

Original Article

Effect of Stromal Vascular Fraction and Platelet-Rich Plasma Combination on Polymorphonuclear Cells in Wistar Rats Anal Trauma Model

Erwin Hadi Chandra¹, Sulmiati^{2,3}, Nita Mariana^{2,3}, Fonny Josh^{4,5}, Sachraswaty Rachman^{4,5}, Firdaus Hamid⁶

¹*Department of Surgery, Faculty of Medicine, Universitas Hasanuddin, Makassar, Indonesia*

²*Division of Pediatric Surgery, Department of Surgery, Faculty of Medicine, Universitas Hasanuddin, Makassar, Indonesia*

³*Division of Pediatric Surgery, Department of Surgery, Wahidin Sudirohusodo Hospital, Makassar, Indonesia*

⁴*Division of Plastic and Reconstructive Surgery, Department of Surgery, Faculty of Medicine, Universitas Hasanuddin, Makassar, Indonesia*

⁵*Division of Plastic and Reconstructive Surgery, Department of Surgery, Wahidin Sudirohusodo Hospital, Makassar, Indonesia*

⁶*Department of Microbiology, Faculty of Medicine, Universitas Hasanuddin, Makassar, Indonesia*

Corresponding Author:

Name: Erwin Hadi Chandra

Email: erwinhadi.candra@gmail.com

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ABSTRACT

Introduction: By relying on polymorphonuclear (PMN) cells, stromal vascular fraction (SVF) and platelet-rich plasma (PRP) contribute significantly in the healing process. Through anal trauma model, this study examined the effect of SVF and PRP combination on PMN counts in vivo. **Method:** Three groups of twenty-eight eligible Wistar rats anal trauma model randomly assigned to receive SVF and PRP combination (Group A), normal saline (Group B), or healthy controls (Group C). On days 1, 7, and 14, we examined at PMN cell counts in Groups A and B. Group C were sacrificed early to acquire baseline data on PMN cells. **Results:** On the first day, Group A had more PMN cells than Group B, but this difference faded by days 7 and 14. On day 1, the test

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*revealed a significant increase in PMN cells ($p=0.002$).
Conclusion: Combining SVF and PRP can improve PMN cells' acute phase of anal damage repair in vivo.*

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1. INTRODUCTION

Because of their anatomy, anorectal trauma incidents in adults and children are uncommon. Because of the anus's superficial placement, anal trauma is very prevalent.¹ Anorectal injury was found in 0.2 percent of children in Utah between 2003 and 2012. The anus was engaged in 43% of these cases, the rectum was involved in 38%, and the anorectal complex was implicated in 19%.² Accidents or sexual abuse are the most common causes of anorectal damage in children.³

Anorectal trauma is diagnosed and treated with a mix of examination and treatment that focuses on the primary and secondary surveys, as well as the site and severity of the injury.⁴ Several practitioners employ the 4D paradigm in addition to surgery (Debridement, Proximal Diversion, Drainage, and Distal Washout).^{2,4,5}

Approximately of 19% of fecal incontinence cases is caused by disturbance during the anal trauma recovery phase.² If left untreated, fecal incontinence might result in a 3–10% mortality rate.⁵ The anal sphincter is injured during or after surgery, resulting in fecal incontinence. Anal stenosis is another common complication of anal trauma, and it is caused by incomplete post surgery healing. Stenosis of the anal is caused by the growth of cicatricial tissue, which decrease the diameter of the anal lumen.⁶ The use of stem cells to treat this problem has been demonstrated to be effective.⁷

Growth hormones, cytokines, and chemokines regulate tissue regeneration, which is a compelling process involving reciprocal action between immune cells and the tissue stromal component.^{8–10} Simple wounds go through an inflammatory phase that lasts 1–2 days and helps to avoid infection. This phase consisting of vascular hemostasis and cellular reaction.⁸ Tissue edema is a symptom of this inflammatory process.¹¹ After 2–10 days, the proliferative phase begins with abundant production of granulation tissue and angiogenesis.⁸ The latest phase is remodeling process, which can take anywhere from 2–3 weeks to 2 years depending on the extent of the damage.⁹ Under normal circumstances, the wound heals normally, leaving behind avascular scar tissue.¹²

In comparison to the skin, the bowel organ has three unique healing process: repair, proliferation, and cell differentiation. The bowels have mono-layered columnar

epithelial cells, higher collagenase activity, and specific microenvironment with aerobic and anaerobic microbes, thus require optimal tissue perfusion in the course of the healing process.^{13,14}

Among different types of leukocytes, neutrophils or polymorphonuclear (PMN) cells are the most prevalent to be found. In the bone marrow, neutrophils being massively produced and get in the circulation to penetrate the tissues to accomplish their tasks as effector cells.^{15,16}

A wide spectrum of neutrophil responses is triggered physiologically by transcriptional process and modulate the production of surface protein. Only a small percentage of neutrophils show these phenotypic alterations, implying a large deal of variation among neutrophils.¹⁶ This study would examine the outcome of a combination SVF and PRP injection on PMN counts in anal trauma model.

2. METHODS

In an experiment with two experimental groups and one healthy group, we engaged the animal in a post-test only control group design. The animals were procured and handled at the Animal Laboratory of Universitas Muslim Indonesia's Faculty of Medicine in Makassar, Indonesia. We performed the biopsy and microscopic investigation at Hasanuddin University Medical-Research Center (HUM-RC). All procedures were approved by the Medical Ethics Committee of Universitas Hasanuddin (670/UN4.6.4.5.31/PP36/2021).

Population and Sample

Twenty-eight healthy male Wistar rats (*Rattus norvegicus*), aged 16–24 weeks old, weighed 170–260 grams, were divided into three groups (using Federer's Formula). After surgery, Group A and Group B received a locally injected of SVF and PRP combination and normal saline, respectively. The PMN cells count were evaluated on days 1, 7, and 14 as the time course of the healing process. Four healthy rats were sacrificed to get baseline PMN cell counts in Group C, which had not been exposed to any stress.

Preparation of the SVF

Josh et al.¹⁷ presented a method for preparing SVFs that we used. Adipose tissue was taken from the Wistar rats' bilateral inguinal regions, diced, rinsed completely in phosphate-buffered saline (Gibco-BRL, USA), and following placed in the different tube. We centrifuged a 0.15 percent collagenase solution for 30 minutes at 37 degrees Celsius (Wako Pure Chemical Industries, Ltd., Japan). Equivalent amounts of Dulbecco's Modified Eagle Media solution (Gibco-BRL, USA), 1 percent antibiotic-antimycotic solution (Gibco-BRL, USA), and 10% fetal bovine serum were added to compensate for the collagenase activity (Gibco-BRL, USA). The suspension was centrifuged at 1,500 rate per minutes (rpm) for 5 minutes. The lowest layer (pellet) was removed, the SVF cells were extracted (approximately 5×10^4 SVF cells) as the final SVF product.

Preparation of the PRP

The blood specimen gathered from the donor rats was stored in an EDTA-filled tube. A two-step centrifuge technique was performed to collect the PRP.^{17,19} Initially, a three-layered solution was created by centrifuging the material for 10 minutes at 2,400 rpm. We made a 2-layered solution by centrifuging the superficial layer (supernatant) and the intermediate layer (buffy coat) for 15 minutes at 3,600 rpm. As the final PRP, we used the lowest layer (infranatant buffy coat).¹⁷

Preparation of the SVF and PRP Combination

A final volume of roughly 0.5 mL solution was made by mix the final SVF product with activated PRP by added 10% CaCl₂.

Wistar Rat Anal Trauma Model

We induce anal trauma model on the Wistar rats based on previous study with some modification.¹⁸ Initially, the rat was placed in a supine position followed by sedated with ether inhalation. Aseptic and antiseptic treatments were performed on the perineal and anal areas prior to surgery. A 6-French urinary catheter was utilized as a marker and a manual massage was performed to emphasize the rectum. Using a vertical incision (10 mm) in the anterior side of the perianal region, the adipose tissue was identified and dissected through the submucosal layer without injure the anal mucosal layer. The submucosa and muscle layer had been repaired with interrupted stitches and absorbable sutures.

In Group A, SVF and PRP were injected locally between the serous intestinal and subcutaneous layers with a total amount of 0.5 ml, with 0.25 mL split doses on both sides of the surgical site. Following the identical method as Group A, we injected 0.5 mL of normal saline into Group B. The skin dissection was repaired using interrupted stitches and absorbable sutures, and the anal trauma wound was cleansed with normal saline. All experimental rats were monitored and placed in a cage after regaining consciousness. Antibacterial (amoxicillin) and analgetic (paracetamol) treatment were given for three days after. There were no restriction for the rats to access the food and tap water.

Rat Termination and PMN Examination

Before being sacrificed, the rats were sedated with ether and then secured on top of the operating table. To acquire a blood specimen from the heart tip, we performed a thoracotomy surgery. Anal rats were biopsied, and the tissue was transported to HUM-RC for further analysis. A histopathology examination was performed to measure the PMN cells count using one field of view (400x magnification) with a light microscope (Olympus EX51, Japan).

Statistical Analysis

The obtained numerical data were reported as mean \pm standard deviation (SD) if they normally distributed. Alternatively, the data were reported as median \pm interquartile range if they not normally distributed. The data analysis was accomplished with the application SPSS version 26.0 (IBM Corp., New York). Repeated Measures test was used to compare the PMN cells count in the different follow-up period. We consider a p-value of less than 0.05 as significant result. Tables and graphs are used to present the findings.

3. RESULTS

The PMN cells count in the three rats group in different time period were shown in Table 1 and Figure 1. It was revealed that the Group A had higher PMN cells on the first day than the Group B, with a p-value of 0.002. On days 7 and 14, the Group A has fewer PMN cells than the Group B.

Table 1. PMN Cell Count

| Day examination | Group | N | PMN Cell Count (Mean ± SD) | p-value |
|-----------------|-------|----|----------------------------|---------|
| D1 | C | 4 | 1 ± 0.75 | 0.002* |
| | B | 4 | 60.5 ± 16.3 | |
| | A | 4 | 123 ± 16.4 | |
| D7 | B | 4 | 84.75 ± 9.60 | 0.074 |
| | A | 4 | 12 ± 12.5 | |
| D14 | B | 4 | 11 ± 8.2 | 0.10 |
| | A | 4 | 2.7 ± 2.2 | |
| Total | | 28 | | |

A: treatment group; B: placebo group; C: healthy control group; N: sample number, SD: standard deviation.

*significant with independent sample T-test

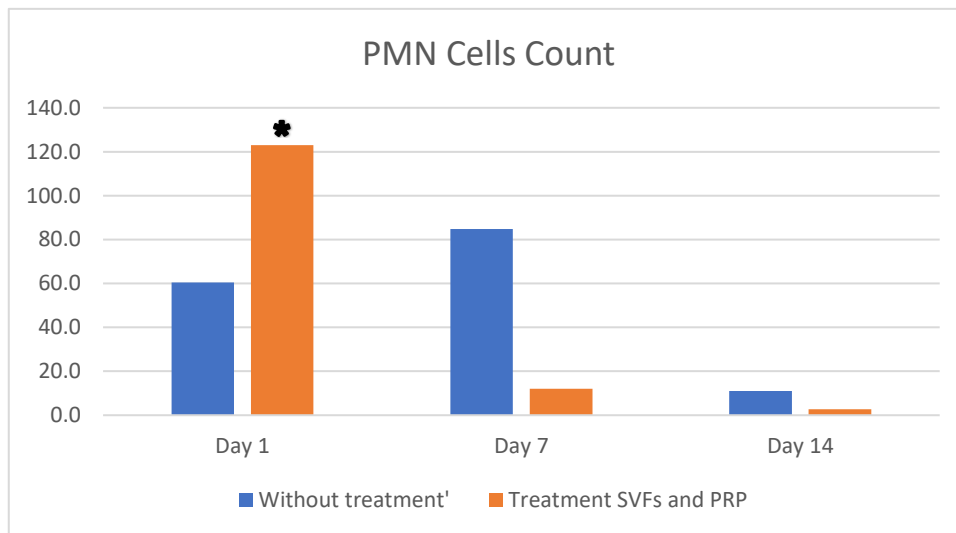


Figure 1. PMN Cells count on day 1, 7 and 14 show that PMN cell is higher in the treatment group on the first day.

*significant with independent sample T-test

All of the data was subsequently examined to assess the requirement for Repeated Measures Test. Based on the Shapiro-Wilk Normality Test, the data was normally distributed. With a p-value of 0.002, the statistical test

demonstrates that PMN was significantly higher on the first day in the treatment SVFs and PRP group than in the without treatment group. On the seventh day, PMN cell count was lower in the treatment SVFs and PRP group than in the without treatment group but statistically insignificant (p-value 0.074). In addition, PMN cell count was lower in the treatment SVFs and PRP group than in the without treatment group on the fourteenth day but statistically insignificant (p-value 0.10). Figure 2 depicts a representative image of each group's histopathological examination. PMN cells were discovered in greater numbers on the treatment group's trauma tissue than on the placebo group's on the first day. Furthermore, when the study was repeated on days seven and fourteen, PMN cells were discovered in the placebo group rather than in the treatment group's trauma tissue.

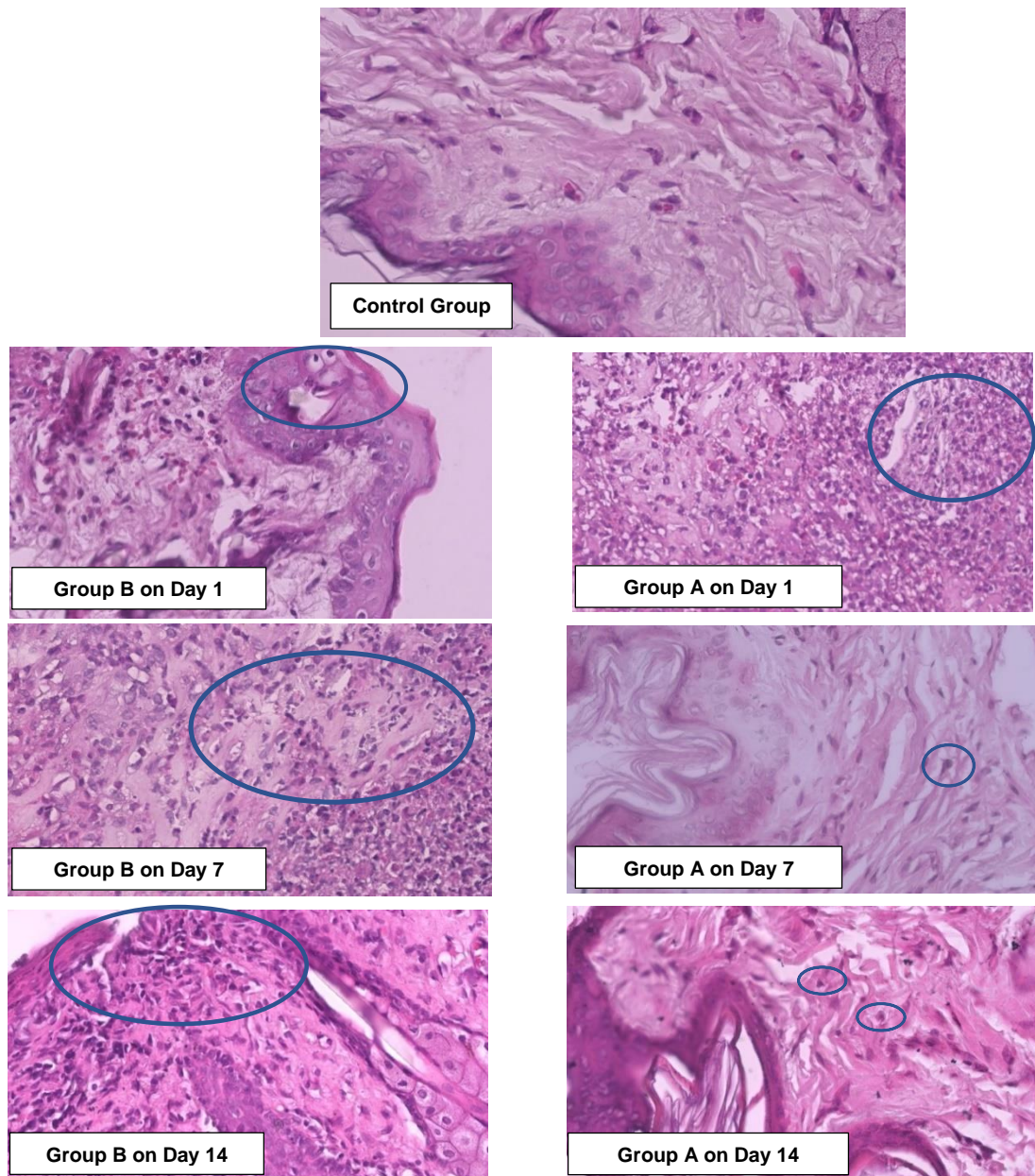


Figure 2. Representative images of histopathology examination. PMN cells are within the blue circle
 Note: A: *treatment group*; B: *placebo group*

4. DISCUSSIONS

The polymorphonuclear (PMN) cell is the first to arrive at the wound site. The numbers will swiftly rise and peak in the next 24-48 hours. The main function is to kill incoming bacteria via phagocytosis processes, increase blood vessel permeability, release prostaglandins, and stimulate neutrophil migration in the presence of chemotactic components such as complement factors, platelet factor 4, tumour necrosis factor- α (TNF- α), interleukin-1 (IL-1), or all-facet bacterial products.¹⁵

Josh et al.¹⁷ report on a study of the combination of SVFs and PRP for wound healing, specifically the Malondialdehyde indicator (MDA) and Nitric Oxide (NO), with the result that the combined PRP and SVFs against deep dermal burns show lower MDA and NO levels, which are metabolite products of lipid peroxidase, free radicals that cause macromolecular oxidative damage to cells.

Compared to vaseline administration, Laidling et al.²⁰ reported that the combined SVFs and PRP enhanced tissue granulation, re-epithelization, angiogenesis, and the count of PMNs. The combination significantly increased the count of PMNs in the first 48 hours compared to controls (vaseline).

Although the interaction mechanism between SVFs and PRP in tissue regeneration is unknown, various implications and studies have been documented. As a result, SVFs and PRP are mixed in a single culture medium. Compared to basal media, PRP media has a lot of proliferation activity. In human SVFs, PRP can also induce osteogenic proliferation and dissemination.²¹

PRP treatments and SVFs on rat skin graft transplantation tests were performed by Yeol et al. in 2014, and the neovascular structure was obtained eight weeks after transplantation, with no symptoms of hemorrhage or tissue contracture.²² For a long period, ASCs have been extensively explored. With complete consideration, knowledge of immunological characterization and possible differentiation grows. ASCs have been extensively promoted at many international medical conferences.²³

Cardoso et al.'s delivery of SVFs to full-thickness burns in mice was also investigated in PMN research. Microscopic and macroscopic evaluations were also looked at in the study. Collagen, hemorrhage, hyperemia, fibrin, PMN, and angiogenesis are evaluated microscopically. Necrosis, crust, granulation, and fibrosis are all evaluated macroscopically. On day three after receiving SVFs, the PMN cells count was significantly increase compared to the group without SVFs treatment, and on day 7, PMN levels were lower than controls, and on day 30, PMN levels were the same in both groups.²⁴ This finding is consistent with this study.

An increase in PMN in the SVFs group lowered the inflammatory response. Under the stimulation of Th 17, macrophages secrete various pro-inflammatory cytokines, and the production of IL-17 invites additional PMNs to come. Cardoso et al. also discovered that giving SVFs to mice with burn injuries changed the inflammatory mediator's macrophage activity to a wound healing mediator. In the SVFs group, increased extracellular matrix formation and anti-inflammatory cytokines IL-10 can control and diminish the inflammatory process.²⁴

This study has the advantage that it can show the effect of the combination of SVF and PRP on the healing of anal trauma wounds in the early phase of wound healing. This phase is the most critical phase in wound regeneration. In addition, this study offers the novel alternative technology for adult stem cell therapy which is relatively easy to obtain, abundant, and reliable for tissue engineering and regenerative medicine applications.^{11,25} However, this study has a limitation because it only measures one parameter, namely PMN cells. Whereas as has been known, the wound healing process is determined by many factors such as the presence of cytokines, chemokines, growth factors, and other cellular components. Further studies are needed to assess the potential of this combination stem cell therapy in anal trauma using more complete parameters.

5. CONCLUSION

The efficacy of combining SVF and PRP in accelerating the healing process was demonstrated, as measured by the amount of PMN in the treatment of anal trauma model. When compared to rats who did not get treatment, PMN counts were considerably greater on day one and decreased on days 7 and 14. This research shows that combining PRP and SVFs is a therapy with a bright future. The administration of SVFs and PRP altered PMN's role as a pro-inflammatory mediator into a mediator that expedited wound healing and reduced inflammation. It is hoped that this study will serve as a model for other researchers and contribute to the advancement of medicine in the future.

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Conflict of Interest Statement:

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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