LAPORAN PENELITIAN

The Effect of ADSC-CM on Wound Healing in Sprague-Dawley Rat

Oleh:
dr. Twidy Tarcisia, M.Biomed.
dr. Marcella Rumawas, M.Sc., Ph.D.

FAKULTAS KEDOKTERAN
UNIVERSITAS TARUMANAGARA
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Abstrak

Latar belakang: Penyembuhan luka terdiri dari fase hemostasis, inflamasi, proliferasi dan remodeling yang berjalan saling tumpang tindih. Dua proses utama dalam penyembuhan luka kulit adalah epitelisasi dan kontraksi luka. Epitelisasi dievaluasi melalui jumlah lapisan epidermis dan ketebalan jaringan epidermis baru. Kontraksi luka dievaluasi dengan penilaian secara matematika menggunakan rumus *Wound Contraction Index* (WCI). Beberapa penelitian menunjukkan bahwa pemberian ADSC-CM dapat meningkatkan stimulasi keratinosit dan fibroblas yang terjadi pada proses epitelisasi dan kontraksi luka. Mengingat pentingnya peran ADSC-CM, penelitian ini dilakukan untuk mengetahui efek ADSC-CM pada penyembuhan luka melalui parameter histologi epitelisasi dan kontraksi luka.

Metode: *Full tickness wound* dibuat pada 18 tikus Sprague-Dawley dan terbagi menjadi luka baluran ADSC-CM, luka kontrol negatif, luka kontrol medium kultur dan luka kontrol pelarut. Tikus dikorbankan pada hari ke-3, 7 dan 14. Luka kemudian dievaluasi dengan parameter histologi menggunakan *Image Raster software*.

Hasil: Luka baluran ADSC-CM memiliki lapisan epidermis terbanyak dibanding luka lain dan terdapat perbedaan bermakna secara statistik pada parameter ketebalan epidermal (dalam μm) pada hari ke-14 (p = 0,04).

Kesimpulan: ADSC-CM memiliki potensi signifikan dalam proses penyembuhan luka melalui parameter histologi epitelisasi dan kontraksi luka

Abstract

Back ground: Wound healing consists of four interrelated phases, namely hemostasis, inflammation, proliferation and remodeling. Two major processes in wound healing are epithelialization and wound contractions, which stimulates keratinocytes and fibroblast. Epithelialization is evaluated through thickness and multiple layers of new epidermal tissue, while wound contractions are evaluated mathematically by the Wound Contraction Index (WCI) formula. A body of literature suggests the potential role of ADSC-CM in stimulating keratinocytes and fibroblasts. However, the potential mechanism of ADSC-CM on wound healing remains unclear. We examined the effects of ADSC-CM on wound healing through histological parameters of epithelialization and wound contraction.

Methods: Full tickness wound was made in 18 Sprague-Dawley rats and divided into ADSC-CM wound, negative controls wound, control of culture medium wound and solvent control wound. Rats were sacrificed on day 3,7 and 14. Wounds were evaluated by histological parameters using Image Raster software.

Result: ADSC-CM treated wound had the highest multiple layer of epidermis compare to others wounds and there were statistical significant difference on the epidermal thickness (in μm) on day 14 (p = 0,04).

Conclusion: ADSC-CM has a significant potency on wound healing through histological parameters of epithelialization and wound contraction.

Background

Wound healing is a series of cellular and molecular mechanisms to regenerate damaged tissue.[1,2] Wound healing is necessary to sustain life. Many studies have been conducted to explain the mechanisms of wound healing.[1,3] Wound healing consist of 4 simultaneous phases: hemostasis, inflammation, proliferation and remodeling. Platelet activation initiates hemostasis phase at the beginning of wound healing. The inflammatory phase occurs at the first 24 hours until the 48 hours of injury. In this phase, inflammatory cells will produce various mediators to stimulate the inflammatory process and initiate the proliferation phase.[4] Proliferation phase is composed of angiogenesis, fibroplasia, epithelialization and wound contraction. The final phase of wound healing is remodeling that regulates the balance between synthesis and apoptosis cells. [4,5]

Two major processes in skin wound healing are epithelialization and wound contraction. These processes construct a new intact epidermal tissue and reduce wound size.[2,6] After injury, keratinocyte and fibroblast migrate from wound edge to begin epithelialization and wound contraction. Keratinocyte begin to migrate, proliferate and differentiate to restore epidermal tissue, which called epithelialization. Meanwhile, fibroblast will migrate and transform into myofibroblasts to conduct wound contraction. Eventually, myofibroblasts will disappear through the process of apoptosis, and ultimately will form a scar tissue, indicating the epithelialization process is completed.[5,7,8,9,10] Epithelialization can be assessed based on the thickness of new epidermal tissue. In this study, epidermal thickness was evaluated according to the numbers of layers and the size in μm

epidermal tissue, while wound contractions was measured by mathematical assessment based on the Wound Contraction Index (WCI). [10,11,12,13,14] Both processes of epithelialization and wound contraction can be used as parameters for optimal wound healing.

Adipose stem cell conditioned media (ADSC-CM) has been reported as an alternative therapy due to tissue repair factors.[1,15,16,17,18] The role of ADSC-CM in wound healing has been investigated by Hu et al. with the results of an increased in keratinocytes and fibroblast migration with ADSC-CM stimulation.[19] Furthermore, a study by Yuan et al. reported that DSC-CM also stimulated fibroblasts in the early phase of wound healing and simultaneously increased the apoptosis of fibroblast as a negative feedback mechanism in the final phase.[20] Considering the important role of ADSC-CM in wound healing, we studied the effects of ADSC-CM on skin wound healing in rat through histological parameters of epithelialization and wound contraction.

Methods

This experimental study included 18 Sprague-Dawley rat. Ethical approval for experimental animals was obtained from the Ethics Committee of the School of Medicine, University of Indonesia (No. 142 / UN2. F1 / ETIK / 2015). The research methods comprised: ADSC-CM preparation, rat injury, wound dressing, tissue processing and HE coloring.[21] Rat skin wounds (full thickness wound) were treated with either ADSC-CM (100%), complete culture medium, basal medium or without treatment (negative control).

Histological evaluation was conducted by using Image Raster software, and the evaluation was subsequently documented by using the Optilab Advanced Plus software. Epithelialization was assessed on days 3, 7 and 14 by counting the number of epithelial layers in newly formed epidermal tissue and measuring the thickness of the new epidermis in μm.[10,11,12,13,14] On days 3, 7 and 14, wound contractions were calculated mathematically according to the wound contraction index (WCI), as the sum of SCI (superficial contraction index) and DCI (deep contraction index). SCI was obtained from (L-S) / L, while DCI was obtained from (N-D) / N, with results varying from 0 and 1.[12]

We used SPSS v22 statistical software to analyze the data. The parametric tests of ANOVA and Chi-square had been applied, or alternatively the Kruskal-Wallis non-parametric test when the data failed to meet the parametric test requirements even after transformation. Statistical significance was defined at the level of $p < 0,05$.

Result

Eighteen rats were injured on the first day of experiment. One rat was death after day-1 injured, and excluded from the next follow-up. **Figure 1** showed how to evaluate the parameters of epithelialization on the follow up days, which were the thickness in μm (the white line) and the numbers of epithelial layer (green dots). The thickest epidermal measurement on day 3 and 14 were observed in the control-wound group (median: 7,27 μm on day 3 and 70,47 μm on day 14, respectively), whereas the thickest in μm on day 7 was found in the ADSC-CM treated wound group (median: 98,36 μm) (**figure 2**). Furthermore, for the new epidermal layer,

ADSC-CM treated wound attained the thickest layer, which indicated by the widest range of epidermal layers on day 7 and day 14 (7-19 and 7-12 layers, respectively) (**table 1**). However, statistical significant difference was found only on day 14, which was for the parameter of epidermal thicknes in µm (p = 0,04).

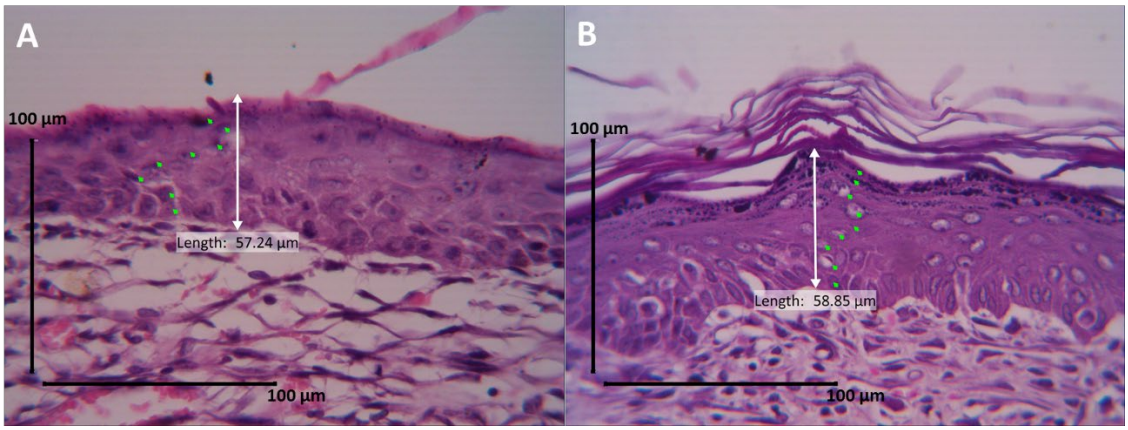


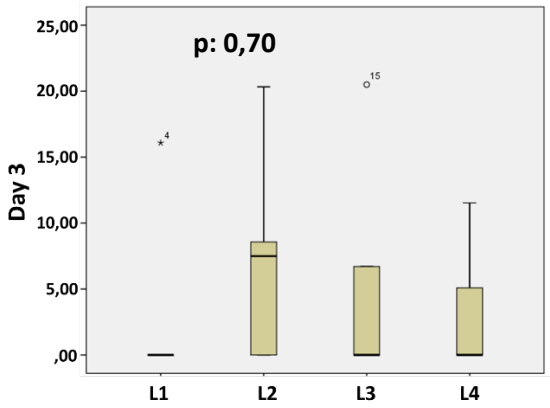
Figure 1. epithelialization parameters with HE staining (400 times magnification) (A) Day 7 (B) Day 14.

Caption: The white line described the measurement of epitheliazation in µm and the green dot illustrated the multiple layers of new epidermal.

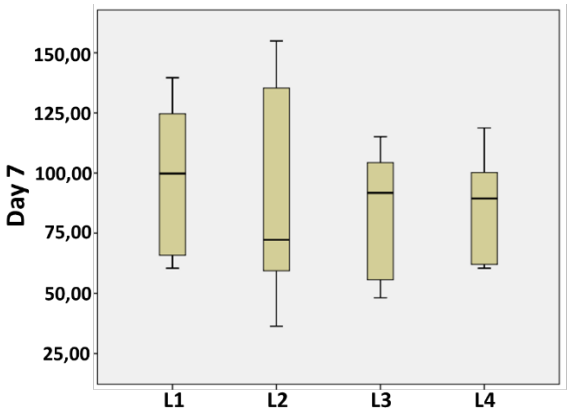
Table 1.Range of epidermal layer in rat skin wound preparation.

Wound	L1	L2	L3	L4	p
Day 3	0-3	0-4	0-4	0-3	0,68
Day 7	7-19	6-15	7-15	8-13	0,52
Day 14	7-12	7-11	7-10	7-11	0,13

*L1: ADSC-CM treated wound; L2: control wound; L3: culture medium treated wound; L4: basal medium treated wounds



p: 0,65



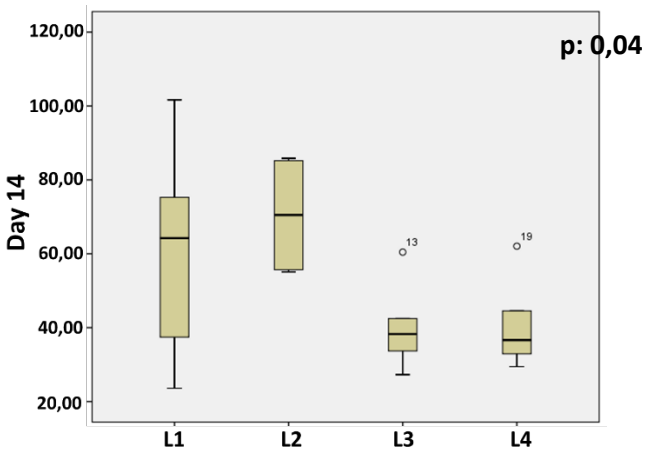


Figure 2. Epidermal thickness in µm on days 3, 7 and 14.

Caption: L1: ADSC-CM-treated wound; L2: control wound; L3: Culture medium treated wound; L4: Basal medium treated wound

Wound contraction was calculated by WCI on day 3, 7 and 14. As for example, on **figure 5** we showed the calculation of WCI on day 7 as a function of SCI (purple arrow and green curve) and DCI (white and black arrows). On day 3, 7 and 14, the groups of ADSC-CM wound and the basal medium treated wound indicated a consistent increment in WCI in contrast to the culture medium treated wound group (figure 4). The control wound group seemed to have the highest WCI on day 3 and 7, however, this events started to decrease on day 14. In spite of these findings, there were no statistical significant difference on WCI within the four groups ($p \geq 0{,}05$)

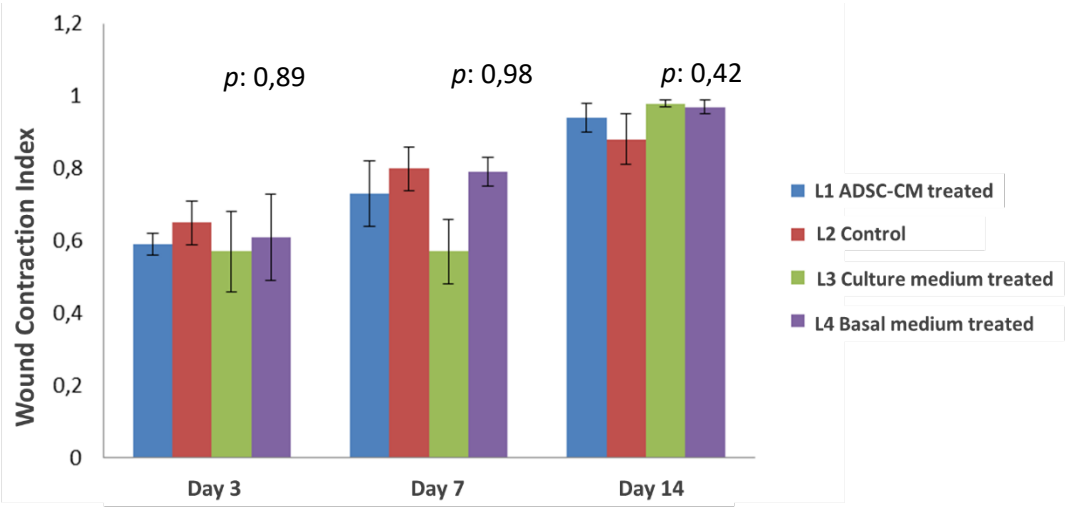


Figure 4. Wound contraction index in four groups on day 3,7 and 14

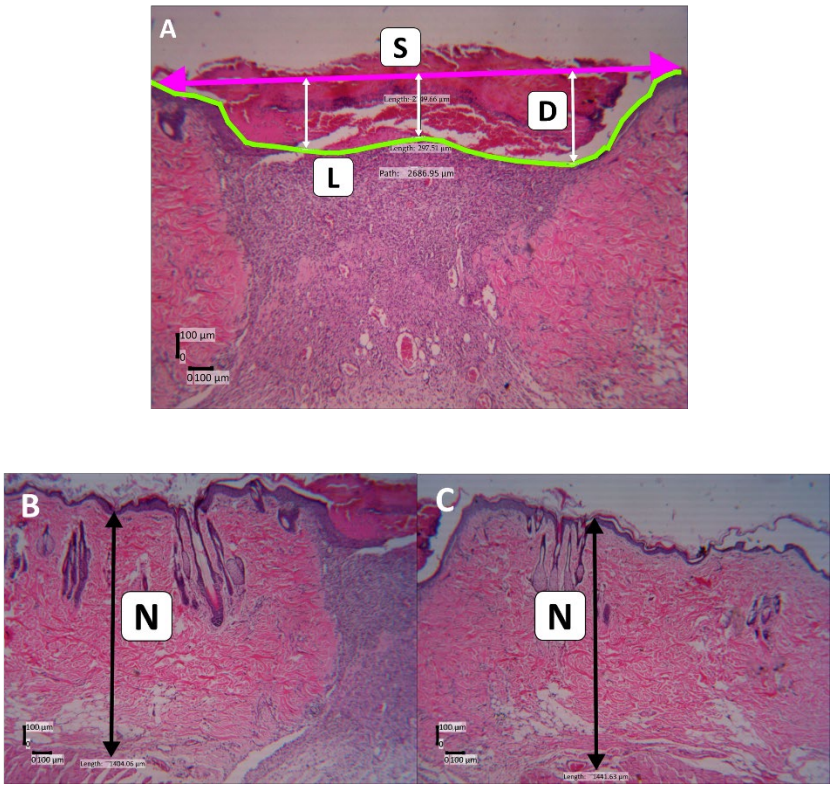


Figure 5. Day 7 WCI with HE staining (40 times magnification). (A) Preparation of wound gaps; (B) Preparation of the edge of the left wound; (C) Preparation of the right wound edge. Caption : purple arrow shows the distance between the two edges of the wound (S), the white arrow shows the depth of the wound (D), the black arrow shows the normal dermis thickness (N), the green line shows the length of the epithelial area (L), the light blue arrow shows the measurement limit . The distance between the edges of the wound shows the sum of the sum of the purple arrows in the two images; wound depth shows the deepest black arrow from both images; the length of the epithelialization area is calculated by adding up the length of the green line in both images.

Discussion

There was significant difference between the ADSC-CM treated wound and the other wounds in epidermal measurement on day 14. Furthermore, the ADSC-CM treated wound showed the thickest layer of epidermal as compared to that in the other groups, which indicated a potential wound healing stimulation through the epithelial process.

Previous studies suggested that ADSC-CM comprised various growth factors and cytokines such as VEGF, EGF, KGF, PDGF, bFGF and TGF β to stimulate wound healing process. EGF, as the main regulator of epithelialization, was discovered to stimulate migration and proliferation of epidermal cell.[18,22,23,24] Epidermal thickness was first seen on the 3rd day of injury, increased until the 7th day of injury and finally decreased on day 14. In our study, the thickest layer of epidermal was found in the ADSC-CM group (**table 1**). Despite this latter finding, there was a conflicting result regarding to the two indicators of epidermal thickness, i.e the numbers of epidermal layers and measurement of epidermal in μm. At day 14, the greatest measurement in μm was found in the control group, while the thickest layer of epidermal was shown in the ADSC-CM group (**figure 6**). In the control group, we observed that although the layers were fewer, the cells were bigger in size, implying a hypertrophy. Consistent with the previous findings, we suspected that the keratinocytes in control group might still in active proliferation stage.[25] Thus, unlike the other groups, this indicated that the control group has not yet completed the epithelialization process. Nonetheless, further studies are warrant to confirm this potential inference.

Wound contraction, as measured by Wound Contraction Index (WCI) is an indicator of wound healing.[12] Wound contraction is mainly supported by myofibroblasts. i.e.an activated fibroblast that requires α-SM actin expression and are essentially affected by TGF β.[5,7,8,9] Since ADSC-CM was reported to comprise several growth factors including TGF β, a number of research has been done to explore the effect of ADSC-CM in fibroblast, especially in myofibroblast. Early studies demostrated that ADSC-CM application in both invitro and invivo experiments were able to stimulate migration, proliferation and contractility of fibroblast.[18,19,25,26] Eryani et.al revealed that a repeated usage of topical ADSC-CM was capable to promote the acceleration of wound clossure.[26] Despite the statistically significant results regarding the effect of ADSC-CM treatment compared to its counterparts that was observed in this study, this difference was rather marginal. A single application of ADSC-CM on the skin wounds and/or the sample size could be the explanation of this trivial trend.

Clinically, ADSC-CM potentially escalated the wound healing process through epithelialization and wound contraction. This phenomenon emerged from the thickest layer of new epidermal, and independent from the measurement of epidermal. Thus, WCI analysis in our study showed a variety of results between the wound groups (**figure 4**). When compared to the control and culture medium groups, both ADSC-CM and basal medium groups demontrated an accelerated WCI within increasing days. Furthermore, reduction of WCI in the control group at day 14 and the culture medium group at day 7 might indicate the present of a stressor

that could significantly reduce the wound healing process.[27] Additional experiments are needed to investigate the prospective influence of these factors.

In this study, significant difference in epidermal thickness parameter among the four compared groups was shown only on day 14. However, due to the lack of sampel size, we were not able to continue with the multiple comparison analysis in order to determine which group contributed specifically to the observed difference. Indeed, the significant trend found in this study was an essential affirmation with regard to the beneficial effect of ADSC-CM on wound healing.

In conclusion ADSC-CM implies a potential wound healing capacity, both statistically and clinically. Thus, further studies are remain essential to confirm the mechanism of ADSC-CM on wound healing.

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