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Wound Remodelling Anal Trauma in Wistar Rat After Application of Combination Platelet Rich Plasma (PRP) and Stromal Vascular Fraction Cells (SVFs)

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ABSTRACT

Anal trauma is a very rare condition. Although rare, anal trauma has the potential to cause anal stenosis, ectropion, or incontinence that can interfere with a person's quality of life. This problem can also be caused after surgery on the anus, which is the primary modality of this case. More severe complications can include peritonitis, sepsis, to death. Therefore, an effective non-invasive alternative therapy is urgently needed in healing anal trauma. This research was conducted using an experimental laboratory method on male Wistar rats using a post-test control group consisting of three groups treated with PRP+βSVF (each, n=4), three groups treated without PRP+SVF (each, n=4), and one control group (sacrifice on day 0). All the treatment groups were sacrificed on days 1, 7, and 14 post-treatment. The anal trauma was made through a longitudinal incision from distal to proximal with a depth of 5 mm from serosa to intraluminal. TGF-β was measured using the ELISA method. Data represented the mean +/- standard deviation and was further analyzed by the one-way Anova test. There was a significant difference between the mean TGF- β levels in the intervention group and the control group on day 1 (31.07±1.65 vs. 25.6±1.02, p=0.01) and day 14 (31.47±0.51 vs. 27.42±1.17, p= 0.01). Meanwhile, on day 7, there was no significant difference (29.37±2.27 vs. 26.44±2.61, p=0.18). A combination of SVF and PRP effectively accelerates the healing process of anal trauma in vivo, which is assessed based on TGF-β levels.

1. Introduction

Anal trauma is a rare case. According to epidemiological data, anal trauma is mainly reported in military

settings [1]. Cases of anal trauma alone (isolated) are rare. Several case reports have reported that anal trauma is often accompanied by trauma to the rectum and perineum [2], [3]. Several conditions have the potential to cause anal trauma, including childbirth, sexual intercourse, iatrogenic, ingested foreign bodies, and all types of both blunt and penetrating injury to the anus [4].

Although rare, anal trauma has the potential to cause anal stenosis, ectropion, or incontinence that can interfere with a person's quality of life. This problem can also be caused after surgery on the anus, which is the primary treatment modality of this case. More severe complications can include peritonitis, sepsis, and even death [5]. Therefore, an alternative non-invasive therapy is urgently needed that is effective in healing anal trauma.

Currently, stem cell therapy is the most studied therapeutic modality for mucosal epithelial regeneration. A meta-analysis conducted by [6] also shows much of the literature on the development of this therapeutic modality in patients with anal trauma who experience incontinence. Stem cells are primitive cells that can differentiate to replace cells that have been damaged, either due to disease or trauma [7]. Platelet Rich Plasma (PRP) is a stem cell therapy often used to heal mucosal wounds. The mechanism of PRP in wound healing is through the initiation of angiogenesis and the proliferation of undifferentiated stem cells [8]. Stromal vascular fraction (SVFs) is another stem cell that can improve wound healing by increasing cell proliferation and vascularization, strengthening inflammation, and increasing fibroblast activity [9].

Recently, only a few research data have directly described the effectiveness of using a combination of PRP and SVFs in cases of anal trauma. [10] conducted a study on sixteen male rats to assess the effectiveness of PRP in healing the intestinal mucosa and showed satisfactory results. In addition, [11] revealed that ADSC (of which SVF is one of its derivatives) could play a role in reducing traumatized cell apoptosis, decreasing intestinal permeability, increasing intestinal mucosal recovery, and increasing neovascularization in hypoxic conditions.

Based on the tremendous potential given by PRP and SVF on healing the gastrointestinal mucosa, the authors were interested in conducting this study that aims to see the effectiveness of the combined use of PRP and SVFs in cases of anal trauma by using the Transforming Growth Factor (TGF) as an indicator of anal mucosal repair.

2. Materials and Methods

This research was conducted using an experimental laboratory method on Wistar rats using a post-test control group of one group treated with PRP+SVF and without PRP+SVF (sacrifice on days 1, 7, and 14 post-treatment), and one control group (sacrifice on day 0). Healthy male Wistar rats, aged 16-20 weeks and weighing 170-260 grams were included. The exclusion criteria were damage biopsy sample, infection on the incision wound, and early death of the rats during treatment. This study followed the Helsinki Declaration guideline and was approved by the Ethical Committee of Health Research Medical Faculty of Hasanuddin University with registered number 654/UN4.6.4.5.31/PP36/2021.

2.1 Animal Preparation

Twenty-five male Wistar rats (*Rattus norvegicus*) were obtained from the Veterinary Laboratory of the Faculty of Medicine, Hasanuddin University. Before the treatment procedure, adaptation was carried out for two weeks by being kept in cages (40x20x20 cm^3), which each cage containing 4-5 rats. The temperature in the cage was set at room temperature (temperature 28 ± 2 ^{0}C, humidity 5-60%). Room lights with a 12-hour cycle on and off for 12 hours. The cage was cleaned every day. The rats were fed with 20 grams of standard

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AD2 pellets every day, and drinking water was given ad libitum.

The rat was shaved on the rat's back, then anesthetized using ether. In the donor group, a thoracotomy was performed until the heart was visible. The identification of the apex heart was then performed by puncture and aspiration of its blood contain using a three cc 25G syringe. It was continued for fat retrieval in both inguinal Wistar rats.

2.2 Preparation of PRP and SVFs

Wistar rats were shaved on the back of the rats; then inhalation anesthesia was performed using ether. The donor group underwent a thoracotomy until the heart was visible. Identification of the apex of the heart then aspirated blood from the apex using a three cc needle 25G syringe. Blood was collected from all donor rats via cardiac puncture to prepare PRP. The drawn blood was transferred to a tube containing EDTA. Blood was centrifuged for 10 min at 2400 rpm (450 g) for the first centrifugation. Plasma supernatant with the buffy coat was collected and centrifuged at 3,600 rpm (850 g) for 15 minutes. The infranatant buffy coat was suspended to prepare the final PRP product [12], [13].

SVFs preparations were derived from adipose tissue collected from all donor rats. Rat fat was taken from the left and right inguinal folds using a surgical knife. The fat taken was then washed with phosphate buffer saline and then chopped into small pieces until smooth and then put into a 15 cc size tube. 0.15% collagenase was added into a tube containing adipose and incubated at 37ºC for 30 minutes. The control medium Dulbecco Modified Eagle Media (DMEM) was filled with 10% FBS, and 1% antibiotic-antimycotic added to neutralize the activation of collagenase, then centrifuged at 1,500 rpm for 5 minutes. Cell pellets were resuspended with distilled water then the number of SVFs cells was counted using Trypan blue and the Neubauer counting chamber [13]. SVFs cells of 50,000 cells added with 0.5 aqua dest were transferred to Eppendorf tubes for the final SVFs product [14], [15]. The PRP + SVFs group used SVFs of 50,000 cells added with 0.5 ccs of PRP to become the final product of PRP+SVFs for the treatment group [12- 15].

2.3 Induction of Anal Trauma Rat Models

The rats were anesthetized using ether inhalation technique. The mouse was supine with room temperature and warm air condition. The antisepsis was performed on the operating area, particularly the perineal and anus region. The anal canal was emptied, and a 6 Fr catheter was inserted for marking. An anterior perianal incision of about 10 to 15 mm was made after the adipose tissue was identified, followed by sharp and blunt dissection. Then a 5 mm deep incision was made through the muscular layer to reveal the submucosa without injuring and penetrating the mucosal layer (catheter as marking). An interrupted suture was made on the mucosa [16]. After that, the submucosal and muscular layers were sutured using absorbable 6.0 sutures, followed by applying PRP and SVF injections on each side of the surgical wound. The cutaneous layer was sutured with absorbable sutures. Finally, the surgical wound was washed with 0.9% sodium chloride and covered with a dressing. The rats were then observed and returned to the cage when they were awake. The administration of antibiotics and postoperative analgesics and eating and drinking was arranged as usual.

2.4 The Procedure of PRP and SVFs Application

Four edges of the wound and one in the middle of the wound were marked using gentian violet to facilitate the evaluation of wound healing. In the treatment group, 0.1 cc PRP+SVFs were injected subcutaneously at the four edges of the wound (12, 3, 6, 9 hours direction) and in the middle of the wound, each with a total volume of 0.5 cc PRP + 50,000 SVFs cells for each experimental animal. In the control group, 0.1 cc

placebo solution was injected subcutaneously at the four edges of the wound (12, 3, 6, 9 hours direction) and in the middle of the wound with a total volume of 0.5 ccs for each experimental animal. The wound was covered with a transparent film, and then a girdle was placed in a circle covering the wound in the four groups. Antibiotics and analgesics were given to the whole rat drink. Rats were sacrificed on days 0, 7, 14, and 21 with thoracotomy procedure.

2.5 Measurement of Transforming Growth Factor beta (TGF-β)

During the thoracotomy procedure, the whole blood from the apex of the heart was aspirated using a 3 ml syringe. The blood sample was then examined in the laboratory for the enzyme-linked immunoassay (ELISA) test with standard procedure.

2.6 Statistical Analysis

All collected data were analyzed using SPSS software for Windows 21 (IBM SPSS Statistics for Windows, Version 21.0. IBM Corp., Armonk, NY). The TGF levels were tested for the data's normality distribution using the Shapiro Wilk method. Data distribution was presented in mean ± standard deviation (SD) for normal distribution and median ± range for not normally distributed data. Comparison of TGF levels between the three groups was tested using the one-way Anova test by first assessing homogeneity with the Levene method. Post hoc analysis was carried out when a significant difference was found in the One Way Anova test to determine which treatment groups had significant differences specifically. The p-value < 0.05 was considered significant.

3. FINDINGS AND DISCUSSION

This experimental study was conducted in the animal laboratory of the Faculty of Medicine, Universitas Muslim Indonesia, and the Laboratory of HUM-RC, Faculty of Medicine, Universitas Hasanuddin Indonesia, using 28 rats. The blood sample was examined using the Elisa kit in the HUM-RC laboratory of Hasanuddin University to obtain the TGF-β level. The comparison of TGF-β levels between the three groups was presented in Table 1. There was a significant difference (p = 0.001) between the three groups, namely the control group, the untreated group, and the treated group.

Table 1. Comparison of TGF-β levels in the control group, without treatment, and PRP + SVFs treatment based on the day of observation.

Day of observation	Group	TGF-β level (unit measure)	p-value[1]
Day-1	Control	27.72 ± 0.54	
	Without treatment	25.90 ± 1.02	0.01*
	PRP + SVFs treatment	31.07 ± 1.65	
Day-7	Control	27.72 ± 0.54	
	Without treatment	26.44 ± 2.61	0.18
	PRP + SVFs treatment	29.37 ± 2.27	
Day-14	Control	27.72 ± 0.54	
	Without treatment	27.42 ± 1.17	0.001**
	PRP + SVFs treatment	31.47 ± 0.51	

Data presented on mean ± standard deviation , [1]One-way Anova test, * p-value < 0.05,** p-value < 0.01

There was a significant difference on day 1 (p = 0.001) and day 14 (p = 0.001) between the treatment groups and non-treatment groups. The Bonferonni Post Hoc test (Table 2) was carried out on data analysis to determine the significance obtained in the one-way Anova test on day 1 and day 14. Significant differences on day-1 were shown between the control group vs. the treatment group (p = 0.01) and the non-

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treatment group vs. the treated group (p = 0.001). This significant result was in line with the findings on day 14 with differences in the control group vs. the treatment group (p = 0.001) and the non-treatment group vs. the treatment group (p=0.001).

Table 2. Post Hoc Bonferonni test on day 1 and day 14 observation.

Day Observation			p-value[1]
H1	Control	Non-treatment	0.16
		Treatment	0.01**
	Non-treatment	Control	0.16
		Treatment	0.01**
	Treatment	Control	0.01
		Non-treatment	0.01**
H14	Control	Non-treatment	1
		Treatment	0.01**
	Non-treatment	Control	1
		Treatment	0.01**
	Treatment	Control	0.01**
		Non-treatment	0.01**

[1]Post Hoc Bonferroni test, *p-value <0,01

Wound healing is a complex and dynamic interaction process between cell types, ECM, cytokines, and growth factors. PRP contains many growth factors, including VEGF, HGF, bFGF, EGF, TGF-β, IGF-1, and PDGF. Likewise, ADSC/SVF contains many growth factors and cytokines, which play a role in influencing angiogenesis, mononuclear infiltrates, fibroblasts, and collagen production. Several previous studies have reported that the administration of SVF and PRP can accelerate the wound healing process. Although the mechanism is not yet fully understood, the potential for this combination is capable of triggering progenitor cells and assisting in immune modulation, and rapidly overcoming the acute wound state leading to a complete healing phase.

Regarding the healing process itself, the research of reported that PRP combined with Mesenchymal stem cells (MSC) promotes wound closure and vascularization by increasing the production of TGF-β1 and PDGF at the transplant site [17]. In this study, differences in TGF were found between treatment and non-treatment groups, particularly on day 1 and day 14 after the intervention. These results indicate that the combination of PRP and SVF can produce higher TGF. A study supported these results that found TGF-β levels with PRP intervention were four times higher than Platelet Poor Plasma (PPP) [18]. This result is also in line with the study of [19] who reported that administration of PRP significantly increased the expression of TGF-β. Likewise, the study conducted by [20] in burned diabetic mice comparing PRP therapy and keratinocyte-like cells derived from locally derived MSC adipose tissue also found a pattern similar to the results of this study. They found that EGF (epidermal growth factor) levels increased on day 3, decreased on day 7, rose again on day 10, and peaked on day 14, similar to the results of this study. This process indicates the initiation of migration and proliferation at the beginning of the first week and after entering the second week.

This study also found a significant relationship between TGF levels in the treatment group (PRP + SVF) and the non-treatment group. Likewise, the results showed that, by day, there was a significant difference between the treatment group and the non-treatment group on day 1 and day 14 after the intervention p<0.05. These results are in line with the research of [21] in Wistar rats treated with anal trauma and looking at growth factor levels, namely EGF. The results showed a significant relationship between EGF levels in the

intervention and control groups on day 14. Fluctuations in the increase in TGF values during the first day were associated with the early stages of the healing process. Growth factors play a role in the process of restitution and cell proliferation. The process of migration, restitution and proliferation in this phase lasts until the end of the first week until re-epithelialization and remodeling occur in the area of the wound defect due to new tissue closure. TGF itself functions to increase the angiogenic properties of endothelial progenitor cells to facilitate blood supply to the traumatized site and stimulate fibroblast contraction for wound closure. The decrease in TGF value in the intervention group on day 7 could be associated with restitution and proliferation that turned into a re-epithelialization process. As the cell layer undergoes a re-epithelialization process, the growth factor release slows down, and its concentration decreases. The Day 7 was a transition period between the proliferation and remodeling phases, and the level of growth factor (EGF) reached the lowest level. Then, on the 14th day, the growth factor value rose again and approached the expected value. The wound defect has closed while the re-epithelialization phase has been reached, and TGF levels will return to normal limits.

The regulation of inflammation, cell proliferation, remodeling, extracellular matrix deposition, angiogenesis, and epithelialization plays an essential role in skin wound healing. SVF contains many types of cells, such as ADSCs, pre-adipocytes, fibroblasts, endothelial cells, macrophages, and lymphocytes. These cells use growth factors in PRP and secrete many growth factors and cytokines that modulate wound healing [22]. It is not just normal wound healing; [23] have reported that the combination of SVF and PRP also contributes to inhibiting the local response in burns by reducing the levels of malondialdehyde (MDA) and nitric oxide (NO). Several other studies, including this study, also support the superiority of the combination of SVF and PRP for wound healing or tissue regeneration.

3.1 Constraint

The are several limitations of the present study. This study is based on animal studies with the Wistar rat model, which has differences in humans, so further clinical studies are needed before being applied to daily practice. This study only focused on TGF-β levels and did not perform a clinical or histopathological assessment of anal trauma in a Wistar rat model. However, this study has some advantages. The combination of SVF and PRP in a Wistar rat model by assessing the outcome in the form of TGF-β levels is a novelty thing. The research method was carried out in a structured procedure with minimal bias risk. In addition, the results showed the potential of the combination of SVF and PRP as a modality for wound healing and post-traumatic tissue regeneration.

4. Conclusion

This study has revealed the effectiveness of using a combination of SVF and PRP in accelerating the healing process in the treatment of anal trauma in Wistar rats. The levels of TGF-β on the administration of a combination of SVF and PRP therapy were significantly higher on day 1 and day 14. This proves the combination therapy of PRP and SVF to be a promising therapy in the future. Furthermore, this research can be used as a reference for other research and is expected to assist in developing advanced medical technology later.

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