Effects of methylene blue, pentoxyphylline and enoxaparin on postoperative adhesion formation and markers of angiogenesis in a rat uterine horn model

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Summary

Objective: Postoperative adhesions still remain as a common and serious problem leading to morbidity, mortality and economic loss. Adhesions are the major cause of postoperative intestinal obstruction, infertility, and chronic pelvic pain. In this study, we aimed to compare adhesion prevention effects of pentoxyphylline, enoxaparin and methylene blue and to investigate the effects of these agents on angiogenesis, which is suggested as an important step in wound healing, in rat a uterine horn model. *Material and Methods:* Forty female Wistar albino rats were randomized into four subgroups and underwent laparotomy. Adhesions developed following cauterization at the anti-mesenteric surfaces of both uterine horns. After 14 days, adhesions were investigated by using macroscopic, histopathological and immunohistochemical [vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor (TGF-β), platelet-derived growth factor (PDGF)] methods. *Results:* We found that enoxaparin significantly reduced adhesion formation. Pentoxyphylline had no significant effect on adhesion formation, whereas methylene blue had a significant decreasing effect on histopathologically determined adhesion markers and it may affect angiogenesis through PDGF. *Conclusion:* Among three agents, which were intraperitoneally given by a single dose manner in order to prevent postoperative adhesions, methylene blue and enoxaparin exhibited a positive effect, while no such effect was shown with pentoxyphylline.

Key words: Rat; Uterus; Adhesion; Methylene blue; Pentoxyphylline; Enoxaparin; Angiogenesis.

Introduction

Adhesions are the structures which contribute to revascularization of tissues with impaired blood supply, limit infection and prevent leakage from anastomosis during the healing period of traumatized tissues [1, 2]. Pathological adhesions are the primary cause of postoperative intestinal obstruction, infertility and chronic pelvic pain [3].

By microscopic evaluation, it was shown that there are very thin vascular structures, namely angiogenesis, within adhesions [4]. Angiogenesis is a complex phenomenon involving migration, proliferation, maturation and organization of endothelial cells within capillary tubes by angiogenic stimulation. Angiogenic stimulation occurs via release of pro-angiogenic cytokines and growth factors from inflammatory cells, pericytes and tumor cells, mainly from leukocytes, macrophages and mast cells. Some of these factors induce proliferation and migration of endothelial cells by directly binding to surface receptors, whereas others stimulate local stromal and inflammatory cells to induce angiogenesis [5, 6]. Some of these are vascular endothelial growth factor (VGEF), basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF).

The role of VGEF, through effects on fibroblast functions, has been demonstrated in the restoration process of tissues, such as early inflammatory response, wound repair and remodeling [7]. Basic fibroblast growth factor (later called bFGF), also termed as FGF-2, is a well documented angiogenic growth factor and induces endothelial cell replication, migration and extracellular proteolysis [8-10]. PDGF-BB is involved in neo-vessel stabilization and functionalization by inducing anastomoses and recruiting pericytes. Vessel stabilization has been shown to be dependent on expression of PDGF β-receptors, which are expressed by fibroblasts, endothelial cells and smooth muscle cells [11, 12]. PDGF-BB also stimulates production of extracellular matrix proteins from pericytes, including fibronectin, collagen and proteoglycans which are necessary for the basal membrane of capillaries. In addition, PDGF-BB increases expression levels of VEGF in mural cells and stimulates fibroblasts to produce and secrete collagenases, which are key factors for cell migration in the angiogenesis [13]. TGF-β may play a role in the formation and maintenance of fibrous adhesions following intraperitoneal injury [14].

Production of oxygen radicals, inhibition of nitric oxide synthase (NOS) and K channels can be specified among pharmacological effects of methylene blue (MB) [15]. It was shown that 1% concentration of MB was effective in the prevention of adhesion formation [16]. However, it was mentioned that both higher (5-7%) and lower (0.1-0.5%) concentrations were ineffective [17].

Pentoxyphylline is a non-selective phosphodiesterase inhibitor, which has significant immunoregulatory and anti-inflammatory effects. Pentoxyphylline inhibits activation and adhesion of peripheral blood T lymphocytes in

Revised manuscript accepted for publication August 4, 2011

vitro [18]. It was reported that it reduced formation of intraperitoneal adhesions in the anastomosis area in rats after intestinal resection [19].

Enoxaparin is a low molecular weight heparin (LMWH). LMWHs are fragments of heparin, which can no longer be fragmented, and produced by controlled enzymatic and chemical depolymerization [20]. It was observed that heparins inhibited capillary tube formation by human endothelial cells from a macrovascular bed, which is stimulated by proangiogenic factors such as FGF-2 and VGEF; it was also suggested that this inhibitory capacity of heparin is dependent on its molecular weight [21].

In this study, it was aimed to evaluate the effects of methylene blue, pentoxyphylline and enoxaparin on adhesions in a uterine horn model by using macroscopic, histopathological and immunohistochemical methods.

Materials and Method

Forty female Wistar albino rats (10-12 weeks old; 200-220 g weight) were used. They were housed five animals per cage with the appropriate diet and water ad libitum. All rats were observed for several days to ascertain health before operations. All procedures were approved by and performed under the guidelines of the Animal Care and Use Committee of Cumhuriyet University.

Each rat was anesthetized by using 40 mg/kg intravenous ketamine hydrochloride before the surgery, the abdomen was shaved and prepared with povidone iodine solution. Using a sterile technique, a 3 cm midline vertical incision was made and both uterine horns were exposed; then 2 cm segments of each uterine horn were traumatized at ten spots on the anti-mesenteric surface using unipolar cautery (Elman Surgitron, Leofarma, Istanbul, Turkey). Care was taken to avoid gross bleeding from injured sites. Handling of other tissues was minimized. Rats were randomly assigned into four groups each consisting of ten rats. Treatment groups were as follows: (C) control group, 2 ml saline solution only; (E) enoxaparin group; (MB) methylene blue group; (P) pentoxyphylline group.

Enoxaparin (Clexane, Sanofi Aventis, Istanbul, Turkey) solution were obtained by diluting 50 anti-Xa IU/ml of enoxaparin. Methylene blue (Methylene blue, Sigma, USA) was diluted to obtain 1% solution and pentoxyphylline (Trental ampul, 100 mg/5 ml, Aventis Pharma, Istanbul, Turkey) solution was diluted to obtain a solution of 5 mg/ml. Before abdominal closure, all therapeutic agents (2 ml) and saline (2 ml) were instilled onto uterine horns. The incision was closed in a single layer, excluding the peritoneum with a running 4-0 monofilament delayed absorbable suture. The total operative time was less than 10; a 2-week recovery period was allowed.

On postoperative day 14, animals were sacrificed by cervical dislocation. A transverse sub-costal incision was made above the cephalad extent of the midline laparotomy site, and the abdominal cavity was inspected for the presence of adhesions. The extent and severity of adhesions in the operation site for each uterine horn were evaluated and recorded by an investigator blinded to the treatment group according to criteria proposed by Linsky *et al*. [22]. The extent of adhesions was graded as follows: 0, no adhesion; 1, 25% of traumatized area; 2, 50% of traumatized area; 3, total involvement. The severity of adhesions was graded as follows: 0, no resistance to separation; 0.5, some resistance (moderate force required); 1, sharp dissection needed. Total adhesion score (TAS) was recorded as arithmetic sum of severity and extent of adhesion [23].

Biopsy materials of all four groups were fixed by using 10% formaldehyde solution to perform histological evaluation and histochemical and immunohistochemical staining. Hematoxylin-eosin stained slides obtained from paraffin blocks

Table 1. — *Histological adhesion score*.

	Inflammation	Fibroblastic activity	Foreign body reaction	Collagen formation	Vascular proliferation
Grade 0	None	None	None	None	None
Grade 1	25% mixed inflammation	Mild	Mild	Mild	Mild
Grade 2	50% mixed inflammation	Moderate	Moderate	Moderate	Moderate
Grade 3	75% mixed inflammation	Marked	Marked	Marked	Marked
Grade 4	Massive inflammation	Massive	Massive	Massive	Massive

Table 2. — *Macroscopic median adhesion scores of all groups, (minimum-maximum)*.

	Control (n = 10)	Enoxiparin (n = 10)	Methylene blue (n = 10)	Pentoxyphylline (n = 10)
Adhesion extent score	3 (1-3)	1.5 (1-3)[a]	3 (1-3)	3 (2-3)
Adhesion severity score	1 (0.5-1)[b]	0.5 (0-0.5)	1 (0-1)	1 (1-1)
Total adhesion score	3 (1-3)	2 (1-3.5)[c]	3 (1-3)	3.5 (2.5-3.5)

[a] $p < 0.05$; vs pentoxyphylline group.
[b] $p < 0.05$; vs enoxiparin group.
[c] $p < 0.05$; vs control, methylene blue and pentoxyphylline group.

Table 3. — *Histopathological and histochemical median findings of all groups (minimum-maximum)*.

	Control (n = 10)	Enoxiparin (n = 10)	Methylene blue (n = 10)	Pentoxyphylline (n = 10)
Inflammation score	1 (0-3)	3 (2-4)[a]	2 (1-3)	1.5 (0-3)
Fibroblastic activity score	2 (2-4)	2 (1-4)	1 (0-2)[b]	2 (2-3)
Foreign body reaction score	1.5 (0-3)	3 (1-4)	3 (1-4)	1 (0-3)
Collagen formation score	2.5 (2-4)	1.5 (1-4)	1 (0-4)[c]	2 (1-3)
Vascular proliferation score	2 (1-3)	2 (1-3)	1 (0-3)[d]	2 (1-4)
MT score	2.5 (1-4)	2 (1-4)	0.5 (0-2)[e]	2 (2-3)

MT, Masson's trichrome.
[a] $p < 0.05$; vs control, methylene blue and pentoxyphylline group.
[b,c,d,e] $p < 0.05$; vs control, methylene blue and pentoxyphylline group.

Table 4. — *Immunohistochemical adhesion scores of all groups*.

	Control (n = 10)	Enoxiparin (n = 10)	Methylene blue (n = 10)	Pentoxyphylline (n = 10)
VEGF score	0.5 (0-4)	0.5 (0-2)	0 (0-1)	0 (0-3)
PDGF score	2.5 (1-4)	2 (1-4)	1 (0-2)[a]	1 (1-3)
TGFβ score	2 (0-4)	2 (0-3)	1 (0-3)	2 (0-3)
bFGF score	2 (0-4)	2 (1-4)	1 (0-2)	2 (0-4)

VEGF: vascular endothelial growth factor; PDGF: platelet derived growth factor. TGF-β: transforming growth factor; bFGF, basic fibroblast growth factor.
[a] $p < 0.05$; vs control, methylene blue and pentoxyphylline group.

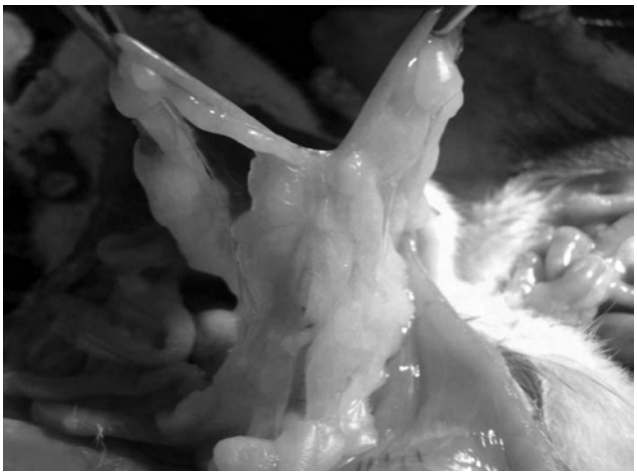

Figure 1. — Intensive omental adhesions.

Figure 2. — Adhesion with moderate resistance involving 50% of the horn.

which were prepared by routine tissue management, were assessed in blind manner to macroscopic adhesion scores. The method proposed by Kanbour-Shakir *et al*. [23] was used to semi-quantitatively grade (grade 0 to 4) inflammation on the serosal surface, fibroblastic activity, foreign body reaction, collagen formation and severity of vascular proliferation (Table 1). Moreover, Masson's trichrome histochemical staining was performed to make severity of collagenization more pronounced in all preparations. Results were scored as 0, 1+, 2+ and 3+; 4 μm thick sections were obtained to interpret angiogenesis, a step in the formation of adhesions. VEGF Ab-7 (mouse monoclonal antibody, Cat # MS-1467-R7, Neomarkers, USA, 2007), bFGF (mouse monoclonal antibody, Cat # AM 359-5M, Biogenex, USA, 2007), PDGF (rat monoclonal antibody clone, Cat # RB-9257-R7, Neomarkers, USA, 2007) and TGF- 3 (rabbit monoclonal antibody, Cat # RB-9262-R7, Neomarkers, USA, 2007) markers were used in the immunohistochemical evaluation. Results were scored as 0, 1+, 2+, 3+ and 4+.

Statistical Analysis

Data are presented as median (min-max). Kruskall Wallis ANOVA and Tukey post hoc test were used to compare scores of adhesion extent, adhesion severity, total adhesion, inflammation, fibroblastic activity, foreign body reaction, collagen formation, vascular proliferation, MT, VGEF, PDGF, TGF and aFGF between groups. A p value < 0.05 was considered as significant.

Results

There was no mortality in the study group. Forty female rats recovered without incident after operation and resumed preoperative physical activity and feeding patterns postoperatively. All animals appeared healthy; they were evaluated and there were no signs of impaired wound healing or bleeding complications.

Table 2 presents macroscopic adhesion scores of all groups (extent, severity, and total score of adhesions). The extent scores of adhesions in the control, enoxaparin control, enoxaparin, methylene blue and pentoxyphylline groups were observed as 3 (1-3), 1.5 (1-3), 3 (1-3), and 3 (2-3), respectively. The severity scores of adhesions in the control, enoxiparin control, enoxaparin, methylene blue and pentoxyphylline groups were observed as 1 (0.5-1), 0.5 (0-0.5), 1 (0-1), and 1 (1-1), respectively. As shown in Table 2, the adhesion extent score of the enoxaparin group was found to be significantly lower than the score of the pentoxyphylline group ($p < 0.05$). When adhesion severity scores of the control, enoxaparin, methylene blue and pentoxyphylline groups were compared, the score of the control group was found to be significantly higher than the score of the enoxaparin group ($p < 0.05$). When total adhesion scores of the control, enoxaparin, methylene blue and pentoxyphylline groups were compared, only the score of the enoxaparin group was found to be significantly lower than those of the control, methylene blue and pentoxyphylline groups ($p < 0.05$) (Figures 1 and 2).

Table 3 shows histopathological (inflammation, fibroblastic activity, foreign body reaction, collagen formation, and vascular proliferation) and histochemical (Masson's trichrome scores) findings of all groups. When inflammation scores of the control, enoxaparin, methylene blue and pentoxyphylline groups were compared, only total adhesion score of the enoxaparin group was found to be significantly higher than scores of the control, methylene blue and pentoxyphylline groups ($p < 0.05$). The fibroblastic activity score of the methylene blue group was found to be significantly lower than scores of the control, enoxaparin and pentoxyphylline groups ($p < 0.05$). When foreign body reaction scores of the control, enoxaparin, methylene blue and pentoxyphylline groups were compared, no significant difference was found among groups. The collagen formation score of the methylene blue group was found to be significantly lower than scores of the control, enoxaparin and pentoxyphylline groups ($p < 0.05$) (Figure 3). The vascular proliferation score of the methylene blue group was found to be significantly lower than scores of the control, enoxaparin and pentoxyphylline groups ($p < 0.05$). The Masson's trichrome (MT)

Fig. 3A

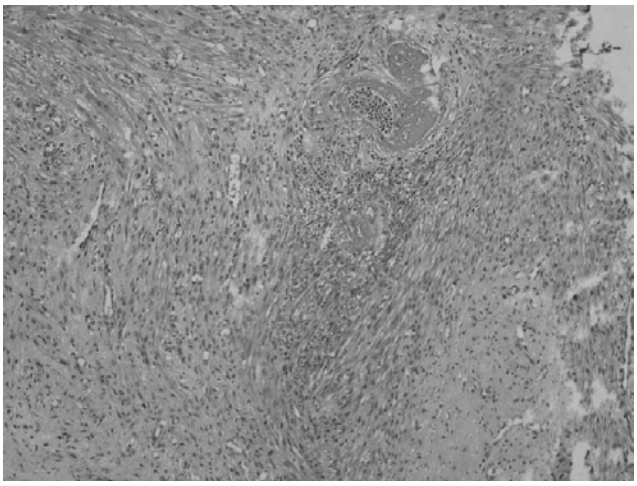

Fig. 3B

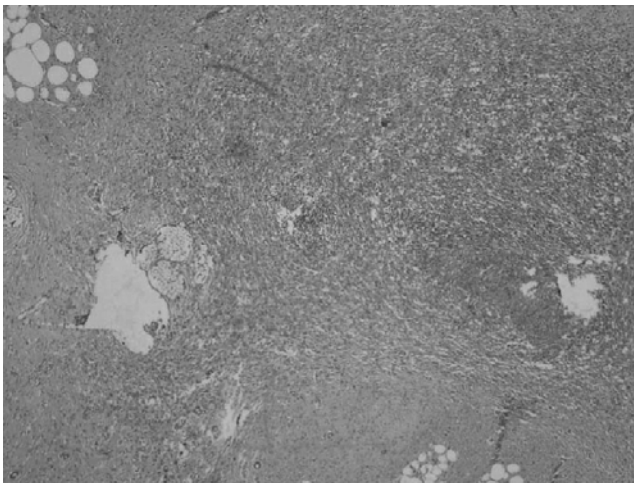

Fig. 3C

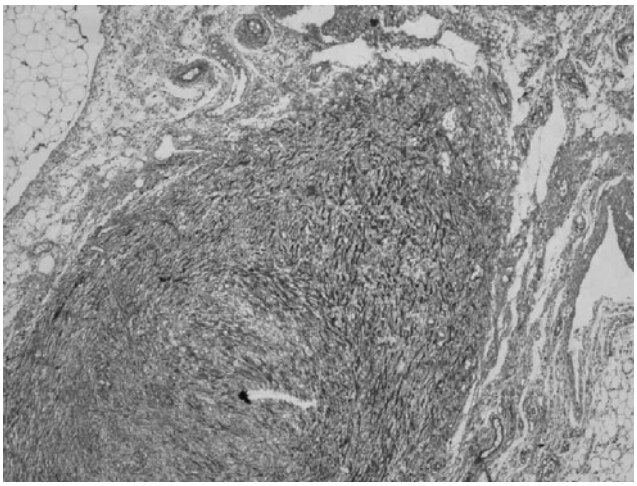

Fig. 3D

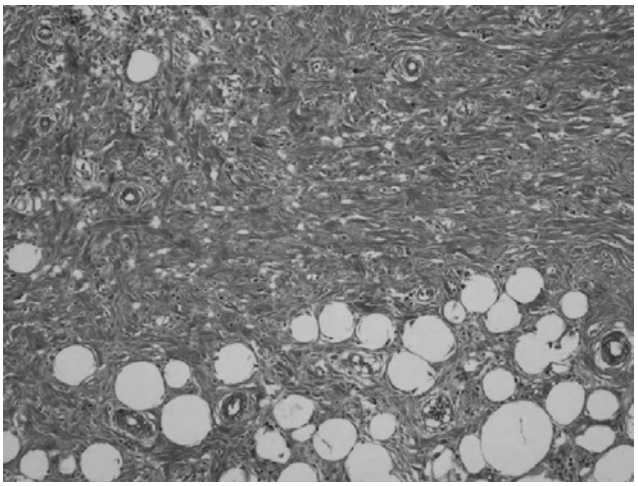

Figure 3. — Histopathological images of groups. A) Grade 3 fibroblastic activity; Grade 2 vascular proliferation in the control group (HE x 10). B) Grade 4 inflammation and foreign body reaction in the enoxaparin group (HE x 10). C) Grade 1 collagenization in the enoxaparin group (MT x 40). D) Grade 4 collagenization in the enoxaparin group (MT x 20).

scores of the control, enoxaparin, methylene blue and pentoxyphylline groups were compared and only the MT score of the methylene blue group was found to be significantly lower than scores of the control, enoxaparin and pentoxyphylline groups ($p < 0.05$).

Table 4 presents immunohistochemical adhesion scores (VEGF, PDGF, TGF and aFGF) of all groups. When VEGF, TGF and aFGF scores of the control, enoxaparin, methylene blue and pentoxyphylline groups were compared, no significant difference was found between the groups, whereas only PDGF score of the methylene blue (MB) group was found to be significantly lower than scores of the control, enoxaparin and pentoxyphylline groups ($p < 0.05$) (Figure 4).

Discussion

There is no in vivo study that has investigated the effect of MB on angiogenesis in the rat adhesion model in the literature. In our study, we found no significant reduction in total adhesion score in rats, of which 1% MB was administered intraoperatively; however, there was a significant reduction in adhesion severity score compared to controls. When fibroblastic activation, collagen formation, collagenization and vascular proliferation scores were compared, a significant reduction was observed in the MB group. However, it was shown that MB had no effect on cytokines linked to angiogenesis, such as VGEF, bFGF and TGF, although there was a significant reduction in PDGF compared to other groups. We think that MB has a reducing effect on adhesions and may be related to reduction in fibroblastic activation, collagen formation, and vascular proliferation that occurs through angiogenesis. In the literature it was shown that MB had an anti-angiogenic effect in chicken chorioallantoic membranes [24]. However histological examination of the lungs showed that VEGF-positive cells were decreased in rats treated with MB, in which hepatopulmonary syndrome developed due to common bile duct ligation and the authors concluded that MB treatment decreased the proliferation of alveolar capillary vessels and angiogenesis [25].

Fig. 4A

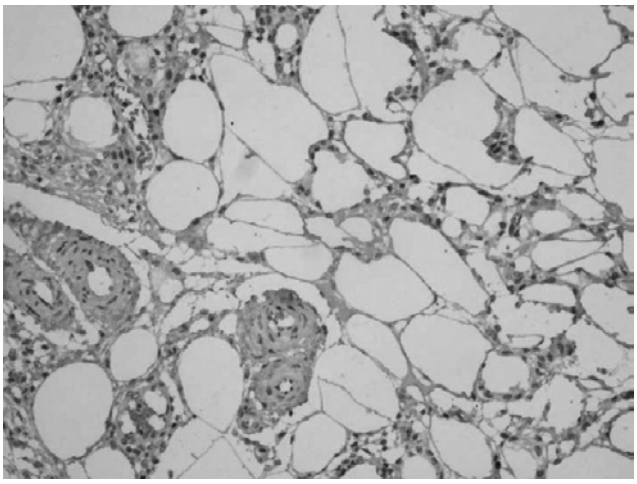

Fig. 4B

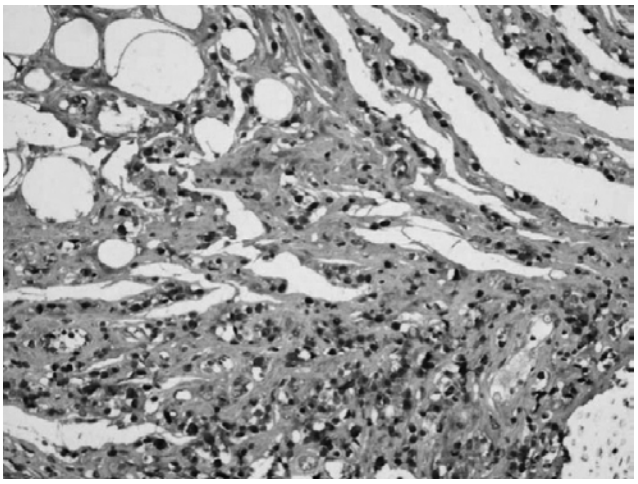

Fig. 4C

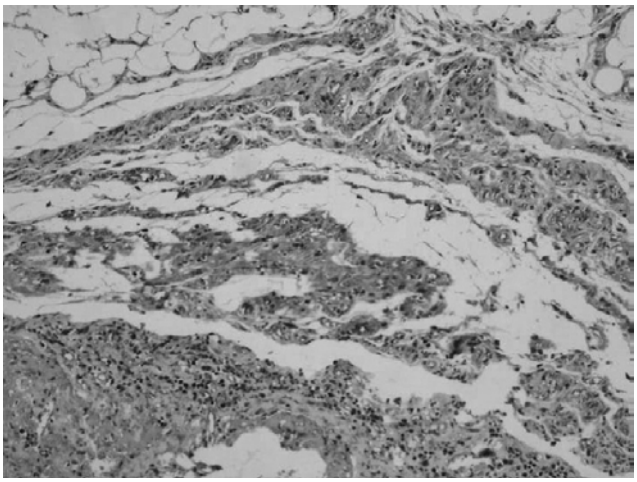

Fig. 4D

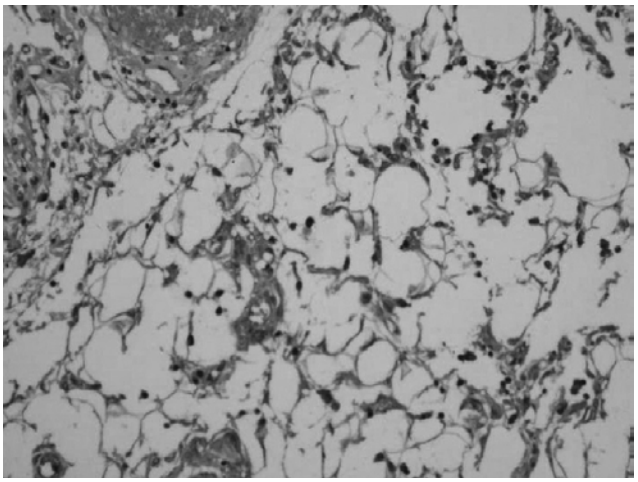

Fig. 4E

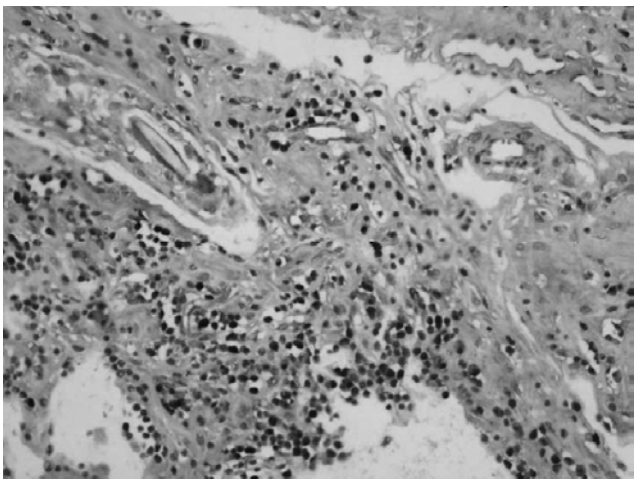

Fig. 4F

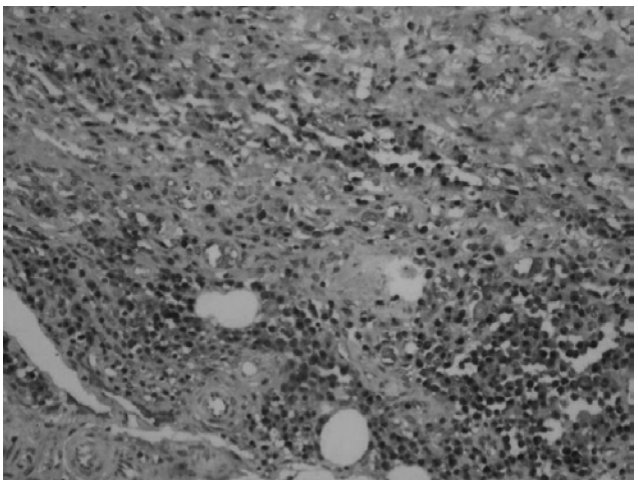

Figure 4. — Immunohistochemical images of groups. A) VEGF-negative in the control group (IHC x 40). B) VEGF–4+ in the control group (IHC x 20). C) TGF-β-1+ spindle cells in the control group (IHC x 10). D) TGF-β-3+ spindle cells in the control group (IHC x 20). E) bFGF-2+ mononuclear cells in the pentoxyphylline group (IHc x 20). F) bFGF-4+ mononuclear cells in the enoxaparin group (IHC x 20).

The administration of intraoperative single dose MB leads to fragmentation of fibrinous proto-adhesions by up-regulating the fibrinolytic system during the consequent 24 hours [26]. Also it was reported that MB stimulated NADPH production via pentose phosphate and an increase occurred in (tissue plasminogen activator) (tPA) secretion after day 2 or 4 [27]. This can also explain the adhesion prevention effect of MB by fibrinolysis. In addi-

tion, it was reported that methylene blue, as an inhibitor of superoxide generation by xanthine oxidase [28], was very effective in preventing formation of peritoneal adhesions. Its activity is probably through inhibition of free-radical generation [29]. In addition to all this information, our results and other studies [24, 25] showing an angiogenesis inhibition effect of MB suggest that adhesion preventive effect of MB can occur through inhibition of angiogenesis.

Gude *et al*. [30] found that pentoxyphylline inhibited angiogenesis induced by tumoral activity. This effect was probably through a decrease in endothelial cell proliferation and levels of urokinase type PA, which are released from these cells. In a study by Barros *et al*. [31] phosphodiesterase inhibitors, cilostazol and pentoxyphylline were administered over seven days after operation by gavage, and they showed that both agents decreased angiogenesis in sponge-induced intraperitoneal adhesions in mice; however, it was also shown that there was a more significant decrease in the levels of VEGF and TGF-β within adhesion tissue at high doses (500 mg/kg for pentoxyphylline). In our study, it was shown that single dose intraoperative administration had no significant effect on adhesion formation and markers of angiogenesis. Pentoxyphylline increases blood flow by reducing blood viscosity and increasing fibrinolytic activity of plasma. It was reported that intravenous and intraperitoneal administrations of pentoxyphylline decreased adhesions induced by cecal abrasion with gauze by altering fibrinolytic activity [32]. Kaleli *et al*. [33] found that single dose intraperitoneal pentoxyphylline decreased intraabdominal adhesions in a rat horn model, which was developed by denuding the serosa on the proximal antimesometric area with a scalpel until macroscopic punctate bleeding was observed over most of the uterine horn surface. In addition, the peritoneum was stripped from the lateral abdominal wall over an area of 1 x 1 cm and adhesion was achieved by suturing after bleeding. The results of the last two studies seemed to be inconsistent with our findings; we believe that this inconsistency might be caused by the difference of the adhesion formation models (we used a cauterization method which causes ischemia in tissues instead of bleeding in our adhesion model). In rats, it was shown that pentoxyphylline reduced intraperitoneal adhesion formation at the anastomosis site after intestinal resection, but single dose intraperitoneal and 50 mg/kg intramuscular administration had no effect on the non-anastomotic regions [19]. Parra-Membrives *et al*. showed that there was a positive effect on healing by increasing fibrosis on adhesion tissue in the model of ischemic colon anastomosis [34]. In addition, Steinleitner *et al*. [35] showed that pentoxyphylline reduced adhesion development after adhesiolysis by administering 2.5 mg/kg intravenous doses six times with 12-hour intervals in a rabbit uterine horn model. In light of these data, we think the that adhesion model should be considered when assessing the efficiency of pentoxyphylline on preventing adhesion formation, and also drug dose and administration route are important factors that can affect results.

Intraperitoneal heparin administration was found to be effective in animal models [36, 37]. Arıkan *et al*. [38] found that enoxaparin decreased intraabdominal adhesions without compromising wound healing. However, Diamond *et al*. [39] reported that heparin, administered by intraperitoneal lavage, intravenous injection or the intraabdominal route was ineffective in preventing adhesion formation in a rabbit uterine horn model. Similarly, it was reported that administration with Interceed was effective, but not with carboxymethyl cellulose or dextran 70 [39]. It was detected that 500 and 1000 USP units of heparin, which were administered to horns of each rabbit, had significant efficiency in preventing adhesions [40]. Both unfractionated heparin and LMWH alter the bioavailability and activity of growth factors. LMWH affects fibrin structure and inhibits angiogenesis in vitro [41]. It was observed that heparins inhibited capillary tube formation by human endothelial cells from a macrovascular bed, which is stimulated by proangiogenic factors such as FGF-2 and VGEF; it has also been suggested that this inhibitory capacity of heparin is dependent on its molecular weight [21]. When total adhesion scores were considered, it was seen that the enoxaparin group had the lowest score among groups. However, it was detected that it decreased inflammation scores among adhesion markers, which were evaluated in a histopathological manner, but had no significant effect on fibroblastic activity score, foreign body reaction score, collagen formation score, vascular proliferation score and MB (collagenization) score. In our study, it was shown that single dose intraperitoneal enoxaparin administration had no significant effect on growth factors related to angiogenesis, although it decreased adhesions. Although a number of studies have sought to identify a broad spectrum of the biological effects of heparin, many challenging questions remain unanswered [42].

In conclusion, our results demonstrated that MB had a positive effect on prevention of postoperative adhesions and this effect could be through inhibition of angiogenesis via PDFG. Intraperitoneal administration of single dose pentoxyphylline had no significant effect on the formation of adhesions and enoxaparin had no significant effect on growth factors related to angiogenesis, although it led to a decrease in adhesions. Prevention of postoperative adhesions has complex and multifactorial characteristics. We believe that combination treatment or multifactorial effect treatment is more successful than the treatments related to one of these factors. At present, many more specific and original studies are necessary for the purpose of improving effective and inexpensive treatment methods.

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