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Tavşan karotis arterlerinde yapılan anastomozlarda bosentanın intima hiperplazisi üzerine olan etkisi

Effect of bosentan on intimal hyperplasia of carotid artery anastomoses in rabbits

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ÖZET

Amaç: Tavşan karotis arterlerinde yapılan anastomozlarda bosentanın intima hiperplazisi üzerine olan etkisi araştırıldı.

Çalışma planı: Çalışmamızda 18 adet Yeni Zelanda tipi erkek tavşan ilaç alan (Grup B) ve almayan (Grup A) olmak üzere iki gruba randomize edildi. Tüm deneklerin sağ karotis arteri kesildikten sonra 10/0 polipropilen dikiş ipliği ile uç uca anastomoz yapıldı. Sol karotis artere bu uygulama yapılmadı. Grup B deneklere ameliyattan üç gün önceden başlayarak ve 21 gün süre ile 30 mg/kg/gün oral bosentan verildi. Grup A deneklere ise herhangi bir ilaç verilmedi. Ameliyat sonrası 28. gününde anastomoz bölgesi ve karşı kontrol tarafı çıkartılıp parçalar hazırlanarak histomorfometrik olarak incelendi.

Bulgular: Anastomoz yapılan arterlerde anastomoz yapılmayan sol tarafa kıyasla anlamlı derecede intima hiperplazisi geliştiği saptandı. Bosentanın ilaç almayan gruba kıyasla intima alanını (Grup A: 48.3 µm2 [37.1 µm2 - 65.7 µm2], Grup B: 31.4 μm^2 [12.2 μm^2-63.2 μm^2], [p=0.04]) ve intima/medya alan oranını (Grup A: 0.49 [0.13-0.74], Grup B: 0.22 [0.09-0.37], [p=0.024]) anlamlı şekilde azalttığı saptandı.

ABSTRACT

Objectives: We investigated the effect of bosentan on intimal hyperplasia of carotid artery anastomoses in rabbits.

Study design: Eighteen New Zealand male rabbits were randomized into two groups, as drug (Group B) and non-drug (Group A). The right carotid artery of all the subjects was transected and anastomosed end-to-end with 10/0 polypropylene suture. The left carotid artery was left intact. Group B subjects received 30 mg/kg/day oral bosentan for 21 days, starting 3 days before the operation. Group A subjects did not receive any medication. After 28 days, the anastomoses site and the contralateral control site were removed, and samples were investigated histomorphometrically.

Results: Significant intimal hyperplasia was observed at all anastomoses compared to the non-anastomotic left side. Bosentan decreased significantly the intimal area [Group A: 48.3 μm^2 (37.1 μm^2-65.7 μm^2), Group B: 31.4 μm^2 (12.2 μm^2-63.2 μm^2), (p=0.04)] and intimal/medial area ratio [Group A: 0.49 (0.13-0.74), Group B: 0.22 (0.09-0.37), (p=0.024)] compared to the non-drug group.

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Sonuç: Bosentanın tavşan karotis arter modelinde gelişen intima hiperplazisini azalttığı görülmüştür. Damar girişimlerinde bosentanın klinik kullanımının yararını desteklemek amacıyla ileri çalışmalara ihtiyaç vardır.

Conclusion: According to our investigation, bosentan decreased the intimal hyperplasia developed in a rabbit carotid artery model. Further investigations are needed to support the potential clinical utilization of bosentan after vascular interventions.

Abbreviations:

ET-1 *Endothelin-1*
ETA *Endothelin A*
ETB *Endotelin B*
GA *Gluteraldehyde*
MMP *Matrix metalloproteinase*

Nowadays occlusive arterial diseases are a serious cause of mortality, and morbidity. In the treatment of these diseases, revascularization, and medical treatment have important roles. The most important reason of failure in the early post-revascularization period is development of thrombosis on the site of anastomosis. However, frequently intimal hyperplasia is held responsible for failed cases observed in the long run. Intimal hyperplasia is an arterial accomodation mechanism against stressors. In addition, it is a part of a healing process, observed following any event of damage. Histologicallly, intimal hyperplasia is a uniform structure where smooth muscle cells, myofibroblasts, and scarce number of myofibriles are incorporated in a large stroma.[1]

Endothelin-1 (ET-1) is a polypeptide released mainly from endothelial cells, and it is the most powerful vasocontrictor agent known so far.[2] This polypeptide induces vasoconstriction via endothelin A, and B receptors localized on smooth muscle cells. In vitro studies have detected that ET-1 stimulates proliferation of smooth muscle cells.[3] In addition, since ET-A, and ET-B receptors are found in fibroblasts, and on the membranes of endothelial cells, stimulation of these cells by ET-1 also promotes expression of leukocyte adhesion molecules. Thus, they mediate interaction between leukocytes, and endothelial cells.[4] ET-1 acts as a cytokine which suggest its potential role in various vascular pathologies. This hypothesis is substantiated by higher levels of ET-1 in patients with aterosclerotic vessel disease associated with intimal hiperplasia.[5] In many literature studies, it has been emphasized that formation of neointima can be prevented by systemic inhibition of ET-1 via antagonizing ET receptors.[6]

Bosentan is an oral specific endothelin receptor antagonist. *In vitro* studies have demonstrated that bosentan-like ET antagonists strongly inhibit ET-1 .[5] In this study, we aimed to investigate potential inhibitor effect of bosentan on post-anastomotic carotid artery intimal hyperplasia, and proliferation of smooth muscle cells in rabbits.

MATERIAL AND METHOD

This study was a randomized controlled experimental investigation. After approval, and permissions of the ethics committee on animal experiments were obtained, the study was realized in research laboratory of experimental animals.

Subjects

In our study, randomly selected 18 New Zealand male rabbits each weighing 2900-3100 g (3000 ± 100 g) were used. The

subjects were randomized in two groups. Rabbits in Group 1, were not given any drug. This group was fed only with rabbit food. Rabbits in Group B received bosentan (Tracleer, Actelion Pharmaceuticals, USA) as oral gavage at daily doses of 30 mg/kg for 21 days, beginning from three days before the operation. During the study, accomodation, and feeding conditions of all rabbits were standardized (laboratory with ambient temperature, climatization equipment under exposure of sun light)

Surgical procedure

On the day of the experiment, the rabbits were sedated with intramuscular injections of ketamine (50 mg/kg), and xylazine (5 mg/kg). To avoid infection, after shaving the incision site, necessary conditions of asepsis, and antisepsis were met. After skin cleaning, experimental animals were placed in supine position, and a longitudinal neck incision was made. In all rabbits, for anastomosis, right carotid arteries of the rabbits were used. Following administration of heparin at a dose of 100 IU/kg through internal jugular vein, atraumatic vascular clamps were used to clamp carotid artery from its proximal, and distal parts to provide a bloodless area (Figure 1). Carotid arteries were transected. Then the transected carotid arteries were end-to-end anastomosed with interrupted 10/0 polypropylene sutures under microscope. Then the tissue layers were closed respecting proper anatomical planes (Figure 1). Left carotid arteries were not intervened. At the end of the 28 days the non-anostomosed left side carotid artery segments were extracted, fixated in gluteraldehyde, and sent to the histopathology laboratory for analysis. Afterwards the animals were sacrificed using higher doses of pentothal.

Figure 1. Anastomising transected carotid artery after clamping its distal, and proximal ends with interrupted 10/0 polypropylene sutures)

Histological evaluation, and morphometry

The extracted vascular tissues were fixated with gluteraldehyde (GA), and sent to hsitology laboratory. Then prepared tissue samples were cut in 0.5 µm- thick sections using Leica Reichert Supernova microtome (Reichert Microscope Services, USA). The specimens were placed on slides, stained with toluidine blue dye, and

readied for analysis. The analyses were performed using Eclipse E400 POL (Nikon Corporation, Japan) light microscope, and digital images were obtained with the aid of Zeiss AxioCam ICc3 R3 Digital Camera (Carl Zeiss MicroImaging GmbH, Germany). The morphometric characteristics (luminal area, intimal area, ratio of intimal /medial areas) of the sections in the digital media were analyzed, calculated, and evaluated using Image Tool 3.0 (UTSCSA; Image tool version 3.0) program.

Statistical Method

Data were evaluated using "SPSS for Windows 11.0" program. The fitness of the data obtained to normal distribution was evaluated with Kolmogorov-Smirnov test. In multiple groups where data do not fit normal distribution pattern Kruskal-Wallis test, and in comparisons between two groups Mann-Whitney U non-parametric tests were used to investigate the presence of statistical significance. P values less than 0.05 were considered as statistically significant

RESULTS

In the study, 18 New Zealand strain male rabbits were used. During the study period none of the rabbits were lost. Wound site infection, and neurological problems were not encountered in any experimental animal. At the end of the study, carotid arteries which were (right side) or were not (left side) anastomosed were harvested, and sent to histopathology laboratory for analysis. Sections obtained from the harvested carotid artery segments were analyzed as for the thickness of luminal, and intimal layers, and

Results of the histopathological analysis

Analysis of the specimens harvested from the rabbits in Group A, detected vascular lumens which were nearly completely occluded in some sections, and occasional areas of recanalization (Figure 2a). When serial sections were evaluated, indented, and rugged lumens with very narrow diameter, and patchy areas of adhesions were observed (Figure 2b). Extreme intimal hyperplasia as a result of proliferation of the smooth muscle cells, and increased amount of diffuse connective tissue was seen (Figure 2b).

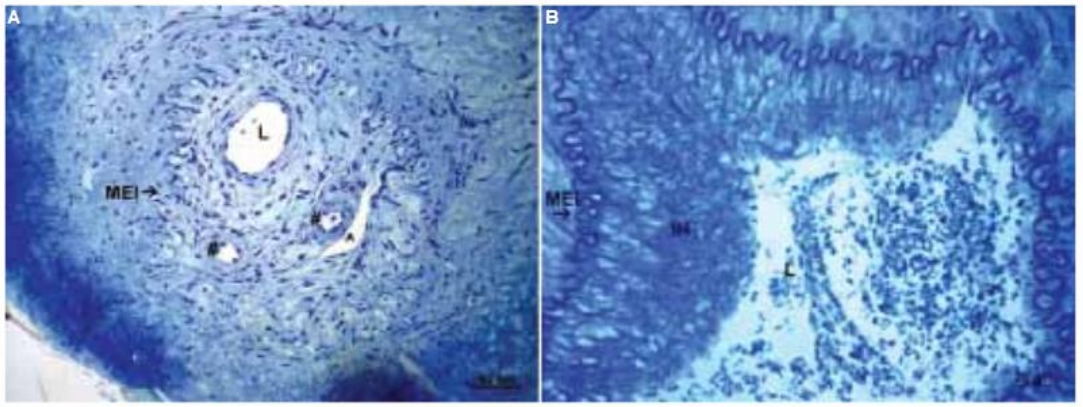

Figure 2. Histological appearance of Group A. (A) Diffuse intimal hyperplasia is occluding the lumen (L). Recanalized areas (#) are seen in between hyperplasic tissue Impairment (^) of intensity, and integrity of smooth muscle cells (Toluidine blue x20). (B) Multilayered diffuse intimal hyperplasia (IH) developed on membrana elastica interna (MEI) (Toluidine blue x40).

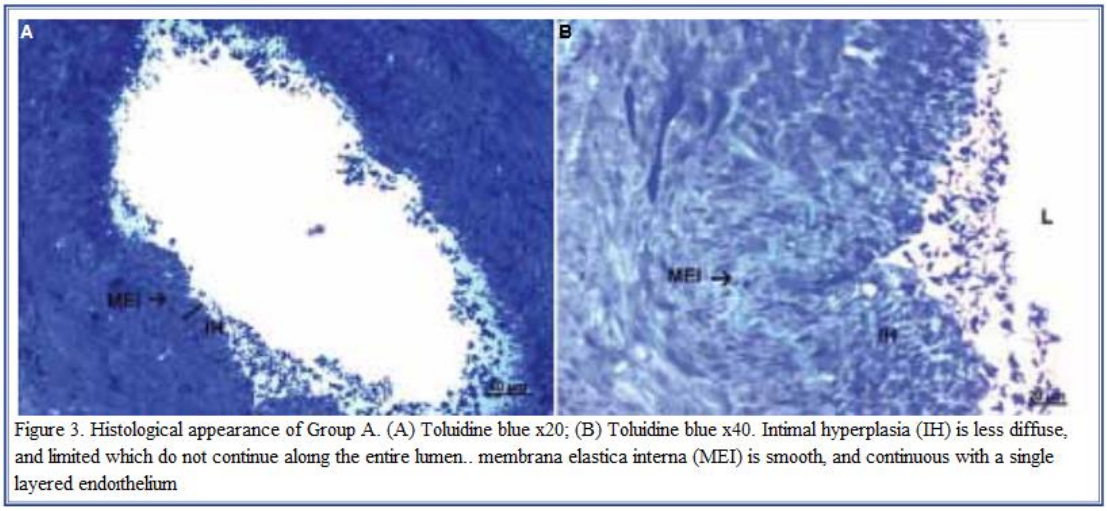

Figure 3. Histological appearance of Group A. (A) Toluidine blue x20; (B) Toluidine blue x40. Intimal hyperplasia (IH) is less diffuse, and limited which do not continue along the entire lumen.. membrana elastica interna (MEI) is smooth, and continuous with a single layered endothelium

However in Group B, vascular lumens of the experimental animals were relatively smoother, and larger than those of Group A with a statistically significant difference between groups. (p=0.014). However proliferation of smooth muscle cells in the intimal layer was at a relatively lesser extent, and development of intimal hyperplasia secondary to the increase in connective tissue cells was seen. (Figure 3).

Histomorphometric comparisons (Table 1)

In Group A (the group which didn't receive bosentan) right carotid lumen area (21.2 µm2 [3.4 µm2-46.0 µm2]) was markedly decreased when compared with the left carotid lumen area (106.5 µm2 [88.6 µm2-136.2 µm2]) of the same rabbits with a statistically significant intergroup difference . (p<0.001). Besides, right carotid artery intimal area of the Group A (the group which didn't receive bosentan) (48.3 µm2 [37.1 µm2-65.7 µm2]) was predominantly higher than the the left carotid artery intimal area (20.6 µm2 [19.4 µm2-25.5 µm2]) with a statistically significant intergroup difference. (p<0.001). Right carotid artery intimal/medial area ratio of Group A (the group which didn't receive bosentan) (0.49 [0.13-0.70]) was markedly increased when compared with intimal/medial area ratio of the left carotid artery (0.10 [0.07-0.14]) with a statistically significant intergroup difference (p<0.001).

When anastomosed (right) , and non-anastomosed (left) sides were compared in the untreated group (Grup A), right carotid artery had a larger intimal area, higher intimal/medial area ratio (p<0.001) , and a smaller luminal area (p<0.001) This finding has demonstrated that in the same group of experimental animals, applications of vascular access, and anastomoses led to development of intimal hyperplasia.

When we compared Groups A, and B, bosentan restricted development of intimal hyperplasia, decreased intimal thickness (Grup A: 48.3 µm2 [37.1 μm^2-65.7 μm^2], Grup B: 31.4 μm^2 [12.2 μm^2-65.7 μm^2], p=0.04), and intimal/medial area ratio (Grup A: 0.49 [0.13-0.70], Grup B: 0.22 [0.09-0.37], p=0.024), but increased luminal area (Grup A: 21.2 μm^2 [3.4 µm2-46.0 µm2], Grup B: 73.9 µm2 [15.0 μm^2-112.2 μm^2], p=0.014) were seen.

Tablo 1. Comparisons of luminal, and intimal areas, intimal/medial area ratio

Group	Median (min.-max. luminal area (μm²)	Median (min.-max. intimal area (μm²)	Intimal/medial area ratio
A	21,2 (3,4-46,0)	48,3 (37,1-65,7)	0,49 (0,13-0,74)
B	73,9 (15,0-112,2)	31,4 (12,2-65,7)	0,22 (0,09-0,37)

A statistically significant difference was not detected between Groups A and B as for non-anastomosed left carotid arteries.

DISCUSSION

Intimal hyperplasia is a physiological process at first which is characterized by the thickening of the intimal layer with the involvement of the media layer of the vascular system, but becomes an unwanted pathological development if intimal layer gradually continues to increase in width. Decreasing or prevention of intimal hyperplasia which affects the outcomes of revascularization procedures (percutaneous interventions, anastomoses) , and leads to restenosis resulting in failed procedures, reduced procedural quality, and requirement of additional procedures is still a a matter of debate.

ET-1 which is a potent vasoconstrictor, and mitogen has been associated with the pathogenesis of intimal hyperplasia, and restenosis.[6] Majority of the studies cited in the literature on alleviation of intimal hyperplasia has been focused on selective ETA blockage, and controversial results have been reported. In a rat carotid artery intimal hyperplasia model, Douglas et al.[7] found that selective ETA blockage was ineffective in alleviation of intimal hyperplasia. Similarly, Azuma et al .[8] demonstrated that selective ETA blockage could not decrease intimal hyperplasia created in rabbit carotid artery. However in pig models, favourable outcomes of selective ETA antagonism have been reported.[9] On the contrary, Kitada et al .[10] created arterial damage with balloon dilatation, and demonstrated that selective ETB antagonism increased the severity of intimal hyperplasia. They have advocated that this condition developed realized via higher amounts of ET-1 in the circulation due to overactivity of ETA receptors during selective blockage of ETB

In studies performed on rat, and pig models with non-selective ETA/ETB receptor antagonists, favourable outcomes have been reported.[11,12] Interestingly, in studies performed with human cells dual antagonsim with ETA/ETB , and selective ETB antagonism, decrease in intimal hyperplasia without any effect of selective ETA blockage on neointimna have been detected..[13] We hope that the results obtained from this study will contribute to relieve diversities cited in the literature.

Various models have been proposed for the creation of experimental intimal hyperplasia. Some methods used to induce endothelial damage, and stimulate intimal hyperplasia may include, inflation of an intraluminal balloon, placement of a ring around the periphery of an artery, ligation of the artery, and incision of the

lumen with a catheter. A bypass operation for carotid arteries with a synthetic graft, and interposition of a venous graft have been described as effective methods in the creation of an intimal hyperplasia..[14] In this study “transection, and anastomosis” model was preferred with the presumption that complete transection of carotid artery would affect all vascular layers with resultant higher potential of intimal hyperplasia. Besides, restenosis due to intimal hyperplasia developing in the periphery of anastomosis is an important consideration of cardiovascular surgery. End-to-end anastomosis following transection of carotid artery in rabbits has been tried with various drugs, and successful, and significant outcomes have been achieved..[15]

More et al.[16] induced intimal damage using balloon dilation in rats, and reported that cellular proliferation had started from the first postprocedural day, and reached the highest level in intima at 7. , and in media at 14. day, and gradually declined afterwards. Besides reendolization was achieved completely at 14. days. Schwartz et al.[17] advocated that this neointima might herald development of atherosclerosis, and restenosis in the future. In the light of this information, the postprocedural 28. day where intimal hyperplasia reaches its highest level, has been especially preferred for histopathological, and histomorphometric analyses.

Sanmartín et al.[12] investigated impact of bosentan on intimal hyperplasia in a pig coronary artery model. Based on previously conducted successful studies, and drug information provided by the manufacturer, bosentan was used for 21 days, at daily doses of 30 mg. In our study development of severe intimal hyperplasia on the carotid artery anastomoses in our untreated rabbits (Group A) was detected. In the same rabbits thicker intimal layer, and an increased intima /media ratio were detected when compared with non-anastomosed left carotid arteries. ($p<0.05$). Besides luminal diameter decreased.. Bosentan therapy (Group B) which is a dual antagonist of ETA/ETB receptors has been demonstrated to decrease intimal hyperplasia by preventing intimal thickening on carotid artery anastomoses. This decrease in intimal hyperplasia was found to be statistically significant when compared with the untreated group (Group A) ($p<0.05$). In addition, in the bosentan therapy gfrp (Group B) decrease in intima/media ratio ($p=0.024$) , increase in luminal cross-sectional area ($p=0.014$) was observed when compared with the untreated group (Group A).This outcome supports the viewpoint that bosentan prevents development of intimal hyperplasia.

Reel et al .[18] demostrated increased activation of gellatinases (MMP-2, MMP-9) which belong to the group of matrix metalloproteinases (MMPs) in intimal hyperplasia in a rabbit carotid artery model. These enzymes have been asserted to be very effective in the proliferation of smooth muscle cells, and especially their migration after intercellular matrix injury. Reel et al. used non-selective nedothelin receptor antagonist TAK-044, and observed considerable amount of decrease in the activation, and levels of these enzymes, and intimal hyperplasia. Also bosentan which is a non-selective endothelin receptor antagonist with an activity similar to MMP inhibitors reinforces their antiproliferative effects.

In this study, formation of intimal hyperplasia in the arterial system has been investigated. Majority of vascular grafts has been harvested from venous system. Dashwood et al.[19] demonstrated that

distribution of ETA receptors in the saphenous vein of pigs are more diffuse when compared with carotid arteries. Based of their findings, as a more accurate assumption saphenous vein grafts implanted to the arterial system are more prone to intimal hyperplasia which develops through an endothelin pathway, Relatively lower incidence of neointimal formation in autogen artery grafts used in coronary bypass realtive to use of autogen vein grafts supports this thesis..[20]

In various studies, many pharmacological agents have been used with the intention to prevent development of intimal hyperplasia. Among them, calcium channel blockers, β-blocker, statins, angiotensin-converting enzyme (ACE) inhibitor, gene transfer, biphosphonate have yielded favourable results. However the same success rates have not been demonstrated in clinical applications. Presence of more than one pathway in the development of intimal hyperplasia, and the information obtained so far have not elucidated this pathological process which prevent finding of accurate treatment approach

In conclusion, in the rabbit carotid artery model, it has been confirmed that intimal hyperplasia is formed by vascular anastomosis, and oral bosentan significantly decreases intimal hyperplasia. This effect of bosentan is thought to support the thesis that ET-1 plays an important role in the pathogenesis of intimal hyperplasia, and restenosis. In the future, long-term, and larger scale studies are needed related to its clinical use after vascular interventions.

Conflict of interest: None declared

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