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Original Investigation

Investigation of Antithrombotic Drug Effects on Preventing Thrombosis Emerging in Gluteus Maximus Flaps before Anastomosis

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Abstract

Aim of this study was to investigate the effects of thromboprophylactic agents at pre-anastomotic period of thrombosis in gluteus maximus muscle flaps. Forty five male Sprague-Dawley rats that have 289 g average weight were randomly diveded into 9 experiment groups and, each group was consisted of five rats. The first group was a control (sham) and not treated with antithrombotic agents. The other groups were consisted of rats treated with SC heparin, SC LMWH, oral aspirin and the combinations of these groups with IV heparin, and only treated with IV heparin and flaps of these groups were washed with % 10 heparin solution. For the ninth group, we didn't give any thromboprophylactic agents but washed flaps with % 10 heparin solution. The presence of fibrinogen, vWf and the thrombomoduline were investigated in the flap tissues. A diffuse thrombosis was evaluated in the sham group. There was found the lowest thrombosis findings in SC heparin group. A mild to moderate degree of thrombosis was seen in the SC LMWH, oral aspirin or IV heparin injected group. In this animal model, preoperative SC heparin treatment has significant thrombroprophylactic effect when used alone but not in combination.

Keywords: flaps, thrombosis, antithrombotic drugs

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Introduction

Pathogenesis of venous and arterial thrombosis is complex and multifactorial. The classic Virchow's triad includes vascular endothelial injury, reduced blood flow, and alterations in the constitution of the blood. The interactions among these factors play a role in activation of the hemostatic system and thrombus formation. Comprehension of the mechanism of pathological thrombus formation by the hypercoagulable states requires an understanding of normal hemostasis, which consists of two important processes: the platelet plug formation and fibrin clot formation that occurs concurrently at a site of vascular injury. The formation of a platelet plug covers adhesion, activation, and aggregation of platelets. Platelets adhere to the vascular subendothelium exposed collagen fibers directly via collagen receptors and indirectly by attaching to collagen bound von Willebrand factor (vWF) molecules at a site of vascular injury. Once adhered, platelets are activated by several agonists shuch as thrombin, collagen, epinephrine, and thromboxane A2 and release their dense-granule contents, causing further platelet activation, and aggregation to form the platelet plug. Transient or reversible conditions may occur in case of pregnancy, use of oral contraceptives, hormone replacement therapy, immobilization, trauma or major surgery, and prolonged travel [1].

Microsurgical failure rates vary from 5 to 10 percent of free flaps. Surgical technique is most important factor in patency alone. However, various pharmacologic therapies have been proposed to improve success for anastomosis. Altought pharmacologic prophylaxis and intervention are extensively used for microsurgical operations, an unified algorithm is not present in the literature. This may led to confusing and nonstandardized practice due to anecdotal experiences [2].

For minimizing the risk of postoperative thrombosis as a cause of flap failure, numerious agents are routinely used by microvascular surgeons at the preoperative period, through the surgical procedure, and the postoperative course. The frequently used drugs for surgical anticoagulation include the heparin-based anticoagulants (heparin and enoxaparin), the antiplatelet agents (aspirin, ketorolac), fibrinolytics [streptokinase, tissue plasminogen activator (t-PA)] and the plasma volume expanders Dextran[3].

In this study, our objective was to reveal the most effective drug administration protocol for preventing thrombosis emerging in gluteus maximus flaps at pre-anastomotic period by

immunohistochemically determination of fibrinogen, von Willeabrant Factor (vWF) and thrombomodulin levels.

Materials and Methods

After approval of our study protocol by local Ethic Committee for experiment animals of Inonu University, forty-five Spraque Dawley rats were enrolled in the study. All experimental process was performed in Inonu University, Experiment Animals Reproduction and Research Center. The rats were weighted and randomly divided into nine groups. Each group was consisted of 5 animals. The first group was a control (sham) and not treated with any antithrombotic agents. The other groups of rats were subjected to frequently used antithrombotic agents. From the second to eighth group of rats were treated with oral aspirin, oral aspirin and intravenous (IV) heparin, subcutaneous (SC) heparin, SC heparin and IV heparin, SC low-molecular weight heparin (LMWH), SC LMWH and IV heparin, IV heparin alone, respectively. Flaps of these groups were additionally washed with 10% heparin solution. The ninth group was not subjected to oral or IV antithrombotic agent administration but their flaps were washed with 10% heparin solution alone.

Surgical Procedures

All surgical procedures were performed by the same surgeon and the right side gluteus maximus muscle flaps were used. While flaps were taken from the first group (sham) of rats and the washed with isotonic NaCl at the first day of experiment, the second group of rats was treated with oral aspirin (15 mg/kg). Next day, while the same surgical procedure was applied to the second group of rats, the third group of rats was treated with oral aspirin. One day later, fourth group of rats were treated with subcutaneous unfractioned heparin (100IU/kg) whereas the third group of rats underwent surgical operation. These procedures were applied to the other groups in subsequent days. I.V. heparin was administrated to the rats in I.V. heparin injected groups via tail vein using bolus injection just before before cutting the pedicle of flaps. Except from sham group, flaps were washed 10% heparin solution immediately before taking from gluteus maximus muscle of rats. During the experiments, daily 10 rats underwent surgical intervention to prevent tiredness of surgeon.

Before surgery, the animals were anesthetized with intraperitoneal injections of ketamine (50 mg/kg) and xylazine (5 mg/kg). Each animal's back was shaved and disinfected with Betadin solution.

After the surgical preparation, gluteus maximus muscle trace was marked on the skin. From its upper edge, vertebral origin of muscle as viewing fascia was cut longitudinally and underside of muscle was put out. Turning from this point to lover edge, it was achieved to pedicle of muscle by cutting tensor fascia lata and vastus lateralis on the lateral side of muscle and, flap was removed [4]. Except from sham group, flaps were washed with 10% heparin solution just before removal then fixed in 10% formalin for histopathological examinations.

Imminohistochemistry

Paraffin blocks were prepared from the proximal, medial and distal areas of pedicle of flaps fixed in %10 formalin. Three tissue sections were taken from each block at 5 µm thickness. For staining, the tissue sections was kept at 60 ^{0}C for one hour in an incubator and then treated with xylol and ethanol solutions. Trombomodulin (Mouse clonal Ab, ab33513, abcam, Cambridge, UK), von VVillebrand factor (rabbitanti-human Ab, GeneTex), fibrinogen (goat anti-rat Ab, Nordic, Tilburg, Netherlands) staines were used to Streptoavidin-biotin histochemical analysis for determining these parameters.

Treated tissue sections were visualized by using light microscopy. The tendancy for thrombosis were evaluated by staining rates of fibrinogen, vWf and trombomodulin. Unstained sections were accepted negative (0) whereas the low, moderate and high stained sections were graded as weak (1), medium (2) and intense (3), respectively. Additionally, widespread of staining was noticed to evaluate fibrinogen and vWf.

Percent values of stainig rates were calculated for variables in experimental groups and then compared with those of control groups.

Results

Staining rates of fibrinogen in the flaps were indicated in table 1. When assessed histopathological findings, the presence of fibrinogen was intensely detected on all tissue sections in sham group. However, this finding was found weak on sections in SC heparin-injected groups. SC heparin and İV heparin injected group has the 80% weak and 20% medium staining rates (Fig 1a-b) whereas oral aspirin adninistrated group has 40% medium and 60% intense staining rates (Fig 1b-c). When IV heparin added to oral aspirin administrated group, 80% weak and 20% medium staining rates were determined (Fig1 a-b). On the other hand, SC LMWH injected group showed 80% weak and 20% medium staining rates (Fig 1a-b). When IV heparin administrated to this group, these staining rates were found 60 % and 40 % (Fig 1a-b). It was found that staining rate of fibrinogen was 100% weak in only IV heparin injected group whereas this rate was %100 medium in flaps only washed with 10% heparin solution (Fig 1a-b).

When viewed staining rates of vWf in table 2, staining rate was intense on all flaps of sham group whereas that was weak on all flaps of only SC heparin injected group. Staining rate was 80% weak and 20% medium (Fig 2a-b) in SC heparin and IV heparin injected group. On the other hand oral aspirin administrated group showed 40% medium and 60% intense staining rates (Fig 2b-c). When IV heparin added to the oral aspirin group, these findings remained unchanged.

SC LMWH injected group showed 80% medium and %20 intense staining rates (Fig1 b-c) but IV heparin addition changed these ratios to weak 60% and medium 40%, respectively (Fig 1a-b) (Table 1). Staining rates for vWf was found 100% weak in only SCH injected groups whereas this rate 100% medium in only IVH group (Table 2).

For the determination of thrombomodulin, staining rate was also intense on all flaps of sham group whereas the flaps of only SC heparin injected group did not show any reactivity (Table 3). Staining rate was only 20% weak (Fig 3a) in SC heparin and IV heparin injected group. On the other hand oral aspirin, LMWH and Heparin washed (HW) administrated groups were showed 100% medium (Fig 3b) staining rate. When IV heparin added to the oral aspirin group, that rate was completely weak. And an 100% intense staining rate was found in Aspirin, LMWH and Heparin washed administered groups (Fig 3c).

SC LMWH injected group showed 100% medium (Fig 2b) staining rate but IV heparin addition changed that to 800% weak staining rate (Fig 2a) (Table 2). Staining rates for thrombomodulin were similar to staining rates of vWf in only IV heparin injected group and only heparin washed (sham) group.

During the experiment, subcutaneous hematoma was detected in two animals in SC LMWH injected group and one animal in SC standard heparin injected group. No death had occurred during experimental process.

Table 1. Tissue fibrinogen levels in study groups

Group	**Weak**		**Medium**		**Intense**		**Total**	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Sham	**0**	0.0	0	0.0	**5**	**100.0**	5	100.0
Aspirin	**0**	0.0	**3**	**60.0**	**2**	**40.0**	5	100.0
SCH	**5**	**100.0**	0	0.0	**0**	0.0	5	100.0
SCH+IVH	**4**	**80.0**	**1**	**20.0**	**0**	0.0	5	100.0
Aspirin+HIV	**4**	**80.0**	**1**	**20.0**	**0**	0.0	5	100.0
LMWH	**0**	0.0	**4**	**80.0**	**1**	**20.0**	5	100.0
LMWH+IV	**3**	**60.0**	**2**	**40.0**	**0**	0.0	5	100.0
IVH	**5**	**100.0**	0	0.0	**0**	0.0	5	100.0
HW	**0**	0.0	**5**	**100.0**	**0**	0.0	5	100.0
Total	**21**	46.6	16	35.5	**8**	17.9	45	100.0

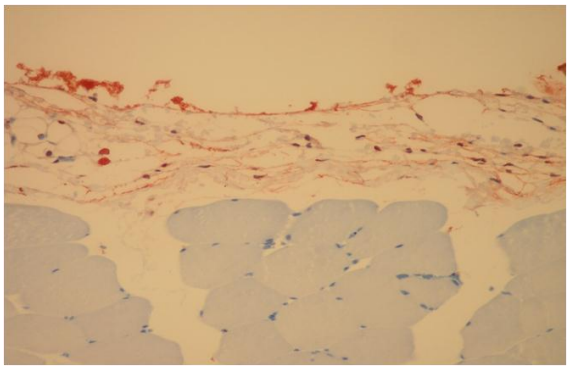

Figure 1a

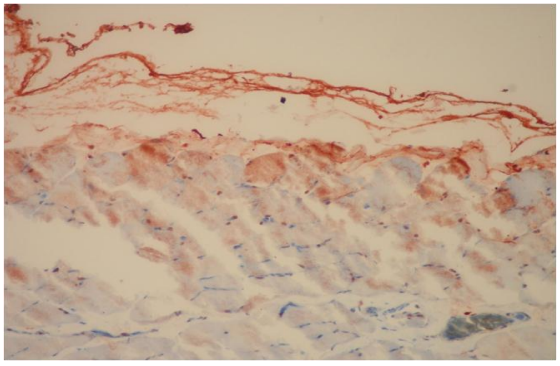

Figure 1b

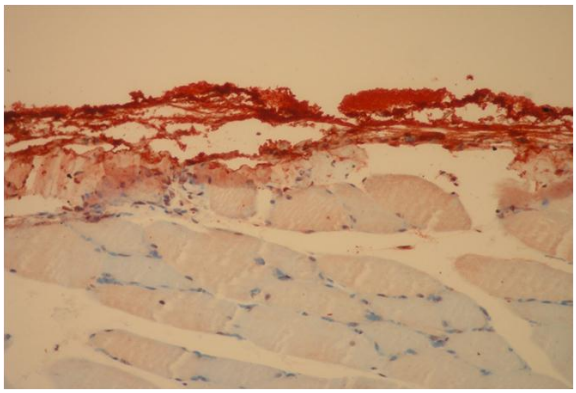

Figure 1c

Figure 1. a) Low staining rate b) medium staining rate c) intense staining rate for tissue fibrinogen (View x 400)

Table 2. Tissue von Willebrant factor levels in study groups

	Weak		**Medium**		**Intense**		**Total**	
Group	**N**	**%**	**N**	**%**	**N**	**%**	**N**	%
Sham	**0**	0.0	**0**	0.0	**5**	**100.0**	5	100.0
Aspirin	**0**	0.0	**2**	**40.0**	**3**	**60.0**	5	100.0
SCH	**5**	**100.0**	**0**	0.0	**0**	0.0	5	100.0
SCH+IVH	**4**	**80.0**	**1**	**20.0**	**0**	0.0	5	100.0
Aspirin+IVH	**0**	0.0	**2**	**40.0**	**3**	**60.0**	5	100.0
LMWH	**0**	0.0	**5**	**100.0**	**0**	0.0	5	100.0
LMWH+IVH	**0**	0.0	**5**	**100.0**	**0**	0.0	5	100.0
IVH	**0**	0.0	**5**	**100.0**	**0**	0.0	5	100.0
HW	**0**	0.0	**1**	**20.0**	**4**	**80.0**	5	100.0
Total	**9**	**20**	**21**	46.6	**15**	33.3	45	100.0

SCH: Subcutaneous Heparin; IVH: Intravenous Heparin; LMWH: Low Molecular Weight Heparin; HW: Heparin Washed

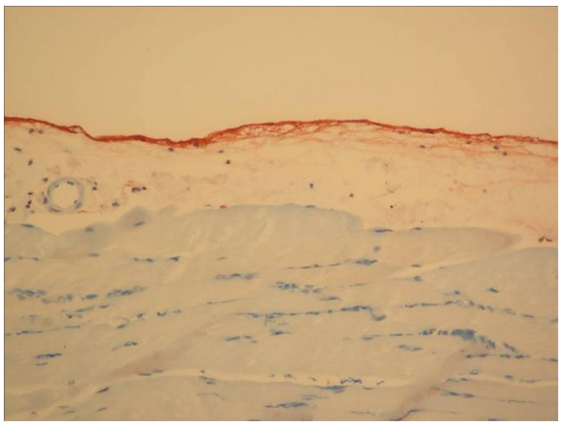

Figure 2a

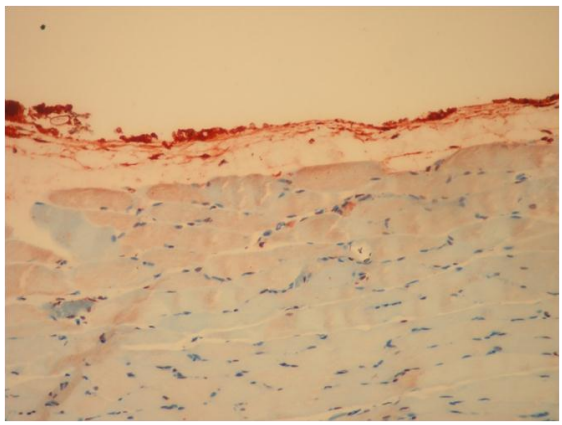

Figure 2b

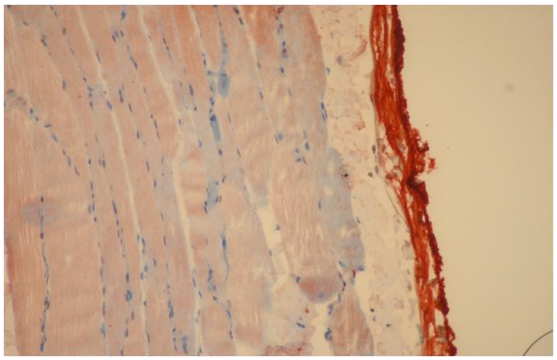

Figure 2c

Figure 2. a) Low staining rate b) medium staining rate c) intense staining rate for tissue von Willebrant factor (View x 400)

Table 3. Tissue thrombomodulin levels in study groups

	None		Weak		Medium		Intense		Total	
Group	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Sham	0	0.0	0	0.0	0	0.0	5	100.0	5	100.0
Aspirin	0	0.0	0	0.0	5	100.0	0	0.0	5	100.0
SCH	5	100.0	0	0.0	0	0.0	0	0.0	5	100.0
SCH+IVH	4	80.0	1	20.0	0	0.0	0	0.0	5	100.0
Aspirin+IVH	0	0.0	5	100.0	0	0.0	0	0.0	5	100.0
LMWH	0	0.0	0	0.0	5	100.0	0	0.0	5	100.0
LMVH+IVH	0	0.0	5	100.0	0	0.0	0	0.0	5	100.0
IVH	0	0.0	5	100.0	0	0.0	0	0.0	5	100.0
HW	0	0.0	0	0.0	5	100.0	0	0.0	5	100.0
Total	9	20.0	16	35.5	15	33.3	5	11.1	45	100.0

SCH: Subcutaneous Heparin; IVH: Intravenous Heparin; LMWH: Low Molecular Weight Heparin; HW: Heparin Washed

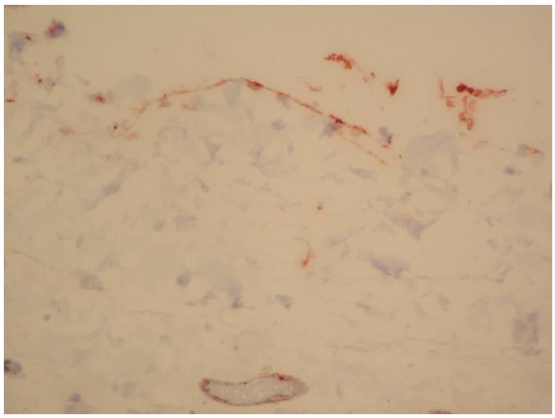

Figure 3a

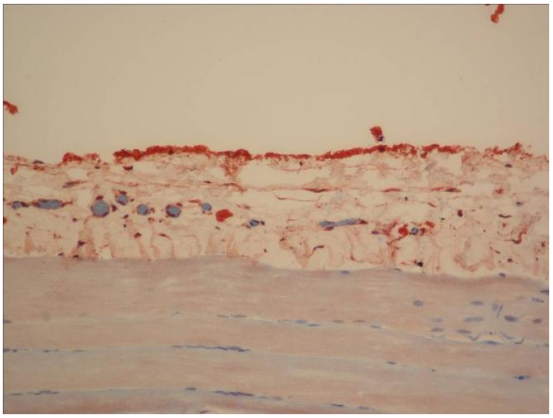

Figure 3b

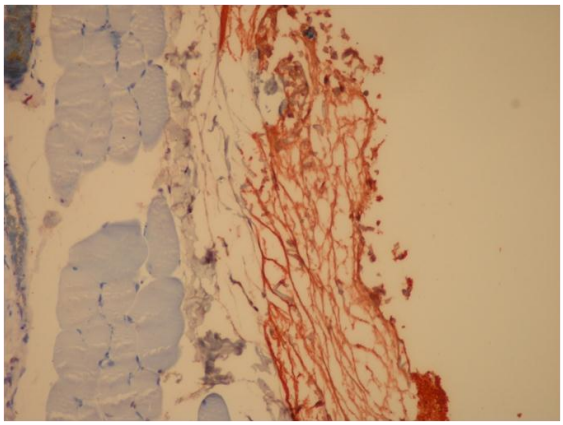

Figure 3c

Figure 3. a) Low staining rate b) medium staining rate c) intense staining rate for tissue thrombomodulin (View x 400)

Discussion

Despite major improvements and significant refinements of techniques, microsurgical anastomosis still carries a significant risk of failure due to microvascular thrombosis. The key for success of microvascular surgery may be achived by pharmacologic control of thrombus formation. In this process, glycoprotein IIb/IIIa is a highly abundant platelet surface receptor that plays a major role in platelet aggregation by binding platelets to each other through the coagulation factor fibrinogen [5].

Endothelium is much important in regulation of coagulation cascade. It has procoagulant activity by producing several cofactors including High molecular weight kininogen, factor V and VII, von Willebrand factor, and tissue factor [6]. Thrombin converts fibrinogen to fibin and promotes platelet activation and upregulates the activity of coagulation cascades by activating factor V and VIII. Hence, thrombin inhibition is a very important antithrombotic strategy [7].

Weksler et al. (1983), [8] studied the ability of a single oral dose of aspirin to inhibit prostacyclin synthesis by human arterial and venous tissue and to inhibit thromboxane A2 synthesis by platelets in 70 patients who were undergoing aortocoronary bypass. It was shown that a low dose of aspirin (40 to 80 mg) can largely inhibit platelet aggregation and thromboxane synthesis but has much less effect on prostacyclin production in arterial and

venous endothelium. Esclamado et al. (1999),[6] also revealed that oral aspirin administration had not superiority effect on rat microvascular free flaps in preanastomotic pedicular damages when comparedd to controls. In our study, we found that preoperative aspirin administration was not enough to reduce thrombosis alone and, this result was similar to previous one. On the other hand, Chien et al. (2005), [9] also showed that oral aspirin and IV heparin administration had not more effective than the other treatment regimens on thrombosis in microvascular free flaps using for head and neck reconstruction. Our results indicate that this combination has litlle effects on preventing thrombosis in the gluteus maximus flaps. However, Peter emphasized that low dose aspirin (5mg/kg systemically) inhibits anastomotic venous thrombosis and improves microcirculatory perfusion in their rat model after provided quantitative data confirming and clarifying the beneficial effects of low dose [10].

In 817 patients, Norgren et al. (2004), [11] studied that wheather LMWH could be replaced with standard heparin for peripheral artery repair, and they did not found any significant difference between them. In Samama' et al study, 201 consecutive patients scheduled for femorodistal reconstructive surgery under general anesthesia were enrolled in an open randomized multicenter (n = 14) study (from November 1990 to November 1992). Just before arterial cross-clamping, patients were given an intravenous bolus of either enoxaparin (ENX), 75 anti-Xa IU/kg (n = 100), or unfractionated heparin (UFH), 50 IU kg (n = 101). Meanwhile, the saphenous vein or a prosthetic graft was flushed with ENX (25,000 anti-Xa IU) or UFH (25,000 IU) in 250 ml of saline solution. Subsequent treatment consisted of subcutaneous administration of ENX, 75 anti-Xa IU/kg, or UFH, 150 IU kg, beginning 8 hours after the intravenous injection and then every 12 hours thereafter for 10 days. Analysis of patients on an intention-to-treat basis (patients who received at least on injection of ENX or UFH and who had at least one end-point evaluation) showed that graft thrombosis occurred in 30 of 199 cases: eight (8%) in the ENX group and 22 (22%) in the UFH group (p = 0.009). Among the 131 patients who were evaluated by arteriography before day 12, twelve (9.1%) had graft thrombosis: four (6%) in the ENX group and eight (12.5%) in the UFH group (NS). There were no significant differences between the two groups in terms of safety--that is, there were 12 major hemorrhages in each group, and during the follow-up period five patients in the ENX group died compared to nine in the UFH group (NS). These results indicate that ENX is as safe as but more effective than UFH when used for the prevention of early graft thrombosis in patients undergoing femorodistal reconstructive surgery [12].

In our study, we found that LMWH had lower antithrombotic effect when used alone, but effectiveness of LMWH inceased to moderate level when combined with IV heparin injection. In a study related to LMWH, Hadlock et al. (2003), [13] had revealed that LMWH was not effective in preventig of thrombus formation. However, a standard microarterial anastomosis tuck injury was created in both femoral arteries of 25 Long Evans retired breeder rats. Thirteen animals received a subcutaneous injection of 50 IU/kg of enoxaparin 2 hours before the procedure, while 12 control animals received vehicle (isotonic sodium chloride solution) alone. Sites of injury/repair were assessed 2 hours after the procedure for anastomotic patency or thrombosis. Six (23%) of 26 vessels in the drug-treated group developed an arterial thrombosis at the site of repair, while 6 (25%) of 24 vessels in the control group developed thrombosis. There was no statistically significant difference at the 95% confidence interval between the 2 groups based on a comparison-of-proportions test. So, the preoperative subcutaneous administration of 50 IU/kg of enoxaparin did not alter the rate of arterial thrombosis following the creation of a thrombogenic tuck injury/repair of the rat femoral artery. Montalescot et al., 2006, [14] emphasized that, in elective percutaneous coronary intervention (PCI), a single intravenous bolus of 0.5 mg of enoxaparin per kilogram is associated with reduced rates of bleeding, and a dose of 0.75 mg per kilogram yields rates similar to those for unfractionated heparin, with more predictable anticoagulation levels.

In our study, Immunohistochemical parameters were all weakly stained in three SC heparin injected groups but, staining rate increased to high when I.V. heparin added intraoperatively. Altought SC heparin is satisfactory to decrease the tendency of thrombosis, this achievemnt decreases with intraoperatively IV heparin administration.

Andresen et al. (2002), [7] found that heparin is an effective antithrombotic substance when used in arterial microsurgery and, they suggested that local administration of heparin is superior to systhemic administration, having an equal antithrombotic effect, but less influence on systemic parameters. Contrary to Andresen' findings, our results showed that local administration of heparine did not show adequate beneficial antithrombotic effects in the flaps of only heparin washed group. Unlikely, SC heparin administration is most effective for preventing thrombosis in flaps when combined with local administration of heparin.

Conclusions

When compared to the literature, our study seems to be the most extent study in this field, and it is the first study in which the thrombosis had been investigated in flaps by using immunohistochemical methods. Among the antithrombotic and anticoagulant drugs, preoperatively SC heparin administration brings down thrombosis to the lowest degree. Additionally, IV heparin administration decreases the effect of SC heparin in gluteus maximus muscle flaps. LMWH has lower antithrombotic effect when used alone, but effectiveness of LMWH inceases to moderate level when combined with IV heparin injection.

Conflict of interests

None declared.

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