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ORIGINAL ARTICLE

The Effect of Platelet-Rich-Plasma on the Healing of Left Colonic Anastomosis in a Rat Model of Intra-Abdominal Sepsis

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ABSTRACT

Objective: The aim of this study was to investigate the effect of platelet-rich plasma (PRP) on the healing of colonic anastomosis in the presence of sepsis. *Materials and Method:* Fifty Wistar-albino male rats were used. Ten healthy rats were euthanized to prepare PRP, the rest were subjected to colonic anastomosis and randomly allocated into four groups of 10 rats each as anastomosis without PRP (C), without PRP in sepsis (SC), anastomosis with PRP (C-PRP), and with PRP in sepsis (S-PRP). Sepsis was induced by cecal ligation and puncture procedure. All animals were euthanized on postoperative day 7. The body weight change, anastomotic bursting pressure (ABP), tissue hydroxyproline (TH) and histopathological examination of each group were analyzed by using one-way analysis of variance (ANOWA) and Tukey's HSD post-hoc test to assess the differences between the groups. *Results:* There was no statistical difference among the groups in terms of body weight changes. The ABP was measured at a mean value of 179.5 ± 10.3, 129.3 ± 14.2, 209 ± 14.4, and 167.5 ± 7.5 mm-Hg, in group C, SC, C-PRP, and S-PRP, respectively. The ABP and TH of C-PRP group was significantly higher than three groups ($p < .05$, for each comparison). In sepsis, PRP significantly raised the mean ABP and TH levels up to the levels of C group. Tissue regeneration was significant with increased collagen formation in C-PRP group than the other groups ($p < .05$). The healing effect of PRP in the presence of sepsis was significant than S-group ($p < .05$), while similar to C group ($p = .181$). *Conclusion:* PRP application to colonic anastomosis promotes the healing process in rats with intra-abdominal sepsis.

Keywords: Platelet-rich-plasma; colonic anastomosis; intra-abdominal sepsis; surgery

INTRODUCTION

Anastomotic leakage still continues to be a major surgical complication after intestinal surgery. The acceptable rate of anastomotic leakage ranges from 3 to 6% for modern surgery, however, further studies are still being performed to reduce these rates [1]. In recent studies, the application of growth factors on intestinal anastomosis seems to offer promising therapeutic results [2–7]. Furthermore, it may seem to be reliable and clinically applicable.

Platelet-rich plasma (PRP) is known as an autologous platelet concentrate suspended in plasma which contains growth factors including platelet-derived growth factor AB (PDGF-AB), transforming growth factor β-1, β 2 (TGFβ-1, 2), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and insulin-like growth factor (IGF) [6]. When platelets become activated, these growth factors are released and accelerate wound healing process. PRP is prepared from the centrifuge of autologous whole blood and combined with thrombin and calcium chloride to

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produce a viscous coagulum gel capable of being introduced as a surgical graft material [7]. PRP has been successfully used since 1985 in different clinical fields for various treatments [8]. The significant efficacy of PRP on the healing process encourages surgeons to improve healing of intestinal anastomosis. Recently, the effect of PRP on intestinal anastomotic healing has been evaluated in several well-designed experimental models, however, in the absence of unfavorable conditions which leads to impair wound healing [6,7,9].

According to our extensive literature search, there is no study evaluating the effect of PRP on the healing of colonic anastomosis in the presence of intra-abdominal sepsis to date. This study was designed as an experimental model since the difficulty of designing a controlled, randomized, prospective clinical study. The purpose of this study was to investigate whether PRP has a positive effect on the healing process of colonic anastomosis in the presence of intra-abdominal sepsis induced by cecal ligation and puncture procedure.

MATERIALS AND METHOD

Ethical Considerations/Animals

This study was established at the Experimental Research Center of Mersin University after obtaining the ethical committee approval of Mersin University Medical Faculty (Approval number: 2014/26). Fifty Wistar-albino male rats, weighing 280–330 g were used in the present study. The animals were maintained at 2°C, humidity at 40–60% with a 12 hr light/dark cycle and allowed free access to water and standard chow during the study. All animals were observed closely and weighed on days 7 after surgery. This research was carried out in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, 1985).

PRP Preparation and Activation

PRP was prepared followed by two-stepped centrifuge of whole blood taken from healthy rats. Nine milliliters of whole blood was drawn from 10 healthy rats through cardiac puncture into ten 10-ml PRP tubes (Cence Medical, Istanbul, Turkey) containing 0.5 ml buffered sodium citrate with a ratio 9:1. Centrifuge levels were set according to the manufacturer's instruction (Cence Medical). The whole blood was centrifuged $400 \times g$ and 20°C for 10 min. Blood was separated into three layers as; red blood cells at the bottom, buffy coat layer in between, and acellular plasma in the supernatant. Subsequently, the supernatant was transferred with a sterile pipette to another 10 ml centrifuge tube and recentrifuged at $800 \times g$ and 20°C for 10 min. About 1 ml of PRP was collected from the bottom of the tube. To activate PRP and obtain a viscous coagulum gel that can be applied to anastomosis, 1 ml of PRP was mixed with the activator including 50 μl of 10% calcium chloride (B. Braun Medical SA) for neutralizing the anticoagulation effect of the citrate and 1 ml of thrombin (DiaMed, Morat, Switzerland; PPT-Reagent) to initiate the clotting process.

Study Design/Surgical Procedure

Whole blood was drawn from 10 healthy rats through cardiac puncture to prepare PRP. The rest of the rats were randomly allocated into four groups of 10 rats each, as follows:

Group 1: Control group (C): subjected to colonic anastomosis without PRP application.
Group 2: Septic control group (SC): subjected to colonic anastomosis without PRP application in sepsis.
Group 3: Control PRP group (C-PRP): subjected to colonic anastomosis covered by PRP.
Group 4: Septic PRP group (S-PRP): subjected to colonic anastomosis covered by PRP in sepsis.

After an overnight fast, the rats were anesthetized by intramuscular injection of ketamine 50 mg/kg (Ketalar; Parke Davis, Eczacibasi, Istanbul,Turkey) and xylazine 10 mg/kg (Rompun; Bayer AG,Leverkusen, Germany). Animals were allowed to breathe spontaneously during the surgery. A heating lamp was used to preserve the body temperature at 37°C. All operations were performed by the same surgeon to ensure technical uniformity. The abdominal skin of the rat was shaved, and under aseptic condition a 4 cm midline incision was made. In septic groups, intra-abdominal sepsis was induced by cecal ligation puncture (CLP) procedure. CLP was performed according to the guidelines of Wichterman et al. [10]. The cecum was exteriorized and filled with feces by milking stool back from the ascending colon. The distal end of the cecum was ligated about 15 mm proximal to cecal pole with 3/0 silk suture without stricture of the ileocecal valve. The ligated cecum was punctured twice with an 18-gauge needle, and is gently squeezed to extrude a small amount of stool from the perforation sites to induce peritonitis. The cecum was then replaced in its original position and the abdomen was closed with continuous 3/0 silk suture. The rats received a single dose of ciprofloxacin 20 mg/kg plus metronidazole 20 mg/kg subcutaneously and isotonic saline (0.5 ml) into the peritoneal cavity every 1 hr for compensation of perioperative fluid loss. Relaparotomy was performed after 8 hr of CLP. The ligated cecum was excised and the peritoneal cavity was irrigated by 50 ml of warm sterile saline. The left colon was transected approximately 2.5–3.5 cm above the peritoneal reflection and an end to end hand-sewn anastomosis was performed using one

layer of interrupted 4/0 silk sutures. Activated 0.5 ml PRP gel was applied to the anastomosis as a film layer of 2 mm width and 1 mm thickness. The muscle-fascial layer was closed with a single layer continuous absorbable 3/0 suture (Vicryl, Johnson, and Johnson) and the skin was closed with interrupted 3/0 silk suture. In aseptic groups (groups 1 and 3), the operations were performed without puncturing the cecum.

Postoperative course was assessed every 6 hr within the first 48 hr and every 8 hr for five days as described Zantl et al. [11]. No postoperative analgesics were used since we observed no symptoms associated with pain including discomfort, agitation or itching of wound side. On postoperative day 7 (POD 7), all animals were euthanized by intraperitoneal overdose of 2 ml pentobarbital sodium (200 mg/ml, KU Life, Copenhagen, Denmark). The anastomosis was carefully found in order to avoid interrupting of the mesenteric blood supply or adhesions to determine the exact anastomotic bursting pressure (ABP) in vivo. The anastomosis was resected with a margin of at least 3 cm on each side with regard to histopathologic examination and tissue hydroxyproline level followed by measuring the ABP.

Anastomotic Bursting Pressure Measurement

All rats were anesthetized by the same drugs at the same dose as in the first surgery before relaparotomy. The abdomen was opened followed by euthanasia. ABP was measured in vivo to evaluate the integrity and strength of the anastomosis. A 14-gauage silicon catheter was inserted from both sides of the anastomosis with a distance of 3 cm followed by cleaning feces with isotonic saline. Both sides were occluded with 4/0 silk suture. Isotonic saline was administered at 4 ml/min rate with an infusion pump while the pressure within the lumen was monitored via the transducer of a pressure monitoring system connected to the other catheter. ABP was defined as the pressure at which saline leak was observed and corresponded with the maximum pressure attained just before rupture of the anastomosis.

Histopathological Examination

A 1-cm-long sample including colonic anastomosis was immediately fixed in 4% formaldehyde. The samples were dehydrated, embedded in paraffin, and cut in 4-μm-thick slices. Histopathologic sections were stained with hematoxylin-eosin (H&E) for inflammation parameters and picrosirius red for collagen. A conventional binocular Leica DM 2000 light microscope (Leica Microsystems, Wetzlar, Germany) was used for analysis. The samples were assessed in a blinded manner by two pathologists to avoid bias.

Inflammation parameters including necrosis, polymorphonuclear cells, lymphocytes, macrophages, edema, state of epithelial layer, and bridging of submucosal-muscular layer were scored according to Verhofstadt scale [12]. Collagen formation was assessed as decreased deposition (0), normal (1), or increased deposition (2).

Hydroxyproline Level Measurement

After measuring ABP, 1 cm of the anastomosis including 0.5 cm proximal and distal from the anastomosis were excised and blunt dissection was performed to clear the anastomotic line from adherent tissues. The concentration of hydroxyproline was measured using a modified procedure based on alkaline hydrolysis of the tissue and subsequent determination of the free hydroxyproline in hydrolysate [13]. Chloramine- T was used to oxidize the free hydroxyproline for production of a pyrrole. The addition of Ehrlich reagent resulted in the formation of a chromophore that can be measured at 550 nm. Results were expressed as micrograms of hydroxyproline per gram of dry tissue (μg/mg, dry tissue).

Statistical Analysis

Statistical Med Calc 9.3.9.0 software was used for sample size calculation. The primary outcome variable was ABP with a difference rate of 10% between the groups. Using a power, 80% α-error and 5% β-error, a sample size of eight rats were calculated for each group to show a statistical significant difference. Considering the possible casualties during the study, 10 rats were allocated for each group. The data obtained were summarized in a computerized spreadsheet and statistical analyses were performed by using SPSS 15.0 for Windows. Numerical data were presented as mean $\pm$ standard deviation (SD). The mean values of ABP, the levels of tissue hydroxyproline and histologic scores were analyzed with using one-way analysis of variance (ANOWA) and Tukey's HSD post-hoc test were used to assess the differences between the groups. Differences were considered statistically significant at $p < .05$.

RESULTS

General Observations

All animals survived surgery except two rats in SC group which died from anastomotic leakage on POD 4. Septic animals displayed clinical features of illness from 12 hr from surgery including a decrease in activity, reduced alertness, ruffled fur, and hunched posture. On POD 2, septic animals were lethargic and de-

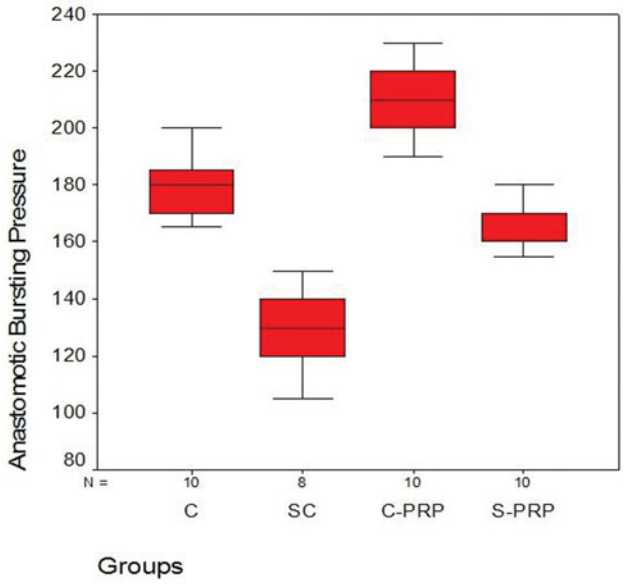

FIGURE 1 Colonic anastomotic bursting pressures of the groups.

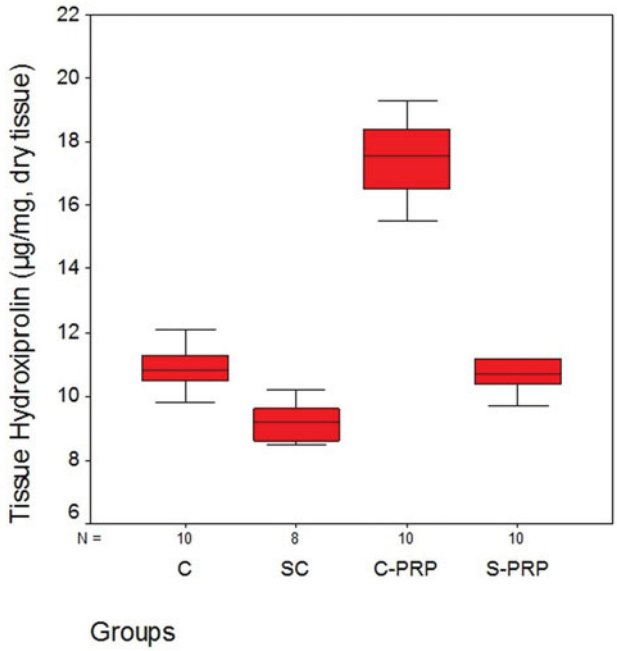

FIGURE 2 Tissue hydroxyproline levels of the groups.

creased response to exogenous stimuli was observed. Control animals displayed no signs of septic symptoms and all survived. No local or systemic complications related to PRP application were observed. The initial body weight was almost similar in four groups. Although the mean body weights were reduced in all groups during the study period, there was no statistical difference among the groups in terms of body weight changes on POD 7.

Anastomotic Bursting Pressure

The ABP was measured at a mean value of 179.5 ± 10.3, 129.3 ± 14.2, 209 ± 14.4, and 167.5 ± 7.5 mmHg, in group C, SC, C-PRP, and S-PRP, respectively. Considering the results, a significant increase of ABP in both PRP groups was detected. PRP application on healthy intestinal anastomosis (C-PRP) significantly increased the ABP when compared with group C (209 ± 14.4 vs. 179.5 ± 10.3, $p = .01$), group SC (209 ± 14.4 vs. 129.3 ± 14.2, $p = .01$), and group S-PRP (209 ± 14.4 vs. 167.5 ± 7.5, $p = .01$). Sepsis (SC) significantly reduced the ABP when compared with the C group (129.3 ± 14.2 vs. 179.5 ± 10.3, $p = .01$). However, it is notable that PRP application in sepsis (S-PRP) raised the measure of ABP up to the levels of C group (167.5 ± 7.5 mmHg vs. 179.5 ± 10.3, $p = .128$). Furthermore, it was found to be significantly higher when compared with SC group (167.5 ± 7.5 vs. 129.3 ± 14.2, $p = .01$). These results revealed that PRP application to colonic anastomosis significantly prevented the sepsis-induced reduction of ABP (Figure 1).

Hydroxyproline Levels

Hydroxyproline is an essential amino acid in collagen formation which is frequently used to determine the collagen concentration of the tissue. Tissue hydroxyproline levels on POD 7 were significantly lower in S-group when compared with the other three groups (C, C-PRP, S-PRP, $p = .01$, $p = .012$, and $p = .031$, respectively). In C-PRP group, tissue hydroxyproline levels were increased significantly when compared with the other groups (C, SC, S-PRP, $p = .023$, $p = .01$, and $p = .012$, respectively). The tissue hydroxyproline level of S-PRP group was similar to C group ($p = .959$). It was a data which revealed the positive effect of PRP on the healing of colonic anastomosis in the presence of sepsis (Figure 2).

The body weight change, ABP and hydroxyproline levels of the groups were summarized in Table 1.

Histopathological Examination

Verhofstad scale is a good scoring system for grading histological changes in the tissue that allows us to evaluate the healing on cellular base and tissue remodeling. There were statistically significant differences among the groups, particularly in terms of mucosal healing (Table 2). Particularly in C-PRP groups, the healing of anastomosis was observed as almost excellent with increased collagen formation when compared with the other groups (C, SC, and S-PRP, $p = .032$, $p = .013$, and $p = .031$; respectively). Moreover, the healing effect of PRP in the presence of sepsis was significant

TABLE 1 Body weight change, anastomotic bursting pressure, and hydroxyproline levels of the groups

Parameters	Control ($n = 10$)	Septic ($n = 8$)	Control PRP ($n = 10$)	Septic PRP ($n = 10$)
Initial body weight (g)	298 ± 10.5	299 ± 16.4	298 ± 11.1	301 ± 11.7
Body weight on POD7	297 ± 7.8	294 ± 9.2	298 ± 7.1	296 ± 6.14
Anastomotic bursting pressure (mmHg)	179 ± 10.3	129 ± 14.2	209 ± 14.4	168 ± 7.5
Hydroxyproline levels (μg/mg tissue)	10.8 ± 0.67	8.98 ± 1.04	$17,4 \pm 1.21$	10.6 ± 0.52

when compared with S-group ($p = .027$), while similar to C group ($p = .181$). The data were summarized in Table 2. Microscopically, there was no statistically significant difference in terms of collagen formation between the groups except a significant reduce in S group when compared with C group and C-PRP group ($p = .048$ and $p = .01$, respectively) likely due to the deleterious effect of sepsis on intestinal anastomotic healing. However, although increased collagen formation was noted in S-PRP group, no statistically significant difference was detected between S and S-PRP group ($p = .132$) (Figure 3).

DISCUSSION

In the present study, the anastomotic healing effect of PRP was assessed depending on the results of four valid determinant factors: body weight change, ABP for anastomotic strength and integrity, tissue hydroxyproline levels (TH) for biochemical evaluation and histopathological examination for evaluating tissue regeneration. It is well-known that body weight reduction is associated with impaired wound healing [14]. In the present study, the mean body weights were reduced in all groups and no statistical difference were detected in terms of body weight loss among the groups likely due to the short period of our study. We conclude that the mean weight reduction in our study is not sufficient to cite malnutrition as a cause of impairment in healing of anastomosis. Our data revealed a significant increase in the levels of ABP and TH in C-PRP group when compared with the other groups. On histopathological examination, the major difference was increased collagen formation that contributes better tissue regeneration in C-PRP group. These results were compatible with the previous studies evaluating the healing effect of PRP on intestinal anastomosis in the absence of sepsis [6,7,9].

Comparing with the septic control group, PRP gel application to anastomotic line significantly improved the healing process of colonic anastomosis in the presence of abdominal sepsis. The results of S-PRP group were almost similar when compared with the C group in terms of ABP, TH, and histopathological score. It is likely due to the fact that PRP stimulated the collagen production of the healing tissue site. It is well known that collagen formation is a main indicator of the healing process under septic conditions [15]. Considering the positive healing effect of PRP, it is interesting that there is no published data evaluating the anastomotic healing effect of PRP in the presence of sepsis to date in the literature. To our knowledge, our study is the first. Therefore, according to our results, we concluded that PRP application to intestinal anastomosis significantly promotes the healing process of anastomosis by accelerating tissue regeneration and remodeling either under normal or under septic condition.

Delayed diagnosis of colonic perforations caused by trauma, diverticulitis, tumor, inflammation, or ischemia usually results in intra-abdominal sepsis. The surgical approach to left colonic disease complicated by perforation and following intra-abdominal sepsis is still a subject of debate whether to perform primary anastomosis or colostomy. Considering the recent reports in the literature, there is a growing tendency toward resection with primary anastomosis in parallel with advances in surgical techniques and critical care support whereas a significant number of surgeons still have reasonable concerns about performing primary anastomosis when faced with peritonitis [16–18]. Distal colonic anastomosis is particularly more prone to

TABLE 2 Histologic scores of the groups

Parameters	Control ($n = 10$)	Control PRP ($n = 10$)	P value	Septic ($n = 8$)	Septic PRP ($n = 10$)	P value
Necrosis	0.3 ± 0.48	0.2 ± 0.42	0.971	1.1 ± 0.64	0.7 ± 0.48	0.303
PMNs	1.3 ± 0.94	0.7 ± 0.67	0.208	1.5 ± 0.53	1.2 ± 0.42	0.325
Lymphocytes	1.0 ± 0.47	1.0 ± 0.47	1	1.6 ± 0.51	1.3 ± 0.48	0.013
Macrophages	1.0 ± 0.00	1.6 ± 0.51	0.048	1.1 ± 0.83	1.5 ± 0.52	0.918
Edema	0.4 ± 0.51	0.3 ± 0.48	0.977	1.2 ± 0.71	0.4 ± 0.51	0.014
Mucosal epithelium	0.3 ± 0.48	0.3 ± 0.48	1	1.0 ± 0.53	0.6 ± 0.51	0.179
Bridging	0.6 ± 0.51	0.2 ± 0.42	0.348	1.2 ± 0.71	0.8 ± 0.42	0.149
Total	4.9 ± 1.28	4.3 ± 1.33	0.728	9.8 ± 1.12	6.1 ± 1.37	0.012

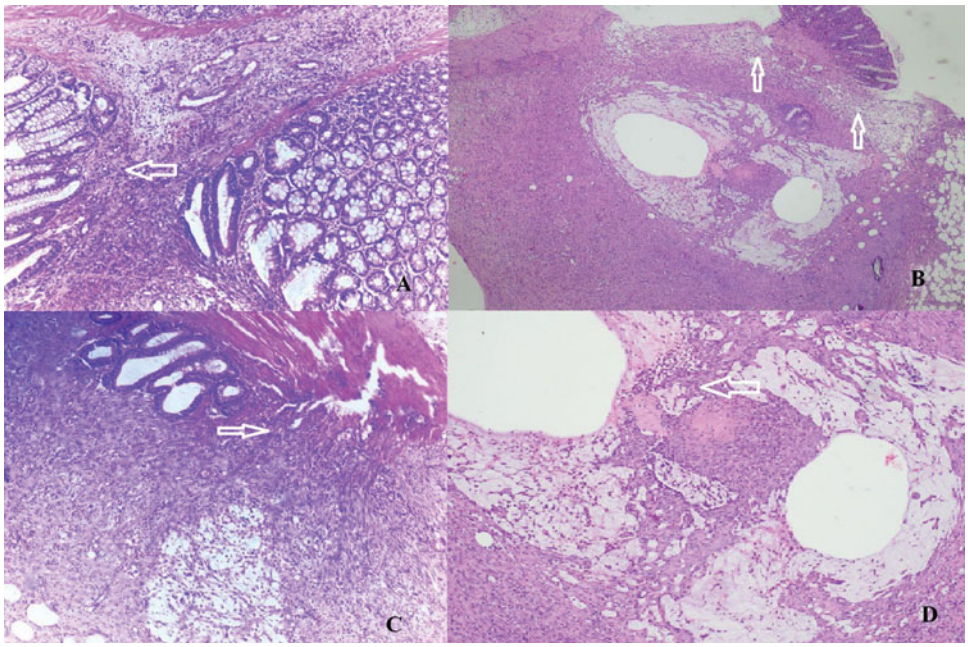

FIGURE 3 Histopathological examination of the samples. (**A**) Control group, moderate inflammatory cell infiltration with moderate fibrosis (H&E ×20). (B) Septic group: Massive infiltration of inflammatory cells with poor fibrosis (H&E ×20). (C) Control PRP group: Less inflammatory cell infiltration with intensive fibrosis (H&E ×20). (D) Septic PRP group: Moderate inflammatory cell infiltration with moderate fibrosis (H&E ×20).

dehiscence when compared with the other colonic segments due to fecal loading and technical difficulty in constructing anastomosis at this level [19]. The presence of peritonitis impairs the healing process of intestinal anastomosis due to apoptosis, disrupted blood flow and microvasculature failure which is caused by well-known mediators of inflammation [16]. Hence, the anastomosis becomes more vulnerable to dehiscence in the presence of sepsis. Due to this fact, many surgeons still prefer performing colostomy followed by colonic resection in patients with sepsis. In clinical practice, our expectation from this study was to investigate whether we can construct safer colonic anastomosis in the presence of sepsis by promoting the healing process of the anastomosis by using PRP gel. Thus, we expected to prevent some unnecessary colostomy operations which may lead to possible complications including impaired quality of life and the risks of reoperation for colostomy closure.

Another point we want to emphasize that, considering our clinical practice, under normal circumstances, the majority of the surgeons accomplish the anastomosis in a standard manner without using any further agents. However, the question is whether to perform primary anastomosis or not in a contaminated field. Therefore, in our opinion, the anastomotic healing effect of any agents including PRP should be evaluated under unfavorable conditions which may lead to impair wound healing, particularly in the presence of intra-abdominal sepsis. We stress that this condition seems to be closer to the clinical practice and yields more reliable results. Accordingly, we designed this study as a sepsis model. In this study, CLP model was preferred to the other techniques inducing sepsis since we share the same view as Maier et al. [20].

The wound healing and tissue regeneration effect of certain growth factors such as VEGF, PDGF, TGFβ-1, 2, EGF, and IGF has been discussed in several studies [6,7]. PRP contains the concentrations of these growth factors that constitute the theoretical basis of the use of PRP in wound healing and tissue regeneration. In accordance with understanding the significant healing effect of PRP from clinical practices in various clinical fields, PRP has been used in general surgery to solve one of the major problems of the surgeons: intestinal anastomotic leakage. PRP initiates 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, TGF β, VEGF, and EGF [6,21]. These growth factors are active polypeptides which facilitate regeneration of injured tissue through acceleration of cell proliferation and matrix formation [9,22]. The structure of PRP gel is almost similar to the natural fibrin clot [23]; however, it should be activated by adding calcium chloride and thrombin before its use. Calcium chloride acts as a clot activator that promotes the physiological clotting process which provides tight attachments to the anastomotic line that enables tight scaffolds between the cells and also sustains delivery of growth factors. Thrombin plays an important role in keeping the vi-

ability of platelets in PRP, thus provides continuous degranulation of α-granules to release viable growth factors [9].

It is well established that the anastomotic healing is adversely affected in the presence of sepsis, particularly because of the increased activation of TNF-α and IL-1β which incite the production of free radicals that causes cell damage [24,25]. In wound healing, the proliferation phase starts after 4 days from injury and macrophages become the predominant inflammatory cells that promote the release of growth factors which are essential for tissue repair [26]. TNF-α is mainly released by macrophages and plays a potential role in wound healing. TNF-α inhibits collagen synthesis by stimulating the collagenase production during this phase [27]. Particularly in sepsis, the intestinal anastomosis becomes more vulnerable to dehiscence during proliferation phase possibly due to increased activity of collagenase to destroy unorganized collagen fibrils. Thus, although the debate continues about the postoperative day for measuring the ABP, we evaluated the bursting pressure on POD 7 since anastomotic leakage is often diagnosed in the first postoperative week in clinical practice.

Although PRP is described as an autologous concentrate of platelets in a small volume of plasma, the use of homologous PRP seems to be the limitation of our study. Unfortunately, in small animal models such as rats, approximately a total amount of 8–10 ml whole blood can be obtained from each rat, thus we initially sacrificed 10 healthy rats. As Marx [21] stated that the use of donor blood could cause overt immune response which could lead to false-negative results. However, no negative local or systemic effect related to PRP application were observed in this study likely due to the absence of leukocytes in the content. Furthermore, despite this concern, the positive effect of PRP was demonstrated in both PRP groups. Second limitation was that we evaluated the overall biological effect of PRP on anastomotic healing. Unfortunately, we could not be able to identify the specific growth factor or cytokines in PRP that actually promotes the anastomotic healing because of the lack of our facilities.

CONCLUSION

In conclusion, PRP gel application to colonic anastomosis promotes the healing process of anastomosis in rats with sepsis. Although this study was experimental, our results encourage the further clinical application of PRP on intestinal anastomosis to reduce the frequency of anastomotic leakage in patients with intra-abdominal sepsis. However, this suggestion needs to be supported by controlled, randomized, and prospective clinical studies.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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