The effects of heparin and pentoxifylline on prevention of intra-abdominal adhesions in rat uterine horn models: histopathological and biochemical evaluations

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SUMMARY

This experimental study was designed to evaluate the degree of adhesion formation and to determine the uterine tissue malondialdehyde (MDA), nitric oxide (NO) and reduced glutathione (GSH) contents as well as the activities of catalase (CAT) and glutathione peroxidase (GPx) in an intraperitoneal adhesion formation model (uterine horn and abdominal wall scraping) in adult rats receiving or not simultaneous intraperitoneal instillation of heparin or pentoxifylline. The sham animals (group S, n = 7) were only submitted to laparotomy and intraperitoneally received 0.9% NaCl (2 mL), whereas in the 30 other rats, abrasions of the left horn were performed coupled to the intraperitoneal administration of 0.9% NaCl (group C), 500 UI of Heparin (group H) or 25 mg/kg of pentoxifylline (group PTX) (n = 10 in each group). All rats were sacrificed on day 14 and the macroscopic adhesion score, the histopathological examination and the measurements of oxidant and antioxidant markers were performed in the left horn samples. Compared to the not treated surged controls, the adhesion formation was significantly lowered in the H group but was only slightly reduced in the PTX group. However, the intensity of the mononuclear cell infiltrate of the uterine mucosa was significantly reduced after PTX treatment and the fibrosis was significantly diminished with heparin. In addition, these 2 treatments have reduced the tissue MDA accumulation and sustained the antioxidant systems, although not significantly but they have significantly prevented the NO formation and the activities of catalase (CAT) and glutathione peroxidase (GPx). These results show that both heparin and pentoxifylline can limit the occurrence of the surgery-induced oxidative stress and differentially regulate the inflammatory reaction but also that heparin is in fact more efficient than pentoxifylline in preventing the intra-abdominal adhesion formation in this rat model.

Keywords: Rat, heparin, pentoxifylline, intra-abdominal adhesions, inflammatory reaction, oxidative stress, antioxidants.

Introduction

In intra-abdominal surgery, the development of intraperitoneal adhesions is a serious postoperative complication. Post surgical adhesions are lead to small-bowel obstruction, chronic abdominal pains and pelvic pains and female infertility [18]. A wide variety of therapeutics has been used in order to decrease postoperative adhesion formation [6, 11-13, 16, 27]. However, according to our knowledge, no biochemical or histopathological changes in adhesion tissue using heparin and pentoxifylline treatment have been yet reported.
Heparin has been used as a prophylactic and therapeutic anticoagulant for many years. The most likely explanation for the mechanism of action of heparin is that it acts as an anticoagulant and activates antithrombin III, resulting in a reduction of fibrin clots [6, 25, 30]. It is also possible that the fibrin matrix is imperfect secondary to the heparin effect, making it more susceptible to the plasminogen activated into plasmin. Indeed, heparin directly stimulates the plasminogen activator activity and increases the plasmin action, which would enhance fibrinolysis [25, 30]. It also probably stimulates macrophages to secrete the plasminogen activator [6, 7].

Pentoxifylline (PTX) is a synthetic methylxanthine derivate commonly used in the treatment of peripheral vascular diseases [8, 16, 22, 29]. PTX has a vasodilatory effect and improves microcirculatory blood supply, decreases platelet aggregation, neutrophil infiltration, increases thrombolytic, thus avoiding small-vessel obstruction, improves the erythrocyte deformability and reduces the blood viscosity [22]. Furthermore, few animal studies have reported the capacities of PTX in preventing postoperative intraperitoneal adhesions [16, 28, 29].

The purpose of this study was to assess the anti-adhesive effects of intraperitoneal administration of heparin and PTX for preventing the intra-abdominal adhesion formation in the rat uterine horn model.

**Materials and Methods**

**ANIMALS AND PROTOCOL DESIGN**

The experiment, approved by the Ethic committee of the Firat University, Turkey, was carried out in 37 female Sprague-Dawley rats (4.5 month-old, weighing between 200 and 220 g) housed in a climate-controlled animal care facility (relative humidity of 40-60% and temperature between 21 and 24°C), with a 12 hour light/dark cycle. They had free access to water and to standard rodent food.

The rats were randomly divided into four groups and anaesthetized with single intramuscular injection of 85 mg/kg ketamine hydrochloride (Parke-Davis, Ketalar, ketamine hydrochloride, 50 mg/mL) and 6 mg/kg xylazine hydrochloride (Bayer, Rompun, Xylazine hydrochloride, 23.32 mg/mL). The abdomens of all rats were shaved and prepared with 1% antiseptic povidone-iodine solution (Kim-Pa, Poviiodeks, 10% hydrochloride, 50 mg/mL) and 6 mg/kg xylazine hydrochloride (Parke-Davis, Ketalar, ketamine hydrochloride) and a 3 cm midline laparotomy was made.

Antiseptic povidone-iodine (Kim-Pa, Poviiodeks, 10% hydrochloride, 50 mg/mL) and 6 mg/kg xylazine hydrochloride (Parke-Davis, Ketalar, ketamine hydrochloride) were instilled in the peritoneal cavity of animals from the groups C, H and PTX, respectively (n = 10 in each group). The abdominal incision was closed with usual manner.

**HISTOPATHOLOGICAL EXAMINATIONS**

The rats were anaesthetized as mentioned before and the abdomen was exposed through a U-shaped incision providing maximal exposure on the postoperative day 14. All animals were sacrificed via removing blood from vena porta puncture. In the macroscopic examination, the adhesions were graded in a blinded fashion using the classification system described by NAIR et al. [21] (Table I).

The left uterine horn and any adherent material from rats were immediately collected after sacrifice for histopathological examinations. The tissue samples were fixed in 10% neutral formalin buffer, embedded in paraffin wax, cut into 5 µm sections and stained with haematoxylin and eosin [20]. For the semi-quantitative analysis, severity of the histological changes (fibrosis, oedema and cell infiltration) under light microscope on 5 microscopic fields (magnification x 10) was evaluated. These changes were scored as mild (1+), moderate (2+) and severe (3+).

**BIOCHEMICAL EXAMINATIONS**

Some pieces from each tissue were washed twice with cold saline solution, placed in glass bottles and stored at -30°C until processing within a maximum delay of 10 hours. After weighing, the tissue (1 g) was placed on ice, cut into small pieces with scissors and homogenized (2 minutes at 3000 x g) in ice-cold Tris-HCl buffer (50 mM, pH 7.4) (1:5, w/v) using a glass-Teflon homogenizer (Caliskan Cam Teknik, Ankara, Turkey). All preparation procedures were performed at 4°C. After addition of butylhydroxytoluol (4 µL / mL), the tissue homogenates were used for immediate nitric oxide, lipid peroxidation, GPx, CAT and GSH measurements.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Adhesion characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Complete absence of adhesion</td>
</tr>
<tr>
<td>1</td>
<td>Single band of adhesion between viscera from one viscus to the abdominal wall.</td>
</tr>
<tr>
<td>2</td>
<td>Two bands, either between viscera or from viscera to the abdominal wall</td>
</tr>
<tr>
<td>3</td>
<td>More than two bands between viscera or from viscera to the abdominal wall</td>
</tr>
<tr>
<td>4</td>
<td>Multiple dense adhesions, or viscera directly adherent to the abdominal wall and extent of adhesive bands</td>
</tr>
</tbody>
</table>

**Table I: Grading of postoperative adhesions according to NAIR et al. [21].**
The lipid peroxidation intensity in tissue homogenate were measured with the thiobarbituric acid reaction by the method of PLACER et al. [23] evidencing the MDA (Malondialdehyde) formation. The glutathione (GSH) content of the tissue homogenate was measured at 412 nm using the method of SEDLAK and LINDSAY [26]. The tissue glutathione peroxidase (GPx) activities were spectrophotometrically measured at 37°C and 412 nm according to the method of LAWRENCE and BURK [17]. Results were expressed as µmol/g of protein.

The tissue protein content was measured by the method of LOWRY et al. [19] with bovine serum albumin as standard. The catalase (CAT) activity was assayed in tissue homogenate by the AEBI method [1] and results were expressed as katal/g of tissue. The tissue NO content was measured according to method of CORTAS and WAKID [9] and results were expressed as µmol/g of tissue.

STATISTICAL ANALYSIS

A Kruskal-Wallis test was used to compare the mean values among the groups and the Mann-Whitney U test was performed for establishing differences in the value distribution between two groups. All analyses performed with the statistical package for social science (SPSS) version 11.0 (Chicago, Illinois). Results were given in the text as mean ± standard error (SE) and differences were considered as significant when $P$ values were less than 0.05.

Results

Macroscopically, the proportion of adhesion-free animals was significantly higher in heparin treated rats (70%) than that observed in the C group (10%) ($P < 0.05$) whereas the percentages of animals without macroscopic adhesions in the PTX group (20%) and in the C group were similar. Besides, slight bleeding was even observed in 3 animals of the H group. Moreover, after grading the adhesion intensity, it was observed that the mean adhesion grade of the H group has not significantly differed with the mean score achieved in the S (sham) group while it was significantly depressed compared to the mean value obtained for the not treated control rats ($P < 0.05$) (Table II). The mean adhesion grade was also slightly lower in the PTX group than in the C group, but not significantly (Table II).

Pathological symptoms were not observed in microscopic examination of the uterus from the S group animals. In the C group, adhesions between uterus and neighbour tissues were visible. Oedema, thickness and capillary vascular proliferations were noted in adhesion regions. There were also focal micro-abscesses, fibrosis and intensive mononuclear cell infiltrations in the muscular layer of the uterus (figure 1). Oedema, fibrosis, mononuclear cell infiltrations in the adhesion regions, as well as the thickness of the uterine serosa and capillary vascular proliferation were also observed 14 days after surgery in sections of uterus from rats treated with heparin (figure 2) or with pentoxifylline (PTX) (figure 3). However, these pathological elements of the inflammatory reaction were less severe than in the C group: compared to the not treated animals, fibrosis was significantly less intense in the H group ($P < 0.05$) and cell infiltrations in the uterine mucosa were alleviated in the PTX group ($P < 0.05$) (Table II).

As shown in Table III, the superficial scrapping of the anti-mesenteric surfaces of the left uterine horn and of the left abdominal wall was coupled to slight increases of the lipid peroxidation intensity evidenced throughout the tissue MDA contents on the day 14 post surgery. However, the variations of the tissue MDA concentrations compared to the sham control group (group S) were not statistically significant even for the not treated group (group C) in which the mean MDA concentration was the highest. Additionally, the tissue GSH content and the antioxidant enzyme GPx and CAT activities tended to increase in the rats treated with heparin or with pentoxifylline (groups H and PTX) whereas these antioxidant systems were remained roughly constant in the not treated group. However, compared to the sham group, the fluctuations in the tissue antioxidant capacities were not significant. Nevertheless, the tissue NO content was significantly increased in the C group compared to the sham controls ($P < 0.05$) whereas in the 2 other operated groups, peritoneal instillations with heparin or with pentoxifylline have significantly prevented the NO accumulation in the uterine horn (Table III).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group S</th>
<th>Group C</th>
<th>Group H</th>
<th>Group PTX</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macroscopic findings</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adhesion grade</td>
<td>0.28 ± 0.18$^a$</td>
<td>1.90 ± 0.37$^c$</td>
<td>0.50 ± 0.30$^{ab}$</td>
<td>1.10 ± 0.23$^{bc}$</td>
</tr>
<tr>
<td><strong>Histopathological findings</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrosis</td>
<td>Absent</td>
<td>2.40 ± 0.16$^a$</td>
<td>2.00 ± 0.00$^b$</td>
<td>2.20 ± 0.13$^{ab}$</td>
</tr>
<tr>
<td>Oedema</td>
<td>Absent</td>
<td>2.70 ± 0.15</td>
<td>2.50 ± 0.16</td>
<td>2.40 ± 0.16</td>
</tr>
<tr>
<td>Cell infiltration</td>
<td>Absent</td>
<td>2.80 ± 0.13$^a$</td>
<td>2.60 ± 0.16$^{ab}$</td>
<td>2.30 ± 0.15$^b$</td>
</tr>
</tbody>
</table>

*Different superscripts $^{a,b,c}$ in the same row indicate significant difference between groups ($P < 0.05$).*

Table II: Macroscopic and histological traits of adhesions formed 14 days after superficial scrapping of the anti-mesenteric surfaces of the left uterine horn and of the left abdominal wall and intraperitoneal instillation of 0.9% NaCl solution (group C), 500 UI Heparin (group H) and 25 mg/kg of pentoxifylline (group PTX) (n = 10 rats in each group). In the group S (n = 7), laparotomy without mucosa scrapping was performed. Results were expressed as mean ± standard error.
HEPARIN AND PENTOXIFYLLINE EFFECTS ON INTRA-ABDOMINAL ADHESIONS IN RATS

Discussion

There are many experimental models for constituting peritoneal adhesions [7, 11-13, 16]. As the uterine horn model mimics abdominal surgery [7, 28], the anti-mesenteric surfaces of the left uterine horn and of the left abdominal wall were scrapped until petechial spots appeared for developing intra-abdominal adhesions. Different classifications were proposed for grading peritoneal adhesions [4, 6, 21, 29]; among them, the NAIR model scoring adhesions from 0 to 4 according to their severity [21] was retained in the present study because of its simplicity.

When a fibrin matrix between serosa tissue surfaces is insufficiently developed, the adhesion formation is prevented [29]. The effect of heparin on adhesion formation may be accounted by its actions on platelet aggregation and macrophage functions leading to enhanced fibrinolysis after antithrombin III and plasmin activation [6, 7]. It was previously demonstrated that heparin has a local action on the deperitonealized surfaces and directly accelerates fibrinolysis process [7]. In the present study, the intraperitoneal heparin adminis-

<table>
<thead>
<tr>
<th>Tissue parameters</th>
<th>Group S</th>
<th>Group C</th>
<th>Group H</th>
<th>Group PTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT (katal / g of tissue)</td>
<td>18.15 ± 1.71</td>
<td>19.57 ± 1.76</td>
<td>20.31 ± 1.42</td>
<td>22.97 ± 2.70</td>
</tr>
<tr>
<td>GPx (µmol / g of protein)</td>
<td>100.07 ± 3.62</td>
<td>108.10 ± 11.06</td>
<td>110.64 ± 9.91</td>
<td>119.29 ± 16.17</td>
</tr>
<tr>
<td>GSH (µmol / g of protein)</td>
<td>5.17 ± 0.49</td>
<td>4.59 ± 0.33</td>
<td>6.00 ± 0.65</td>
<td>6.30 ± 1.07</td>
</tr>
<tr>
<td>MDA (µmol / g of protein)</td>
<td>2.23 ± 0.20</td>
<td>2.55 ± 0.10</td>
<td>2.48 ± 0.21</td>
<td>2.49 ± 0.38</td>
</tr>
<tr>
<td>NO (μmol / g of tissue)</td>
<td>33.46 ± 0.08a</td>
<td>34.96 ± 1.25b</td>
<td>33.53 ± 0.11a</td>
<td>33.12 ± 0.05a</td>
</tr>
</tbody>
</table>

CAT: catalase; GPx: Glutathion peroxidase; GSH: glutathione; MDA: malonedialdehyde. Different superscripts a,b in the same row indicate significant difference between groups (P < 0.05).
tration has markedly reduced the adhesion formation in the rat uterine horns compared to the not treated controls \((P < 0.05)\) but, the haemorrhage risk due to the high heparin dosage used (500 UI) remains a problem. It was also observed that heparin played a major role in significantly reducing the fibrosis reaction compared to the not treated and surged controls \((P < 0.05)\).

On the other hand, pentoxifylline improves the tissue oxygenation and endothelial function and inhibits pro-inflammatory cytokine production, chemotaxis, platelet aggregation, tumour necrosis factor production by macrophages and neutrophil degranulation [22]. In the uterine horn model in rats [16, 29] and in rabbits [28], it was previously reported that PTX is able to reduce the adhesion formation after a primary injury. In this study, the adhesion formation was slightly decreased in animals treated with PTX but, there was no significant reduction in the macroscopic adhesion grade after intraperitoneal PTX instillation following injury. Macrophages play an important role in inflammation and in the subsequent adhesion formation. Following surgery, the number of macrophages increases and switch in the cell functions is observed [18]. KISHI et al. [15] reported that PTX attenuated reperfusion-associated membrane injury and tissue oedema and that the methylxanthine derivates suppressed leukocyte adhesion and improved hindlimb blood flow during the reperfusion period. In the present study, the mean oedema score was slightly diminished in the PTX group, but also, the mononuclear cell infiltration in the uterus mucosa was markedly and significantly reduced compared to the surged control group \((P < 0.05)\). PTX may have partly prevented the formation of intraperitoneal adhesion by reducing the oedema formation and mainly limiting the macrophage influx in the uterine mucosa.

It has been postulated that free oxygen radicals are involved in adhesion development following surgery [2-4]. The prevention of adhesion formation after strengthening the antioxidant superoxide dismutase and catalase activities or with free radical chelators like dimethyl sulfoxide and alloporinol has been observed in some animal studies [2, 27]. In rats pretreated with antioxidants, the severity and the extent of the adhesions after complete regional intestinal ischemia have been significantly reduced [2]. In this way, it was already reported that heparins can act as endogenous antioxidants in synergy with other free radical chelators [14, 25]. PTX is a potent free-radical scavenger, diminishing neutrophil degranulation and reducing the superoxide release [15]. QING et al. [24] reported that PTX significantly suppressed the MDA production while the SOD activities in grafted liver tissue at the end of cold preservation and 1 hour after reperfusion remained elevated. However, PTX may have no preventive effects in ischemic liver [10] and kidney [22]. In the present study, although the variations of the biochemical markers were not significant, it was recorded that the horn uterine surgery has induced a slight MDA accumulation in tissues 14 days after and that, contrary to untreated rats, glutathione and the antioxidant glutathione peroxidase and catalase activities were slightly increased when heparins or pentoxifylline were intraperitoneally administered. Consequently, it is probable that the antioxidant effects of heparin and PTX, even limited here, have partly helped to reduce intraperitoneal adhesions between serosa surfaces.

Nitric oxide (NO) is a free radical gas molecule that is produced from L-arginin oxidative scission catalyzed by the enzyme nitric oxide synthase (NOS) [2-4]. Increased NO content, especially associated with oxidative stress, is a harmful condition for tissue [2]. Although the studies on the role of NO in adhesion formation are limited, it was reported that intraperitoneal administration of aminoguanidine [3], melatonin [4] and resveratrol [27] decreases the NO formation as well as the incidence and extent of peritoneal adhesions. It was previously shown that heparins at high doses can significantly affect the vascular reactivity \textit{in vivo} by down-regulating the expression of the constitutive endothelial NO synthase (eNOS) [5]. In addition, heparins may also directly interact with NO, leading to the impairment of the NO functions [30]. As far as pentoxifylline is concerned, this compound suppresses the inducible NO synthase at the mRNA level [8] and may also directly inhibit NO, contributing in this way to the apoptosis inhibition [31]. The present study has clearly evidenced that heparin or PTX intraperitoneal administrations have markedly decreased the surgery-induced NO accumulation. By limiting in this way the intensity of the oxidative stress, heparin and PTX may play important roles in reducing the adhesion formation.

As a conclusion, the intraperitoneal instillations of heparins or pentoxifylline during uterine horn surgery have limited the intensity of the induced oxidative stress, mainly by reducing NO release and by strengthening the local antioxidant systems at a lesser extend. Nevertheless, the inflammatory reaction has persisted but PTX has significantly limited the mucosa macrophage infiltration and heparins have notably alleviated the fibrosis reaction. Although the adhesion formation was reduced with the 2 treatments, the heparins were more efficient than the PTX administration.

References

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