/water for 2 times and then was immersed in PBS which contains penicillin and streptomycin ($200 \text{ U} \cdot \text{L}^{-1}$), shaking for 2 d, and replacing the PBS every 12 h. Finally, treatments with chemical in sequence including 1% Triton X-100 PBS for 4 h, lipase/PBS ($2\,000 \text{ U} \cdot \text{L}^{-1}$) for 10 h and DNAase/PBS ($2\,000 \text{ U} \cdot \text{L}^{-1}$) for 4 h at 37°C ^[12]. After the decellularization, the obtained dHAM was cut into pieces at $2.0 \text{ cm} \times 2.0 \text{ cm}$ and stored at 4°C for the surgery operation.

1.2 The preparation of eggshell membrane

Egg shell with the membrane were separated from chicken eggs (50-70 g), rinsed with water and then 75% alcohol/water. The shell membrane was carefully peeled off with tweezers which were prior disinfected by 75% alcohol/water. The membrane was washed in 75% alcohol/water for 2 times and rinsed in PBS containing penicillin and streptomycin ($200 \text{ U} \cdot \text{mL}^{-1}$) for 2 times. After the membranes were cleaned totally, they were dried naturally at room temperature and stored at 4°C for the following experiment.

Both dHAM and EGM were embedded in optimum cutting temperature compound (OCT, Sakura, America), and sectioned at $4 \mu\text{m}$ on a Microtome. The sample sections were stained with hematoxylin-eosin (H&E) dye and observed under microscope in order to verify

the decellularization and membrane texture (Fig. 1).

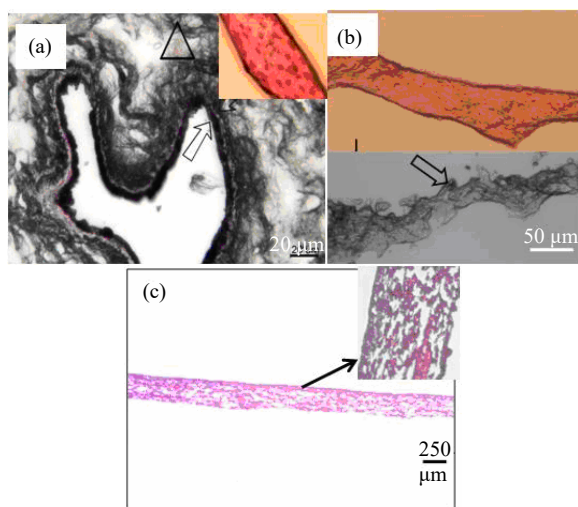


Fig. 1 H&E staining to show the accellular HAM morphology (a), comparing with untreated HAM^[12] (b), and egg shell membrane (c)

1.3 Full-thickness skin defects and the wound treatments

Twenty male rats (3-4 months, 250-400 g) were divided into 2 groups with 10 animals for each group ($n=10$). They were anesthetized with 3% pentobarbital sodium solution (intra-peritoneal injection, $30 \text{ mg} \cdot \text{kg}^{-1}$ animal). The furs were shaved off; the nude skin was disinfected with Iodophor. Two full-thickness defects (square, $1.7 \text{ cm} \times 1.7 \text{ cm}$) on two sides of the rats' back were symmetrically made using surgical scissors, the same size of which were drawn beforehand on the skin.

dHAM or EGM ($2.0 \text{ cm} \times 2.0 \text{ cm}$ for each piece) were used to cover completely the wound, followed by package with medical gauze finally. The rats with same wounds (the opposite) but only covered with gauze were used as the control. The membranes were used once. The rats were fed normally and kept in cages individually. Each rat was given penicillin ($160\,000 \text{ U} \cdot \text{mL}^{-1}$) through intra-peritoneal injection once a day at the initial 3 d post operation. The gauze was removed 2 d later. The wounds were then disinfected with iodine within the first week, once for each day.

For the measurement of white blood cells post-operation, another 12 male rats were used, with two wounds but the same treatments on each rat.

1.4 Appearance of wound

The wound healing was followed. The appearance was observed, for example, the color at wound site, bleeding, infiltration fluid, granulation tissue growth, scab and scar formation etc. The wound size was recorded every day. The wound healing rate was calculated as $(\text{initial wound area} - \text{wound area at some day}) / \text{initial wound area} \times 100\%$.

1.5 Measurement of white blood cell

White Blood Cell (WBC) level of three animal groups (dHAM, EGM and the control group), with 2 wounds on each rat and 4 rats for each group, were detected by hemocyte analyzer (Boehringer mannheim BM830, China). Blood (0.5 mL) was collected from caudal vein at 1, 3, 5, 7, 9, 11 and 17 d. The data at the operation day was recorded as the basic WBC level (1 d, N_0). The all WBC data at the testing day (N_1) shall be deducted by this basic value of the same rat, which was used to calculate the WBC alteration. That is, the increased percentage of $\text{WBC} = (N_1 - N_0) / N_0 \times 100\%$. This value was averaged from 8 wounds (on 4 rats) and expressed as mean $\pm$ standard deviation.

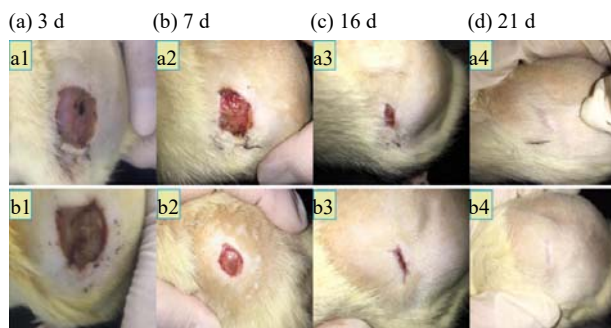
2 Results and discussions

As literatures [13-14] reported, the progress of wound healing is complex, continuous and dynamic. The whole regeneration of defected skin usually includes three phases, i.e. inflammation, proliferation and remodeling, in which various cells, cytokines and growth factors work together and interact with each other to promote skin's proliferation and remodeling. Surely, the better repairing at the preliminary stage would greatly help the skin's healing in later period. Thus, we conducted the animal experiment with our materials to investigate the wound repairing from appearance, inflammation and healing rate.

2.1 Appearance of wound region

The wounds treated with EGM started to shrink since the 3rd day, when some of the white egg membrane left and was observed. The wound exhibited

clear with no pus formation and no bleeding. Some scabs formed. At day 7, the scab fell off; the wound was clear and became smaller, much smaller than that of the control rat (Fig. 2, a2 & b2). There was still bloody secretion and slight edema on the control rat (a2). At day 21, the wound appeared cured; the scar was not seriously obvious (Fig. 2, b4). In the control animals, the wound area became smaller with the time passing (Table 1). It almost cured at day 21 with small scar formation, though the healing rate was smaller than that of the EGM group.



a: Control; b: EGM treatment.

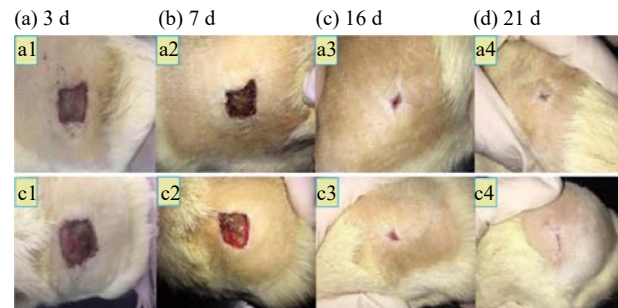
Fig. 2 Appearance of wound treated with EGM

Table 1 Wound area ($\bar{x} \pm s$) and healing rate of EGM group

Time/ d	EGM group		Control group	
	Wound area/ cm ²	Wound healing rate/%	Wound area/ cm ²	Wound healing rate/%
0	2.256±0.024		2.354±0.048	
1	2.178±0.052	3.46±2.30	2.242±0.095	4.76±4.04
3	1.832±0.035	18.79±1.55	2.083±0.037	11.51±1.57
5	1.817±0.029	19.46±1.29	1.916±0.034	18.61±1.44
7	1.615±0.024	28.41±1.06	1.747±0.041	25.78±1.74
9	1.297±0.038	42.51±1.68	1.384±0.038	41.21±1.61
11	0.705±0.045	68.75±1.99	0.847±0.048	64.02±2.04
13	0.302±0.038	86.61±1.68	0.472±0.086	79.95±3.65
15	0.194±0.072	91.40±3.19	0.206±0.053	91.25±2.25
17	0.037±0.049	98.36±2.17	0.131±0.057	94.43±2.42
21	0.013±0.009	99.42±0.40	0.063±0.037	97.32±1.57

In the case of dHAM treatment group, it exhibited the similar tendency of wounds to that of EGM group (Fig. 3). The wound appeared better in dHAM group

than in the control group from wound area, contraction, exudation and scar formation. The wound area alteration with time also supported this result (Table 2).



a: Control; c: dHAM treatment.

Fig. 3 Appearance of wound treated with dHAM

Table 2 Wound area ($\bar{x} \pm s$) and healing rate of dHAM group

Time/ d	dHAM group		Control group	
	Wound area/ cm ²	Wound healing rate/%	Wound area/ cm ²	Wound healing rate/%
0	2.352±0.035		2.363±0.018	
1	2.386±0.023	-1.44±0.98	2.274±0.031	3.76±1.31
3	2.085±0.074	11.35±3.15	2.152±0.051	8.93±2.16
5	1.874±0.042	20.32±1.79	1.956±0.048	17.22±2.03
7	1.538±0.028	34.61±1.19	1.795±0.063	24.04±2.67
9	1.168±0.036	50.34±1.53	1.238±0.031	47.61±1.31
11	0.689±0.034	70.70±1.45	0.715±0.045	69.74±1.90
13	0.367±0.046	85.39±1.96	0.397±0.036	83.20±1.52
15	0.147±0.058	93.75±2.47	0.179±0.046	90.43±1.95
17	0.073±0.025	96.89±1.06	0.096±0.038	94.93±1.61
21	0.025±0.013	98.94±0.55	0.052±0.037	97.39±1.57

With regard to the wound healing rate calculated from the wound area alteration, animals treated with EGM had higher rate than those with dHAM treatment in the early stage (Fig. 4), which was attributed to the anti-bacterial and anti-inflammatory nature from lysozyme in egg white. The less serious inflammation helped tissue to close the wound cut and to recover better from the injury. While in the middle stage, the rate of dHAM group was higher than EGM group, which might be attributed to the rich nutrients from HAM like proteins and growth factors beneficiary for the skin regeneration. Anyway, all animals recovered from the

skin wound at the late stage, after 13 d post-operation. Comparatively, the control animals recovered after 15 or 17 d (Table 1 & 2).

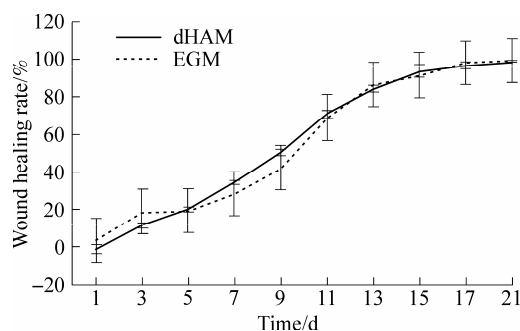


Fig. 4 Healing rate of dHAM and EGM

As can be seen, the wound healed better and the skin regenerated faster of both EGM and dHAM treatments than those of the control animals. However, the wound repairing tendency or the healing progress between EGM and dHAM treatment seemed of little difference from the wound appearance and the wound healing rate.

2.2 WBC level

WBC level is directly proportional to the wound inflammation in clinic. Thus, the vein blood of animals treated separately by dHAM and EGM were collected and analyzed, with the control group as the negative reference. Fig. 5 showed that the WBC values of both dHAM and EGM group increased after the surgery, reached the peak at day 9, and then decreased gradually to the initial level. In the case of the control group, the alteration tendency was the same, but the value was always higher than those of the dHAM and EGM groups during the test time, though the values of all animals returned to the similar level, namely the animal's basic level. This indicated that the inflammation on animals treated with dHAM or EGM was less serious than those of the control group. The less serious inflammation must be beneficiary for the skin's proliferation and remodeling in the later period. Therefore, the wound on animals of dHAM and EGM treatments healed faster and smoother than the control animals in the study interval. However, there was no significant divergence between the animals with either dHAM or EGM

treatment. Thus, we speculated that both dHAM and EGM were biocompatible materials.

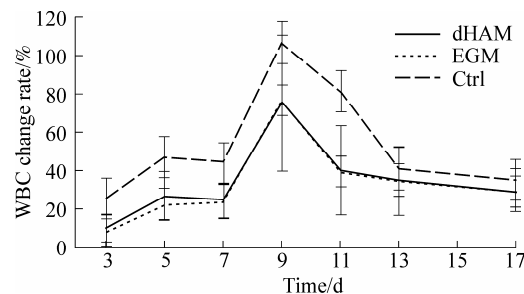


Fig. 5 WBC change rate in three group animals

3 Conclusions

The wound repairing progress was followed and analyzed with SD rats being used as the experimental animals and EGM or dHAM cover being applied for treatments. The results were compared correspondently with the control animals. The wound appearance, area size and WBC level were tested, and the wound repairing rate was calculated. These results showed that both EGM and dHAM promoted the wound repairing greatly during the whole testing period, 21 d in the animal model. The wounds recovered better than those of the control animals. However, the wound repairing tendency or the healing progress between EGM and dHAM treatment seemed of little difference from the present study. The egg membrane is easy to obtain with low cost. The process is also simple. Together with these experimental evidences, EGM would be a more promising wound dressing in clinic application. More detailed research will be conducted in the near future.

References:

- [1] Bi H, Jin Y. Current progress of skin tissue engineering: Seed cells, bioscaffolds, and construction strategies[J]. *Burns & Trauma*, 2013, 1(2):63-72.
- [2] Harder J, Schröder J M, Gläser R. The skin surface as antimicrobial barrier: Present concepts and future outlooks[J]. *Experimental Dermatology*, 2013, 22(1):1-5.
- [3] Hu D H, Wang Y C. Lay more stress on the study and application of covering materials for wounds[J]. *Chinese Journal of Burns*, 2012, 28(5):323-326.
- [4] Sheikh E S, Sheikh E S, Fetterolf D E. Use of dehydrated

- human amniotic membrane allografts to promote healing in patients with refractory non healing wounds[J]. International Wound Journal, 2014, 11(6):711-717.
- [5] Yang J Y, Chuang S S, Yang W G, et al. Egg membranes as a new biological dressing in split-thickness skin graft donor sites: A preliminary clinical evaluation[J]. Chang Gung Medical Journal, 2003, 26(3):153-159.
- [6] Nakano T, Ikawa N I, Ozimek L. Chemical composition of chicken eggshell and shell membranes[J]. Poultry Science, 2003, 82(3):510-514.
- [7] Boyce S T, Lalley A L. Tissue engineering of skin and regenerative medicine for wound care[J]. Burns Trauma, 2018, 6(4):1-10.
- [8] Kunjalukkal S P, Salvatore L, Gervaso F, et al. Synthesis and characterization of collagen scaffolds reinforced by eggshell derived hydroxyapatite for tissue engineering[J]. Journal of Nanoscience and Nanotechnology, 2015, 15(1): 504-509.
- [9] Cordeiro C M, Esmaili H, Anash G, et al. Ovocalyxin-36 is a pattern recognition protein in chicken eggshell membranes[J/OL]. PLoS One, 2013, 8(12):e84112 [2018-03-21]. <https://doi.org/10.1371/journal.pone.0084112>.
- [10] Arias J L, Fernandez M S, Dennis J E, et al. Collagens of the chicken eggshell membranes[J]. Connective Tissue Research, 1991, 26(1/2):37-45.
- [11] Russo A, Bonci P, Bonci P. The effects of different preservation processes on the total protein and growth factor content in a new biological product developed from human amniotic membrane[J]. Cell and Tissue Banking, 2012, 13(2):353-361.
- [12] Shi P, Gao M, Shen Q, et al. Biocompatible surgical meshes based on decellularized human amniotic membrane [J]. Materials Science and Engineering: C, 2015, 54:112-118.
- [13] Gambichler T, Pljakic A, Schmitz L. Recent advances in clinical application of optical coherence tomography of human skin[J]. Clinical, Cosmetic and Investigational Dermatology, 2015, 8:345-354.
- [14] Pereira R F, Barrias C C, Granja P L. Advanced biofabrication strategies for skin regeneration and repair [J]. Nanomedicine, 2013, 8:603-621.

两种材料修复全层皮肤缺损的初期动物实验研究

竺亚斌¹, 方佳喜¹, 朱成涛¹, 沈美婷¹, 周爱娜¹, 周密¹, 侯瑞霞¹, 葛纷纷²

(1. 宁波大学 医学院, 浙江 宁波 315211; 2. 宁波市医疗中心李惠利东部医院, 浙江 宁波 315000)

摘要: 鸡蛋膜(EGM)是一种天然的生物材料, 被作为烧伤或损伤皮肤修复的敷料或绷带进行研究. 经脱细胞处理后的人羊膜(dHAM)对皮肤组织的再生具有很好的促进作用. 考虑到羊膜和鸡蛋膜二种材料的相似性, 将羊膜和鸡蛋膜在对皮肤伤口修复的作用方面进行检测和对比. 以 SD 雄性大鼠为实验动物, 在动物背上做好全层皮肤缺损的模型, 然后对伤口分别进行消毒、EGM 或者 dHAM 覆盖, 最后再覆盖上医用纱布, 同时, 以同样的伤口但只作医用纱布覆盖的动物作为对照, 在伤口外观、尺寸随时间变化及血液白细胞检测等方面进行比较分析, 发现与对照组相比, 分别用 EGM 和 dHAM 处理的动物伤口尺寸变小更快、动物体内白细胞(WBC)值更小、恢复到基础值也更快. 因此得出结论: 在 21 d 的实验周期内, 大鼠缺损的皮肤都基本得到修复, 羊膜和鸡蛋膜处理的伤口修复显著优于对照组动物, 但 EGM 和 dHAM 两者之间未发现显著差异. 这些信息对于深入研究皮肤再生具有重要的参考价值.

关键词: 鸡蛋膜; 脱细胞人羊膜; 伤口修复; 生物材料; 皮肤再生

(责任编辑 韩 超)