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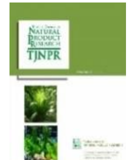

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Original Research Article

Effectiveness of Dental Pulp Stem Cells-Secretome on Superficial Burn Wounds

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ABSTRACT

Dental Pulp Stem Cells (DPSCs) represent a new therapeutic alternative for treating superficial burn wounds. This study aimed to evaluate the effectiveness of DPSC-derived secretome on superficial burn wounds in an animal model. The study employed a post-test-only controlled group design using 15 rats with superficial burns. Rats were divided into a negative control group of 0.9% NaCl (K1), a positive control group of moist-exposed burn ointment or MEBO (K2), and a treatment group of DPSC-secretome (K3). The effectiveness of the DPSC-secretome was assessed by wound area percentage and histopathological observations on days 5, 14, and 21. DPSC-secretome significantly reduced the burn wound area ($p<0.05$). Histology data showed that the DPSC-secretome promoted re-epithelialization, fibroblasts, and collagen formation. Polymorphonuclear cells were also reduced after 21 days, indicating that the DPSCs-secretome has an anti-inflammatory effect. In addition, the DPSC-secretome and MEBO showed equal effects on angiogenesis after 14 days. The DPSC-secretome was more effective than MEBO on superficial dermal burns. This study showed that the DPSC secretome has therapeutic potential for burn wounds.

Keywords: Stem cell, Secretome, Dental pulp, Superficial wounds

Introduction

Burns are a significant public health problem throughout the world. The high prevalence of cases and the risk of morbidity and mortality have caused significant impairment in appearance and function, resulting in loss of jobs and uncertainty about the future.[1] Southeast Asia has the highest incidence rate of burns. About 27% of burns cases lead to death. Research conducted by the Plastic Surgery Division of Sanglah General Hospital found that grade II burns are the most often treated.[2] Second-degree burns with a wound area of less than 20% are more often treated in general surgery.[3]

Second-degree burns affect the epidermis and dermis, causing redness of the skin, pain, blistering, tenderness, and paleness.[4] The superficial burns are the current classification of burn depth that emphasizes the possibility of spontaneous re-epithelialization in the context of re-epithelialization capacity.[5] Precise integration between various biological processes is required for efficient tissue repair.[6] These biological processes include various phases of homeostasis, inflammation, proliferation, and remodeling.[7]

Recently, molecular biology has led to an advancement in the medical field through the use of stem cells.[8] Secretomes are bioactive chemicals secreted by stem cells.[9] The secretome refers to the diverse collection of chemicals produced by stem cells, which consists of protein molecules, lipids, extracellular vesicles (exosomes, microvesicles, and apoptotic bodies), and nucleic acids (miRNA, mRNA, and lncRNA), which is involved in the regulation of physiological processes.[10]

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Current research has shown that secretome play a vital role in cell communication.[11] The secretome can be modulated by genetically modifying stem cells.

Vascular endothelial growth factor (VEGF), primary fibroblast growth factor (b-FGF), and antiapoptotic factors are present in the secretome and are essential for angiogenesis, wound healing, cell survival, and the prevention of tissue fibrosis and inflammation.[12]

Some studies have shown that Dental Pulp Stem Cells (DPSCs) can differentiate, regenerate, and proliferate faster than mesenchymal and adipocyte stem cells.[13] Recently, DPSCs have been a promising cell source for many applications in regenerative medicine, and are being investigated for tissue repair. Some studies have shown that stem cells are possible treatment alternative for acute and chronic wounds, however, there have been no reports on the DPSCs secretome in superficial burn wounds. Therefore, this study aimed to develop burn wound therapy using DPSCs secretome.

Materials and Methods

Animal model

This study employed a post-test-only control-group design. The research subjects were 15 male Wistar rats aged 2-3 months, weighing between 150-250 g. The rats were acclimatized to the laboratory condition of 12/12 h light/dark cycle at a consistent temperature of 23±2°C for one week. The rats were divided into three groups of five rats each. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Universitas Prima Indonesia, Medan, Indonesia, with approval reference No. 032/KEPK/UNPRI/VIII/2023.

Preparation of DPSCs

Dental pulp stem cells (DPSCs) were obtained from the Laboratorium Riset Terpadu Fakultas Kedokteran Gigi Universitas Gadjah Mada. DPSCs were harvested from the third molar of a 23-year-old female patient at Soedomo Hospital, stored in a refrigerator, thawed, and cultured in Petri dishes containing alpha-MEM medium, 20% fetal bovine serum, streptomycin, and penicillin fungizone for 4-6 weeks until the third cycle before being used for the experiment. Dental pulp stem cell cultures were stored in an incubator at 37°C and 5% CO_2.

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Isolation of DPSCs secretome

After the dental pulp stem cell culture reached 80-85% confluence, the cell density was determined using a microscope. The medium was then replaced with 10 mL of serum-free Dulbecco's Modified Eagle's medium (DMEM), and antibiotics were added (100 units/mL Penicillin G, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B). Stem cells were incubated in media for 48 h. Cultured cells within the conditioned media are defined as the secretome. After 48 h of incubation, the secretome was centrifuged at 440xg, at 4°C for 5 min. The supernatant was separated, centrifuged at 17,400 × g at 4°C for 3 min, and filtered. If it is to be stored, the medium is centrifuged at 1500 rpm for 5 min followed by 15,000 rpm for 1 min to remove debris and dead cells. A total of 5 mL of culture medium was mixed with 45 mL of absolute ethanol, incubated at -20°C for 1 h, and then centrifuged for 15 min at 15,000 rpm at 4°C to separate the supernatant. The precipitate was resuspended in cold 90% ethanol and centrifuged at 15,000 rpm and 4°C for 15 min. The resulting precipitate was then frozen at -80°C and stored in a refrigerator.[14]

Creation of superficial burn wounds and treatment

The rats were anesthetized by intraperitoneal administration of 0.1 mL/100-200 g of a mixture of 1 mL ketamine (10%) and 10 mL NaCl (0.9%). An iron measuring 2 cm × 2 cm was heated on a naked flame for 2 min and then placed on the middle back of the rat for 5 s. Then, the resultant burn was cleaned using 0.9% NaCl before being smeared with 0.1 mL NaCl 0.9% (K1), 0.5 g MEBO (K2), and 0.1 mL DPSCs secretome (K3).[15–17]

The treatments were performed twice daily (8 am and 5 pm) for 21 days. The wound area was observed regularly with a ruler and a digital camera on the 5th, 14th, and 21st day, followed by histopathological skin preparation and interpretation using an electron microscope.[18–20]

Histopathological and microscopic examination

Skin samples were collected on days 5, 14, and 21 by scrapping the burnt part.[21] Skin tissue samples were fixed in a 10% formaldehyde buffer solution for 3 h and then washed with tap water. Tissue samples were then dehydrated with 70% alcohol (3 ×) for 30 min, 96% alcohol (3 ×) for 30 min, absolute alcohol for 1 h, followed by clearing with xylol (1:1) for 1 h. Paraffin impregnation was performed for 60 min in an oven at 65°C, followed by staining with HE and Masson's trichrome. Paraffinized tissues were cut into 5 µm-thick cross sections.[22]

Histopathological analysis was done using a microscope at 40x and 200x magnification at five fields of view for each specimen, according to the scoring parameters[23,24,25] as presented in Table 1.

Statistical analysis

The normality test was performed using the Shapiro-Wilk test. Analysis of variance was performed using Levene's test. Significant differences between means were determined by One-way ANOVA followed by *Bonferroni* post hoc *test*. The significant difference was established at $p < 0.05$ and $p < 0.01$.[26]

Table 1: Microscopic scoring parameters

	Parameters (per-view of field)	Score
a	Polymorphonuclear cells	
	1-5 PMN cells	3
	6-10 PMN cells	2
	11-15 PMN cells	1
b	Collagen	
	Collagen density is more than normal tissues	3
	Collagen density is equal to normal tissues	2
	Collagen density is less than normal tissues	1
c	Epithelialization	
	Normal epithelialization	3
	Some epithelialization	2
	No epithelialization	1
d	Vascularization	
	More than two new vascular blood	3
	1-2 new vascular blood	2
	No new vascular blood	1
e	Fibroblasts	
	More than 50 cells	3
	10-50 cells	2
	5-10 cells	1
	No cells	0
f	Re-epithelialization	
	Complete, the epidermis layer is fully formed and has no thickening.	3
	Incomplete, the epidermis layer is formed, and there is thickening.	2
	Starting, the epidermis layer begins to form.	1
	Absence and complete damage are found in the epidermis layer.	0

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Results and Discussion

Percentage of wound healing

Using Image J software, as depicted in Figure 1, the average area of burn wounds in K1 was 50%, K2 was 75%, and K3 was 98%. The normality test result with the Shapiro-Wilk test showed that the decreasing wound area in the K1, K2, and K3 groups were normally distributed ($p > 0.05$). Variance analysis with Levene's test indicated that all groups were homogenous ($p > 0.05$). Moreover, there were significant differences among the groups ($p < 0.01$). These results indicated that DPSC secretome had a significant effect on burn healing.

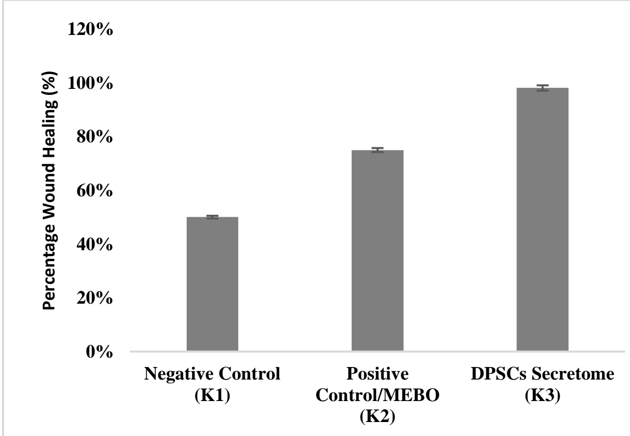

Figure 1: Percentage wound healing measured by mean decrease in wound area. Error bars represent the standard error of Mean

Physiological observations

Visual observation of changes in superficial dermal burns were done on days 1, 5, 14, and 21 as shown in Table 2. From the photographs displayed in Table 2, particularly in the K2 group, scabs appeared to come off more quickly on the 14th day compared o the K3 and K1 groups. Meanwhile, the K1 experienced slower healing than the K2 and K3 groups based on decreased burn area, discolouration of the burn wound, and scab detachment. On the 21st day, it was seen that healing in the K3 group was superior to the control groups (K1 and K2). The area of the burn wound decreased by almost 99% of the burn area, the scabs were released, and the colour of the wound appeared to have blended with the normal skin colour.

On the 5th day in the K3 group, the wound colour changed to dark brown, and the scabs formed was thicker and looked dry. Whereas, in the K2 and K1 groups experienced a changed in wound colour, and formation of a thin brown scabs. The formation of scabs indicates that the wound-healing process has entered the initial stage of the proliferation phase. The wound is filled with collagen, fibroblasts, and inflammatory cells to form granulation tissue, a reddish tissue with an uneven surface.[5] On the day 14, it was observed that a portion of the scabs in the K3 group had fallen off and was reddish brown; the scabs had peeled off all over the burn wound's surface, and the K2 group's skin had turned reddish brown; meanwhile, the K1 group had lost one-third of its scabs and had dark brown skin. On the 21st day of observation, it was seen that the K3 group had no scabs, and their skin colour looked normal, while the K2 group had formed scabs again, and their skin colour was yellowish. The K1 still had scabs remaining, and their skin colour was reddish.

The DPSCs-secretome was proposed to have anti-inflammatory effect because it could speed up the healing process of burn wounds, in line with previous research which treated excision wounds in mice with intradermal injection of the secretome of gingival fibroblasts, which resulted in a decrease in cell inflammation.[13,27] In this study, it was observed that the K3 group still had scabs that had not been removed, and the burn wounds looked drier than in the K1 group. The K3 group revealed that the DPSCs-secretome had a less moisturizing effect on superficial dermal burns.[21] This condition is due to the DPSCs' secretome being a pure substance that contain no skin moisturizers.[27]

Meanwhile, the K2 group appears to be fast in exfoliating burn wounds because it contains substances like Phellodendri cortex, which has antimicrobial activity,[28] Coptidis rhizoma with antimicrobial activity,[29] Berberine and Scutellariae radix with anti-inflammatory activity[30,31] as well as Sesame oil and cera flava which provide anti-inflammatory and moisturizing effects on wounds.[32,33]

Table 2: Physiological Observations on Superficial Wound

Day (s)	0.9% NaCl	MEBO	DPSCs secretome
1	Size: 4.61 cm^2 Colour: Brownish white Scab forms: - Scab comes off: -	Size: 6.59 cm^2 Colour: Brownish white Scab forms: - Scab comes off: -	Size: 6.99 cm^2 Colour: Brownish white Scab forms: - Scab comes off: -
5	Size: 4.95 cm^2 Colour: Brownish white Scab forms: yes	Size: 6.87 cm^2 Colour: Brown Scab forms: yes	Size: 4.43 cm^2 Colour: Sepia Scab forms: yes

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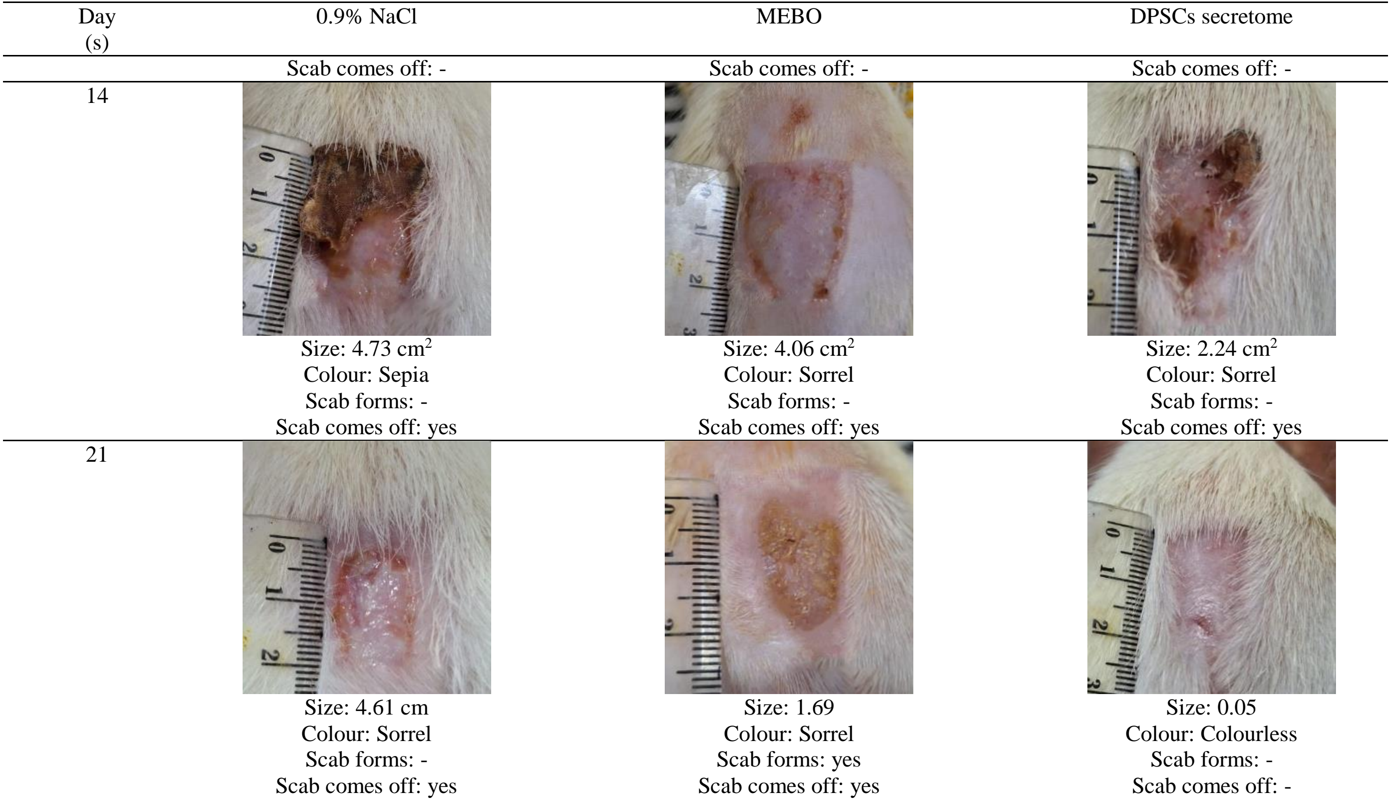

Day (s)	0.9% NaCl	MEBO	DPSCs secretome
	Scab comes off: -	Scab comes off: -	Scab comes off: -
14	Size: 4.73 cm^2 Colour: Sepia Scab forms: - Scab comes off: yes	Size: 4.06 cm^2 Colour: Sorrel Scab forms: - Scab comes off: yes	Size: 2.24 cm^2 Colour: Sorrel Scab forms: - Scab comes off: yes
21	Size: 4.61 cm Colour: Sorrel Scab forms: - Scab comes off: yes	Size: 1.69 Colour: Sorrel Scab forms: yes Scab comes off: yes	Size: 0.05 Colour: Colourless Scab forms: - Scab comes off: -

Histopathological and microscopic features of the burn wound

On histopathological examination, the number of epithelialization formations, Poly Morpho Nuclear (PMN) cells, new blood vessel formation, collagen formation, and fibroblast formation was observed on days 5, 14, and 21 (Table 3). A scoring method, as presented in Table 4, was used to evaluate the epithelialization, PMN cells, vascularizations, collagen, and fibroblast formation.

The microscopic findings were corroborated by a prior study that found that all wounds had full re-epithelialization on day 14, with the percentage of re-epithelialization being higher on days 3 and 7 following hepatocyte Growth Factor (hGF)-treated wounds in comparison to the control groups. Collagen III expression increased in response to hGF and hGF-CM on day 7 compared to the control group. However, on day 14, all treatments resulted in similar levels of collagen III expression.[34] Another study also supported the observation that the expression of transforming growth factor-beta increased on the third day,[35] which is in line with a study on burn recovery, which showed that epithelialization and fibroblast formation were complete in all treatment groups after 14 days.[36]

Regarding the formation of fibroblasts, the DPSCs had more than 50 cells on day 14. *In vitro* study with Adipose Tissue Secretome (ASCs) showed a higher concentration of fibroblast growth factor (FGF), which is known to be involved in wound healing and regeneration.[37,38] Keratinocytes migrate during the proliferation phase to create a fresh epithelial layer to cover the wound. Epidermal growth factor (EGF), heparin-binding epidermal growth factor (HB-EGF), and transforming growth factor-α all play a role in keratinocyte migration. In addition, fibroblasts are crucial in synthesizing collagen, which supports vascular tissue.[38]

On microscopic examination of polymorphonuclear cells, there was a decrease in PMN cells in the K3 group. It can be seen here that the DPSCs secretome has an anti-inflammatory effect on superficial dermal burns. Previous *in vitro* studies with Human Adipose Mesenchymal stem cells (MSCs) have shown the ability of MSCs to modulate myeloid cell differentiation towards a phagocytic and anti-inflammatory profile as well as modulate the M0 secretome of non-polarised macrophages (Mphs) and mature dendritic cells (mDCs), as demonstrated by the upregulation of anti-inflammatory and reparative factors and the decrease in pro-inflammatory factors.[39]

However, the formation of blood vessels or the angiogenesis process of all groups (K1, K2, and K3) were the same in the proliferation phase of the wound healing process. The angiogenesis process ensures that damaged tissues and organs heals properly, and their functions are maintained.[40,41]

Table 3: Histopathology images of all groups on the 5th, 14th, and 21st day

	Magnification 40x		Magnification 200x	
Groups	HE	Masson's Trichrome	HE	Masson's Trichrome
Day 5				
The negative control group of 0.9% NaCl (K1)				

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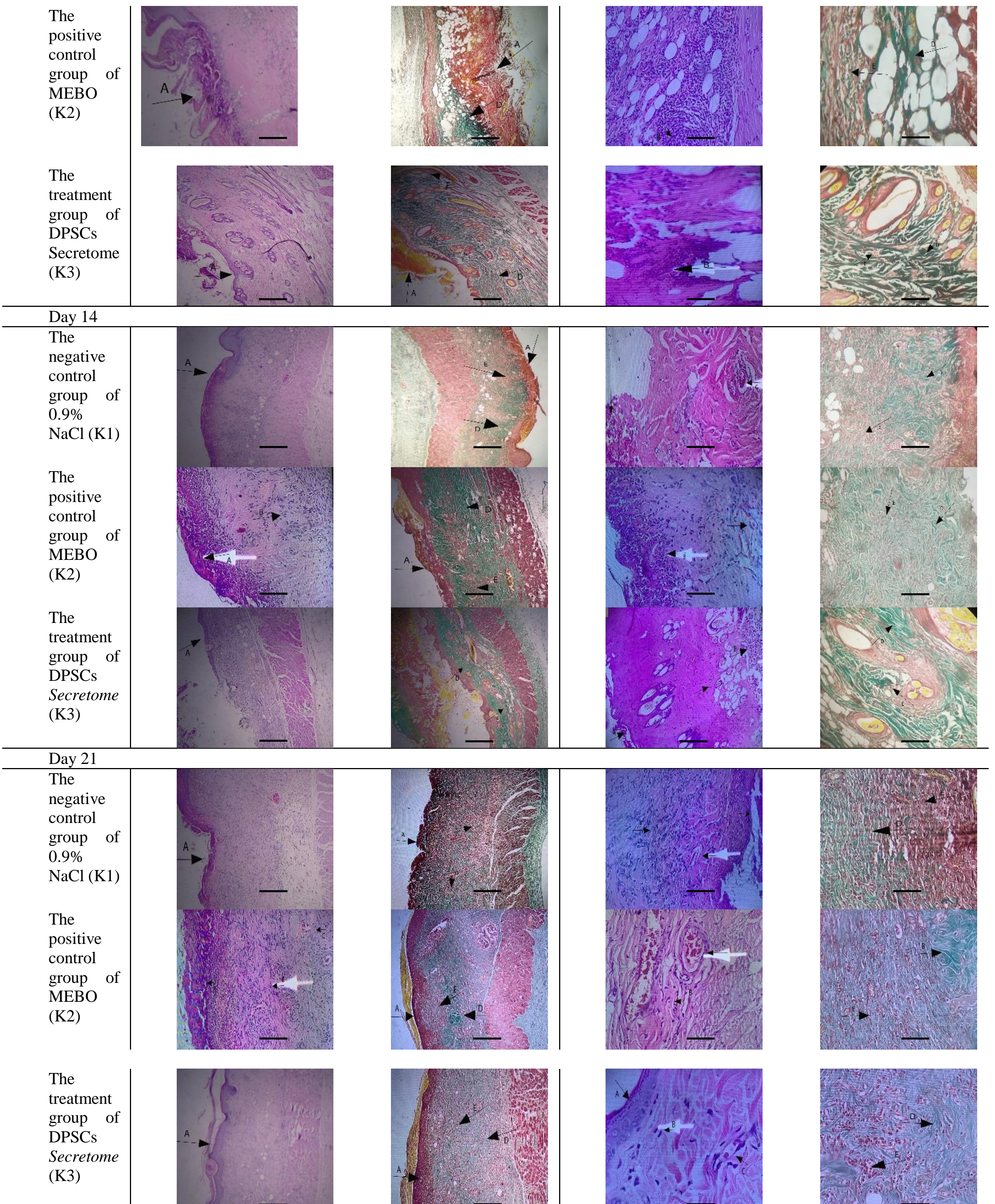

The left panels are HE and MT stainings at 40x magnification, scale bars = 50. The right panels are HE and MT stainings at 200x magnification, scale bars = 10 µm. Image labels represent the presence of (A) epithelialization, (B) polymorphonuclear cells, (C) new vascular, (D) collagen, (E) and fibroblasts.

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Table 4: Microscopic Scoring Analysis

	Epithelialization	**PMN cells**	**Vascularization**	**Collagen**	**Fibroblasts**
Day 5					
0.9% NaCl (K1)	1	1	1	1	2
MEBO (K2)	2	1	1	1	2
DPSCs Secretome (K3)	2	1	1	2	1
Day 14					
0.9% NaCl (K1)	1	1	3	1	3
MEBO (K2)	2	1	3	2	3
DPSCs Secretome (K3)	3	1	3	2	3
Day 21					
0.9% NaCl (K1)	2	1	3	2	3
MEBO (K2)	3	3	3	3	3
DPSCs Secretome (K3)	3	3	3	3	3

Conclusion

This study has demonstrated the therapeutic potential of DPSCs-secretome on superficial burns. During the 21 day treatment period, DPSC secretome showed better effect in reducing wound area or increasing healing percentage compared to moist exposed burn ointment (MEBO). Physiological observations and histopathological analysis with microscopic scoring methods revealed that DPSCs-secretome could decrease PMN cells and increase the collagen density, epithelialization, vascularization, fibroblasts, and complete form of epidermis layer. However, the MEBO exhibited a better moisturizing effect on burn wounds and better scab exfoliation than the DPSCs-secretome treatment, which did not provide a moistening effect on burn wounds.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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