The Effect of Ascorbic Acid on Proliferation of Vascular Smooth Muscle Cells and Intimal Hyperplasia

Askorbik Asidin Anastomoz Sonrası Gelişen Vasküler Düz Kas Hücre Proliferasyonu ve İntimal Hiperplazi Üzerine Etkisi: Tavşan Modeli

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ABSTRACT Objective: Proliferation of smooth muscle cells leading to intimal hyperplasia developing at vascular intervention sites plays a major role in the development of restenosis. Ascorbic acid is a potent anti-oxidant with antiproliferative properties. The aim of this study was to investigate such effect of ascorbic acid on intimal hyperplasia. **Material and Methods:** Twenty-one male white New Zealand rabbits weighing 2-3 kg were selected. The animals were allocated to three groups each consisting of seven rabbits. Group 1 was the sham group. Group 2 was the control group and Group 3 consisted of rabbits receiving ascorbic acid. The right carotid arteries of the subjects in Groups 2 and 3 were transected and re-anastomosed. A daily dose of 100 mg ascorbic acid per kg body weight was administered intraperitoneally for 14 days to rabbits in Group 3. Rabbits in Group 2 were not subject to any pharmaceutical agent. All the subjects were sacrificed at the end of postoperative day 28. Their right carotid arteries were resected and were subject to histopathologic examination for smooth muscle cell proliferation and intimal hyperplasia. The arterial segments were fixed in 10% buffered formalin solution until the time of histological analysis. Each vessel was sectioned serially in 2-mm increments from the prepared paraffin blocks. Sections of 5 μm thickness were stained with hematoxylin and eosin (H&E) and Masson Trichrome. The diameters of vessels and their luminal areas, and the areas of tunica intima and tunica media were measured with 10X magnification. The thicknesses of tunica intima and tunica media were measured with 20X magnification. The morphometric measurements were then compared between groups. **Results:** Intimal thickness and intimal area were significantly lower in Group 1 when compared with the other groups (p=0.004, p=0.003). In Group 3, the ratios of tunica intima thickness/tunica media thickness and area of tunica intima/area of tunica media were significantly lower than those of Group 2 (p=0.015, p=0.046). **Conclusion:** Ascorbic acid reduces the intimal hyperplasia developing after vascular anastomoses.

Key Words: Ascorbic acid; muscle, smooth; hyperplasia

ÖZET Amaç: Düz kas hücre proliferasyonu ve bunun ardından gelişen intimal hiperplazi, vasküler girişimden sonra meydana gelen restenozda önemli rol alır. Askorbik asit antiproliferatif özelliği olan güçlü bir antioksidandır. Bu çalışmanın amacı, askorbik asidin bu etkilerinden yola çıkarak intimal hiperplazi üzerindeki etkisini incelemektir. **Gereç ve Yöntemler:** Rastgele seçilen 21 adet ortalama 2-3 kg ağırlığında Yeni Zelanda tipi erkek tavşan, her birinde 7 tavşan bulunan 3 gruba ayrıldı. Birinci gruba (Grup 1) hiçbir girişim yapılmadı. İkinci gruba (Grup 2) anastomoz yapıldı, ancak hiçbir ilaç verilmedi. Üçüncü gruba (Gup 3) ise anstomoz yapılıp askorbik asit verildi. Grup 2 ve Grup 3'deki deneklerin sağ karotis arterleri transekte edilip tekrar anastomoz edildikten sonra Grup 3'deki deneklere 14 gün süreyle her gün 100 mg/kg/gün dozunda intraperitoneal yoldan askorbik asit yapıldı. Grup 2'ye ise herhangi bir ilaç verilmedi. Tüm denekler 28.gün sonunda öldürüldü; sağ karotis arterleri çıkartıldıktan sonra düz kas hücre proliferasyonu ve intimal hiperplazi açısından histopatolojik olarak incelendi. Arter segmentleri %10'luk tamponlanmış formalin solüsyonu içerisinde histolojik incelemeye kadar bekletilerek fikse edildi. Hazırlanan parafin bloklardan her damar ardışık 2'şer mm'lik arayla kesitler halinde dilimlendi ve 5 μm kalınlığındaki kesitler hematoksilen-eozin (H&E) ve Masson Trikrom ile boyandı. Damar çapları, lümen alanları, intima ve media tabakalarının alanları 10X büyütmeli objektif ile ölçüldü. İntima ve media tabakalarının kalınlıkları da 20X büyütmeli objektif ile ölçüldü. Ardından morfometrik ölçümler gruplar arasında karşılaştırıldı. **Bulgular:** Grup 1'in intima kalınlıkları ve intima alanları diğer gruplara göre istatistiksel açıdan anlamlı ölçüde daha düşük bulundu (p=0,004, p=0,003). Grup 3'deki deneklerin tunica intima kalınlığı/tunica media kalınlığı ve tunica intima alanı/tunica media alanı oranları Grup 2'ye göre anlamlı düzeyde daha düşük bulundu (p=0,015, p=0,046). **Sonuç:** Askorbik asit, vasküler anastomozlardan sonra gelişen intimal hiperplazinin azaltılmasında etkilidir.

Anahtar Kelimeler: Askorbik asit; düz kas; hiperplazi

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Reconstructive surgery is a common treatment modality in obstructive arterial disease. Recently, the success of these interventions is worse then expected due to spontaneous thrombus formation or development of stenosis. Contrary to acute occlusion immediately after vascular reconstructive interventions, where acute thrombus formation takes place, neointimal hyperplasia -developing after migration of smooth muscle cells and accumulation of collagen- plays an important role in the pathogenesis of constriction or restenosis at a later period.[1,2] Intimal hyperplasia is both a normal adaptation mechanism of arteries against hemodynamic stress and a characteristic step in the healing process of arterial injuries.[3] Inhibition of the hyperplastic response may provide prolongation of the patent period of the bypass grafts and of the native vessels after balloon angioplasty.[4] Free oxygen radicals contribute to intimal hyperplasia as shown previously.[5-7] Due to its cytotoxic effects, ascorbic acid is a potent antioxidant with antiproliferative properties and thus may be effective on the inhibition of intimal hyperplasia. In this study, we investigated the effects of ascorbic acid on intimal hyperplasia.

MATERIAL AND METHODS

This study was conducted according to the "Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care)" and we obtained approval from the Local Animal Ethics Committee of the Dokuz Eylül University. The study was held at the laboratories of the Dokuz Eylül University, Institute of Health Sciences, Department of Laboratory Animals Science between May and July 2010.

During the course of the study, all rabbits were routinely fed with rabbit chow and tap water ad libitum. The rabbits were maintained on a 12:12-h light:dark cycle in an environmentally monitored room with a ventilation system at a temperature of 20±2°C. The study included 21 male white New Zealand rabbits weighing 2-3 kg.

The animals were allocated to three groups each consisting of seven rabbits. Group 1 was the sham group; the rabbits were not subjected to any intervention. Group 2 was the control group where the rabbits underwent anastomosis but received no medication. Group 3 consisted of rabbits receiving ascorbic acid after the anastomosis procedure. Rabbits in Group 1 were sacrificed at the end of 28 days using 150 mg of thiopental sodium per kg body weight (Pental, İbrahim Ethem Ulagay, İstanbul-Turkey) and their right carotid arteries were resected. In Group 2 and 3 the auricular marginal veins of the rabbits were cannulated preoperatively and all animals received intramuscular injection of ketamine hydrochloride (Ketalar, Eczacıbaşı, Istanbul-Turkey) 50 mg/kg body weight and xylazine hydrochloride (Rompun, Bayer, Istanbul-Turkey) 5 mg/kg body weight. Intravenous cephazolin sodium (Sefazol, Mustafa Nevzat, Istanbul-Turkey) 50 mg/kg body weight was administered for antibiotic prohylaxis. Under aseptic conditions, a vertical right jugular incision was performed and a 3-cm segment of the carotid artery was explored. Then, heparin sulphate (Liquemine, Roche, Turkey) 100 IU/kg body weight was administered intravenously and carotid artery was clamped proximally and distally with bulldog clamps. The same artery was then transected and re-anastomosed using 8/0 polypropylene suture. Subcutaneous and cutaneous tissues were closed. No additional drug was administered to the animals in Group 2 and they were then sacrificed at the end of postoperative day 28 using 150 mg of thiopental sodium per kg body weight. Their right carotid arteries were then resected. The animals in Group 3 received intraperitoneal injections of ascorbic acid (Redoxon, Bayer, Istanbul-Turkey) 100 mg per kg body weight for 14 days following the operation and were sacrificed at the end of postoperative day 28 as described above.

HISTOPATHOLOGICAL EXAMINATION

The marked anastomosed segment of the right carotid artery from group 2 and 3, and a segment from the carotid artery of group 1 were isolated. The arterial segments were fixed in 10% buffered formalin solution until histological analysis. Each vessel was sectioned serially in 2-mm increments

from the prepared paraffin blocks. Sections of 5 μm thickness were stained with hematoxylin and eosin (H&E) and Masson Trichrome. The images were analyzed with a computer-assisted image analyzer system consisting of a microscope (Olympus BX-51, Japan) equipped with a high-resolution video camera (Olympus DP71, Japan). Digital image analysis software (UTHSCSA; Image tool version 3.0) was used for image processing. The diameters of vessels and their luminal areas, and the areas of tunica intima and tunica media were measured with 10X magnification. The thicknesses of tunica intima and tunica media were measured with 20X magnification. The morphometric measurements were then compared between groups.

STATISTICAL ANALYSIS

Results were presented as median. All data were analyzed with the Kruskal-Wallis test followed by the Mann Whitney test. P<0.05 was considered statistically significant.

RESULTS

In Groups 2 and 3, where vascular injury was formed by anastomosis, median intimal thickness was significantly higher than that of Group 1, where the native arteries were kept without surgical intervention (p=0.004, p=0.003) (Table I). The median intimal thickness was measured <0.01 mm in Group 1, whereas it was 0,10 mm (0.08-0.16) and 0.08 mm (0.04-0.13) in Group 2 and 3, respectively. The median intimal area was 0.03mm^2 (0.01-0.05) in Group 1, 0.13 mm^2 (0.05-0.2) in Group 2 and 0.1 (0.04-0.16) mm^2 in Group 3. This difference was also statistically significant (p=0.004, p=0.010). This increase in intimal thickness and intimal area was seen to originate from increased intimal collagen accumulation and proliferation of smooth muscle cells due to vascular injury (Figures 1a, 1b, 1c).

Although the median intimal thickness of the subjects in Group 3 (anastomosis + ascorbic acid) was lower than that of subjects in Group 2 (anastomosis only), this difference was statistically insignificant (p= 0.116) (Table I). In addition, the median thickness of tunica media of the subjects in Group 3 was higher than that of subjects in Group 2, but with no statistical significance (p=0.475) (Table I). Nevertheless, in Group 3, the median ratio of tunica intima thickness/tunica media thickness [0.54 (0.27-0.78)] was significantly lower than that of Group 2 [0.82 (0.64-0.89)] (p=0.015) (Table I). This difference between the two groups showed that intimal hyperplasia decreased in the group that received ascorbic acid when compared to the group

TABLE 1: Histopathological measurements related to tunica intima and media.

	Group1 (Sham) (n=7) Median(min-max)	Group 2 (Control) (n=7) Median(min-max)	Group 3 (Vit.C) (n=7) Median(min-max)		Significance (p)	
İntima thickness (μm)	7.56 (6.48 - 8.18)	105.10 (89.70-169.36)	81.45 (46.04-133.21)	0.004	Group 1-Group 2	0.004
					Group 1-Group 3	0.003
					Group 2-Group 3	0.116
Media thickness (μm)	176.36 (161.06-179.94)	144.23 (105.14-190.74)	154.84 (123.16-216.55)	0.380		
İntima/Media ratio	0.043 (0.04 - 0.05)	0.82 (0.64 - 0.89)	0.54 (0.27 - 0.78)	0.001	Group 1-Group 2	0.004
					Group 1-Group 3	0003
					Group 2-Group 3	0015
İntima area (mm^2)	0.0307 (0.0187-0.0545)	0.1364 (0.0595-0.2001)	0.1095 (0.0455-0.1627)	0.009	Group 1-Group 2	0.004
					Group 1-Group 3	0.010
					Group 2-Group 3	0.253
Media area (mm^2)	0.5326 (0.4447-0.5494)	0.4252 (0.2786-0.5994)	0.5487 (0.2982-0.7373)	0.373		
İntima area/Media area	0.057 (0.42 - 0.99)	0.337 (0.09- 0.52)	0.192 (0.12 - 0.26)	0.002	Group 1-Group 2	0.004
					Group 1-Group 3	0.003
					Group 2-Group 3	0.046

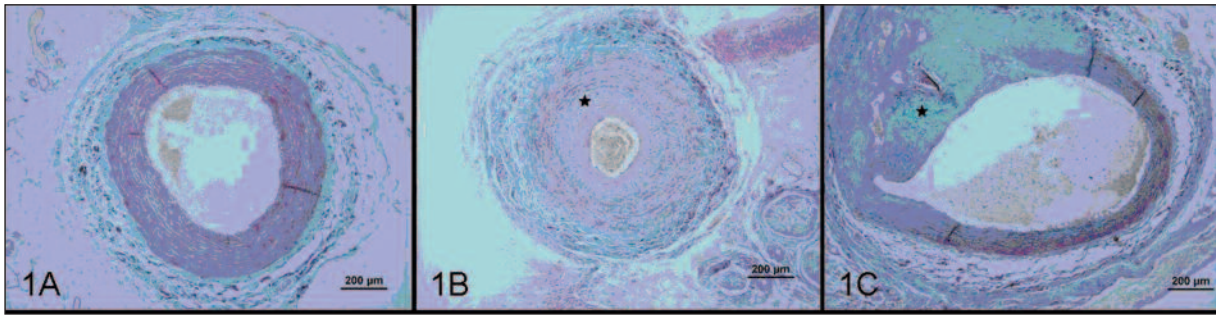

FIGURE 1: Histological sections of the carotid artery (Masson trichrome stain). **1A)** Group 1, **1B)** Group 2, **1C)** Group 3. Carotid artery sections obtained from control group showed thin and intact lamina and collagen-rich adventitia **(1A)**. Accumulation of intimal collagen in the remaining two groups **(1B, 1C)**.
*=collagen
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that did not. Although the median intimal area in Group 3 was higher than that in Group 2, this difference was statistically insignificant (p=0.253) (Table I). On the other hand, the median media area in Group 3 was lower than that in Group 2, but with statistical insignificance (p= 0.568) (Table I). The median area of tunica media was 0.54 (0.29-0.73) mm^2 in Group 3 and 0.42 (0.27-0.59) mm^2 in Group 2; this difference was statistically insignificant. However, the most significant parameter is the ratio of tunica intima area/tunica media area for intimal hyperplasia. This ratio was 0.19 (0.12-0.26) and 0.33 (0.09-0.52) in Groups 3 and 2, respectively with statistical significance (p=0.046) (Table I).

In histological sections ascorbic acid seemed to inhibit smooth muscle cell proliferation and collagen accumulation significantly, thus reducing the ratio of intima thickness/media thickness (Figures 2a,2b,2c).

DISCUSSION

Although there are several other methods as perivascular silicon collar insertion, two most common methods used to create vascular injury in experimental settings regarding intimal hyperplasia were balloon catheterization and anastomosis models, which allowed the development of more prominent intimal injury.[1,8-11] Balloon catheterization causes intimal injury mostly whereas the anastomosis model injures all layers of the vessel wall. During balloon catheterization, the catheter balloon is inflated manually and this causes heterogeneity in terms of pressure applied. It is also impossible to locate the exact vessel segment where the balloon is inflated in, which may result with the resection of the uninjured segment of the vessel for histological examination. Many studies showed that the application of higher pressures in-

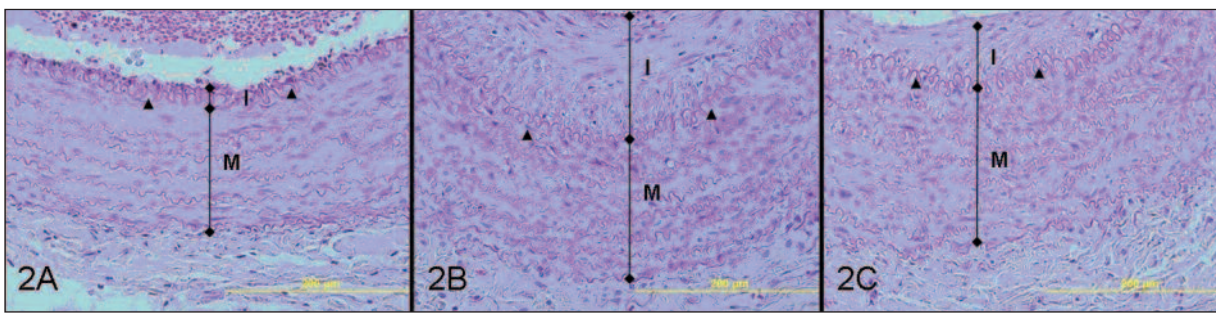

FIGURE 2: Histological sections of the carotid artery (Hematoxylin and eosin stain). **1A)** Group 1, **1B)** Group 2, **1C)** Group 3. The ratio of intimal thickness/media thickness was lower in Group 3 than that of Group 2. Arrowheads point at internal elastic laminae. I: Intima, M: Media.
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creased the restenosis rates.[12] Another advantage of the anastomosis model over balloon catheter is the involvement of all layers of the vessel wall, thus – we think- causing more severe intimal injury. Therefore, with the thoughts that balloon catheterization may cause problems in terms of standardization of the experimental model we preferred the anastomosis model. Since estrogen diminishes neointimal response, we used male rabbits.[13] Our study revealed that intimal hyperplasia developed in both anastomosis groups compared to the control group with no anastomosis, which indicated that the anastomosis model created a successful vascular injury.

Ascorbic acid is a water-soluble vitamin and a carbohydrate derivative. Ascorbate -as an antioxidant- is a scavenger for peroxyl radicals. Particularly, hydroxyl and superoxide radicals react with ascorbic acid. Transportation of either an electron or a hydrogen ion from ascorbate to the radical constitutes the antioxidant function. By this mechanism, ascorbate removes the free oxygen radicals and prevents the possible injuries caused by them.[14,15]

Free oxygen radicals play a role in the etiology of the intimal hyperplasia as reported previously.[5,7] In one of those studies, Huynh *et al.* interposed a venous graft into rabbit carotid artery and administered intraoperative local polyethylene glycol superoxide dismutase as antioxidant therapy, which ultimately reduced the intimal thickening.[7] Many studies discovered the positive effect of ascorbate on endothelial functions due to its potent antioxidant effect.[16,17]

Although vitamin C generally has an antioxidant effect, it may act as a pro-oxidant by converting the free radicals to hydrogen peroxide. Hydrogen peroxide, if not neutralized by a cellular enzyme catalase, may harm cell membranes and DNA. At adequate concentrations, vitamin C can produce hydrogen peroxide at cytotoxic levels. Tumor cells usually lack catalase and therefore are more sensitive to hydrogen peroxide. With this feature, vitamin C may be used as an anti-cancer agent if cytotoxic intracellular concentrations are achieved.[18,19]

Other factors demonstrated to play a role in the pathophysiology of intimal hyperplasia are matrix metalloproteinases that particularly stimulate smooth muscle cells.[20] Previous studies have shown that ascorbic acid inhibits the release of matrix metalloproteinases.[21]

In a cell culture study conducted by Ivanov *et al.* cells showed biphasic growth pattern in the presence of ascorbate. Cell growth increased 25% at a concentration of 125 μM ascorbate, whereas at higher concentrations (2mM) cell growth decreased 50%. The effect of ascorbate on DNA synthesis of vascular smooth muscle cells goes parallel with a drop in the cell count. This effect is not associated with the cytotoxicity of ascorbate. A study investigating the direct and matrix-mediated effects of ascorbate on vascular smooth muscle cells showed that in the presence of ascorbate at a concentration of 0.5-2.0 mM, the cell proliferation was interrupted. This effect was dose-dependent but was not associated with the cytotoxic effect.[22] In another cell culture study, Arakawa *et al.* showed that ascorbic acid and its derivatives stimulated differentiation of vascular smooth muscle cells. However, they suggested that animal studies would be required to conclude that ascorbic acid inhibited smooth muscle cell proliferation and intimal hyperplasia.[23] In our study, ascorbic acid attenuated intimal hyperplasia prominently by inhibiting smooth muscle cell proliferation, which probably is due to its antioxidant and cytotoxic properties.

In conclusion, intimal hyperplasia develops with smooth muscle cell proliferation and migration of those cells to tunica intima due to some physiopathological changes. Inhibition of smooth muscle cell proliferation slows down the development of intimal hyperplasia.

We think that ascorbic acid, as a commonly used vitamin C metabolite, may decrease intimal hyperplasia both by inhibiting smooth muscle cell proliferation owing to probable cytotoxic effects and by its role in scavenging free oxygen radicals that are significant in the pathophysiology of intimal hyperplasia.

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