Original Research Article

Effect of Erythropoietin on Microvascular Anastomosis in Rat

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Abstract

Purpose: To investigate the re-endothelialization potential of erythropoietin (EPO) following microvascular anastomosis in rat femoral artery.

Methods: Ninety-six male Sprague-Dawley rats weighing between 300 g and 320 g were allocated randomly into two groups (control and EPO, n = 48). Left femoral artery microvascular anastomosis was performed in the rats. The control group was given 0.5 mL of 0.09 % NaCl subcutaneously 48 h post-operatively while the EPO group was given 150 U/kg recombinant human erythropoietin subcutaneously 48 h post-operatively. Tissue was harvested from each group after 1, 3, 5, and 7 days. All of the rats were sacrificed for morphometric analysis. Each arterial segment was analyzed morphometrically using Clemex Image Analysis software by a single examiner. The cross-sectional area of the media and neointima was measured and the intima/media ratio was calculated. Hematocrit measurement was carried out for all the rats.

Results: The I/M ratio differed significantly (p < 0.05) between the control and erythropoietin groups, at all time-points. The hematocrit was significantly (p < 0.05) lower in the control group than in the erythropoietin groups on day 7, but not on the other days.

Conclusion: This study demonstrates that erythropoietin markedly inhibits neointima formation with accelerated re-endothelialization in rat femoral arteries following microvascular anastomosis.

Keywords: Erythropoietin, Microvascular anastomosis, Re-endothelialization, Hematocrit, Neointima/media ratio

INTRODUCTION

Microsurgical procedures require much labor, time, and funding. Problems that arise with anastomosis account for most of the complications that follow microsurgical procedures. Delayed re-endothelialization at the anastomosis line leads to thrombus formation and anastomotic blockage for various reasons. The main contributor to a successful microsurgical procedure is safe vessel anastomosis. The faster the healing of the anastomosis is, the fewer complications that result.

There are many growth factors and hormones that stimulate angiogenesis. Vascular endothelial growth factor (VEGF) is an important growth factor with strong mitogenic effects on endothelial cells [1-4]. Erythropoietin (EPO) is a

critical regulator of the proliferation of erythroid and endothelial precursors, but its role as a potential re-endothelialization agent in microvascular anastomosis is unclear [5-8]. This study investigated the effect of erythropoietin on re-endothelialization histologically.

EXPERIMENTAL

The study was approved by the Ethics Committee of the Experimental Medical Research and Application Center of Selçuk University and the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985). We used 96 male Sprague–Dawley rats weighing 300 – 320 g. The animals were kept in the same room at 22 ± 1 °C under standard physical conditions and fed the same standard diet.

For the experiment, each animal was given intramuscular anesthesia with 35 mg/kg ketamine (Ketalar® 50 mg/mL vial, Pfizer) and 5 mg/kg xylazine (Rompun® vial, Bayer). The animal was positioned on its back on the operating table. After shaving the left inguinal area, the skin was cleaned with antiseptic solution and a 2-cm incision was made on the left inguinal crease with a no. 15 blade. After careful dissection, the femoral neurovascular bundle was identified. The femoral artery was exposed using an 8× loupe and clamped with an Acland approximator (00409A; S&T, Switzerland), then cut vertically and 0.1 % bupivacaine (Marcaine, AstraZeneca®) was applied locally to reduce vessel spasm.

After cleaning the adventitia, two tension sutures were inserted at 120° angle with 10/0-75 µm, round polyamide needles (Ethicon Ethilon-W2850-J&J). These sutures were hung around the Acland approximator. After repairing the anterior wall, the approximator was rotated 180 degrees. Then, the posterior wall was repaired. After fixing the approximator, the microclamp was removed. An average of six sutures was inserted for each anastomosis. Then, hemostasis was performed and the skin was closed with 4/0 silk sutures. The mean anastomosis time was 30 min, while the total operating time was 40 min for a rat. The animals were divided into control (n = 48) and EPO (n = 48) groups. The control group was given 0.5 mL of 0.09 % NaCl subcutaneously 48 h post-operatively. The EPO group was given 150 U/kg recombinant human erythropoietin (Eprex 2000 IU rHuEPO vial) subcutaneously 48 h post-operatively.

Tissue was harvested from each group after 1, 3, 5, and 7 days. For this, each animal was again

anesthetized with intramuscular ketamine and xylazine, as described above, and placed in the supine position on the operating table. After removing the sutures from the wound, the anastomosis area was dissected with the aid of an 8×190-mm loupe (Prismen Lupe KS, Carl Zeiss, Germany). A 1-mm length was taken from the femoral artery including the anastomosis.

Hematological analysis

Blood samples collected from carotid arteries.The Blood samples were taken from all animals and the hematocrit was determined using Roche Diagnostic Sysmex KX 21-N (Japan 2005).

Histological analysis

The tissue samples were fixed in 10 % formaldehyde, followed by Autotechnicon (Shandon Citadel 2000). After 24 h they were embedded in paraffin blocks vertically and 5-µm cross-sections were prepared from these paraffin blocks using a microtome. The cross-sections were stained with Verhoeff–Van Gieson stain and examined using a Nikon Eclipse E400 microscope.

They were photographed with a Nikon Coolpix 5000 digital camera. The photographs were analyzed using Clemex Image Analysis and the areas of the tunica intima and media were calculated (Figure 3).

Statistical analysis

The data were analyzed using Statistical Package for the Social Sciences (SPSS), version 16.0, for Windows. One-sample Kolmogorov–Smirnov test was used to analyze the distribution of continuous variables. Continuous variables with normal distributions were compared between the two groups using Students t-test. All significance levels were two-tailed and set at $p < 0.05$.

RESULTS

The results (Figs 1 – 3) of our investigation can be summarized as follows. First, in the early stage (1 – 3 days), EPO reduced intimal hyperplasia by increasing endothelialization at the microvascular anastomosis line. Second, at 5 – 7 days, intimal hyperplasia is insignificant due to the completion of endothelialization. Third, the hematocrit did not increase significantly following the use of EPO in the early stage (1 – 3 days), but increased significantly at 5 – 7 days.
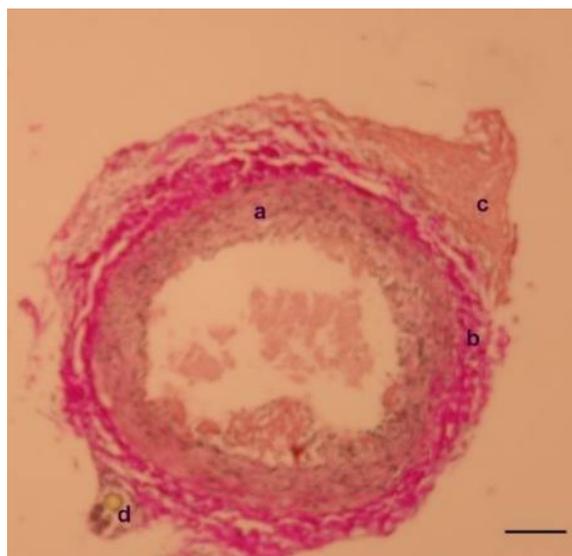
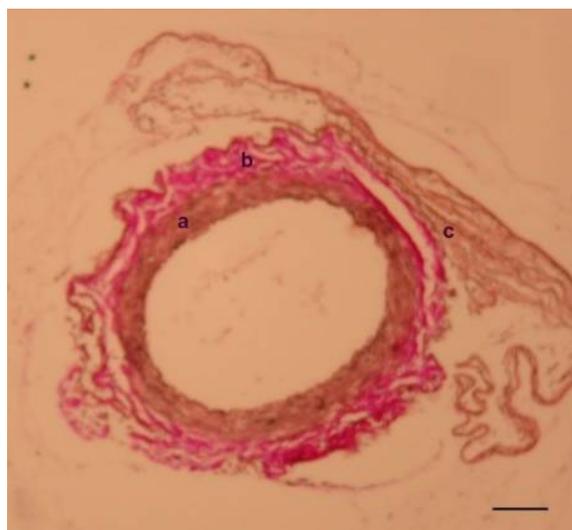**Figure 1:** Appearance of control group on day 7 after Verhoeff-Van Gieson staining: tunica intima. I/M (a/b) **=** 0.78

Figure 2: Appearance of the erythropoietin group on day 7 after Verhoeff-Van Gieson staining. I/M (a/b) **=** 0.69
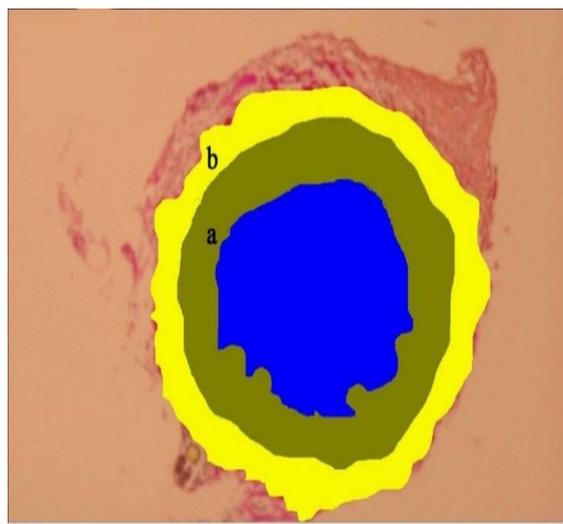

Figure 3: Tunica intima and tunica media areas. I/M (a/b) **=** 0.78

The mean intima/media (I/M) ratio, hematocrit and p-values of all groups are shown in Table 1. I/M ratio differed significantly ($p < 0.05$) between control and erythropoietin groups, at all-time points. Hematocrit was significantly ($p < 0.05$) lower in the control group than in the erythropoietin groups on day 7, but not on the other days.

DISCUSSION

The role of microvascular surgery in plastic surgery has been increasing gradually. The main condition for a successful microsurgical procedure is a safe vessel anastomosis. The faster the healing of the anastomosis is, the fewer complications there will be. The results of our investigation can be summarized as follows: This study supports other recent studies of angiogenic factors and underlines the importance of EPO in microvascular surgery. Based on these positive effects, we believe that EPO will reduce complications and increase the success rate of microsurgery.

Table 1: Group statistics for I/M and hematocrit

Parameter	Day	Erythropoietin (mean ± SD)	Control (mean ± SD)	*P*-value[a]
Intima/media ratio	1	0.97±0.02	1.04±0.05	**0.001**
	3	0.86±0.009	1.01±0.04	**0.000**
	5	0.69±0.01	0.80±0.02	**0.000**
	7	0.69±0.47	0.76±0.2	**0.000**
Hematocrit	1	40.83±3.27	40.41±2.71	0.737
	3	41.58±2.93	41±2.82	0.625
	5	43.83±3.24	41.25±3.19	0.183
	7	45.33±1.37	43.16±2.16	**0.008**

[a] Student's *t*-test

Many growth factors and hormones stimulate angiogenesis [1-3]. VEGF is an important growth factor that has a strong mitogenic effect on endothelial cells [4]. There are many other angiogenic growth factors and hormones, but only a limited number of studies have examined them. EPO is one of these factors. Cytokines and especially EPO-related hematopoiesis are thought to play a role in angiogenesis as hematopoietic and endothelial cell lines are derived from the same common ancestor [5-8].

Ribatti *et al* showed that cytokines previously thought to be specific for the hematopoietic system affect endothelial cell functions, including angiogenesis [9]. However, there are few studies of the angiogenic effects of EPO compared to other angiogenic factors (VEGF and fibroblast growth factor (FGF)). VEGF is the most important angiogenic factor [10].

In a study of myocardial tissue cultures from 111 patients, Jaquet *et al* showed that the capillary growth rate was 220 % with VEGF versus 230 % with EPO [11]. This study showed that EPO was as effective as VEGF and supports our results. However, Nitta *et al* stated that EPO increased DNA synthesis in cattle glomerular endothelial cell cultures and this stimulated VEGF synthesis [12]. EPO increases the synthesis of endothelin 1, via thrombin and angiotensin, two potential stimulators of ET-1 [5,6}. Salani *et al* studied the role of ET-1 in angiogenesis in human umbilical vein endothelial cell (HUVEC) cultures in comparison to VEGF, and found that ET-1 increases the angiogenesis induced by VEGF [14].

The potential interaction between VEGF and ET-1 was also investigated by Matsuura *et al*, who proved that VEGF increases the ET-1 secretion in endothelial cells [13]. All of these studies show that many factors interact in angiogenesis. In addition to the direct angiogenic effect of EPO, it also has indirect angiogenic effects by increasing the production of other factors.

Vascular injuries cause an increase in extracellular matrix, the proliferation of smooth muscle cells, and formation of neointima via cell proliferation and migration [15,16]. Endothelial cells inhibit intimal hyperplasia by regulating the growth of smooth muscle cells [15,17]. However, rapid re-endothelialization inhibits intimal hyperplasia via thrombus formation [18-23]. These data suggest that there is a reverse relationship between intimal hyperplasia and re-endothelialization.

Based on this information, many studies evaluating re-endothelialization have measured the neointima and media, and their ratio has been accepted as an indicator of re-endothelialization [20-24]. These values are calculated from a histomorphometric analysis and the resulting numerical data indicate that it is a sensitive and an effective method. We used Clemex Image Analysis for the morphometric analysis. Other techniques used to evaluate re-endothelialization are macroscopic photomicrography with Evans blue staining and fluorescence microscopy [21,23]. These techniques are more subjective than morphometric analysis. Morphometric field analysis is useful in the rat femoral artery reanastomosis model because the injury site in the endothelium is so small that it cannot be quantified sensitively using photomicrographic techniques.

Comparing the I/M ratios calculated in the morphometric analysis, it was found that the intima and media areas were lower than in the other groups, but their ratio (I/M) was higher. This shows that the tunica intima responds more rapidly and intensely to the injury at the anastomosis line than the tunica media.

In a histological study, Weinstein *et al* showed that elastic and muscular elements do not regenerate like the endothelium, and the response of the media layer to healing is slower than the intima [25]. Since the intimal hyperplasia in the control group was lower than in the erythropoietin group on day 1, it is thought that even a single dose of EPO has powerful positive effects on the endothelium. In both groups, the intima and media areas increased on the third day, while their ratio decreased. This showed that the intimal hyperplasia decreased in both the control and erythropoietin groups.

On the third day, the intima was thinner than on the first day; therefore, the re-endothelialization activity was rapid on the third day [25,26]. Less intimal hyperplasia was seen with the EPO activity in this group. On days 5 and 7, there was no substantial increase in the intima and the I/M ratio was low because re-endothelialization was complete [26-28]. The I/M values were significant in all groups ($p < 0.05$). This showed that erythropoietin decreased intimal hyperplasia by increasing re-endothelialization.

In this study, EPO was administered to the experimental animals subcutaneously at 48-hour intervals. Macdougall *et al* reported that the subcutaneous administration of EPO was the best treatment [29]. Following subcutaneous

injection, to maintain a high EPO concentration in blood, the injection must be repeated every 48 h. The hemoglobin increases markedly when EPO is used in chronic renal failure for long periods [30]. The main side effect is hypertension in patients treated with EPO [31]. However, lower-dose EPO treatment and careful selection of the hemoglobin target decreases the incidence of hypertension [31]. In a study of healthy individuals, McMahon *et al* did not observe hypertension in any of the subjects [32]. Saray *et al* reported that there was no significant difference in arterial blood pressure and hematocrit with short-term EPO use, but there was a significant difference with long-term use [33]. Therefore, we used low-dose EPO. In our study, the hematocrit was minimally higher in experimental groups 1 to 3 than in the controls, but the difference was not significant, while the difference was significant in group 4. These values might have resulted from the low-dose, short-term EPO use.

CONCLUSION

The findings of this study support other recent reports of angiogenic factors and underline the importance of EPO in microvascular surgery. It appears that EPO has two positive effects on microsurgery. First, it increases endothelia-lization on the anastomosis line and, secondly, it enhances flap vascularity. Based on these positive effects, it seems likely that EPO will reduce complications and increase the success rate of microsurgery. In this way, patients will not lose the opportunity for treatment, while physicians will be able to increase their success rates.

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