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Effects of intra-arterial heparin on cytokine levels in the ischemic tissue

Research Article

Sami Karapolat[1]*, Bilgehan Erkut[2]

[1] *Department of Cardiovascular and Chest Surgery, State Hospital, 13000 Bitlis, Turkey*

[2] *Department of Cardiovascular Surgery, Faculty of Medicine, Ataturk University, 25080 Erzurum, Turkey*

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Abstract: The purpose of this study was to evaluate the effects of systemic and intra-arterial application of heparin by measuring tissue levels of inflammatory cytokines. Twenty-one adult male Wistar albino rats were divided into three groups (Group A, B and C). All the rats had undergone ligation of the right femoral artery with 4-0 silk suture to induce limb ischemia. Group A was the control group. In Group B, unfractionated heparin of 1500 U/kg/day was given through the tail vein for 10 days, the same dose was given to distal part of ligated right femoral artery for 10 days in Group C. On the 3rd, 5th, and 10th days, biopsies were taken from rectus femoris muscle on the ischemic extremities. Tumor necrosis factor-α, interleukin-1β, and vascular cell adhesion molecule levels in muscle tissue were measured by a standard enzyme-linked immunoabsorbent assay method.An increase in tumor necrosis factor-α level was found in all three groups throughout the duration of the experiment. The increase in Group C was statistically significant as compared with the other groups. The significant increases that occurred in tumor necrosis factor-α level as a result of intra-arterial application of heparin can be postulated to be one of the results of angiogenesis induced by the heparin in ischemic extremities. This might delay the formation of a necrosis in ischemic extremities, depending on the increased angiogenesis response by means of intra-arterial heparin application and may result in extended vitality of an extremity.

Keywords: *Ischemia • Heparin • Angiogenesis stimulators • Cytokines*

1. Introduction

Atherosclerosis and peripheral arterial occlusion have become one of the most important health problems of our age, largely because of improper nutrition and smoking. Both the wide spread of these pathologies and increasing development of diagnostic devices have resulted in an increase in the number of diagnoses, atherosclerosis is one of the diseases most frequently encountered by vascular surgeons. Local and systemic inflammations occur as a result of ischemic damage, increasing levels of plasma cytokines, arachidonic acid derivatives, complement and free oxygen radicals, inflammation also brings about neutrophilic activation. Finally, it can cause dysfunction of the vital organs as well as severe ischemia, threatening the viability of the extremities [1].

In general, vascular collaterals develop in response to an ischemic stimulus. Thus, vascular endothelial growth factor can stimulate the development of collateral arteries. Collateral vessel development in ischemia-induced neovascularization contributes to more rapid recovery of blood flow in the ischemic areas. However, the collaterals formed are insufficient to accomplish complete recovery from the ischemia. Therefore, an angiogenesis that develops rapidly and more intensely may be postulated as a way to negate the existing disease condition.

Angiogenesis, the formation of new blood vessels, is important in wound healing, inflammation, and ischemic disease. Monocytes-macrophages are key angiogenesis effector cells in these settings. Angiogenesis is driven by microvascular endothelial cells and accompanied by perivascular inflammation. Cytokines are a group of proteins and peptides used in organisms as signaling

* *E-mail: samikarapolat@yahoo.com*

compounds. These chemical signals are similar to neurotransmitters and allow one cell to communicate with another. Among these, tumor necrosis factor-α (TNF-α) is produced primarily by macrophages, it promotes numerous inflammatory reactions associated with atherosclerosis, including the induction of vascular adhesion molecules and the recruitment and proliferation of monocyte-macrophages [2,3]. Interleukin–1β (IL–1β) is a pro-inflammatory cytokine, and is produced by macrophages and monocytes. It is important in the regulation of hematopoiesis. In addition, the vascular cell adhesion molecule (VCAM) has a considerable role in the human immune system. It contains six or seven immunoglobulin domains, and is expressed on both large and small vessels only after the endothelial cells are stimulated by cytokines.

Thromboembolic disorders are driven by hypercoagulable, hyperactive platelet, proinflammatory, endothelial dysfunction, and proangiogenesis states. Heparin can effectively modulate all of those multifactorial components [4]. It is an anticoagulant drug used for prevention and treatment of thrombosis. Khorana et al. suggested that, the relation with heparin and angiogenesis is dependent to dose and molecular weight of heparin [5].

The purpose of this study is to investigate whether intra-arterial treatment of heparin will show any difference from standard intravenous heparin application from the standpoint of tissue ischemia and angiogenesis. For this purpose, tissue cytokine levels in rats receiving heparin treatment were compared. If any significant increase in the cytokine levels occurs as a result of angiogenesis in the treatment group, it would be possible to suggest a new method for the treatment of the peripheral arterial occlusion and tissue ischemia frequently encountered in clinics.

2. Material and Methods

Twenty-one adult male Wistar albino rats with an average of 220–250 g body weight from the same colony were used. The reasons for using this strain of rats are easy availability, safety and used often for similar experiments 6). The rats were cared for in accordance with the Guide for the Care and Use of Laboratory Animals. They were kept at 21–23°C, with controlled humidity, and a dark-light cycle of 12 h to 12 h. Food and water were available *ad libitum*. The experimental protocol was approved by Ataturk University, School of Medicine, Animal Care and Use Committee.

Rats were randomly assigned to three groups.: Group A (n=7) was the control, Group B (n=7) was treated with systemic heparin, Group C (n=7) was treated with intra-arterial heparin. All rats were anesthetized by administering ketamine hydrochloride (Ketalar, Pfizer, Istanbul, Turkey) 50 mg/kg/intraperitoneal and xylazine hydrochloride (Rompun, Bayer, Turkey) 3 mg/kg/ intraperitoneal according to their body weights. During the surgery, rats were put into the supine position, the right femoral region was shaved and disinfected using povidone iodine. All the rats underwent ligation of right femoral artery with 4-0 silk suture by longitudinal femoral incision to induce limb ischemia, and the layers were closed properly after bleeding control. During the first 24 hours, rats were given Buprenorphine 0.03 mg/ kg subcutaneously every 8 hours. Antibiotic therapy with cefazolin sodium (15 mg/kg/intramuscular) was given for 10 days.

In Group B, unfractionated heparin of 1500 U/kg/day (Liquemine, Roche, Brazil) was administered through the tail vein for 10 days. In Group C, the same dose of heparin was administered to the distal part of ligated right femoral artery for 10 days. Heparin was administered at one dose per day. In Group C, before this procedure, rats were given ketamine hydrochloride 10 mg/kg/ intraperitoneally, the incision in the femoral region was opened from bottom and the femoral artery was located. Heparin was then administered intra-arterially, and the incision was closed.

During the 10-day follow-up period, muscle biopsies from the rectus femoris muscle were taken from the ischemic extremities of the rats on the 3rd, 5th, and 10th days. Before this procedure, rats were given ketamine hydrochloride 10 mg/kg/intraperitoneally, the bottom part of the muscle was accessed through another incision opened at right thigh distal, and muscle tissue samples were taken.

Wound infection developed in two rats (1 in Group A and 1 in Group C) and they were excluded from the experiment protocol. Rats were sacrificed at the end of 10th day by administering lethal ketamine hydrochloride intraperitoneally.

TNF-α, IL-1β, and VCAM levels in muscle tissue cuts were measured using a standard enzyme-linked immunoabsorbent assay method (TNF-α, IL-1β and VCAM ELISA kits, R&D systems, USA) according to the manufacturers' instructions. Each tissue was stored in a separate bowl at -80°C until analysis. A Tris tampon of 10 mL was added per one gram of frozen tissues. Homogenates were centrifuged at 10.000 x G for 10 minutes after homogenization. Supernatants were kept in stock at -80°C until analysis. The levels of cytokines were expressed as picograms per tissue wet weight.

The results were evaluated statistically, with all results calculated as mean ± SEM for each study group.

Figure 1. Levels of TNF-α on the 3rd, 5th and 10th days in Group A, B and C. A significant difference on the 3rd and 10th days was observed between Group B and C (p<0.05).

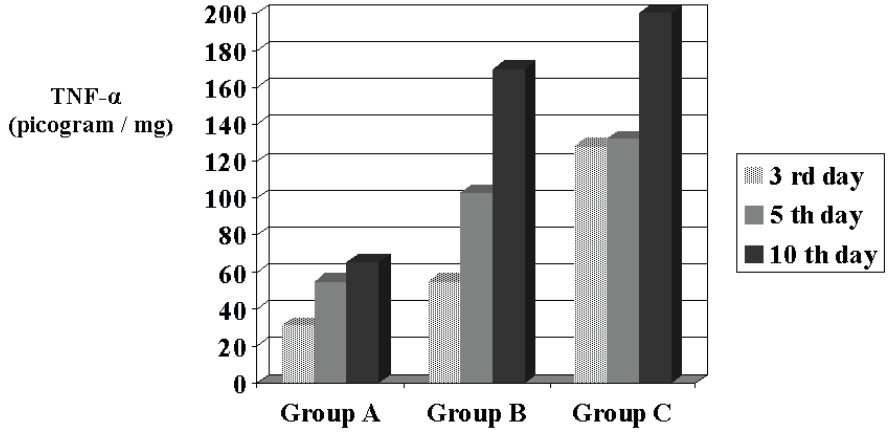
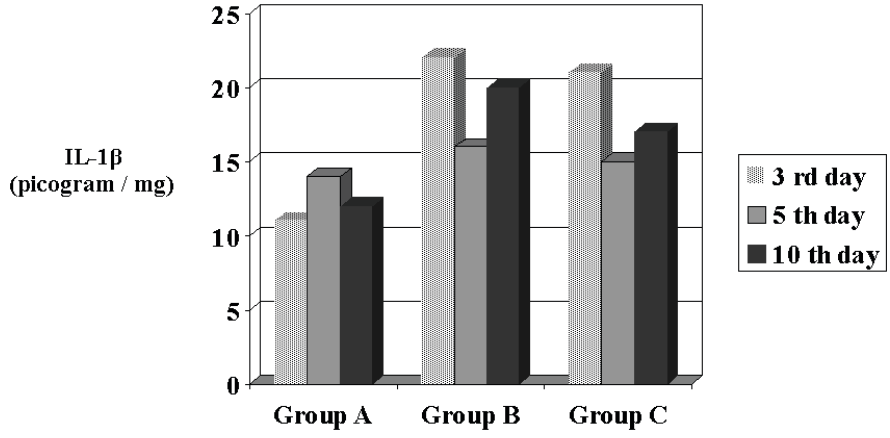


Figure 2. Levels of IL-1β on the 3rd, 5th and 10th days in Group A, B and C. There was no statistically significant difference throughout the whole follow-up period (p>0.05).



All statistical analyses were carried out using SPSS 10.0 statistical software (SPSS Inc, Chicago, IL). To evaluate the difference among experiment groups, the Nonparametric Mann Whitney-U test was used, with p<0.05 accepted as significant in this test.

3. Results

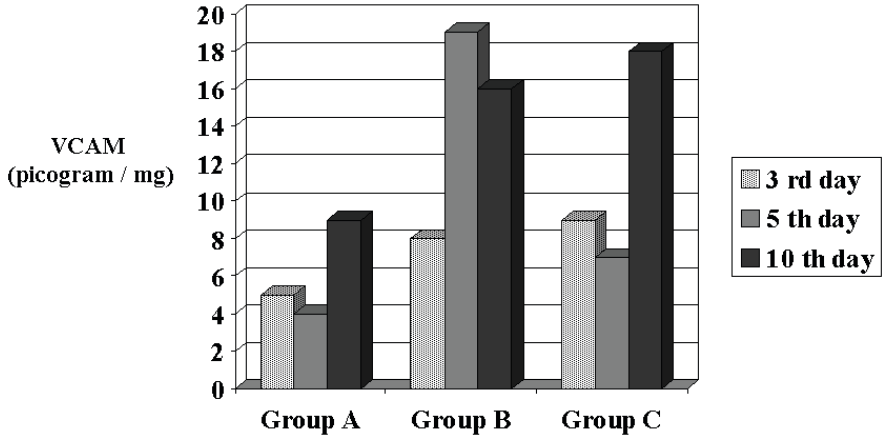
TNF-α levels were 30.34 ± 2.19 in Group A, 54.21 ± 4.83 in Group B, and 127.96 ± 3.93 for Group C on the 3rd day, 55.43 ± 1.11 in Group A, 103.74 ± 2.57 in Group B, 132.74 ± 1.58 for Group C on the 5th day, and 64.32 ± 2.22 in Group A, 169.17 ± 2.88 in Group B, 201.1 ± 2.65 for Group C on the 10th day (Figure 1).

The values of the parameters of TNF-α were compared for the following time intervals: 3rd to 5th, 3rd to 10th, and 5th to 10th day. The TNF-α level in Group A increased during the experimental period but these increases were not statistically significant (z=-1.1, p>0.05). In Group B, significant differences were observed for all three time intervals for TNF-α, and the result was significantly different (z=-2.2, p<0.05). In Group C, the results of TNF-α level on the 3rd and 5th days were very close to each other. However, when the values for the time intervals of 3rd to 10th and 5th to 10th days were compared, a statistically significant difference was found (z=-2.3, p<0.05). A significant difference in the values of TNF-α parameters on the 3rd and 10th days was observed between Group B and C (z=-2.3, p<0.05 and z=-2.5, p<0.05, respectively). No significant difference was detected for the samples taken on the 5th day.

IL-1β levels were 10.19 ± 3.11 in Group A, 22.84 ± 3.17 in Group B, 21.75 ± 1.48 for Group C on the 3rd day, 14.44 ± 1.13 in Group A, 15.15 ± 3.77 in Group B, 14.47 ± 3.52 for Group C on the 5th day, and 12.44 ± 3.53 in Group A, 19.99 ± 2.78 in Group B, 17.51 ± 1.91 for Group C on the 10th day (Figure 2).

The values of the parameters of IL-1β were compared for the following time intervals: 3rd to 5th, 3rd to 10th, and 5th to 10th day. There was no statistically

Figure 3. Levels of VCAM on the 3rd, 5th and 10th days in Group A, B and C. In Group B, a statistically significant difference was found the values of the 3rd to 5th and 3rd to 10th day time intervals ($p<0.05$). In Group C, 3rd-10th and 5th-10th days were compared, and there was a statistically significant difference ($p<0.05$).



significant difference in the IL-1β values throughout the entire follow-up period.

Finally, VCAM levels were 5.11 ± 3.61 in Group A, 7.77 ± 3.43 in Group B, 9.64 ± 2.44 for Group C on the 3rd day, 4.34 ± 1.21 in Group A, 19.11 ± 2.35 in Group B, 7.29 ± 2.58 for Group C on the 5th day, and 9.13 ± 1.69 in Group A, 16.36 ± 4.09 in Group B, 18.09 ± 4.96 for Group C on the 10th day (Figure 3).

The values of VCAM parameters were compared for the following time intervals: 3rd to 5th, 3rd to 10th, and 5th to 10th day. When VCAM levels among these intervals were compared in Group A, there was no statistically significant difference ($z=-0.9$, $p>0.05$). In Group B, a statistically significant difference was found when the values of the 3rd to 5th and 3rd to 10th day time intervals were compared ($z=-2.4$, $p<0.05$). However, there was no significant difference between 5th and 10th day values ($z=-1.3$, $p>0.05$). In Group C, when VCAM levels between the 3rd and 5th days were compared, there was no statistically significant difference ($z=-1.2$, $p>0.05$). However, when the VCAM levels between the 3rd and 10th and 5th and 10th days were compared, there was a statistically significant difference ($z=-2.2$, $p<0.05$).

4. Discussion

The present study reveals that there was generally an increase in the TNF-α and VCAM levels studied on the tissue biopsies of ischemic extremity in the postischemic period. This is a result of a systemic inflammatory response occurring at the ischemic extremity. As long as the ischemia continues, an comparable increase in the levels of these cytokines can be expected. TNF-α levels were increased in Groups B and C (treated by heparin). The increase in the treatment with heparin administered intra-arterially (Group C) was greater compared with the systemic application (Group B). There were no significant changes in the levels of IL-1β, and there were even some decreases in these levels in the heparin groups (B and C) at the end of the experiment. When the VCAM levels were compared, the increase in Group B towards the middle of the experiment remained fixed until the end of the experiment, whereas in Group C the initial value showed a decrease on the 5th day, but reached a value twice the initial value at the end of the experiment.

TNF-α is a polypeptide hormone and a pleiotropic inflammatory cytokine. It is an acute phase protein that initiates a cascade of cytokines and increases vascular permeability. This cytokine is involved in the regulation of a wide spectrum of biological processes, including cellular proliferation, differentiation, apoptosis, and coagulation. TNF-α possesses both growth-stimulating properties and growth-inhibitory processes. Furthermore, it promotes the remodeling of injured tissue by stimulating fibroblast growth [7]. TNF-α enhances endothelial cell permeability and increases the production of growth factors. In limb ischemia, it is thought that TNF-α increases primarily in the myocytes of the ischemic muscle. In vascular endothelial cells, TNF-α increases the expression of angiogenic factors, such as the vascular endothelial growth factor, the basic fibroblast growth factor, and interleukin-8 [8] and is angiogenic *in vivo*. The angiogenic properties of TNF-α might be consequent to the production of secondary mediators.

Heparin modulates the binding of many angiogenic factors, such as the vascular endothelial growth factor, fibroblast growth factor, scatter factor, and hence, affects proliferation of endothelial cells [9]. It contributes to the formation of a more porous fibrin matrix. Heparin not

only affects the proliferation of endothelial cells, but also facilitates angiogenesis by altering the structural and mechanical properties of the fibrin network. The similar effects of heparin and TNF-α on such angiogenic factors are likely to be explained by similarities in their effecting mechanisms. The concentration of heparin in the serum must reach a specific point before TNF-α can produce an optimal angiogenesis. This suggests that angiogenesis is induced by heparin, and a resulting positive feedback mechanism produces a certain increase in the levels of TNF-α working towards the same objective. In our study, the group where TNF-α levels increased the most was the C Group, to which heparin was administered intra-arterially. We assume that this increase of TNF-α level induces the angiogenesis that occurred in the ischemic extremity by the intra-arterial administration of heparin. If this is correct, the question of what kind of additional benefits might be obtained if heparin treatment is directly oriented to the distal of the obstructed vessel gains importance. In general, the reduced blood flow in the distal of an obstructed arterial system causes intra-arterial stasis, leads to the increase of existing thromboses, and causes the formation of new thrombi. This may lead to the formation of thrombi that can reach the end points of the branches of the obstructed main artery unless heparin treatment is initiated. We suggest that such a widespread thrombus formation can be prevented, on the condition that heparin treatment is initiated at an earlier stage. In this way, it is clear that an intensive angiogenesis will occur in the end points of the arterial system that can remain more open if we take into account that angiogenesis generally initiates from these vascular ending points. Such an effect in the ischemic extremities might delay the formation of necrosis and preserve the vitality of the extremities for an extended time.

However, it is known that if the levels of TNF-α increase dramatically, its angiogenic effect is reversed and an anti-angiogenic effect is created [10,11]. In this study, the dose of heparin was controlled to a level that can be accepted as sufficient and efficacious for rats and that causes no systemic bleeding [12]. Under these conditions, it was about 50% between the 3rd and 10th days of the experiment, when the TNF-α levels reached the highest level in Group C, and was below the level where anti-angiogenic effect starts.

Another support for these ideas is that the plasma concentration of TNF-α is generally high in the early stage of myocardial infarction, and that the TNF-α levels remain high in persons having a prior history of myocardial infarction. It is thought that this observation is associated with the myocardial remodeling and development of a collateral system in response to the induction of angiogenesis in existing coronary vessels. Heparin has been found to promote angiogenesis in studies of its action in the context of coronary ischemia and cardiac development." In a study by Carroll et al., it was found that myocardial blood flow was restored and brought almost to normal levels in resting conditions, and substantially increased the recovery of coronary reserve in the subjects in whom coronary artery occlusion had occurred and heparin was administered at an early stage [13]. The role of heparin and angiogenesis is clear in these cases.

The upregulation of VCAM in endothelial cells is generally a response to increases in TNF-α and IL-1β levels. We assume that the VCAM increases seen in our study resulted from substantial increases in TNF-α level. This result may bring to mind that until now, no direct relation between angiogenesis and VCAM has been shown. However, considering our results, more research should be carried out on this subject. Another finding from our study is that there is no statistically significant result for the IL-1β levels in each three groups throughout the experiment. This could be interpreted to mean that IL-1β levels are in general independent from angiogenesis. Nevertheless, it is possible that this might be a result of the small number of the subjects in this study.

In a study carried out on heparin and TNF-α by Chamorro et al., 167 ischemic stroke cases were divided into two groups, one group was given unfractionated heparin, and the other group was given acetylsalicylic acid. During the treatment period, the levels of TNF-α and VCAM were measured and compared, and the authors observed that TNF-α levels decreased but VCAM levels increased less in the group to which unfractionated heparin was given. They concluded that high-dose heparin application has an anti-inflammatory effect and thus improves the recovery [14]. The decrease in TNF-α levels in that study is in contrast to our study. It is likely that this situation resulted from the mediator releasing between the extremities and central nervous system, the ischemic end organ and from the differences among their receptors.

In another study by O'Blenes et al., the authors found that heparin is effective at accelerating the pulmonary vascular maturation in newborn rabbits. In this study, it was found that heparin induces expression of the potent angiogenic growth factor, fibroblast growth factor-2, by smooth muscle cells. The authors also found that heparin appears to act as a co-factor in the binding of fibroblast growth factor-2 to its receptor, and increases fibroblast growth factor-2 stimulation of angiogenesis in a dose-dependent manner [15]. These findings support the notion that fibroblast growth factor-2 has an effect on

angiogenesis and smooth muscle cell proliferation as a potential mediator of heparin.

If a 4- to 6-hour effective time of heparin is considered, it is seen that the one dose per day in this study may be insufficient. However, the necessity of anesthetizing for each heparin application and continuous surgical intervention to the incision region made one dose per day application necessary. It was impossible to place a permanent intra-arterial catheter in rats and hold it in its place throughout the experiment. Thus, for this reason, we applied one nonhemorrhagic dose per day.

Other limitations of this study are that we have not performed measurement of the distal perfusion and blood flow in the ischemic extremities, and do not have histopathological proof of angiogenesis formation in these tissues. However, given the results we have obtained on the effects intra-arterial heparin, further studies are indicated. Among them, taking blood flow measurements in ischemic extremities on certain days of the experiment, as well as other invasive and non-invasive tests to show the tissue perfusion would be useful. In addition, pathological studies to verify angiogenesis would make our results obtained by intra-arterial heparin injection more valuable.

5. Conclusion

The significant increases in TNF-α levels in the ischemic extremity tissue due to intra-arterial heparin administration may be an indication of a partial increase in angiogenesis. Therefore, intra-arterial application of heparin in ischemic extremities might delay necrosis. Thus, by intra-arterial application of heparin, the vitality of the extremities might be preserved in the peripheral arterial occlusion cases we encounter frequently in the clinic. Also, the amputation ratio can be reduced and life comfort of the patients would be improved. Additional experimental studies still need to be carried out in this field, and following the histopathological proof of the obtained results may clarify actual results of intra-arterial heparin application through clinical Phase 1 and Phase 2 studies done on humans.

References

[1] Karapolat S, Dag O, Abanoz M, Aslan M. Arterial embolectomy, a retrospective evaluation of 730 cases over 20 years. Surg Today. 2006,36, 416-9

[2] Sugano M, Tsuchida K, Hata T, Makino N. In vivo transfer of soluble TNF-α receptor 1 gene improves cardiac function and reduces infarct size after myocardial infarction in rats. Faseb J. 2004,18, 911-3

[3] Shteinberg D, Halak M, Shapiro S, Kinarty A, Sobol E, Lahat N, et al. Abdominal aortic aneurysm and aortic occlusive disease, a comparison of risk factors and inflammatory response. Eur J Vasc Endovasc Surg. 2000,20, 462-5

[4] Mousa SA. Heparin, low molecular weight heparin, and derivatives in thrombosis, angiogenesis, and inflammation, emerging links. Semin Thromb Hemost. 2007,33, 524-33

[5] Khorana AA, Sahni A, Altland OD, Francis CW. Heparin inhibition of endothelial cell proliferation and organization is dependent on molecular weight. Arterioscler Thromb Vasc Biol. 2003;23: 2110-5

[6] Singh S, Padmanabhan R, Singh G, Kaur G. Effect of maternal hypervitaminosis A during late gestation on pre- & postnatal lung maturation in rat. Indian J Exp Biol. 1983, 3,103-7

[7] Schreyer SA, Peschon JJ, LeBoeuf RC. Accelerated atherosclerosis in mice lacking tumor necrosis factor receptor p55. J Biol Chem. 1996,271, 26174-8

[8] Sugano M, Tsuchida K, Makino N. Intramuscular gene transfer of soluble tumor necrosis factor-alpha receptor 1 activates vascular endothelial growth factor receptor and accelerates angiogenesis in a rat model of hindlimb ischemia. Circulation 2004,109, 797-802

[9] Collen A, Smorenburg SM, Peters E, Lupu F, Koolwijk P, Van Noorden C, et al. Unfractionated and low molecular weight heparin affect fibrin structure and angiogenesis in vitro. Cancer Res. 2000,60, 6196-200

[10] Guo DQ, Wu LW, Dunbar JD, Ozes ON, Mayo LD, Kessler KM, et al. Tumor necrosis factor employs a protein-tyrosine phosphatase to inhibit activation of KDR and vascular endothelial cell growth factor-induced endothelial cell proliferation. J Biol Chem. 2000,275, 11216-21

[11] Roesel JF, Nanney LB. Assessment of differential cytokine effects on angiogenesis using an in vivo model of cutaneous wound repair. J Surg Res. 1995,58, 449-59

[12] Cervera A, Justicia C, Reverter JC, Planas AM, Chamorro A. Steady plasma concentration of

unfractionated heparin reduces infarct volume and prevents inflammatory damage after transient focal cerebral ischemia in the rat. J Neurosci Res. 2004,77, 565-72

[13] Carroll SM, White FC, Roth DM, Bloor CM. Heparin accelerates coronary collateral development in a porcine model of coronary artery occlusion. Circulation 1993,88, 198-207

[14] Chamorro A, Cervera A, Castillo J, Davalos A, Aponte JJ, Planas AM. Unfractionated heparin is associated with a lower rise of serum vascular cell adhesion molecule-1 in acute ischemic stroke patients. Neurosci Lett. 2002,328, 229-32

[15] O'Blenes SB, Merklinger SL, Jegatheeswaran A, Campbell A, Rabinovitch M, Rebeyka I, et al. Low molecular weight heparin and unfractionated heparin are both effective at accelerating pulmonary vascular maturation in neonatal rabbits. Circulation 2003,108, 161-6