ORIGINAL ARTICLE

Effects of Stromal Vascular Fraction and Platelet-Rich Plasma Treatment on Capillary Histopathology During Anal Trauma Healing in Rats: An in Vivo Study

Darwin[a], Sulmiati[b], Fonny Josh[c], Ahmadwirawan[b], Andi Alfian Zainuddin[d], Sachraswaty R. Laidding[c], Nita Mariana[b], Muhammad Faruk[a]

[a]Department of Surgery, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia
[b]Division of Pediatric Surgery, Department of Surgery, Faculty of Medicine, Hasanuddin University - Wahidin Sudirohusodo Hospital, Makassar, Indonesia
[c]Division of Plastic and Reconstructive Surgery, Department of Surgery, Faculty of Medicine, Hasanuddin University - Wahidin Sudirohusodo Hospital, Makassar, Indonesia
[d]Department of Public Health and Community Medicine Science, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia

Keywords
platelet-rich plasma; anal trauma; histopathology; stromal vascular fraction; in vivo study.

Corresponding Author
Dr. Sulmiati
Division of Pediatric Surgery,
Department of Surgery,
Faculty of Medicine, Hasanuddin University - Wahidin Sudirohusodo Hospital, Makassar, Indonesia
Jl. Perintis Kemerdekaan KM 11,
Makassar, 90245, Indonesia.
E-mail : umiandi@yahoo.com

Received: 4th November 2022; Accepted: 13th March 2023

Doi: https://doi.org/10.31436/imjm.v22i2

ABSTRACT

INTRODUCTION: Anal traumas, such as blunt and penetrating injuries to the perineum, can disrupt the anal sphincter and cause considerable morbidity. Non-invasive anal trauma therapies have been widely developed, including growth factors administered as platelet-rich plasma (PRP) and stromal vascular fraction (SVFs) containing adipose-derived stem cells (ASCs) and limited growth factor. This study aimed to assess PRP and SVF effects on capillary histopathology in the anal trauma Wistar rat model. **MATERIALS AND METHODS**: This experimental study used a rat model. Rats were divided into three groups: Group A (n=12) was treated with PRP and SVFs; Group B (n=12) was treated with normal saline (placebo); Group C (n=4) was sacrificed on day 0 to obtain baseline capillary counts. Groups A and B were further divided into three subgroups, sacrificed on days 1, 7, and 14 post-treatment. **RESULTS:** Capillary counts significantly showed higher density between Groups A and B (p=0.037, analysis of variance). On day 7, there was a significant difference between Groups A and B (p=0.001). **CONCLUSION:** Mean capillary density significantly improved in anal trauma treated with a combination of PRP and SVFs compared to control. Our findings support the use of PRP and SVFs to aid recovery of patients with anal trauma.

INTRODUCTION

Anal trauma is a medical term describing any excoriations or lacerations occurring at the lining of the anus. While this condition is generally not a serious injury, it causes pain and discomfort. Anal trauma, such as blunt and penetrating injuries to the perineum, can disrupt the anal sphincter and cause considerable morbidity due to the high rate of concomitant pelvic injuries.[1] In addition to surgical therapy, non-invasive anal trauma therapies have been widely developed, including growth factors administered as platelet-rich plasma (PRP) extracted from the patient's blood and stromal vascular factors (SVFs) - isolated from adipose tissue-contains adipose-derived stem cells (ASCs) and limited growth factor.[2,3]

PRP is a component of the blood plasma fraction rich in platelets and contains growth factors involved in wound healing, such as epidermal growth factor, transforming growth factor (TGF)-α, TGF-β1, TGF-β2, and vascular endothelial growth factor (VEGF).[2,4,5] SVFs are a heterogeneous cell population comprising many cell types, such as erythrocytes, lymphocytes, monocytes, endothelium, fibroblasts, and other progenitor cells used in wound healing therapies.[6–8] SVFs can regenerate tissue through their differentiation and have been shown to increase angiogenesis. SVFs secrete various growth factors that repair pre-existing endothelial cells rather than act on the neovascularization process.[2,9,10]

This study aimed to explore the effects of PRP and SVF on capillary histopathology in the anal trauma using the Wistar rat model.

MATERIALS AND METHODS

This experimental study was approved by the Medical Research Ethics Commission of the Faculty of Medicine at Hasanuddin University (No. 166/UN4.6.4.5.31/ PP36/2022) and performed according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.[11,12] This study was conducted in the Animal Laboratory of the Faculty of Medicine at the Muslim University of Indonesia (Makassar, Indonesia) and in the Anatomical Pathology Laboratory of Hasanuddin University (Makassar, Indonesia).

The number of rats in each subgroup was four based on Federer's formula.[12–14] Twenty-eight rats were divided into three groups. Group A (n=12) was treated with PRP and SVFs (treatment). Group B (n=12) was treated with normal saline (placebo). Group C (n=4) the control group (without anal trauma or treatment) sacrificed on day 0. Groups A and B were further divided into three subgroups (n=4 in each subgroup), sacrificed on days 1, 7, and 14 post-treatment.

Before treatment, Wistar rats were weighed and acclimatized for two weeks. They were kept in cages measuring 40 × 20 × 20 cm^3, with 4–5 rats per cage. The temperature was set to room temperature (28 ± 2°C) and humidity (50%–60%) with a 12-hour light/dark cycle. The cages were cleaned daily. The rats were fed a standard 20-gram pellet every day, and drinking water was given *ad libitum*.

The backs of the Wistar rats were shaved before anesthetization using ether. In the donor rats, a thoracotomy was performed until the heart was visible. The heart's apex was identified and then punctured using a 25G syringe 3 cc needle to aspirate the blood. Fat tissue was taken from both groin regions.

Platelet-Rich Plasma (PRP) Preparation

PRP was prepared from blood collected via cardiac puncture from all donor rats according to the method of Tajima et al.[15]

Stromal Vascular Fraction (SVF) Preparation

SVFs were prepared from adipose tissue harvested from the left and right groin regions from all donor rats according to the method of Tajima et al.[15]

PRP+SVFs Preparation

Rats in Group A were treated with a solution containing 50,000 SVF cells mixed with 0.5 mL of PRP according to the method of Tajima et al.[15]

Anal Trauma Model

The anal trauma model used in this study was adapted from Trebol et al.[12,14,16]

Sacrificing Procedure

Rats from Groups A and B were sacrificed on days 1, 7, and 14 post-treament. All rats were anaesthetized with ether before sacrificed. Next, their wounds were photographed. Then, rats were fixed to the table, and samples were collected from their anus, paying attention to the incision scars made previously, and fixed with formalin for examination at the Anatomical Pathology Laboratory.

Wound Analysis

Anal tissues were histopathologically examined with haematoxylin and eosin (Cat. No. 105175; Merck, Darmstadt, Germany) using standard methods to determine capillary density. Two pathologists assessed and counted overall capillary density using a high-powered microscope at 200x magnification using the Win Roof (v.3.4) image analysis software (US National Institutes of Health, Bethesda, MD, USA).[2]

Statistical Analysis

All data were expressed as a mean ± standard deviation (SD). The normality test (Shapiro–Wilk test) for parametric data was performed with p>0.05, indicating significant. The data were compared using the repeated analysis of variance (ANOVA) test, Mauchly's sphericity test, and independent *t*-tests with the SPSS for Windows software (v.23.0; IBM Corp., Armonk, NY, USA). All results with $p < 0.05$ were considered statistically significant.

RESULTS

Table I shows the capillary count, properties of capillary counts and comparison of capillary counts post-treatment. Capillary count is documented as mean ± SD, as well as min and max; properties of capillary counts in Groups A and B is shown with Shapiro–Wilk normality test, Mauchly's sphericity test, and Repeated ANOVA test; whilst the comparison of capillary counts is shown as independent sample t-test.

For the capillary count, Group A (treated with PRP and SVFs), capillary counts on day 1 were 62.75 ± 38.79, ranging between 18 and 103 across fields of view. On day 7, the capillary counts decreased to 25.50 ± 9.67, ranging between 17 and 37 across fields of view. On day 14, the capillary counts decreased to 10.50 ± 5.80, ranging between 5 and 16 across fields of view. For Group B (treated with normal saline [placebo]), capillary counts on day 1 were 47.25 ± 21.82, ranging between 29 and 78 across fields of view. On day 7, the capillary counts increased to 66.75 ± 28.37, ranging between 40 and 96 across fields of view. On day 14, their capillary counts decreased to 26 ± 9.34, ranging between 16 and 37 across fields of view. Group C (control group) had capillary counts of 21.25 ± 12.52 on day 0.

For the properties of capillary counts, the repeated ANOVA test includes Mauchly's test of sphericity to enable researchers to determine whether groups in their data have a homogeneous variance. The capillary count data showed homogeneous variance across Groups A and B and days 1, 7, and 14 (p=0.762). Furthermore, the repeated ANOVA test indicated that capillary counts differed significantly between Groups A and B and days 1, 7, and 14 (p=0.037).

For the comparison of capillary counts, the independent *t*-tests were used to assess differences between Groups A and B on sacrifice days 1, 7, and 14 post-treatment. Capillary counts did not differ significantly between groups on day 1 (p = 0.118). However, they did differ significantly between groups on day 7 (p = 0.001). Interestingly, capillary counts did not differ significantly between groups on day 14 (p = 0.210).

DISCUSSION

The anatomy of the rat's anus is very similar to humans. The main difference lies in the anal sphincter, while the other rectal layers have similar structures. Previous studies have suggested that wound healing will be enhanced by PRP and SVF treatment.[12,14] While the mechanism is not fully understood, its potential components and

Table I. Capillary count, properties of capillary counts and comparison of capillary counts post-treatment based on the groups.

Day of sacrifice post-treatment	Group (n=4)	Mean ± SD	Min	Max	*p*			
					Shapiro- Wilk normality test	*Mauchly's sphericity test*	Repeated ANOVA test	Independent sample t-test
0	C	21.25 ± 12.52	14	40	-	-	-	
1	A	62.75 ± 38.79	18	103	0.700	0.762	0.037	0.118
	B	47.25 ± 21.82	29	78	0.386			
7	A	25.50 ± 9.67	17	37	0.346			0.001
	B	66.75 ± 28.37	40	96	0.226			
14	A	10.50 ± 5.80	5	16	0.126			0.210
	B	26.00 ± 9.34	16	37	0.832			

Key: SD, standard deviation; *n*, sample number. Group A: treated with PRP and SVFs, Group B: treated with normal saline [placebo], Group C: control

differentiation processes trigger progenitor cells and assist immune modulation to stimulate the rapid healing process. Some studies indicate that capillary density is an indicator of wound healing progress.[2,10,17,18]

Angiogenesis is generally controlled by changes in pro- and anti-angiogenic factor levels called angiogenic switches, first discovered in Folkman's study of tumour angiogenesis. Pro-angiogenic and anti-angiogenic mediators such as fibroblast growth factor, interleukin-8, placental growth factor, TGF-β, and VEGF stimulate angiogenesis, and their production increases after skin injury.[19] Furthermore, Johnson and Wilgus' 2014 study on angiogenesis[20] showed the VEGF response to the wound healing process using two tests: enzyme-linked immunosorbent assays (ELISA) to measure VEGF levels and immunostaining to visually assess cluster of differentiation 31 (*CD31*) expression. ELISA results showed that VEGF production increased between days 3 and 5 post-injury, indicating that new blood vessels were forming, before decreasing and returning to normal between 7 and 14 days. Immunostaining results showed increased staining on day 5 post-injury. CD31 staining indicated that vascular structures began to form and then increased on day 7. However, the number and density of blood vessels decreased on day 14 post-injury, with CD31 levels close to those found in intact skin.[19]

Singer and Clark[21] studied the regulation of wound angiogenesis. Average human skin has markedly increased numbers of capillaries, much higher than in normal tissue. In the initial wound healing phase, between days 1 to 3, the angiogenesis mediator basic fibroblast growth factor (bFGF) begins its function in wound healing. Next, *VEGF* expression will increase from days 4 to 7, and new granulation tissue will form blood vessels. Then, in the antiangiogenic phase, blood capillaries regress between days 7 and 14, making the granulation tissue appear paler. The tissue stroma will form faster when the newly formed granulation tissue is well vascularized. One way to accelerate the onset of angiogenesis is by increasing the production of growth factors involved in the angiogenesis process.[21,22]

The vascular structure formation process is shown in more detail in Figure 1.[23] Briefly, VEGF is an important pro-angiogenic factor in the angiogenesis process that stimulates endothelial cell migration and proliferation.[24] Activated endothelial cells form a tube, and angiopoietin-2 (*ANG2*) expression will increase.[25] Increased *ANG2* expression directly contributes to new vascular structure formation and generally occurs starting on days 3-5.[23]

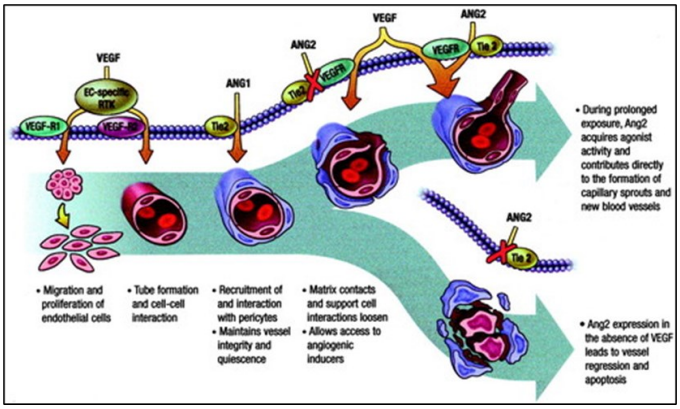

Figure 1. Neovascularization process.[23]

The findings of Hou et al.[26] also support our results, where capillary counts in Groups A (treatment) and B (placebo) began to increase compared to healthy mice on day 1. Capillary counts peaked on day 7 before decreasing on day 14. This decrease is likely due to progressive granulation tissue formation. Furthermore, stem cells have an important role in angiogenesis by releasing angiogenic factors such as VEGF, bFGF, and platelet-derived growth factor (PDGF), stimulating more rapid new vascular formation or influencing vascular physiological processes.

The findings of Johnson and Wilgus,[20] Singer and Clark,[21] Hou et al.,[26] and Rigotti et al.[27] are consistent with our finding that capillary counts did not differ significantly between Groups A (treatment) and B (placebo) on day 1 since the angiogenesis process only begins on days 1–3. Furthermore, capillary counts differed significantly between Groups A (treatment) and B (placebo) on day 7 since the normal angiogenesis process had just begun to form new vascular structures. In contrast, there was a decrease in capillary numbers in Group A (treatment) due to the presence of PRP+SVFs, whose catalytic properties produced angiogenic factors that activated endothelial

cells, accelerating vascular structure formation starting on day 3. Capillary counts did not differ significantly between Groups A (treatment) and B (placebo) on day 14 due to physiologically complete neovascularization and because the formation of new vascular structures in Group A (treatment) was completed before day 14. Consequently, capillary numbers were similar in both groups.[20,21,26,27]

PRP's stimulating effect on angiogenesis has been experimentally shown. Eppley et al.[28] reported that PRP stimulates endothelial cells around the wound area, enhancing new capillary proliferation and formation. In addition, Hu et al.[26] concluded that PRP is a potential donor cell in initiating the angiogenesis process in vitro, recruiting the area's vascular endothelium and initiating bone regeneration. PRP can stimulate undifferentiated stem cell proliferation and differentiation to support tissue regeneration.[29–32] Undifferentiated stem cells migrate to PRP growth factor concentration sites, which trigger their proliferation at the wound site.[33] Adipose-derived SVFs can differentiate into different tissue types, support neovascularization, replace cells, and repair injured tissue. Therefore, SVFs are combined with PRP in one culture medium[34,35] to enhance PRP's strong proliferative activity and induce human SVF osteogenic proliferation and differentiation more rapidly than in basal media.[36] The findings of Rigotti et al.[36] are consistent with the results of this study.

This study's comparisons between Groups A (treatment) and B (placebo) using independent *t*-tests showed the capillary counts did not differ significantly on days 1 (p=0.118) and 14 (p=0.210) but did differ significantly on day 7 (p=0.001). These results indicate that Group A rats treated with PRP and SVFs had more rapid angiogenesis completed on day 7 than Group B rats treated with saline solution on day 14.

However, all rats in Group A treated with PRP and SVFs had completed the angiogenesis process on day 14, indicated by a capillary density that had reached normal levels similar to Group C (control). In contrast, rats in Group B (placebo) underwent a normal angiogenesis process, and their capillary densities did not reach those of Group C (control) by day 14. These differences were shown to be significant using a repeated ANOVA test (p=0.037). This study's results are consistent with those of Ni et al.,[37] who explored diabetic wound healing using PRP and adipose-stromal derived cells (ASDCs). They monitored neovascularization (angiogenesis and vasculogenesis) in diabetic wounds *in-vivo* for 14 days using immunohistochemical staining for vascular markers cluster of differentiation 34 (CD34), CD31, and phosphorylated signal transducer and activator of transcription 3 (p-STAT3) and growth factor VEGF. They found significantly higher CD34, CD31, VEGF, and p-STAT3 levels in the PRP with ASDC group than in the placebo group. Therefore, they concluded that combining PRP and ASDC induced neovascularization.[37]

This study's findings are also consistent with Ahmed et al.[38], who reported that stem cells and PRP promote wound closure and vascularization by increasing TGF-β1 and PDGF production at the wound site. Furthermore, angiopoietin-1 (*ANG1*) and *ANG2* expression, angiogenesis markers, decreased in vivo between days 7 and 20 in the treatment group but not in the control group. In addition, the stem cell and PRP group experienced more rapid re-epithelialization than the control group during days 7–20.

Another in vivo study by Karina et al.[39] found that the number of new blood vessels formed was greater in the PRP with SVFs group than in the PRP or SVFs groups. SVFs have a therapeutic effect on acute and chronic wounds, likely due to their good immunomodulatory effects increasing collagen accumulation and neovascularization, and a cryotherapeutic effect, enhancing the angiogenic response during wound healing.

This study is the first to monitor angiogenesis after anal trauma treated with stem cells (PRP and SVFs). More rapid angiogenesis with than without stem cells highlights their therapeutic potential. However, this study also had some limitations. These included its use of Wistar rats, whose anus has slight anatomical differences from humans. Consequently, the examined treatment may have a different effect when applied to humans. In addition, this study monitored the post-therapy outcome for 14 days.

Therefore, changes occurring more than 14 days post-therapy that might influence its effectiveness were not considered. Finally, this study did not compare alternative treatments.

CONCLUSIONS

Mean capillary density differed significantly by day between rats with anal trauma with or without treatment (PRP & SVF) and controls. Future studies are required to assess the reproducibility of our findings using different administration routes and histopathological characteristics. Large-scale clinical studies are needed to assess the efficacy and safety of administering stem cell therapy in anal trauma and other wounds.

CONFLICTS OF INTEREST

None

FUNDING

No funding was received for this study.

ACKNOWLEDGMENTS

The authors wish to thank all staff from the Animal Laboratory of the Faculty of Medicine at Indonesian Muslim University and Hasanuddin University Medical Research Center (HUM-RC) in Makassar, Indonesia.

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