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38



Combination of platelet rich plasma and stromal vascular fraction on the level of vascular endothelial growth factor in rat subjects experiencing deep dermal burn injury

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ABSTRACT

Background: The healing process of burns includes coagulation, inflammation, and remodeling. Vascular endothelial growth factor (VEGF) is involved throughout this healing process. Stem cells from the platelet-rich plasma (PRP) with stromal vascular fraction (SVF) can increase concentrations of growth factors, including VEGF. This is expected to accelerate burn healing. The aim of this study was to determine the effect of a combination of PRP and SVF on VEGF levels in a rats model of deep dermal burn wound healing.

Materials and methods: This is an experimental research study in rats using a post-test control group design with 4 groups: A) control, B) Vaseline, C) topical PRP and SVF, and D) PRP and SVF injection. Burn wounds were induced according to the modified Guo method.

Results: In a rats model of deep dermal wound healing, topical Vaseline significantly increased serum VEGF compared to control. Topical application and injection of stem cells also significantly increased serum VEGF compared to control and Vaseline. The VEGF concentration was significantly higher following injection of PRP and SVF, suggesting that the injection route is more effective at increasing VEGF levels compared to the topical application of stem cells.

Conclusion: The combination of PRP and SVF, either by injection or topical application, can increase VEGF levels during the healing process from deep dermal burns.

1. Introduction

A burn is defined by injury on the skin or underlying tissue caused by radiation, heat, cold, chemicals, or electricity [1–3]. Many researches have been performed to discover the best method in solving the problem of burns and accelerating the burn healing process. One of them is stem cell therapy [4]. Stem cells are undifferentiated primitive cells that have

the ability to differentiate from just one type of cell (unipotent), or into several types of cells (multipotent). They can even become various types of cells (totipotent). This ability can be utilized to repair body cells damaged by disease or trauma [5].

The stromal vascular fraction (SVF) is a collection of lipoaspirate components derived from fat tissue liposuction [6–9]. Lipoaspirate contains a large number of adipose-derived stem cells (ASCs). SVFs from

20

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fat tissue contain T cell regulators, endothelial precursor cells, pre-adipocytes, anti-inflammation macrophages, superoxide dismutase (SOD), IGF, TGF, FGF, hepatocyte growth factor (HGF), interleukin (IL), hematopoietic stem and progenitor cells, erythrocytes, fibroblasts, lymphocytes, monocyte/macrophages and pericytes are found in the SVF [10–13]. SVFs are known to promote burn healing by increasing cell proliferation and vascularization, enhancing inflammation, and increasing fibroblast activity [14,15].

Platelet-rich plasma (PRP) is a concentrate of thrombocytes in a small volume of plasma. It include growth factors, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), transforming growth factor- β (TGF- β), and insulin-like growth factor-1 (IGF-1), which are secreted after thrombocyte activation [13,15–21].

VEGF is key signal used by oxygen-hungry cells to stimulate blood vessel growth [13]. VEGF is produced in large amounts by keratinocytes, macrophages, endothelial cells, thrombocytes, and fibroblasts during wound healing. Cell disruption and hypoxia, signs of tissue damage, are strong initial inducers of angiogenic factors at the wound site, such as VEGF and its receptor [22,23]. VEGF-A promotes the initial phase of angiogenesis and is important for wound healing. It binds the surface receptors tyrosine kinase Flt-1 (VEGF-1 receptor, or VEGFR-1) and KDR (VEGF-2 receptor, or VEGFR-2). Flt-1 is needed for blood vessel organization, whereas KDR is essential for chemotaxis, proliferation, and endothelial cell differentiation [24]. We aimed to investigate the combination of PRP and SVF can increase the level of VEGF compared to control and Vaseline-treated rats with deep dermal burns.

2. Methods

This was an experimental research study using rats with a post-test control group design comprise a control group and 3 experimental groups (sacrificed on Day 1, 4, 7, 10 and 14 post experiment) conducted in the animal laboratory in our institutions over 2 weeks. The subjects were adult male rats (*Rattus norvegicus*), aged 10 weeks and weighing 150–250 g, obtained from the animal laboratory in our institution. A total of 64 rats were divided into 4 groups. Group A was the negative control in which samples were collected from the blood of healthy rats without burns or treatment (negative control). The 3 experimental groups were: Group B (Vaseline, topical), Group C (PRP and SVF, topical), and Group D (PRP and SVF, injected). Groups B, C, and D consisted of 5 groups of 4 rats, respectively. Group A was a group of 4 rats. This study was conducted in the animal laboratory Faculty of Medicine, Hasanuddin University, and received approval from our local Ethics Commission registration number: 216/UN4.6.4.5.31/PP36/2020. The work was also carried out in line with the ARRIVE guidelines for reporting animal research [25,26].

Table 1
Average change in VEGF level in each group.

Termination Day-	Group A (mean ± SD) (pg/ml)	Group B (mean ± SD) (pg/ml)	Group C (mean ± SD) (pg/ml)	Group D (mean ± SD) (pg/ml)	*p value	**p value
1	65.2 ± 0.7	70.1 ± 1.5	75.5 ± 4.0	83.7 ± 5.0	0.086	<0.001
4	65.2 ± 0.7	75.7 ± 5.1	86.3 ± 4.3	96.1 ± 5.6	0.051	<0.001
7	65.2 ± 0.7	75.8 ± 4.3	87.0 ± 5.2	96.4 ± 4.0	0.046	<0.001
10	65.2 ± 0.7	77.8 ± 4.1	89.8 ± 4.8	98.7 ± 3.9	0.166	<0.001
14	65.2 ± 0.7	78.5 ± 2.4	88.4 ± 7.2	98.6 ± 3.7	0.016	<0.001

*Levene test **One-way Anova test.

2.1. PRP and SVF preparations

For PRP preparations, rats were first shaved on their backs and then anesthetized using ether inhalation. The stem cell donor group underwent a thoracotomy until the heart was exposed. The cardiac apex identification was then conducted by aspirating blood from the cardiac. The blood drawn was transferred to a tube containing EDTA. Briefly, blood was centrifuged for 10 min at 2400 rpm (450 g) for the first centrifugation. The supernatant plasma with a buffy coat was mixed then for 15 min and again centrifuged at a speed of 3600 rpm (850 g). The infranant buffy coat was used to prepare the final PRP product [6, 27].

SVF preparations were derived from fat tissue of donor rats collected by incising the groin area of the four limbs using a scalpel. Then, the fat was washed with phosphate buffer salt and chopped until smooth and transferred to a 15 cc tube. Then, 0.15% collagenase was added to the tube containing fat and incubated for 30 min at 37 °C. To neutralize collagenase activation, Dulbecco Modified Eagle Media (DMEM) with 10% FBS and 1% antibiotic-antimycotic was added and then samples were centrifuged at 1,500 rpm for 5 min. Cell pellets were resuspended with aquadest then treated with Trypan blue dye to count the number of SVF cells in the Neubauer counting room [27,28]. A total of 50,000 SVF cells mixed with 0.5 aquadest were transferred to the Eppendorf tube for the final SVF product.

The combination of PRP and SVF was obtained by mixing 0.5 cc PRP with 50,000 SVF cells [27].

2.2. Deep dermal burn model

In each treatment group, hair was removed in the treatment area using the chemical Jolen (Veet®). Prior to burn injury, rats were anesthetized in a special box using ether inhalation until the state of awareness decreased. The wound area was disinfected with 1% povidone-iodine. Burn wounds were induced according to the modified Guo method of exposure to a hot aluminum metal which its size 10 × 10 mm previously heated in 100 °C water [29]. After modeling the burns, the experimental animals were orally administered an analgesic (sodium dipiron/metamizole, 50 mg/kg BW) and antibiotic (amoxycillin, 15 mg/kg BW) for three consecutive days [29,30].

2.3. Wound analysis

Skin tissue was subjected to histopathological examination to analyzed deep dermal burns using hematoxylin and eosin (H&E) staining. The histopathological slides were analyzed using a light microscope (Leica Microsystems).

2.4. Vaseline and combination stem cell treatment

Vaseline (petroleum jelly) was used as a moisturizer in the burn wound area [31]. In group B, topical Vaseline was applied to the burn area. In group C, the topical combination of PRP and SVFs was applied to the middle of the wound and the four edges of the wound at 6, 9, 12, 3 for a 50,000 SVF cells and total volume of 0.5 cc PRP per experimental rats. In group D, rats received the combination of PRP and SVFs subcutaneously injected into the middle of the wound and the four edges of the wound at 6, 9, 12, 3 for a 50,000 SVF cells and total volume of 0.5 cc PRP per experimental rats.

2.5. VEGF measurements

ELISAs were performed on days 1, 4, 7, 10, and 14 days following treatment. All blood samples were examined using the VEGF ELISA from MyBioSource, Inc. (catalog No: MBS724516) [6,32].

Table 2
Comparison of VEGF levels between groups A and B.

Termination Day-	Group A (Mean ± SD) (pg/ml)	Group B (Mean ± SD) (pg/ml)	*p value
1		70.1 ± 1.5	0.001
4		75.7 ± 5.1	0.026
7	65.2 ± 0.7	75.8 ± 4.3	0.003
10		77.8 ± 4.1	0.008
14		78.5 ± 2.4	<0.001

Independent t-test.

2.6. Statistical analysis

A Levene's test, One-way ANOVA test and paired t-test were employed by using a combination of SPSS statistics 24 (IBM Corp., Armonk, NY) and Excel (Microsoft Corp, Redmond, WA). A p-value of less than 0.05 was considered statistically significant.

3. Results

In this study, 64 rats were divided into 4 experimental groups: A) negative control, B) topical Vaseline, C) topical PRP and SVF, and D) subcutaneously injected PRP and SVF. Groups B, C, and D consisted of 5 groups of 4 rats, respectively. Group A was a group of 4 rats.

Based on Table 1, we can conclude the mean value of changes in VEGF levels for 14 days in 4 treatment groups. The group treated with vaseline, topical PRP and SVFs, and injection PRP and SVFs showed an increase in VEGF levels from day one to day 10 and then decreased on day 14. This indicated that the peak increase in VEGF levels was on day 10. In the PRP and SVFs group, injection in each treatment day had the highest mean value among all groups. In the group treated with vaseline, PRP and SVFs topical, PRP and SVFs injection, and the control group, there were significant differences in changes of VEGF levels with each p

value < 0.001.

3.1. Comparison between group A (negative control) and group B (topical vaseline)

As shown in Table 2, VEGF levels in the Vaseline group were significantly different from the control group (p < 0.05). Based on this result, H0 is rejected and H1 is accepted, suggesting there is a significant effect of Vaseline on VEGF levels in a rat model of deep dermal burns on days 1, 4, 7, 10, and 14. The VEGF levels with Vaseline were highest on day 14 (Fig. 1). This result is similar to a study that reported VEGF serum levels with Vaseline were lower than control on day 7, but higher than control on days 14 and 21 [16]. In another study comparing Vaseline to controls with an angiogenesis parameter, the researchers found that on days 7, 14, and 21, Vaseline was better than the control group [17]. In another study comparing VEGF levels in Pelnac, Vaseline, and a control group found that on day 7, Vaseline was better than control and Pelnac was significantly better than Vaseline [18].

Table 3
Comparison of VEGF levels between groups B and C.

Termination Day-	Group B (Mean ± SD) (pg/ml)	Group C (Mean ± SD) (pg/ml)	*p value
1	70.1 ± 1.5	75.5 ± 4.0	0.047
4	75.7 ± 5.1	86.3 ± 4.3	0.020
7	75.8 ± 4.3	87.0 ± 5.2	0.017
10	77.8 ± 4.1	89.8 ± 4.8	0.010
14	78.5 ± 2.4	88.4 ± 7.2	0.041

^a Independent t-test.

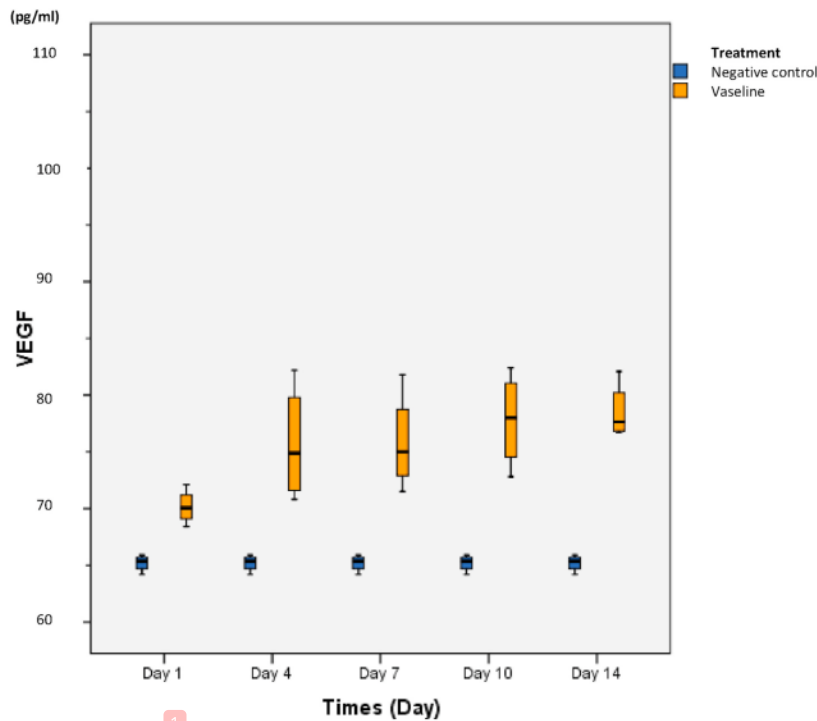


Fig. 1. Comparison result of VEGF levels between groups A and B.

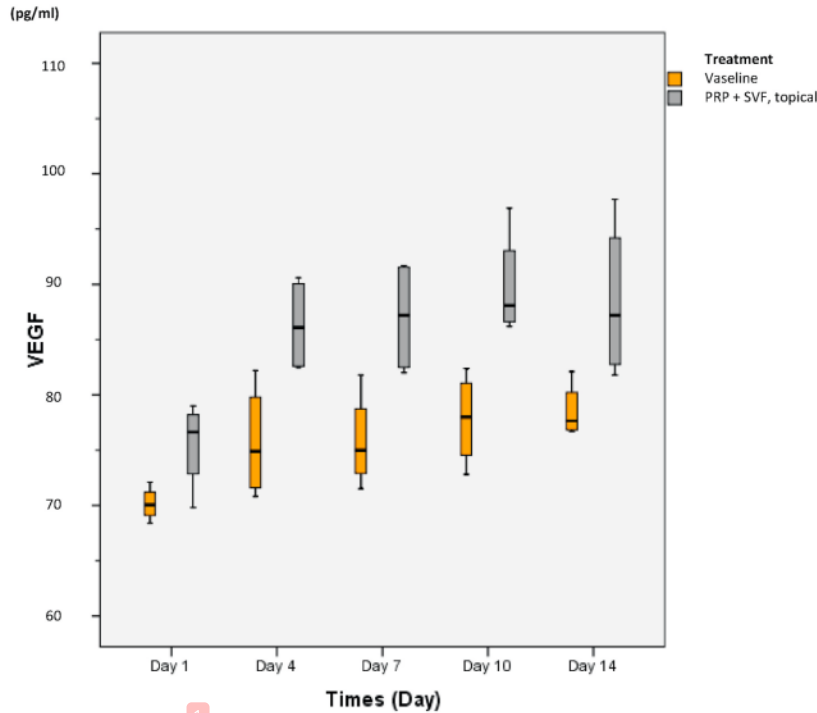


Fig. 2. Comparison of VEGF levels between groups B and C.

Table 4
Comparison of VEGF levels between groups B and D.

Termination Day-	Group B (Mean ± SD) (pg/ml)	Group D (Mean ± SD) (pg/ml)	p value
1	70.1 ± 1.5	83.7 ± 5.0	0.002
4	79.0 ± 2.1	96.1 ± 5.6	0.002
7	77.8 ± 4.1	96.4 ± 4.0	<0.001
10	75.8 ± 4.3	98.7 ± 3.9	<0.001
14	75.7 ± 5.1	98.6 ± 3.7	<0.001

^a Independent t-test.

3.2. Comparison between group B (topical vaseline) and group C (PRP and SVF, topical)

As shown in Table 3, the comparison of VEGF levels after topical PRP and SVF to Vaseline on each day was significantly different ($p < 0.05$) (Fig. 2). Based on this result, H0 is rejected and H1 is accepted, suggesting that topical application of stem cells is more effective than Vaseline for increasing VEGF levels in a rat model of deep dermal burns on days 1, 4, 7, 10 and 14.

3.3. Comparison between group B (topical vaseline) and group D (PRP and SVF, injected)

As shown in Table 4, VEGF levels after stem cell injections were significantly increased compared with Vaseline on each day ($p < 0.05$) (Fig. 3). Based on this result, H0 is rejected and H1 is accepted, suggesting that injections of stem cells are better able to increase VEGF levels compared with Vaseline in a rat model of deep dermal burns on days 1, 4, 7, 10 and 14.

3.4. Comparison between group C (PRP and SVF, topical) and group D (PRP and SVF, injected)

As shown in Table 5, VEGF levels were significantly increased after injection of stem cells and topical stem cells ($p < 0.05$), and there was a significant difference between the two groups (Fig. 4). Based on this result, H0 is rejected and H1 is accepted, suggesting the injection of stem cells is better than topical stem cells in increasing VEGF levels in a rat model of deep dermal burns on days 1, 4, 7, 10 and 14.

4. Discussion

One of the factors that plays an important role in the wound healing process is angiogenesis, which is the process of forming new blood vessels [33,34]. One of the most important and widely studied angiogenic factors is vascular endothelial growth factor (VEGF) [34]. VEGF is one of the most potent mediators of vascular permeability and vascular regulation in angiogenesis. VEGF is also commonly called “vascular permeability factor,” which refers to a specific endothelial cell mitogen secreted as a 45 kDa protein consisting of two subunits that do not induce cell proliferation in other cell types [24,34].

PRP contains many growth factors, including VEGF, HGF, bFGF, EGF, TGF- β , IGF-1, and PDGF [35]. Angiogenesis is particularly stimulated by VEGF, PDGF and basic fibroblast growth factor (bFGF). Angiogenesis is also assisted by pericytes, which in turn depend on the availability of PDGF and VEGF [36,37]. Likewise, ADSC/SVF that was found contain many growth factors and cytokines, which in turn influence angiogenesis, as well as mononuclear infiltrates, fibroblast and production on collagen. VEGF, a potent angiogenic agents, migration of fibroblast and activity stimulators such as TGF, and macrophage chemotaxis agents are regulated by ADSCs [38].

The administration of PRP and SVF, either topically or by injection enhances VEGF levels up to day 10. This result was significant compared

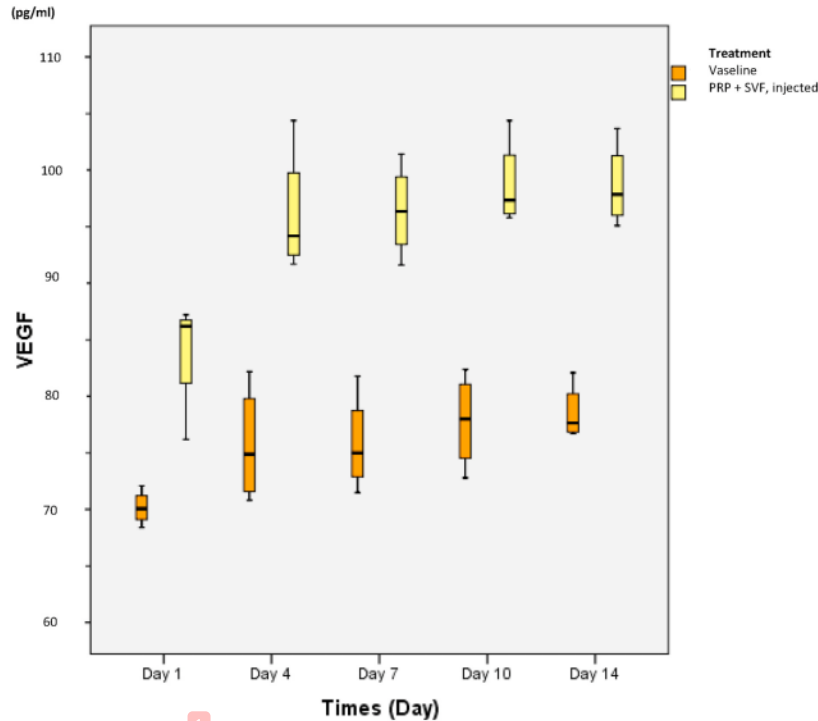


Fig. 3. Comparison of VEGF levels between groups B and D.

Table 5
Comparison of VEGF levels between groups C and D.

Termination Day-	Group C (Mean ± SD) (pg/ml)	Group D (Mean ± SD) (pg/ml)	p value
1	75.5 ± 4.0	83.7 ± 5.0	0.045
4	86.3 ± 4.3	96.1 ± 5.6	0.034
7	87.0 ± 5.2	96.4 ± 4.0	0.030
10	89.8 ± 4.8	98.7 ± 3.9	0.029
14	88.4 ± 7.2	98.6 ± 3.7	0.047

^a Independent t-test.

to Vaseline and control ($p < 0.05$). Our findings are similar to a previous study that reported the benefits of topical PRP gel in treating soft tissue wounds, in which the recovery proceeded faster compared to Vaseline. PRP gel is not only beneficial in chronic wound treatment, but is also effective in acute injury [39]. Another study regarding the administration of the SVF and PRF combination showed significantly better results in the concentration of VEGF levels compared to SVF or PRP alone with VEGF levels peaking on day 7 and slowly declining on days 14 and 28 [40]. Additionally, another study showed that PRP and SVF injection increased VEGF levels on day 1 to day 7 then decreased on day 10 [41].

In our study, we found a significant difference between injection and topical PRP and SVF administration. PRP and SVF by injection is more effective at increasing VEGF levels compared with topical PRP and SVF. The main objective of PRP therapy is to centralize growth factors at the injury site. These factors are considered beneficial in the healing process in soft and hard tissue and are also considered the most effective way to provide a biological stimulus to the tissue [42]. In a previous in vitro study, it was stated that freezing and diluting PRP does not significantly affect VEGF release. There was no significant difference in VEGF levels after freezing for 1 h and 7 days. This study also concluded that kinetic differences in VEGF release are not significantly affected by PRP

preparation; further clinical studies could explore and clarify the PRP effect [35]. Based on our results, it can be concluded that the injection route is significantly more effective than topical application for increasing VEGF release.

In a study analyzing the musculoskeletal effect of PRP and SVF, the authors stated that direct injection of PRP to the joints can control the inflammatory response and promote long-term healing. The growth factor components in PRP have special anti-inflammatory effects and can also reduce pain. Anti-inflammation, immunomodulation, and analgesia are also influenced by soluble factors excreted by SVF or ADSCs, including HGF, VEGF, NGF, EGF, FGF and TGF- β [43].

Inflammation regulation, proliferation of cells, remodeling, deposition of extracellular matrix, angiogenesis and epithelization play important role in skin wound healing. In the mouse skin, PRP and SVFs can reduce inflammatory process, increase collagen deposition, and promote angiogenesis and neurogenesis, thus promote wound healing [44]. PRP and adipose-derived stem cells combination is potent in stimulating increase of growth factor concentration (IGF-1, TGF- β 1, HGF, and VEGF) [27]. PRP is an accessible cell therapy to help treat chronic wounds and severe burns [16,19]. PRP is an autologous cell therapy containing a large number of bioactive factors involved in wound healing and tissue repair. PRP can be used for treating and stimulating wound healing in burns and split-thickness skin grafts, which are often used for the management of burns [45]. 46

Based on this study, further research is needed regarding the effect of the combination of PRP and SVF on VEGF levels in dermal burn healing. Future studies could involve determining the quantity of neo-vascularization in burn healing through histopathological examination.

In addition, our results may be of interest to other researchers investigating the additive and synergistic effects of therapeutic strategies involving stem cells. The fact that the PRP and SVF efficacy data for burn injuries are limited highlights the need for phase I-II clinical trials. We hope that this research can serve as a fundamental resource and an

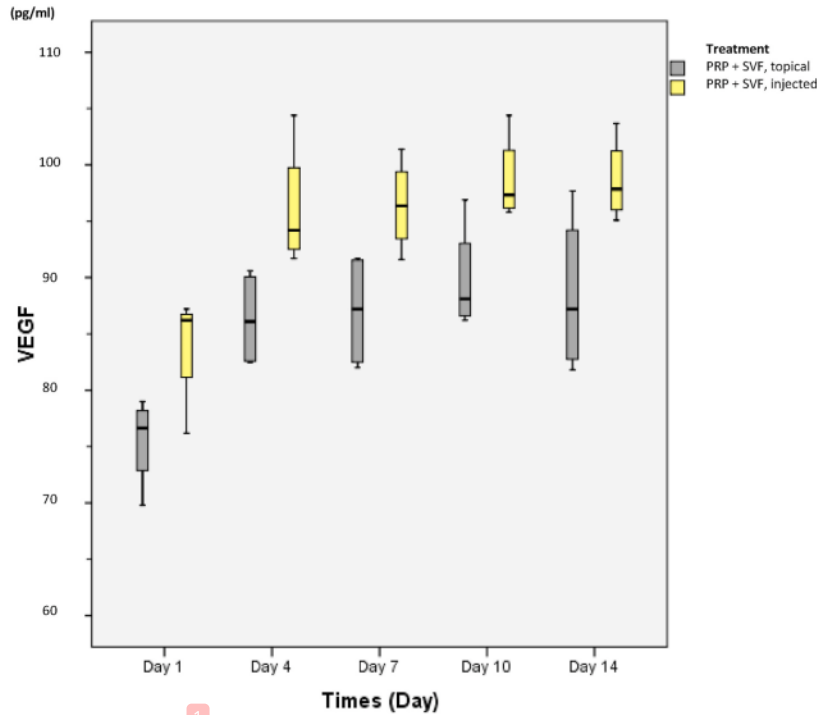


Fig. 4. Comparison of VEGF levels between groups C and D.

additional reference when making decisions about the application of PRP and SVF as a new breakthrough in the management of burn injuries.

5. Conclusion

In our study, we can conclude that the administration of PRP and SVF by injection can significantly increase VEGF levels more effectively than Vaseline in rats with induced deep dermal burns. Further, the administration of topical PRP and SVF significantly increased VEGF levels compared to the Vaseline group. There is a significant difference between PRP and SVF administration by injection and topical application. The injection of PRP and SVF is more effective at increasing VEGF levels compared to topical application.

Provenance and peer review

Our study was non-commissioned and externally peer-reviewed.

Ethical approval

All procedure for Animal experiment has been approved by Ethics Commission Faculty of Medicine, Hasanuddin University Number: 216/UN4.6.4.5.31/PP36/2020.

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Author contribution

SRL, FJ, SB, and MF wrote the manuscript and participated in the study design. SRL, FJ, SB, MF, ASB, WS, MNM, IDS, and AAI drafted and

revised the manuscript. SRL, FJ, SB, and MF performed deep dermal burn model. SRL, FJ, MF, and LJP performed bioinformatics analyses and revised the manuscript. All authors read and approved the final manuscript.

Registration of research studies

None.

Guarantor

Sachraswaty Rachman Laidding.

Consent

This manuscript does not involve human participants, human data, or human tissue.

Declaration of competing interest

The authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.amsu.2021.102254>.

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