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Abolition of anti-adhesiogenic effect of heparin by protamine sulfate

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HIGHLIGHTS

- We aimed studying heparin's anti-adhesiogenic effects by inhibition with protamine.
- Inflammation is significantly lower in heparin group compared to others.
- Fibrosis and vascular proliferation, heparin group was superior to control group.
- It seems that heparin is effective at preventing adhesion in this rat model.
- Abolition of heparin's effect is likely exerted via its antithrombine activity.

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ABSTRACT

Objective: Intraabdominal adhesion is a frequently encountered condition after surgery and can end up in important complications. The objective of this study is to test whether the antiadhesiogenic effect of heparin could be antagonized by administration of protamine in a rat model.

Material and methods: A laparotomy with caecal abrasion model was used in 40 Wistar rats. Single dose of 1 cc saline was injected subcutaneously (SC) in one group (control); 50 IU/kg heparin was injected SC in Group 2; 50 IU/kg protamine SC given to Group 3; 50 IU/kg heparin and 50 IU/kg protamine was given SC to Group 4 for 3 consecutive days. Each group consisted of 10 rats. All rats were sacrificed one week later for macroscopic and microscopic examination and they were scored for adhesion using Mazuji adhesion scale.

Results: There was significant difference in the heparin group with respect to Mazuji adhesion score, histopathological score (fibrosis, inflammation and vascular proliferation) and S-100 staining ($P < 0.05$). Additionally, the inflammation was more severe in the mucosa and submucosa compared to serosa in the heparin group ($P < 0.01$). With respect to fibrosis and vascular proliferation, apart from submucosal fibrosis, heparin group was statistically superior to the control group by means of each layer ($P < 0.01$).

Conclusion: It seems that heparin is effective preventing adhesion in this rat model. Abolition of heparin's antiadhesiogenic effect by protamine administration is likely exerted via its antithrombine activity. Clinical application of our findings in intraabdominal surgery warrants further investigation.

1. Introduction

Postsurgical intraperitoneal adhesions may pose important problems for the patient and the surgeon as well and their management might become pretty much costly to the community. Their physiopathology is characterized by a cascade triggered by injury and composed of coagulation, inflammation and proliferation that end up in fibrin generation and eventual establishment of adhesions in the peritoneal cavity. Thus, its prevention is thought to be possible via reducing inflammation and increasing anticoagulant activity of the intraperitoneal cavity [1–3]. Postoperative abdominal adhesions are seen in 95% of patients after surgery and the rate of re-hospitalization because of those adhesions is reported

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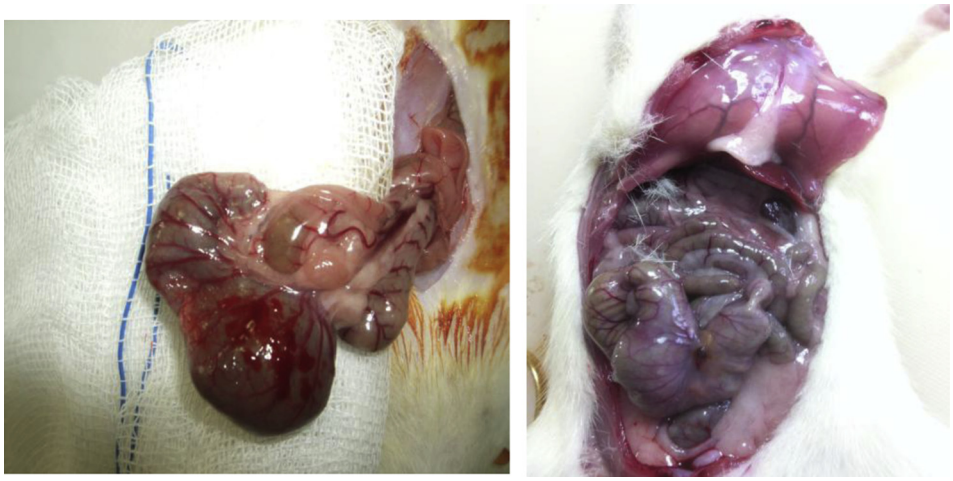

Fig. 1. Establishment of adhesion model (before and after).

to be 6% [4,5]. They are mostly symptomless and/or they may cause light abdominal pain, although occasionally they may lead to obstruction of the bowel or even sometimes of upper urinary tract and they sometimes can be a cause infertility in women as well [6,7].

Heparin use is indicated in the treatment and prophylaxis of venous thrombosis, pulmonary embolism, pectoral angina and acute myocardial infarction, coronary by-pass, vascular surgical procedures, coronary angioplasty, stent applications and in some selected disseminated intravascular coagulation. Main indication to use heparin is its anticoagulation and antithrombotic effects which are exerted via its antithrombine character. Heparin inhibits thrombin, factors Xa, IXa, Xia and XIIa activities [8,9]. On the other hand, protamine sulfate is an antagonist of heparin and it works by cleaving heparin from antithrombine [10].

In this study, we aimed studying heparin's anti-adhesiogenic effects by inhibition of its activity using protamine sulfate.

2. Material and methods

Experimental protocol was approved by the Ethical Board of Marmara University Experimental Animal Research Center (DEHAMER) (5/27/2013, #39.2012.Mar). Forty female rats weighed between 180 and 220 g were obtained from DEHAMER. They were fasted for 12 h and then weighed and they were anesthetized using a combination of intramuscular 3–5 mg chloropromazine (Largactil® 25 mg/5 ml amp Eczacıbaşı, Istanbul, Turkey) and 100 mg/kg ketamine (Ketalar®500 mg flc; Pfizer, Istanbul, Turkey). Surgical procedures were performed with sterile technique. Once general anesthesia induced the abdominal region was cleaned using 7.5% povidone iodine soap (Polyod®) and saline, the area was then shaved and stained with 1% povidone iodine and a laparotomy was done aseptically via 3 cm median incision. Cecum was then found and abrasion was performed onto the cecum by rubbing the surface for 10 times at a 1 cm square surface using dental floss, until subserosal ecchymosis obtained. Subsequently the abdomen was closed using continuous 3/0 silk suture and the surgery was ended. Afterward, four experimental groups were defined, each including 10 rats and the following treatments were performed subcutaneously (SC) for three days to each group starting two hours after the surgery:

- Group 1 (control group) ($n = 10$) SC saline,
- Group 2 ($n = 10$) SC 50 IU/kg heparin (Nevparin 25,000 IU/5 ml flacon, Mustafa Nevzat, Istanbul, Turkey),
- Group 3 ($n = 10$) SC 50 IU/kg protamine (Protamine ICN 5000 IU/5 ml ampoules, MEDA, Istanbul, Turkey),
- Group 4 ($n = 10$) SC 50 IU/kg heparin + 50 IU/kg protamine were administered [11].

During this period, all rats were housed in single cages in a room with controlled temperature (22 ± 2 °C), humidity (50 ± 5%), and a 12-h cycle of light and dark. They were fed laboratory pellet chows and water was given ad libitum. The evaluation of the general condition, food intake, and postoperative movements of rats showed that there was no need of additional analgesic administration.

Seven days after surgical intervention the rats were blindly sacrificed by cervical dislocation and their abdomen was opened in a U shape incision and it was carefully examined. The presence or absence of ascites, intestinal dilatation, hematoma and intestinal obstruction were checked. Adhesion and fibrotic areas were also recorded (Fig. 1) and they were scored based on Mazuji classification [12], which is grade-0: No adhesion; grade-1: very small, irregular adhesion; grade-2: easily separable, moderately rigid adhesion, grade-3: hard and hardly separable regular adhesion; grade-4: Very hard, too hard to separate, homogenous adhesion. Finally, the cecum was excised with the fibrotic area and they were sent for microscopical evaluation.

Histopathological evaluation was performed by one pathologist and histopathological scoring was performed based on fibrosis, inflammation, vascular proliferation and the depth into bowel wall (mucosa, submucosa and serous layers) (Fig. 2). Furthermore a staining with S100 was performed to rule out neural staining [9,10]. Fibrosis was scored as follows: 0: no fibrosis, 1: minimal, loose fibrosis, 2: medium degree fibrosis, 3: dense fibrosis. Inflammation was scored as follows: 0: no inflammation, 1: existence of giant cells, occasional lymphocytes and plasmocytes, 2: giant cells, plasma cells, eosinophil and neutrophils, 3: various inflammatory infiltrates and microabscesses. Vascular proliferation was scored as follows: 0: no vascular proliferation, 1: light vascular proliferation, 2: medium vascular proliferation, 3: severe vascular proliferation.

2.1. Statistics

Mann–Whitney *U* test was used for 2 group comparisons, whereas Kruskal–Wallis H and Kruskal–Wallis H with Bonferroni correction were used for comparison of 3 or more groups between

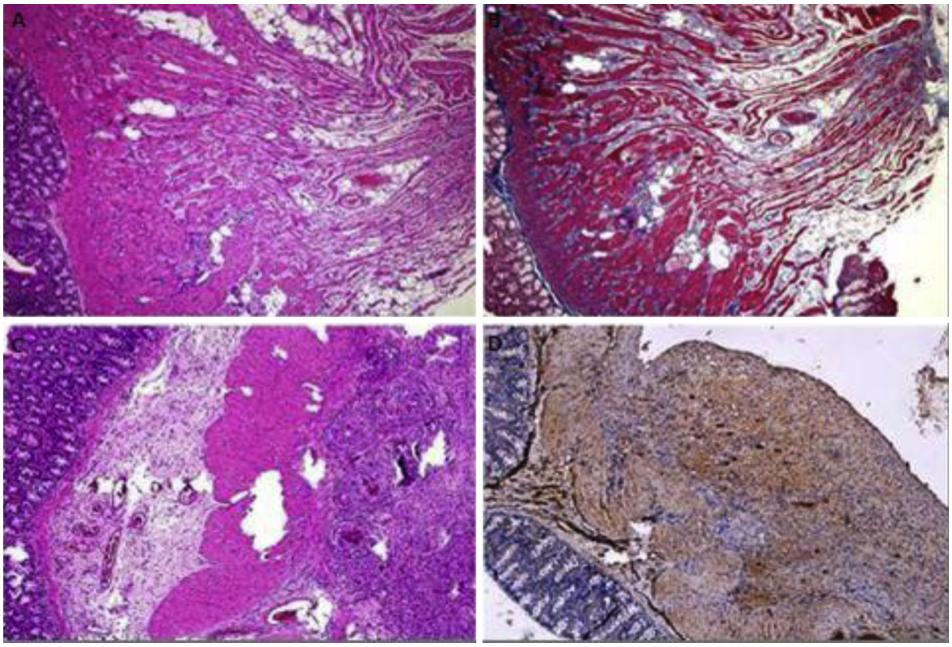

Fig. 2. A: Regular colonic mucosa, light inflammation and accompanying increase in the connective tissue in the peripheral fatty tissue extending into the fibers of muscularis propria (H&Ex100). **B**: Masson's trichrome staining of the same field shows increase in connective tissue in the fatty tissue (masson trikromx100). **C**: Severe inflammation (scored as grade 3) in the fatty tissues consisted of severe infiltration of lymphocytes, polymorphs and histiocytes, other colonic layers look well arranged (H&Ex100). **D**: s100 positive staining at the nerve cuts and also at the severe inflammatory areas in this immunohistochemically prepared specimen (s100x100).

each other. Interdependence among parameters was tested using Fisher's exact test. A $P < 0.05$ and in two group comparisons a $P < 0.01$ were considered significant.

3. Results

No rat mortality was observed. At the exploratory laparotomies minimal ascites was seen only in group 1 and 2 rats, whereas no bowel dilation, no hematoma and no bowel obstruction were recorded. Scoring for adhesion is shown in the Table 1. There was statistically significant difference between control group and heparin group compared to others ($P = 0.006$ and when group 1 and 2 compared, $P = 0.001$). With respect to adhesion rates, there was a difference between control group and group 2 ($P = 0.005$), however no difference was found between control group and groups 3 and 4 ($P = 0.582$). As for S100 staining, there was a difference between control group and group 2 ($P = 0.021$), but no difference observed between control group and groups 3 and 4 ($P > 0.05$).

Histopathological evaluation, fibrosis, inflammation and vascular proliferation and their statistical comparisons are summarized in Table 2. Total Histopathological Score for each rat was calculated as "inflammation + fibrosis + vascular proliferation" and it was found 5.3, 2.4, 5.9 and 5.4 for groups 1,2,3,4 respectively. Therefore Total Histopathological Score was found to be significantly lower in group 2 ($P = 0.0001$). With respect to inflammation, the mucosal and submucosal inflammation was found to be lower in the heparin group compared to others ($P = 0.0001$), but there was no difference in serosal inflammation between groups ($P > 0.01$). A "total inflammation score" was calculated for every rat by summing mucosal + submucosal + serosal inflammation scores, and that was found to be 5.0 in group 1; 1.6 in group 2, 4.5 in group 3 and 4.5 in group 4 respectively. These scores show that inflammation is significantly lower in group 2 compared to others. Furthermore, with respect to fibrosis and vascular proliferation, apart from submucosal fibrosis, heparin group was statistically superior to the control group by means of each layer ($P < 0.01$); mucosal, submucosal and serosal layers (Table 3).

4. Discussion

The use of barriers and pharmacological agents in the research aiming to decrease adhesion formation are mainly based on three

Table 1
Adhesion scoring, rating and S100 staining in all groups.

Group	Rat number	Adhesion score					Adhesion rate (%)	Staining w. S100
		0	1	2	3	4		
1-Control group	10	1	3	4	2	0	9/10 (90)	7
2-Heparin group	10	8	2	0	0	0	2/10 (20)	1
3-Protamine group	10	3	3	2	2	0	7/10 (70)	8
4-Heparin + protamine group	10	3	5	1	1	0	7/10 (70)	7
P value		***P* = *0.006* < *0.05*** ***1–2* = *0.001***, 1–3 = 0.389 1–4 = 0.096 2–3 = 0.015, 2–4 = 0.022 3–4 = 0.578					***P* = *0.005***	***P* = *0.021***

Bold characters point to statistically significant values.

Table 2
Histopathological evaluation of adhesion field.

Histological feature	Group	Score				*P* value and group comparisons
		0	1	2	3	
Inflammation	Group 1	0	2	4	4	***P* = *0.004* < *0.05***
	Group 2	2	6	2	0	***1–2* = *0.004***, 1–3 = 0.168
	Group 3	0	2	8	0	1–4 = 0.511, ***2–3* = *0.007***
	Group 4	0	2	6	2	***2–4* = *0.006***, 3–4 = 0.453
Fibrosis	Group 1	1	4	3	2	***P* = *0.011* < *0.05***
	Group 2	5	4	1	0	1–2 = 0.021, 1–3 = 0.339
	Group 3	0	3	4	3	1–4 = 0.781, **2–3 = 0.002**
	Group 4	0	2	4	4	2–4 = 0.013, 3–4 = 0.497
Vascular proliferation	Group 1	1	3	6	0	***P* = *0.002* < *0.05***
	Group 2	3	5	1	0	1–2 = 0.031, 1–3 = 0.055
	Group 3	0	1	7	2	1–4 = 0.671, ***2–3* = *0.001***
	Group 4	0	5	4	1	***2–4* = *0.009***, 3–4 = 0.154

Bold characters point to statistically significant values.

principles, namely decreasing inflammation, increasing anticoagulation and increasing fibrinolytic. Despite that a lot of research is conducted in this field, their clinical applications remain to be very limited. A lot of chemicals or pharmaceutical agents have been used in preventing intraabdominal adhesions. Some of them are phosphatidylcholine, heparin, tissue plasminogen activator (t-PA), ankaferd, nadroparine calcium (low molecular weight heparin or LMWH), aprotinine, nonsteroid anti-inflammatory agents, corticosteroids, antihistaminics, carboxymethylcellulose, hyaluronic acid derivatives and barriers like Seprafilm… etc. [13–21].

Adhesion formation is a multifactorial process; although we focused mainly on its two aspects, namely on anticoagulation and on antithrombotic effects. Various pharmaceutical agents and/or anticoagulants have been used either intraperitoneally or subcutaneously in previous studies to decrease adhesion formation and some of those studies found heparin to be effective but some of them not [15,20,22], whereas we decided to use it systemically. Since heparin's adhesion decreasing effect could be linked to its anti-inflammatory effects [23], we decided only to investigate its effects on adhesion and fibrin formation by manipulating its antithrombogenic effects using protamine.

Although there are several successful and detailed scoring systems used for extent and severity of adhesion in literature [24] we preferred to use Mazuci MK et al. scoring system (12). We believe that the scoring system of Mazuci MK et al. was also simple and extensive enough.

Inflammation, fibrosis and vascular proliferation are the main aspects of adhesion formation and/or tissue repair [25]. In our study, score of inflammation, fibrosis and neovascularization was significantly lower in heparin group compared to other groups ($P < 0.05$), whereas the same score was high in the protamine group, implying antagonistic effect of protamine to heparin in the last group.

Regarding the degree of inflammation in colonic wall layers, we noticed that heparin was effective decreasing inflammation in the mucosal and submucosal layers but not in the serosal layers. This was surprising to us because our model constituted of serosal injury to the cecum and we expected more inflammation in the serosal layers. Actually, in a study investigating the effect of Ankafert Blood Stopper (ABS) on peritoneal adhesions, Comert M et al. [16], reported less adhesion formation but surprisingly higher degree of serosal inflammation in the ABS group. Even though there was no report or data about fibrosis and vascular proliferation scoring system according to layers in our literature research, fibrosis and vascular proliferation was investigated for each layer; mucosal, submucosal and serosal layers. Apart from submucosal fibrosis, heparin group was statistically superior to the control group by means of each layer.

The results of this study encouraged us to reconsider heparin's indications in the current surgical practice. Currently, it is well known that heparin and its derivatives is recommended and widely used in the perioperative prophylaxis of venous thromboembolism

Table 3
Depth of inflammation, fibrosis and vascular proliferation according to bowel layer and its scoring.

		Inflammation				Fibrosis				Vascular proliferation			
		0	1	2	3	0	1	2	3	0	1	2	3
Mucosal score	Group 1	2	6	2	0	7	3	0	0	5	5	0	0
	Group 2	9	1	0	0	10	0	0	0	9	1	0	0
	Group 3	0	10	0	0	4	6	0	0	0	10	0	0
	Group 4	0	10	0	0	8	2	0	0	4	6	0	0
Submucosal score	Group 1	0	4	4	2	4	3	2	1	1	5	4	0
	Group 2	7	3	0	0	6	4	0	0	6	3	1	0
	Group 3	0	5	5	0	2	3	3	2	0	5	4	1
	Group 4	0	6	4	0	4	5	1	0	1	7	1	1
Serosal score	Group 1	0	2	4	4	1	4	3	2	1	4	5	0
	Group 2	2	5	3	0	5	4	1	0	2	7	1	0
	Group 3	0	2	8	0	0	3	4	3	0	1	7	2
	Group 4	0	2	6	2	1	3	4	2	0	3	6	1
Mucosa (*P* value and group comparisons)		***P* = 0.0001 < 0.05** **1–2 = 0.002 < 0.01**; 1–3 = 1 > 0.01; 1–4 = 1 > 0.01 **2–3; 2–4 = 0.0001 < 0.01;** 3–4 = 1 > 0.01				***P* = 0.027 < 0.05** 1–2 = 0.067; 1–3 = 0.189 > 0.01; 1–4 = 615 > 0.01; **2–3 = 0.004 < 0.01**; 2–4 = 0.146; 3–4 = 0.075 > 0.01.				***P* = *0.001* < *0.05*** 1–2 = 0.057; 1–3 = 0.012 > 0.01; 1–4 = 0.661 > 0.01; ***2–3* = *0.000* < *0.01***; 2–4 = 0.022; 3–4 = 0.029 > 0.01.			
Submucosa (*P* value and group comparisons)		***P* = 0.0001 < 0.05** **1–2 = 0.0001 < 0.01**; 1–3 = 0.403; 1–4 = 0.241 > 0.01 **2–3, 2–4 = 0.001 < 0.01;** 3–4 = 0.661 > 0.01				***P* = 0.084 > 0.05**				***P* = *0.014* < *0.05*** 1–2 = 0.022; 1–3 = 0.426 > 0.01; 1–4 = 0.546 > 0.01; ***2–3* = *0.005* < *0.01***; 2–4 = 0.038; 3–4 = 0.168 > 0.01.			
Serosa (*P* value and group comparisons)		***P* = 0.021 < 0.05** 1–2 = 0.015; 1–3 = 0.510 > 0.01 1–4 = 0.744; 2–3 = 0.017 > 0.01 2–4 = 0.013; 3–4 = 0.737 > 0.01				***P* = 0.011 < 0.05** 1–2 = 0.021; 1–3 = 0.339; 0.01; 1–4 = 0.781 < 0.01; **2–3 = 0.002 > 0.01;** 2–4 = 0.013; 3–4 = 0.497 < 0.01.				***P* = *0.002* < *0.05*** 1–2 = 0.084; 1–3 = 0.028 > 0.01; 1–4 = 0.235 > 0.01; ***2–3* = *0.001* < *0.01***; ***2–4* = *0.006* < *0.01***; 3–4 = 0.264 > 0.01.			

Bold characters point to statistically significant values.

[26]. In view of that, we suggest that the clinical use of heparin in perioperative surgery could be extended in terms of its anti-adhesiogenic effects besides its traditional antithrombotic use.

In conclusion, it seems that heparin is effective in this adhesion model in rats. The protamine's antagonizing effect suggests that heparin's antiadhesiogenic effect is carried out via antitrombine III. As a result, we think that the clinical use of heparin could be expanded in terms of its antiadhesiogenic effects while it is clinically being used for its anticoagulation and antithrombogenic effects at perioperative period.

Ethical approval

Experimental protocol was approved by the Ethical Board of Marmara University Experimental Animal Research Center (DEHAMER) (5/27/2013, #39.2012.Mar).

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Author contribution

Enver Reyhan, study design and writing.
Oktay İrkörücü, study design.
Ali Sürmelioğlu, study design.
Selvinaz Özkara, pathological examination.
Kamuran Cumhur Değer, data collection.
Mehmet Aziret, data collection.
Hasan Erdem, data collection.
Süleyman Çetinkünar, data analysis.
Metin Tilki, data analysis.
Pelin Demirtürk, pathological examination.
Edip Akpinar, edition.

Conflicts of interest

There are no conflicts of interest.

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