Research Article

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Effects of platelet rich plasma on the gastric serosal surface neomucosa formation: an experimental rodent model

Trombositten zengin plazmanın gastrik serozal yüzeydeki neomukoza oluşumuna etkileri: deneysel kemirgen modeli

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Abstract

Background: Autologous platelet rich plasma (PRP) is the platelet concentration obtained from thrombocytes in the plasma. During the healing process, the platelets are activated and then release the granules which stimulate the inflammatory cascade and healing process. Platelet derived growth factor, vascular endothelial growth factor (VEGF), transforming growth factor β (TGFβ), epidermal growth factor (EGF) and fibroblast growth factor (FGF) are valuable markers used for cell regeneration. The aim of this study was to investigate the potential effects of PRP treatment on the neomucosa formation, a potential technique for increasing the intestinal surface area in patients with short bowel syndrome (SBS).

Materials and methods: Thirty-two male Wistar-Hannover rats were divided into: sham, control, PRP-treated and last group for PRP preparation (n = 8). Plasma levels of VEGF, TGFβ, EGF and FGF were quantified by ELISA. En-bloc resection of anastomotic part was performed and stained with hematoxylin-eosin.

Results: VEGF, FGF, TGFβ and EGF levels were found significantly increased in PRP-treated group compared to others ($p < 0.001$). Neomucosa formation was observed in experimental groups but the area increased significantly in PRP group, compared to other groups ($p < 0.001$).

Conclusion: PRP therapy in gastrointestinal anastomoses is truly beneficial and surgically applicable treatment in SBS patients.

Keywords: Growth factor; Neomucosa; Platelet rich plasma; Rat; Short bowel syndrome.

Öz

Amaç: Otolog trombositten zengin plazma (TZP), plazmadaki trombositlerden elde edilen trombosit konsantrasyonudur. İyileşme sürecinde, trombositler aktive olur ve sonra inflamatuar kaskad ve iyileşme sürecini uyaran maddeleri granüllerinden salarlar. Trombosit kökenli büyüme faktörü, vasküler endotelyal büyüme faktörü (VEGF), dönüştürücü büyüme faktörü beta (TGFβ), epidermal büyüme faktörü (EGF) ve fibroblast büyüme faktörü

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(FGF) hücre yenilenmesinde kullanılan değerli belirteçlerdir. Bu çalışmanın amacı, kısa bağırsak sendromlu hastalarda (KBS) intestinal yüzey alanını arttırmak için kullanılan potansiyel bir teknik olan neomukoza oluşumunda TZP uygulamasının olası etkilerini araştırmaktır.

Gereç ve Yöntem: Otuz iki erkek Wistar-Hannover sıçan, şam, kontrol ve TZP uygulanan ve TZP hazırlanan gruplara ayrıldı (n = 8). VEGF, TGFβ, EGF, ve FGF plazma düzeyleri ELISA ile belirlendi. Anastomotik parçaların en-blok rezeksiyonu gerçekleştirildi ve hematoksilen-eozin ile boyandı.

Bulgular: VEGF, TGFβ, EGF ve FGF düzeylerinin TZP-uygulanan grupta diğerlerine göre belirgin bir şekilde arttığı bulundu ($p < 0.001$). Neomukoza oluşumu deneysel gruplarda gözlendi fakat diğer gruplarla karşılaştırıldığında neomukoza oluşum alanı TZP grubunda belirgin şekilde arttı ($p < 0.001$).

Sonuç: KBS'lu hastaların gastrointestinal anastomozlarında TZP tedavisi gerçekten faydalı ve cerrahi olarak uygulanabilir bir tedavidir.

Anahtar Kelimeler: Büyüme faktörü; Neomukoza; Trombositten zengin plazma; Sıçan; Kısa bağırsak sendromu.

Introduction

Patients with short bowel syndrome (SBS) has reduced intestinal absorption according to malabsorption and subsequent malnutrition, which often occurs after due to congenital defects, surgical bowel resection (volvulus, etc.) or loss of absorption associated with the disease [1, 2]. SBS may arise after the resection of more than 50% of small intestine and is certainly emerges after resection of more than 70% or when the less than 100 cm of small bowel left [3]. Increasing nutrient and fluid absorption by slowing intestinal transit time or increase the area of absorption via development of new intestinal mucosa, called neomucosa, must be purpose of the patients' surgical treatments [3, 4]. Neomucosa formation is a potential technique for increasing the intestinal surface area including the growth of new intestinal mucosa, which takes advantage of the regenerative capability of the intestine. The regenerated intestine develops by lateral ingrowth from the surrounding mucosa and is functionally similar to normal intestinal mucosa [5].

Autologous platelet rich plasma (PRP) is the platelet concentration obtained by gradient centrifugation from thrombocytes in the plasma. During the healing process, the platelets are activated and aggregate together and then release the granules which stimulate the inflammatory cascade and healing process [6, 7]. Today, PRP injections have been safely used in many fields including dermatology, sports medicine, orthopedics, cosmetics, fasciomaxillary treatments, urology and neurophysiology and even in treatment of ulcers on extremities [6, 8–11]. In surgery, some growth factor such as platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor β (TGFβ), epidermal growth factor (EGF) and fibroblast growth factor (FGF) are valuable markers used for cell generation or tissue regeneration [12–18].

So far, various animal models have been used to study the growth of intestinal neomucosa in full thickness defects patched with a variety of surfaces, including colonic serosa and abdominal wall [1, 5, 12, 19]. One of the most popular technique is the serosal patch technique. However, because of the limited serosal surface and anatomic factors, in many cases, only short segments of small intestine can be patched [5] and all procedures are still experimental. On the other hand, PRP has never been used in intestinal neomucosa formation with serosal patch technique. Among properties of PRP, there is increase tissue vascularity through increased angiogenesis. Increased angiogenesis leads to increased tissue vascularization, increased collagen synthesis, increased epithelial and granulation tissue production rates, postoperative leaks prevention and pain relief by serving as hemostasis and lymphatic band in the wound. Thus, PRP may help as an ideal paste-like agent in reconstructing the tissue and neomucosa formation since it is biologically compatible, effective and reliable [13].

In this experimental study, our aim was to investigate the potential biochemical and histopathological effects of PRP treatment on the neomucosa formation of the serosal surface of the stomach that was used as serosal patching in terminal ileal defect on rats to grow new intestinal mucosa. Our hypothesis was that PRP application promotes the healing process of intestinal anastomosis and neomucosa formation probably by accelerating tissue regeneration and remodeling through the pathways of VEGF, EGF, FGF, TGFβ factors.

Materials and methods

Experimental design

Thirty-two male Wistar-Hannover rats (300–500 g), obtained from Bagcilar Training and Research Hospital

Animal Center (BADABEM), were housed in cages under controlled room temperature (21 ± 2°C), humidity (60–70%) with 12-h light-dark schedule and were fed with standard pellet, ad libitum (MBD Animal Feed, Kocaeli, Turkey). Animals in all groups were freely fed with diet which contained 21% protein. Fresh drinking water was given every day. All subjects were kept in groups in different cages. Ethical approval were obtained from BADABEM (2016/31).

Study groups and treatment

Rats were randomly divided into four groups (n = 8). Group 1 was assigned as a sham group without surgery. Group 2 was the control group for ileogastric anastomosis performed between mucosal surface of the ileum and serosal surface of the stomach. Group 3 was treated with PRP after operated for the same anastomosis. Group 4 was used only for PRP preparation, used only after 20 days of the experiments for their bloods. All rats were euthanized and bloods of the first three groups (Group 4 was used only for PRP preparation from their blood) were collected by cardiac puncture, centrifuged and stored at −80°C for the measurement of VEGF, FGF, TGFβ and EGF. Also, after midline laparotomy, en-bloc resection of anastomotic part of terminal ileum and stomach was performed and resected tissues were fixed for histopathological examination.

PRP preparation

To execute the experimental procedures, the rats of Group 4 were anesthetized through intramuscular and intraperitoneal injection of xylazine (10 mg/kg) and ketamine (60 mg/kg), respectively. Hearts were exposed to subcostal incision and approximately 5–7 mL blood was collected by cardiac puncture and then the rats were sacrificed. PRP was prepared using the KYOCERA Medical PRP Kit (KYOCERA Medical Corporation, Osaka, Japan). The blood draw occurred with the addition of an anticoagulant, such as citrate dextrose phosphate (ACDA) or sodium citrate to prevent platelet activation. Blood was centrifuged twice; the firstly it was centrifugated at 600 $g \times 7$ min and the secondly at 2000 $g \times 5$ min. Platelet-poor plasma (PPP) was exposed after second centrifugation. The 2/3rd of the solution was composed mostly PPP and the lower portion (1/3rd) composed the PRP. Lower portion was aspirated and then placed into tubes. About 10% of the blood was obtained as PRP depending on the baseline platelet count of rats. The PRP was activated with 0.5 M $CaCl_2$ at 1/10th of the amount of the total PRP according to the manufacturer's instructions. Total time of blood collection and application of PRP did not exceed 1 h, in fact guaranteed the integrity of the blood cells applied on the intestinal mucosa.

Surgical procedure and PRP treatment

Rats were anesthetized by a ketamine/xylazine anesthesia with dosage of 10 mg/kg xylazine and 60 mg/kg ketamine (Ketalar®, Parke-Davis, Istanbul, Turkey, and xylazine 10 mg/kg; Rompun®, Bayer, Istanbul, Turkey) and their abdomens were shaved. Antisepsis and asepsis was provided by 10% povidone-iodine solution and three cm midline abdominal incision was done under aseptic conditions.

Only laparotomy was applied to the Group 1 (sham group) under anesthesia.

Stomach and jejunum of the Group 2 (the control group) were manipulated and exposed.

In the Group 3, 1 cm longitudinal incision was applied on the jejunum. 0.5 mL PRP was injected to submucosal jejunum. The anastomosis of the jejunal defect with the serosal surface of stomach was performed with continuous 6.0 polypropylene sutures after the PRP injection (Figure 1A).

The hearts of rat in the Group 4 were exposed to subcostal incision in order to obtain the PRP. About 5 mL blood were collected and the rats were sacrificed.

Each rat was subjected to relaparotomy under ketamine/xylazine anesthesia on postoperative day 30, and sacrification of the rats was achieved by intracardiac puncture.

Biochemical analysis

Four to five milliliter blood samples collected by cardiac puncture were centrifuged for 10 min in 4000 rpm at +4°C. Serum samples were separated into portions and stored at −80°C. Plasma levels of VEGF, TGFβ, EGF and FGF were quantified by using enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturers' instructions (Boster Biological Technology, Pleasanton, CA, USA). These particular assay kits were chosen because of their high degree of sensitivity and selectivity and inter- and intra-assay precision, and the small amount of plasma sample required to conduct the assay. ELISA assays were evaluated with Biotek GEN5 calculation program by using

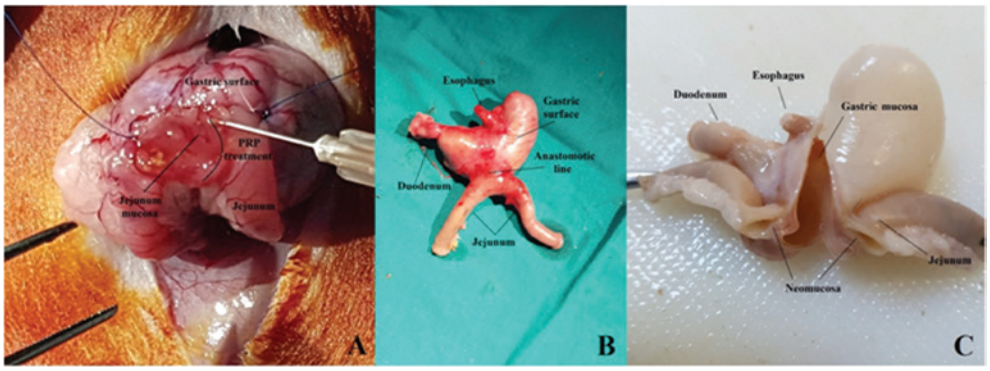

Figure 1: Macroscopic findings of neomucosa formation.
(A) Application of platelet rich plasma (PRP) to the anastomotic line (along the staple line). (B) Anastomotic line between the gastric surface and jejunum. (C) Neomucosa formation on the gastric surface area of the fixed tissue.

Biotek Synergy™ HTX Multi-Mode microplate reader and Biotek 405™ LS microplate washer.

Histopathological analysis

The anastomotic tissues were fixed in 10% formaldehyde and were routinely processed. Four micrometer-thick paraffin sections were obtained and stained with hematoxylin-eosin for the evaluation of inflammatory process, fibrosis, granulation tissue and neomucosa formation by two pathologists.

Chronic inflammation, fibrosis and formation of granulation tissue were classified into the following scales: 0 (minimal), 1 (mild), 2 (moderate), 3 (severe) [20]. The scores were multiplied by 0–3 to reflect 0%–100% extent of the section, respectively, 0–25%, 25–50%, 50–75%, 75–100%. Two sections of the tissue were evaluated and averaged for each animal. Neomucosa formation was given as percentage of newly formed epithelial region in the measured wound area.

Statistical analysis

GraphPad Instat (Version 3.06, 2003, USA) statistical program was used for statistical analysis. Descriptive statistical data (mean, standard deviation) of each group were compared by one-way analysis of variance (ANOVA) and subgroup analysis was performed by Tukey-Kramer multiple comparisons test. ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov test. All groups passed the normality test. p-Values of 0.01 and 0.001 were considered statistically significant.

Results

Macroscopic findings

In macroscopy, anastomotic line was observed between the gastric surface and jejunum on removed tissue of the both group (Figure 1B). After fixation, neomucosa formation on the gastric surface area was indicated along the staple line at both sides especially in PRP treatment group (Figure 1C).

Biochemical findings

ELISA analysis for VEGF and TGFβ of three groups (sham, control and PRP treatment groups) showed that there were significant increases in blood levels of control group compared to the sham group ($p < 0.001$ and $p < 0.01$, respectively). Also, when VEGF and TGFβ levels of sham and PRP treatment groups were compared, treatment group had significantly increased levels ($p < 0.001$ and $p < 0.01$, respectively) (Table 1). EGF and FGF levels also increased significantly in the bloods of the PRP group ($p < 0.001$ and $p < 0.01$, respectively) compared to the control and sham group. However, EGF and FGF levels were not significantly different for the control and the sham group.

Histopathological findings

Microscopic evaluation of the control and PRP treatment groups were presented in Figure 2. In the control group, gastric mucosa on the upper part of full-thickness of stomach wall had anastomotic regenerative intestinal epithelium on both sides. Neomucosa formation was

Table 1: Blood levels (pg/mL) of biochemical parameters (VEGF, TGFβ, EGF and FGF) of the three experimental groups evaluated by ELISA.

pg/mL	Group 1 (sham) (n=8)	Group 2 (control) (n=8)	Group 3 (PRP) (n=8)
VEGF	184.69±10.35	254.59±12.76[a]	369.03±10.85[a]
TGFβ	105.73±0.30	161.86±5.51[b]	269.45±18.64[b]
EGF	39.32±2.89	70.99±4.78	230.20±15.55[a]
FGF	29.93±1.23	31.99±1.28	39.79±1.99[b]

Variables were given as mean±standard deviation.

[a]$p < 0.001$ vs. all groups. [b]$p < 0.01$ vs. all groups.

PRP, Platelet rich plasma, VEGF, Vascular endothelial growth factor; TGFβ, transforming growth factor β; EGF, epidermal growth factor; FGF, fibroblast growth factor; ELISA, enzyme-linked immunosorbent assay.

minimal and granulation tissues were covering the most of the serosal surface (Figure 2A). On the other hand, PRP treatment group had an enlarged gastric mucosa on full-thickness of the stomach wall, and new mucosa formation (neomucosa) on serosa (Figure 2B). The epithelium with their villi were continuously shown on the intestinal neomucosa, with goblet cells and absorptive cells surrounded the intestinal villi.

None of the histopathological processes including inflammatory process, fibrosis, granulation tissue and neomucosa formation were observed in the sham group, coming with zero score (Table 2, Figure 3). Histomorphological evaluations showed similar intensity of chronic inflammation and fibrosis in both control and PRP treated groups (Figure 3). However, average scores of granulation tissue formation showed significant differences between all groups. The mean of granulation increased gradually in the control group and then in PRP treatment group, compared to the sham group ($p < 0.01$ and 0.001, respectively). As the main outcome of the study, neomucosa

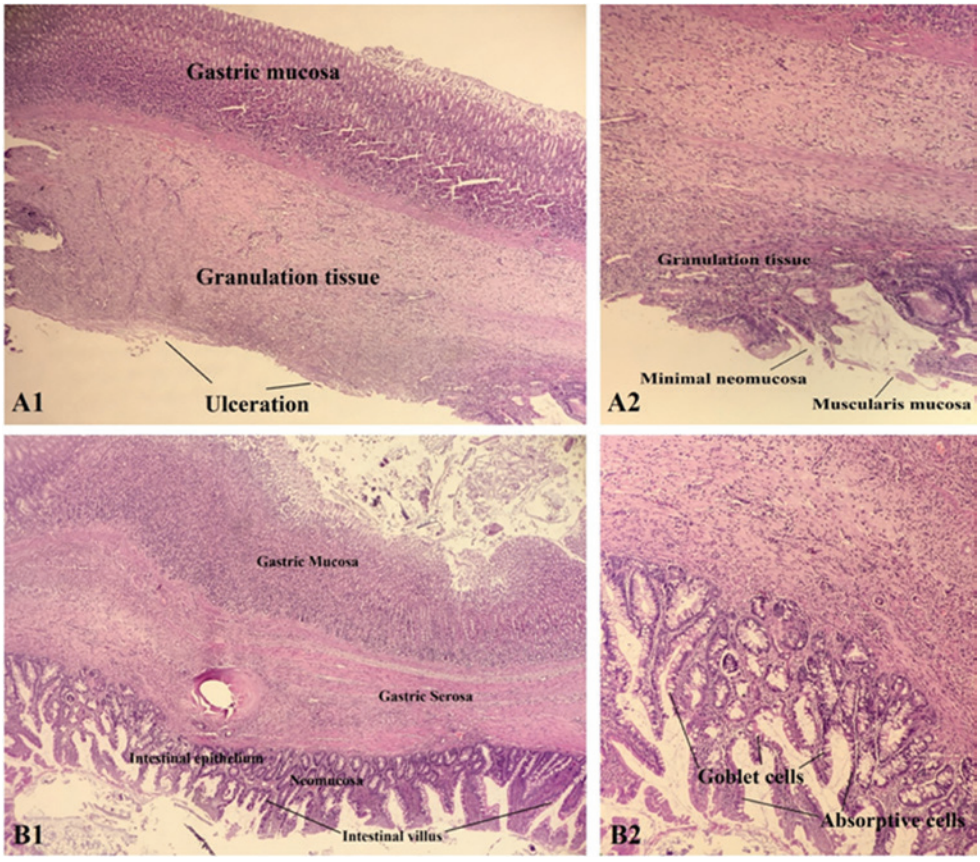

Figure 2: Histopathological and morphological evaluation of control (A1, A2) and PRP treatment groups (B1, B2), Hematoxylin and eosin. (A1) The control group (×100). (A2) Magnified figure of A1 (×200). (B1) The PRP treatment group (×100). (B2) Magnified figure of B1 (×200).

Table 2: Histopathological findings for inflammatory process, fibrosis, granulation tissue and neomucosa formation of the three groups.

		Group 1 (sham) (n=8)	Group 2 (control) (n=8)	Group 3 (PRP) (n=8)
Chronic inflammation	X±SD	0±0[a]	1.38±0.18	1.5±0.19
Fibrosis	X±SD	0±0[a]	1.25±0.25	1.25±0.16
Granulation tissue formation	X±SD	0±0	0.63±0.18[b]	1.38±0.18[a]
Neomucosa formation	%±SD	0±0[a]	39.25±6.04[a]	85.63±4.17[a]

X±SD, Mean±standard deviation; PRP, platelet rich plasma.
[a]$p < 0.001$ vs. all groups. [b]$p < 0.01$ vs. all group.

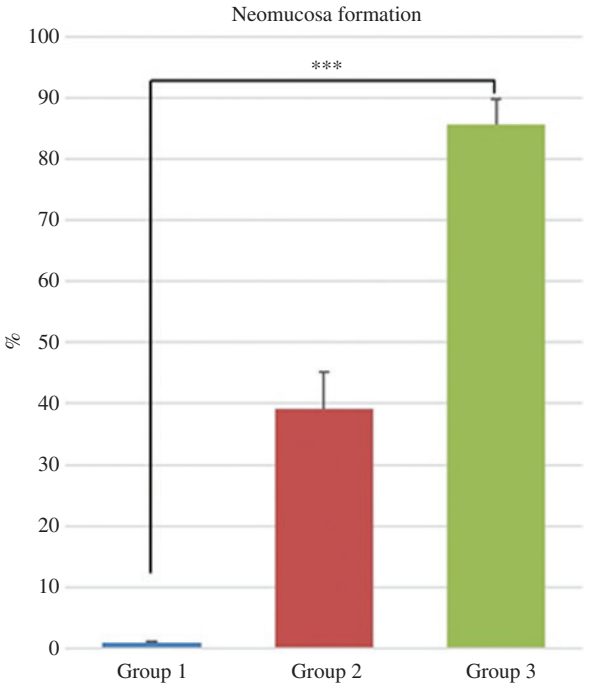

Figure 3: A graphic for the histopathological scores of the sham (Group 1), control (Group 2) and platelet rich plasma (PRP) treatment group (Group 3).
**$p < 0.01$ and ***$p < 0.001$ vs. all groups.

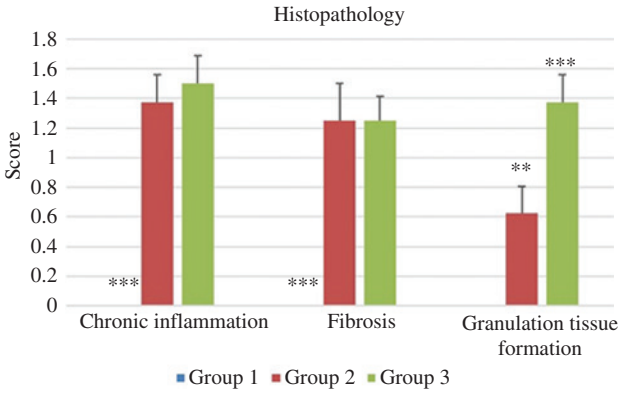

Figure 4: A graphic for the percentage of neomucosa formation showing increased neomucosa ratio both in the sham (Group 1), control (Group 2) and platelet rich plasma (PRP) treatment group (Group 3).
***$p < 0.001$ vs. all groups.

formation was observed in all rats of control and PRP treated groups but the percentage of the total neomucosa area was 85.63% in PRP treated rats whereas the percentage in the control group remained as 39.2% (Table 2, Figure 4). Thus, the mean ratio of neomucosa area increased significantly in PRP group, compared to the control and sham group ($p < 0.001$).

Discussion

In the present study, the main outcome is the anastomotic healing effect of PRP in neomucosa formation in intestinal anastomosis depending on modulating four valid determinant factors, VEGF, EGF, FGF, TGFβ. We affirmed our hypothesis by revealing that PRP application to the intestinal anastomosis significantly promoted the healing process of anastomosis probably by accelerating tissue regeneration and remodeling through increasing VEGF, EGF, FGF, TGFβ factors.

The usage of stomach serosal patching to grow new intestinal mucosa is a technique used for enlargement of the mucosal surface [3, 4]. The regenerated intestinal mucosa develops by lateral ingrowth of the adjacent mucosa and has same functions as normal intestinal mucosa. Up to date, certain surgical procedures have been directed towards increasing the surface and time of absorption. The main autologous intestinal reconstruction procedures are the longitudinal intestinal lengthening and tailoring known as Bianchi Procedure and the serial transverse enteroplasty. They have serious complications like stricture, leakage and bleeding [7]. Thus, we aimed to use growing neomucosa technique as a treatment model of SBS.

Growing factors such as VEGF, PDGF, TGFβ, EGF and IGF have been discussed in several studies according to their healing and regeneration effects [21, 22]. PRP contains different concentrations of these growth factors that constitute the theoretical basis of the use of PRP in healing and neomucosa formation. It has been used in general surgery to solve one of the major problems of

the surgeons: intestinal anastomotic leakage. However, the beneficial effects of PRP have not be elucidated in growing neomucosa method as a treatment model of SBS.

Local bacterial contamination in the anastomosis is caused re-epithelialization of the damaged mucosal borders and secondary inflammatory response. Furthermore, in the intestine several bacterial products, such as the endotoxin lipopolysaccharide produced by Escherichia coli bacteria, influence epithelial homeostasis and wound healing [23]. Production of proinflammatory and anti-inflammatory cytokines such as TGFβ could be observed in this process. According to this findings, the increase in TGFβ levels can be explained by anastomosis and inflammation. Moreover, TGFβ in PRP affects the proliferation of fibroblasts according to their biological features as a paracrine growth factor.

The phases of wound healing are inflammatory, proliferation and maturation phases. TGFβ, an anti-inflammatory/prohealing cytokine, is major regulator of wound healing. In some studies, it was shown TGF presented high levels in the proliferative phase. Inflammation is an supplementary part of wound healing, and the pro-inflammatory cytokines are significant in this treatment. Injury begin an acute inflammatory reaction that enclose the discharge of a number of cytokines including IL-1β, TNFα and IFNγ. Following the intervention after the early injury, there is a replacement in the cytokine profile from pro-inflammatory to anti-inflammatory/prohealing cytokines, including IL-10, IL-13 and TGFβ. The anti-inflammatory cytokines restrict the period and greatness of the inflammatory response that provides the wound accesing to proliferative phase. This shift from an inflammatory environment to a healing one is coordinated by macrophages, likely under the direction of cytokine signals. Redstones et al. showed that, in the dynamic milieu of the healing process, glucagon-like-peptid-2 increased wound IL-1β, IFNγ levels but not TGFβ. Therefore, patients receiving GLP-2 agonist therapeutically who require surgery should not have impaired anastomotic healing [24].

TGFβ impact on the equilibrium between synthesis and breakdown of the ECM, inducing an elevation in production of matrix components and a reduction in proteolytic activity of ECM, resulting in fibrogenesis and differences in the ECM. Also, regulatory T cells are tethered by TGFβ. Therefore, TGF suppression may decrease fibrosis but rises inflammation and tissue damage in the intestinal wall. On the other hand, some studies concern that a common TGF suppression might be followed by inadequate wound healing.

However, in some models, it appears that low concentrations of TGFβ stimulate fibrosis, while high concentrations of TGFβ inhibit fibroblast proliferation and collagen synthesis [25]. In our study, TGFβ was found as significantly increased in PRP treated group compared to non-treated groups. It is suggested that there should be a balance between TGFβ levels and inflammatory factors to accelerate serosal neomucosa formations, as in the case of fibrosis and surgical wound healings.

In our study, EGF and FGF levels were also found significantly increased in PRP treated group compared to control and sham groups. This is based on the characteristic of PRP, initiating the healing process via the degranulation of the α-granules in platelets after their activation; these granules contain synthesized and prepackaged growth factors including PDGF, TGFβ, VEGF and EGF. These growth factors are active polypeptides which facilitate regeneration of injured tissue through acceleration of cell proliferation and matrix formation [26] thus, it is natural to show increased levels of these factors in PRP treatment. Moreover, FGF is known to regulate the tissue homeostasis and vascular branching morphogenesis and also increases the levels of TGFβ [19], so we observed increased levels of FGF in accompany with the elevated levels of TGFβ.

The reason for the treatment of PRP is to reduce the amount of red blood cells that are less useful in the healing process and to increase the rate of platelets amount of to 94% to stimulate healing [27]. Leukocytes produce metalloproteinase, free radicals, reactive oxygen species, and nitrogen, which can cause damage to healing [28]. Limitation of our study may be the presence of small amount leukocytes in PRP.

In years, the number of relevant articles have been published concerning PRP treatment, but comparing the all findings is arduous, because of the differences in processing platelets and using the distinct techniques. Most of them in accordance with positive effect of these substances on anastomosis repair, while the one article showed that applying of PRP could only enhanced fibrosis and granulation tissue, without restoration the breaking strength of anastomotic areas [29]. In our study, the fibrosis degree increased independently of PRP treatment but the level of granulation on the tissue was significantly different between non-treated and treated rats.

Inflammatory, proliferative, and the maturation phases which occurred with overlapping form intestinal healing process. And they take respectively, 0–4, 3–14 and 10–180 day after the surgery [30]. Usually, during the first phase, fibrin contributes to wound healing and strength, but the major strain is allocated to the

sutures. Fibrosis which is the finding of secondary wound healing is composed of the chronic phase of healing. In our study, it was detected at similar density in all tissues, with no significant difference in the histopathological examination that was performed at a late stage. Therefore, fibrosis levels became similar between our non-treated and treated rats since FGF levels in PRP treated rats were also not as high as the levels of other biochemical parameters.

The main outcome of the study is that neomucosa formation in PRP treatment group was observed as approximately two-fold larger than the area of the control group. The use of autologous PRP has been considered a promising advance for new surgical and clinical approaches. Furthermore, recent advances in PRP usage should eliminate the risk of immunological reactions. This type of biological treatment mimics natural tissue healing, while optimizing and reducing the time required for therapy [5]. Our histopathological and biochemical findings reveal that the PRP therapy in gastrointestinal anastomoses is truly a beneficial and surgically applicable treatment.

It is important to point out that PRP therapy can be excellent preventive surgical technique against the anastomotic leakage. Furthermore, treatment with PRP might potentially find their place in a health system situation that is characterized by an increasing shortage of financial resources.

Conflict of interest statement: All authors declared that there are no financial relationships with any organizations that might have an interest in the submitted work; no other relationships or activities that could appear to have influenced the submitted work.

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