Folia Medica Indonesiana Vol. 47 No. 2 April – June 2011 : 137-142

EFFECT OF TOPICAL HYALURONATE AND FREEZE-DRIED AMNION MEMBRANE ADMINISTRATION ON CK 16 PROTEIN EXPRESSION AND THE NUMBER OF EPITHELIAL LAYERS IN SUPERFICIAL WOUND OF MALE WISTAR STRAIN RATS

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ABSTRAK

Reaksi penyembuhan luka merupakan proses dinamis, melibatkan sejumlah mediator, sel darah, matriks ekstra seluler dan sel parenkim. Pada luka epidermal, cytokeratin (CK) 16 berperan mendorong reorganisasi susunan filamen keratin pada saat sebelum migrasi keratinosit ke daerah luka. Hyaluronat berfungsi dalam morfogenesis jaringan, migrasi, diferensiasi serta adesi sel. Penelitan ini bertujuan menjelaskan peran Hyaluronat LMW pada berbagai jumlah lapisan jaringan epidermal dan ekspresi protein CK 16 luka superfisial. Sebanyak 32 ekor tikus jantan galur Wistar dibuat luka superfisial sayatan eksisional pada punggung, kemudian dibagi menjadi kelompok kontrol dan perlakuan. Pada kelompok kontrol diberi terapi membran amnion freeze-dried, sedang kelompok perlakuan diberi hyaluronat LMW 1% dan membran amnion freeze-dried. Kedua kelompok kemudian dibagi menjadi 2 sub kelompok, terdiri dari 8 ekor tikus yang akan dilakukan pengorbanan pada hari ke 3 dan 7 setelah pembuatan luka. Evaluasi histopatologi dilakukan dengan mengukur jumlah lapisan sel epitel dan ekspresi protein CK 16. Hasil menunjukkan terdapat perbedaan jumlah lapisan sel epitel dan ekspresi protein CK 16 antara kelompok terapi membran amnion freeze-dried dengan yang diberi terapi hyaluronat LMW 1% dan membran amnion freeze-dried. Sebagai kesimpulan, pemakaian amnion freeze-dried dan hyaluronat LMW terbukti meningkatkan kecepatan penyembuhan yang ditandai dengan jumlah lapisan jaringan dan reorganisasi filamen cytokeratin 16 di daerah luka.

ABSTRACT

Wound healing is a dynamic process involving mediators, blood cells, parenchymal cells and extracellular matrix. Cytokeratin (CK) 16 in epidermal wound healing, could be to promote reorganization of the cytoplasmic array of keratin filaments, an event that precedes the onset of keratinocyte migration. The essential components of extracellular matrix is hyaluronic acid, which plays a predominant role in tissue morphogenesis, cell migration, differentiation, and adhesion. The aim of this study was to analyze the effects of Low Molecular Weight Hyaluronate on the total of epithelial layer and expression of CK 16 in wound healing. Superficial-thickness excisional wounds were created along the backs of 32 wistar rats. They were divided into 2 groups. One was treated by freeze-dried amnion and 1% Low Molecular Weight Hyaluronate and the other was treated by freeze-dried amnion only as control group. Each of the groups was divided into 2 sub groups. Each of the sub groups composed of 8 wistar rats based on the periode of termination : 3rd and 7th day after wounded. Histological evaluation was done to measure the total of epithelial layer and expression of CK 16. In conclusion, compound of freeze-dried amnion and low molecular weight hyaluronate improved wound healing and reepithelialization on superficial-thickness excisional wounds.

Keywords: *low molecular weight hyaluronate, wound healing, epithelial layer, CK 16*

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INTRODUCTION

Trauma to the skin is quite often encountered in life, both acute and chronic wounds, and can lead to complications of infection, necrosis, ulceration, and even sepsis that can lead to death (Singer & Dagum 2008). More than 1.25 million people suffered burns in the United States and 6.5 million chronic skin ulcers due to venous stasis, or diabetes mellitus (Singer et al. 1999). It is necessary to enable optimal handling of wound closure and aesthetic scar as soon as possible. Several kinds of superficial wound are known to use amniotic membrane (Saputro & Noer 2001, Talmi et al. 1990, Gruss et al. 1978, & Gajiwala Gajiwala, 2006, Singh et al. 2007). Davis first reported the use of fetal membranes for skin transplants in 1910 (Rafii et al. 2007).

Freeze-dried amniotic membrane is the amnion membrane preparations. It is widely used in wound treatment, which has been preserved in freeze-dried so that its use is practical and easy to be distributed without

requiring a specific storage medium and temperature, but the levels of growth factor show a significant decline (Wolbank et al. 2009, Koizumi et al. 2000, Thomasen et al. 2009, Sri Subekti et al. 2009, Ihsan 2009, Lin et al. 2009) so that the amniotic membrane is preserved only as a biological dressing only with the mechanical properties of evaporation that inhibits wound and barrier against bacterial pathogens, like synthetic polymer sheet or a transparent dressing (Pruitt & Levine, 1984, Kumar 2008, Padmani & Perdanakusuma 2008).

Hyaluronic acid is a glycosaminoglycan component of extracellular matrix that plays a role in wound healing process and is produced by fibroblast cells (Jenkins et al. 2005). Hyaluronic is found in large numbers on fresh amniotic membrane. Hyaluronic consists of two groups: High Molecular Weight Hyaluronate (hyaluronic HMW) and its degradation results the Low Molecular Weight Hyaluronate (hyaluronic LMW), where LMW is proven to stimulate angiogenesis, mitosis and cell migration of keratinocytes, fibroblasts and endothelial cells (Shay et al. 2009, Gomes et al. 2004, Hamann et al. 1995, Fraser et al. 1997, West & Fan 2001). LMW hyaluronic also proved to spur growth factor production by macrophages and the inflammatory response of wound healing process. Some research shows that LMW speeds up the process of hyaluronic epithelialization (West & Fan, 2001, King & Hickerson, 1991, Chung et al. 1999).

The evaluation process of epithelialization by immunohistochemical examination using antibodies cytokeratin (CK) 16 is induced if there is injury to the epithelium studded (squamous). CK 16 stimulates the reorganization of the arrangement of keratin filaments in the cytoplasm, which precedes the occurrence of keratinocyte migration towards the injured area (Paladini et al. 1996). Use of combined freeze-dried amniotic membrane and hyaluronic LMW on superficial skin wounds is expected to accelerate the wound healing process and epithelialization.

MATERIALS AND METHODS

White male Wistar rats aged 40-60 days, weight 200-300 grams, were divided into 2 groups, i.e. control and treatment groups by simple random sampling. In both groups tangential excision of superficial wounds was made. Excision performed until it was bleeding or shiny layers of the dermis were exposed. The wound was observed on day 3 and 7 after excision by the histopathologist to determine cell proliferation of keratinocytes and epithelialization stages.

In control, wounds were closed with preserved amniotic membrane, while in treatment group the wound was smeared with a solution of low molecular weight hyaluronate 1%, and then covered with the amnion. The wound was closed with a thick gauze fixed with 4.0 silk sutures on the backs of mice. Histopathologic observation performed on days 3 and 7 at the expense of the mice decapitation.

The parameters tested were the number of layers of epithelial cells and expression of CK 16 between the two groups on days 3 and 7. Examination of protein expression of cytokeratin 16 was done by counting the number of mouse skin epithelial cells that express CK 16 proteins based on the color brown in the cytoplasm around the scar tissue on a slide with immunohistochemical examination using a 40x (400x) objective magnification light microscopy. The number of epithelial cell layer was calculated by adding up the average cell layer that is formed from the stratum corneum to the basement up to 40x (400x) objective in 3 places each dosage at the right edge, left edge and the middle. Statistical test data were performed using Independent t-test when data were in normal distribution, with an error rate of 5% to determine the number of layers of epithelial cells and cytokeratin 16 protein expression between the two groups. The calculation result obtained was regarded significant if $p \leq 0.05$. However, if the data were not in a normal distribution, then they were tested with Mann-Whitney-Wilcoxon test. To test data normality we used Kolmogorov Smirnov test. Furthermore, the data obtained were presented in tabulated form and text as an explanation.

RESULTS

Protein expression of cytokeratin 16

Examination of protein expression of cytokeratin 16 was done by counting the number of mouse skin epithelial cells stained positive (brown color in the cytoplasm of cells) in the scar tissue on a slide with immunohistochemical examination using a 40x (400x) objective magnification light microscopy (Tables 1 and 2):

Table 1. Expression of cytokeratin 16 protein on day 3

Groups	N	*Cytokeratin 16* protein expression			
		Mean	SD	Min.	Max.
Control	8	151.13	15.887	-0.197	0.171
Treatment	8	241.13	33.694	-0.135	0.205

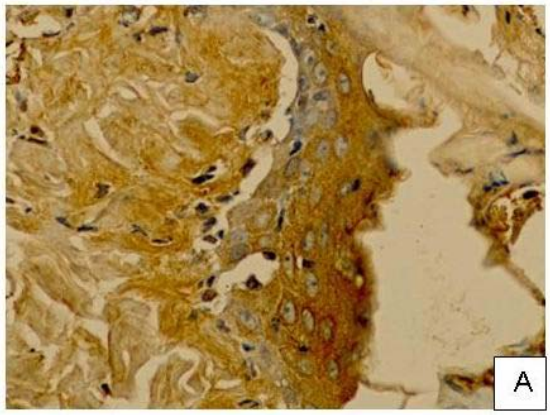
Table 2. Cytokeratin 16 protein expression at day-7

Groups	N	*Cytokeratin 16* protein expression			
		Mean	SD	Min.	Max
Control	8	490.50	22.716	-0.145	0.137
Treatment	8	757.88	33.008	-0.194	0.148

There were significant differences between the expression of cytokeratin 16 protein and treatment control group on days 3 and 7, as evidenced by the test of independent samples t-test of heterogeneous variance on day 3 and the test of independent samples t-test homogeneous variance on day 7 with obtained p-value = 0.000.

The number of layers of epithelial cells

Examination of the epithelial cell layer was done by calculating the average number of formed epithelial cell layer starting from the bottom up to the stratum corneum that was examined with 40× objective in three places each preparation, i.e. the right edge, left and center. The results of calculating the number of layers of epithelial cells is shown in Table 3 and 4. There were significant differences between the number of epithelial cells lining the control group and treatment group on days 3 and 7, as evidenced by the test of Independent samples t-test variance, revealing homogeneous p-value = 0.000 (on day 3) and p = 0.003 (on day 7).



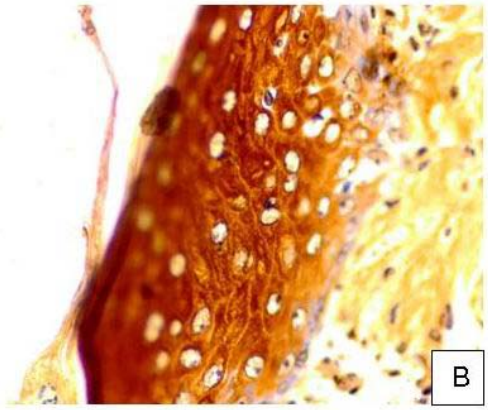
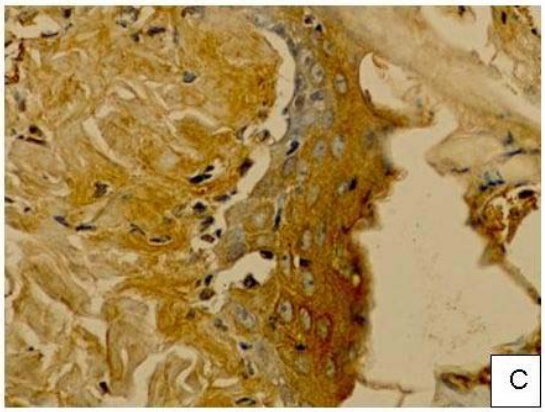


Figure A. Expression of CK 16 positive proteins in the cytoplasm of some keratinocytes on day 3 in control groups (Immunohistochemistry, light microscope 40x objective).

Figure B. Expression of CK 16 positive protein is more in keratinocyte cytoplasm in 3 treatment groups (Immunohistochemistry, light microscope 40x objective).



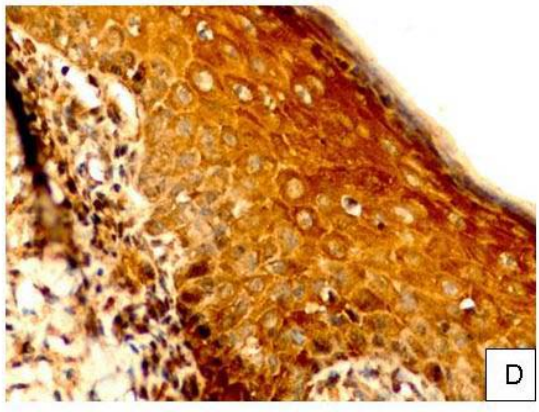


Figure C. Expression of positive CK 16 proteins in the cytoplasm of some keratinocytes in control group on day-7 (Immunohistochemistry, light microscope 40x objective).

Figure D. Expression of positive CK 16 protein in almost all the cell cytoplasm of keratinocytes in treatment group on day 7 (Immunohistochemistry, light microscope 40x objective).

Table 3. The number of layers of epithelial cells on day 3

Groups	N	Number of epithelial cell layers			
		Mean	SD	Min.	Max.
Control	8	3.50	0.535	-0.325	0.325
Treatment	8	5.00	0.756	-0.250	0.250

Table 4. The number of layers of epithelial cells on day 7

Groups	N	Number of epithelial cell layers			
		Mean	SD	Min.	Max.
Control	8	8.13	1.356	-0.203	0.287
Treatment	8	10.13	0.835	-0.228	0.185

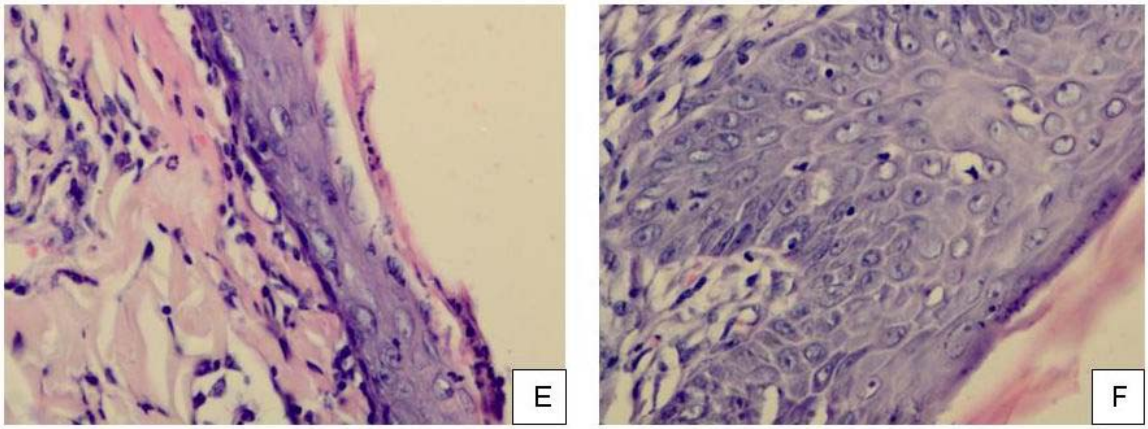


Figure E. The number of epithelial cells lining the control group on day 3 (Hematoxylin eosin, light microscope 40x objective)

Figure F. The number of epithelial cells lining the treatment group (more) on day 3 (Hematoxylin eosin, light microscope 40x objective)

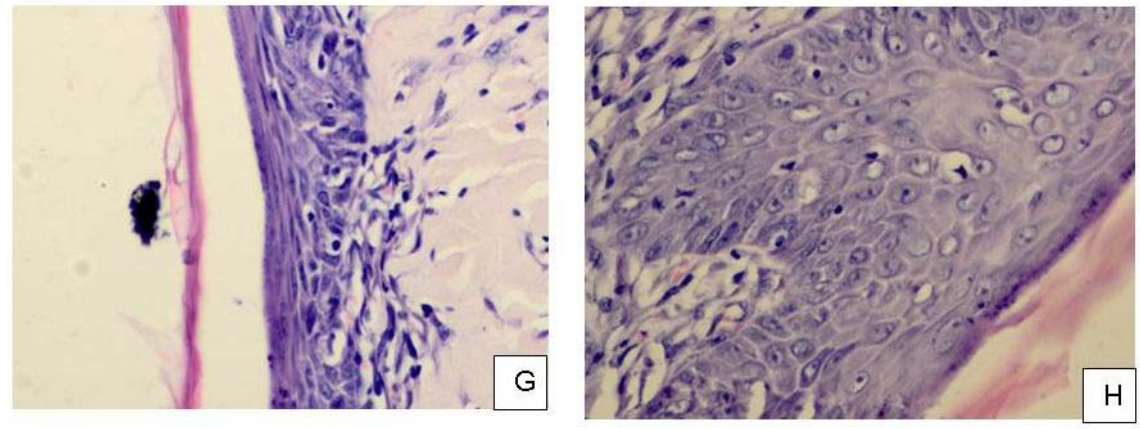


Figure G. The number of epithelial cells lining in control group on day 7 (Hematoxylin eosin, light microscope 40x objective)

Figure H. The number of epithelial cells lining in treatment group (more) on day 7 (Hematoxylin eosin, light microscope 40x objective)

DISCUSSION

Protein expression of cytokeratin 16 keratinocyte cells formed on day 3 and 7 was more remarkable in the treated group, with p = 0.000. The study showed that the protein cytokeratin 16 epithelialization had a role in the activation process by stimulating the reorganization of cytokeratin 16 filaments in the cytoplasm of keratinocytes, which was characterized by an increase in mitosis, cell hypertrophy and increased protein expression of CK 16 in the cytoplasm (Paladini et al. 1996, Takahashi et al . 1994).

In this study there were significant differences between the number of epithelial cells lining in control and treatment groups on day 3 (p = 0.000) and day 7 (p = 0.003). This suggests the hyaluronic role in epidermal wound healing. Many scientific papers explain that hyaluronic is controlling epidermal response to injury through the process of migration, proliferation and differentiation of keratinocytes in a variety of wound healing (Maytin et al. 2004). Maytin obtained important discoveries about the role of hyaluronic as an active regulator of many dynamic cellular processes. Hyaluronic is a component of extracellular matrix and plays a role in the process of migration, proliferation and cellular differentiation. The epidermis contains hyaluronic quite much as the matrix between keratinocytes, especially in the stratum spinosum, basal and corneum, thus plays an important role in cell migration and proliferation. Hyaluronic increases keratinocyte proliferation in response to epidermal injury. Furthermore Maytin concluded that hyaluronic plays an important role in the process of keratinocyte differentiation and wound healing (Maytin et al. 2004), thereby demonstrating that the role of hyaluronic were added to the treatment group, spurring migration, proliferation and differentiation of keratinocytes in the wound healing process (Maytin et al. 2004).

CONCLUSION

Use of combined amniotic and freeze-dried hyaluronic LMW on superficial wound healing improves speed and epithelialization characterized by the number of epithelial layers and the reorganization of cytokeratin 16 filaments in the cytoplasm.

ACKNOWLEDGMENT

The author would like to thank Prof. Dr. H. Sarmanu, drh., MS., As a statistical consultant and Hj. Meianti, dr., in data collecting and processing in this study.

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