Experimental Research

The effect of Platelet-Rich Plasma and Stromal Vascular Fraction combination on Epidermal Growth Factor serum level for anal trauma healing in the Wistar rat model

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ARTICLE INFO

Keywords:
EGF
PRP
SVFs
Anal trauma
Wound healing

ABSTRACT

Introduction: Stromal Vascular Fraction cells (SVFs) and Platelet Rich Plasma (PRP) are clinically proven to aid in cell regeneration and wound healing. The healing effects can be measured by the level of Epidermal Growth Factor (EGF). This study aims to investigate the effect of an SVFs and PRP combination on EGF levels in the anal trauma model.

Method: Twenty-eight adult Wistar rats were divided into 3 groups: Group A consisted of healthy rats as a normal control group; Group B and C underwent modified anal surgical trauma and repair. Group B was treated with saline only and Group C was treated with local injection of a combination of SVFs and PRP after anal surgical repair. The EGF level was subsequently assessed on days 1, 7, and 14.

Results: EGF levels were generally increased in Group C compared to Group B. A one-way ANOVA test result showed significantly increased EGF levels on days 7 (p = 0.038) and 14 (p = 0.018). Based on the linear regression test results, we found that local injection of PRP and SVFs after anal repair on an anal surgical trauma model can increase the EGF level in group C by 36.9% more than that of group B.

Conclusion: The combination of PRP and SVFs can increase the EGF level in the wound healing process of anal trauma. EGF is critical in the anal trauma healing process.

1. Introduction

Anorectal traumas are associated with physical and emotional morbidity. Trauma sustained varies from simple anal laceration to rectal destruction and fistula formation. Anorectal trauma needs to be diagnosed immediately. The treatment varies according to time, trauma severity, location, and intra- or extra-peritoneal wound extension. Proper wound treatment can reduce the risk of bleeding, incontinence, wound dehiscence, stricture, and fistula formation [1,2].

In general, the wound healing mechanism is the center of attention in novel technology and therapy. A series of processes involving tissue regeneration and regulation hold an important role in organ homeostasis. Currently, the intestinal healing process is only partially understood. During the inflammatory phase, macrophages and neutrophils induce local tissue damage by secreting chemical compounds and enzymes, which then trigger the release of pro-inflammatory cytokines and cell-activating peptides that bind with the tissue matrix. If the tissue damage is severe enough, a myofibroblast will migrate to the defected area. This migration function and the ability to contract a wound and produce an extracellular matrix of the myofibroblast cell have an important role in the inflammatory phase. Current remedies for intestinal stricture, fibrosis, and fistulas are unsatisfactory, and selective and powerful therapies are needed to prevent continuous tissue damage to the healing area due to myofibroblast migration modulation and

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https://doi.org/10.1016/j.amsu.2021.102773
Received 3 August 2021; Received in revised form 23 August 2021; Accepted 2 September 2021
Available online 8 September 2021
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extracellular matrix synthesis [3]. There are several variations of growth factors, such as Epidermal Growth Factor (EGF), Transforming Growth Factor (TGF), Fibroblast Growth Factor (FGF), and Insulin-like Growth Factor (IGF), all of which are proven to accelerate recovery time and intestinal epithelial cell proliferation. The whole cell plays a part in the intestinal tissue healing process [4].

Epidermal growth factor (EGF) is an amino acid that has an important role in cell growth regulation, survival, migration, apoptosis, proliferation, and differentiation. EGF is critical in the wound healing epithelization process because it induces fibroblasts to migrate, grow, and accelerate healing. In addition, EGF has also been demonstrated to be an intestinal regulator and mucosal protective factor that helps preserve intestinal barrier integrity, which is essential to nutrient absorption, intestinal cell maturation, and maintaining intestinal cell homeostasis [5]. In several rapidly growing studies, EGF and other growth factors have been associated with attempted application of various forms of therapy, including current trends using platelet autologous therapies such as Platelet-Rich Plasma (PRP) and the use of stem cells [6].

One of the most abundant and easiest-to-harvest stem cell sources is adipose tissue. Stem cells are successfully isolated from adipose tissue 40 times more than from bone marrow. For SVFs, cell population content varies between fibroblasts, hematopoietic cells, endothelial cells, pericytes, preadipocytes, and mesenchymal stromal cells (MSCs) and can differentiate into chondrogenic, myogenic, adipogenic, and osteogenic. Quantitative Polymerase Chain Reactions (PCR) and Reverse Transcriptase PCRs show that the epithelization growth factor of the EGF, chemokines, Stromal Cell-Derived Factor (SDF-1 or CXCL12), Neutrophil-Activating Protein-2 (NAP-2 or CXCL7), and wound healing gene are highly regulated by SVFs. In addition, SVF itself does not require complex processing, complicated insulation, or culture procedures that need complete laboratory facilities, so it is easier to obtain compared to the other stem cell therapies [6,7].

Platelet-rich plasma (PRP) is the autologous platelet concentration above baseline normal platelet count. These large amounts of highly available platelets produce clinical benefits [8]. In platelets, we find growth factors that initiate wound healing such as platelet-derived growth factor, transforming growth factor, vascular endothelial growth factor (VEGF), epithelial growth factor (EGF), insulin-like growth factor (IGF-I, IGF-II), fibroblast growth factor (FGF), endothelial cell growth factor (ECGF), and platelet-derived angiogenesis factor (PDAF). PRP has been proven to encourage healing acceleration and tissue regeneration with its rich growth factor content. Significant use of PRP treatment in this modern era encourages the progression of successful stem cell combination therapy [8,9].

PRP and SVF combination therapy has been widely used in trauma cases to accelerate wound healing and has proven successful. However, PRP and SVF combination therapy in anal trauma cases has not been previously reported. Therefore, this study aims to analyze the effects of PRP and SVF combination therapy on EGF levels as one of the growth factors with a role in the anal trauma healing process.

2. Methods

This study used an experimental post-test control group design in adult male Wistar rats and consisted of 2 experimental groups, 1 control group, and 1 donor group.

All procedures were performed at Hasanuddin University Medical Research Center (HUM-RC) and the Animal Laboratory of Indonesian Muslim University after approval from the Animal Research Ethics Committee (19/UN4.6.4.5.31/PP36/2021) and according to the ARRIVE Guidelines (Animal Research: Reporting of In Vivo Experiments) for animal research [10].

2.1. Population and samples

The subjects were 28 (Federer Formula) male Wistar rats (Rattus Norvegicus) aged 16–24 weeks, weighing (BW) 170–260 g, and obtained from the animal laboratory at Hasanuddin University. Subjects were divided into 3 groups. The first group consisted of four healthy rats—the normal control group, used as baseline data (Group A). The other two groups, addressed as experimental groups (Group B and C, each consisted of 12 rats), underwent anal repair after anal trauma modeling (sacrificed on days 1, 7, and 14 post-treatment; 4 rats from each group were sacrificed for each day). After anal repair, Group B received no injections and Group C was treated with local injection of the SVFs and PRP combination.

We used the trauma sample technique following the anal sphincter trauma research model in rats carried out by Trebol et al. [11], which was later modified to suit this research.

2.2. Platelet-Rich Plasma (PRP) preparation

Blood samples were collected from all rat donors through a heart puncture to prepare PRP. The blood taken was transferred to an EDTA (ethylenediaminetetra-acetic acid) tube. Blood was centrifuged at 2400 rpm (450 × g) for 10 min for the first centrifugation. Supernatant plasma with a buffy coat was collected and centrifuged again at 3600 rpm (850 × g) for 15 min. The infranatant buffy coat was then suspended to prepare the final PRP product [7,12,13].

2.3. SVF preparation

SVF preparation came from fat tissue (adipose) collected from all rat donors. Fat tissues were collected from the left and right inguinal areas. The fats were then washed with phosphate buffer salts (PBS; Gibco-BRL, Grand Island, NY, USA), chopped small until smooth, and then inserted into a tube. We then added 0.15% collagenase (Wako Pure Chemicals) and incubated at 37 °C for 30 min. Next, we inserted media control Dulbecco Modified Eagle Media (DMEM; GIBCO-BRL) with 10% Fetal Bovine Serum (FBS; GIBCO-BRL) and 1% antibiotic-antimicotic (GIBCO-BRL) to neutralize collagenase activation and then centrifuged at 1500 rpm for 5 min. The cell pellet was resuspended with aquade. SVFs were then calculated using Trypan Blue and a Neubauer counting chamber. A total of 50,000 SVFs combined with 0.5 cc aquade were then transferred to an eppendorf tube for the final product [14].

2.4. PRP + SVF preparation

In the PRP and SVF combination group, 50,000 SVFs were combined with PRP until the volume reached 0.5 cc so that the final product was 0.5 cc for each treatment [12].

2.5. Anal trauma modeling and repair procedures

The following procedures were arranged and modified after understanding the morphology of Wistar rats and anal trauma modeling based on Trebol et al. [11]:

1. Wistar rats were given inhalation anesthesia using ether until fully sedated.
2. Rats were positioned in a supine position at room temperature, using warm air if necessary.
3. Operation area antisepsis was performed on the perineal and anal areas.
4. Anal canals were emptied and a 6 Fr Foley catheter was inserted as marking.
5. A vertical anterior perianal incision was performed around 10–15 mm. We then identified and dissected the adipose tissue through the
muscular layer until the sub-mucosal layer was visible without injuring or penetrating the anal mucosal layer with the catheter as a marking. If perforation occurred, interrupted stitches were made on the mucosa.

6. The submucosal and muscular layers were repaired with 3–4 interrupted stitches using absorbable sutures (6-0 RB-1 17 mm 1/2c Taper Coated VICRYL Ethilon).

7. For Group B, no injection was applied and we continued straight to step 8. For Group C, we continued with PRP and SVF injection (0.5 cc) in the tissue between the intestinal serous and subcutaneous layer with a divided dose (0.25 cc) on each side of the surgical wound.

8. The skin incision was then closed with interrupted stitches using absorbable sutures (6-0 RB-1 17 mm 1/2c Taper Coated VICRYL Ethilon).

9. Finally, the surgical wound was washed with 0.9% sodium chloride solution. All rats were observed and returned to the cage after regaining consciousness and were then given antibiotics (Amoxicillin 50 mg/kg BW/day) and analgetic (Paracetamol 10 mg/kg BW/day) until 3 days post-operation with usual eating and drinking habits.

2.6. Sacrifice procedure

Before sacrifice, we gave each rat inhalation anesthesia using ether. Rats were then fixated on top of the surgery table and we performed a thoracotomy. The apex of the heart was identified and punctured using a 25G needle with a 3 cc syringe. Blood was then aspirated and delivered through a catheter. The submucosal and muscular layers were repaired using absorbable stitches (6-0 RB-1 17 mm 1/2c Taper Coated VICRYL Ethilon).

10. Finally, the surgical wound was washed with 0.9% sodium chloride solution. All rats were observed and returned to the cage after regaining consciousness and were then given antibiotics (Amoxicillin 50 mg/kg BW/day) and analgetic (Paracetamol 10 mg/kg BW/day) until 3 days post-operation with usual eating and drinking habits.

2.7. Epidermal growth factor assay procedure

1. All reagents were prepared and all standards and samples were added to Microelisa Stripplate.

2. 50 μl standard were added to standard well.

3. 10 μl testing sample was added, then 40 μl sample diluent was added to testing sample; blank wells remained empty.

4. 100 μl of HRP-conjugate reagent was added to each well and covered with an adhesive strip, and incubated for 60 min at 37 °C.

5. Each well was aspirated and washed; the process was repeated four more times for a total of five washes. Each well was filled with Wash Solution (400 μl) using a squirt bottle and a manifold dispenser. Complete removal of liquid at each step. After the last wash, any remaining Wash Solution was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels.

6. 50 μl Chromogen solution A and 50 μl chromogen solution B were added to each well. Mixed gently and incubated for 15 min at 37 °C and protected from light.

7. 50 μl Stop Solution was added to each well. The color in the wells changed from blue to yellow.

8. Optical density was read at 450 nm using a microtiter plate reader within 15 min.

2.8. Statistical analysis

Data collected were divided according to data type. We selected the appropriate statistical method for each dataset and, finally, data analysis was carried out by IBM SPSS statistic software version 22 for parametric data using a one-way ANOVA test and a linear regression test, which is presented in the form of tables and graphics.

3. Results

There were four rats in each group consistent with the inclusion criteria; 4 control rats were not given any form of treatment (Group A), 12 rats underwent modified anal trauma and repair only (Group B), and 12 rats underwent modified anal trauma, repair, and treatment with a local injection of PRP and SVF combination (Group C). All rats in observation were healthy and active before sacrificed on day 1, 7, and 14.

Based on Table 1, the mean EGF value in Group A was 66.25 ± 3.89. On day 1 in Group B, the mean EGF value was 65.62 ± 3.84, while the mean EGF value for Group C was 73.92 ± 8.23. On day 7, both Group B and C had an overall decreased value. Group B had a mean of 59.40 ± 3.62, while Group C had a mean of 65.52 ± 2.62. On day 14, Group B had a mean EGF of 63.48 ± 4.06 compared to 72.06 ± 1.88 for Group C. Overall, the highest value was obtained from Group C on day 1 (73.92 ± 8.23), while the lowest value was from Group B on day 7 (59.40 ± 3.62).

4. Discussion

Rat anatomy is similar and comparable to human anatomy. The main difference is in the sphincter, while other layers of the rectum have a similar structure. Several previous types of research have posited that wound healing will accelerate when given SVFs and PRP treatment. Although the mechanism is not fully understood, its content potency and differentiation process are capable of triggering progenitor cells and assisting in immune-modulation, so that the combination of PRP and SVFs are able to transform the acute wound state immediately into a chronic wound, which then stimulates the overall healing process. Regarding the healing process itself, in several studies, EGF levels were shown to predict wound prognosis [11].

In this research, a significant difference was found between Group A and C, and between Group B and C. Group B had generally lower EGF levels than Groups A and C, indicating that the combination of PRP and SVF was effective in promoting wound healing. Furthermore, further testing was carried out on each variable every day. A normality test was performed on each group, and we obtained p > 0.05. A Levene homogeneity test was conducted to assess the homogeneity and showed p > 0.05, which indicates homogenous data. We then conducted a one-way ANOVA test for each group, as shown in Table 3, and obtained a significant p-value (p < 0.05) on the 7th- and 14th-day groups. However, the p-value of the first day group variable was not statistically significant (p > 0.05). The post-hoc test determined there was a significant difference on the 7th day between Group B and Group C (p = 0.033), as well as between Group B and Group A (p = 0.020; Fig. 1). On the 14th day post-hoc test, a significant difference was found between Group B (p = 0.006) and Group A (p = 0.040; Fig. 1).

A regression test was conducted to assess the effect of PRP and SVF combination therapy on anal trauma, as shown in Table 4. We calculated a regression test p-value<0.05, indicating that PRP and SVF therapy in anal trauma had a significant effect on EGF value, resulting in a 36.9% increase in EGF. Each PRP and SVF injection increased EGF value by 7.664 pg/mL, consistent with equation (Y = 62.835 + 7.664X).

Table 1

| N  | Mean ± Standard deviation | Min | Max |
|----|--------------------------|-----|-----|
| D1 | Group A 4 66.25 ± 3.89   | 61.81| 70.95|
|    | Group B 4 65.62 ± 3.84   | 60.44| 69.36|
|    | Group C 4 73.92 ± 8.23   | 65.78| 85.37|
| D7 | Group A 4 59.49 ± 3.62   | 54.34| 62.93|
|    | Group B 4 65.52 ± 2.62   | 62.26| 68.57|
|    | Group C 4 72.06 ± 1.88   | 70.28| 74.50|
| Total| 28 66.60 ± 6.09  | 54.34| 85.37|

There were four rats in each group consistent with the inclusion criteria; 4 control rats were not given any form of treatment (Group A), 12 rats underwent modified anal trauma and repair only (Group B), and 12 rats underwent modified anal trauma, repair, and treatment with a
Several previous studies have confirmed this result, such as in Bertrand-
receptors to increase cell proliferation, migration, and laminin deposit
that plays an important role in several cell growth processes. The
found a similar pattern to our study. They found that EGF level increased
2nd week. A study conducted by Hosseini et al. on diabetic rats with
process at the beginning of the 1st week and the end after entering the
EGF level increased on the 2nd day and decreased significantly on the
underwent anastomosis due to full-thickness defect, it was found that
values compared to Group A and Group C. Group C, which had higher
EGF values than the two other groups, demonstrated that the combi-
nation of PRP and SVFs results in higher EGF, thus providing a superior
score on the one way ANOVA statistic test compared to the other group.
Several previous studies have confirmed this result, such as in Bertrand-

| Group   | N   | Mean          | Shapiro-Wilk Normality Test | Levene Homogeneity Test | One-way ANOVA |
|---------|-----|---------------|-----------------------------|-------------------------|---------------|
| Group A | 4   | 66,25 ± 3,89  | 0,98                        | 0,76                    | 0,004         |
| Group B | 12  | 62,83 ± 4,4   | 0,697                       |                         |               |
| Group C | 12  | 70,49 ± 5,95  | 0,16                        |                         |               |
| Total   | 28  | 66,6 ± 6,09   |                             |                         |               |

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| Group   | Group | Mean Difference | Std. Error | P               |
|---------|-------|----------------|------------|-----------------|
| Group A | Group B | −4,24658 | 2,94192 | .161           |
|         | Group C | 3,41731 | 2,94192 | .256           |
| Group B | Group A | −3,41731 | 2,94192 | .256           |
|         | Group C | −7,66897* | 2,08025 | .001*          |
| Group C | Group A | 4,24658 | 2,94192 | .161           |
|         | Group B | 7,66897* | 2,08025 | .001*          |

Table 4

Regression test on PRP and SVF therapy group.

| P-value | R   | R-square | Equation |
|---------|-----|----------|----------|
| PRP + SVFs therapy | 0,002* | 0,607* | 0,369 | Y = 62,835 ± 7,664X |

In a study conducted by Saxena et al. [17] on the ileum of rats that
underwent anastomosis due to full-thickness defect, it was found that
EGF level increased on the 2nd day and decreased significantly on the
8th day. In addition, the most generative epithelial cell migration pro-
cess was found on the 2nd day and decreased gradually until the 12th
day, which proves that the cell migration and proliferation process in
intestinal epithelial regeneration is strictly related to EGF concentra-
tion. This is similar to our study results, which showed a significant increase
in EGF levels at the beginning of the experiment (1st day) and a decrease
on the 7th day, marking the start of the migration and proliferation process
at the beginning of the 1st week and the end after entering the
2nd week. A study conducted by Hosseini et al. on diabetic rats with
burn injuries that compared PRP therapy and keratinocyte-like cells
derived from adipose tissue locally derived mesenchymal stem cells also
found a similar pattern to our study. They found that EGF level increased
on the 3rd day, decreased on the 7th day, rose again on day 10, and
reached its peak on the 14th day. However, in the PRP only group, EGF
also increased on the 3rd day, decreased on the 7th, and then rose again
on day 14 [18].

As previously stated, epidermal growth factor (EGF) is an amino acid
that plays an important role in several cell growth processes. The cellular
effects of EGF are mediated by the ErbB1 receptor. In kerati-
nocyte, activation of ErbB1 by EGF requires the assistance of urokinase
receptors to increase cell proliferation, migration, and laminin deposit
proven to have a significant effect on increasing EGF level, which is defect closing speed can start normally. This was inversely proportional phases, and EGF reached its lowest level. Other intrinsic and extrinsic day was a transitional interval between the proliferative and remodeling normal limit. In this study, each group had a lower EGF on the 7th day, attributed to the fact that the re-epithelization phase has been achieved, cell protection can continue. Prolonged inflammation will lead to cicatricial tissue or fistula formation due to T cell, macrophage, and neutrophil infiltration causing extracellular matrix degeneration [3,4].

Several activation signals are involved in intestinal epithelial injury repair. In the epithelial layer, PI3K and Src signaling cascade work with Rac to trigger intestinal endothelial cell migration in response to EGF. Mesenchymal-epithelial interactions are important for proper gut morphogenesis and intestinal epithelial healing. Epimorphin is expressed on the surface of mesenchymal cells in several organs, including the intestine, and acts in epithelial cell morphogenesis as well as cell restitution under oxidative stress through the activation of EGF and MEK/ERK receptors, PI3K/Akt [4]. The migration, restitution, and proliferation process takes place during the first week until the re-epithelization and remodeling process occurs in the wound defect area due to new tissue closure. Decreased EGF value during the 7th day of our study could be related to the restitution and proliferation process that changes to the re-epithelization process. Similar to Kong et al.'s study [22], we also saw a decrease in EGF level during the 7th day, as well as healing and addition to the previously injured endothelial cell layer. Along with cell layers that have undergone the re-epithelization process, the release of EGF slows down and its concentration decreases, as seen in this study. Then, on the 14th day, the EGF value rises again and approaches a normal value (in this case the EGF value of Group A, which did not receive any form of treatment). This event can be attributed to the fact that the re-epithelization phase has been achieved, the wound defect has been covered, and EGF level will return to its normal limit. In this study, each group had a lower EGF on the 7th day, and the post-hoc test showed that Group B had the lowest value. The 7th day was a transitional interval between the proliferative and remodeling phases, and EGF reached its lowest level. Other intrinsic and extrinsic factors in each individual held a part so that the healing process and defect closing speed can start normally. This was inversely proportional on the 14th day, when Group C was significantly superior to Group A and B. The newly formed cells at the end of the inflammatory phase created an environment ideal for the healing process that caused the EGF level to rise again. When comparing Group A and B, the depletion of EGF levels was significantly lower in Group B.

In this study, EGF levels were only monitored for 14 days (proliferation phase). It’s best to keep a monitor on EGF levels until the remodeling phase so that researchers can figure out if the right treatment was administered in anal trauma healing. Other outcomes in rats, such as clinical appearance, histopathological examination (epithelialization, tissue granulation, and collagen synthesis), different ELISA levels (vascular endothelial growth factor, IGF, TGF, and so on), were not assessed in this study, which supports the assessment of therapy effectiveness. We recommend that further research be carried out by including these variables.

Mechanisms of action and the timing of the intestinal cell healing process vary between living things and are affected by several factors. PRP and SVFs combination therapy, even a single initial dose, was proven to have a significant effect on increasing EGF level, which is critical to the anal trauma healing process. The fact that the PRP and SVF efficacy data for anal trauma healing are limited highlights the need for phase I–II clinical trials. We hope that this research can serve as a fundamental resource and an additional reference when making decisions about the application of PRP and SVF as a new breakthrough in the management of anal trauma healing.

5. Conclusion

This research successfully proves that the application of PRP and SVFs can increase EGF level in the anal trauma healing process even from one dose at the beginning of an injury. EGF level is one of the most important growth factors in the healing process. Therefore, PRP and SVFs combination therapy is a promising treatment to increase growth factor levels.

Provenance and peer review

Not commissioned, externally peer-reviewed.

Acknowledgments

A higher appreciation to all staff from the Hasanuddin University Medical Research Center (HUMRC), Makassar, Indonesia.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.amsu.2021.102773.

Please state any conflicts of interest

The authors declare that they have no conflict of interests.

Please state any sources of funding for your research

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical approval

The study was conducted after obtaining approval from the Animal Research Ethics Committee (19/UN4.6.4.5.31/PP36/2021).

Consent

The research was conducted ethically in line with the ARRIVE Guidelines for Reporting Animal Research.

Author contribution

Ivanna Sirowanto, Fonny Josh, Sulmiati, Ahmadvirawan, Andi Alfian Zainuddin, Muhammad Faruk: study concept and design; Ivanna Sirowanto provision of study material and animal; Ivanna Sirowanto, Sulmiati, Ahmadvirawan, Fonny Josh collection and assembly of data; Ivanna Sirowanto, analysis and/or interpretation of data: Ivanna Sirowanto, Fonny Josh, Andi Alfian Zainuddin; manuscript writing: Ivanna Sirowanto, Final approval of manuscript: All authors critical revision: Fonny Josh.

Registration of research studies

Not applicable.
Ivanna Sirowanto, Fonny Josh, and Muhammad Faruk.

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