EXPERIMENTAL STUDY

A free radical scavenger (Tempol) and its effect on intimal hyperplasia of vein grafts in rats

Arif Türkmen, M.D.,[1] Mehmet Doğan, M.D.,[2] Metin Temel, M.D.[3]

[1]Department of Plastic, Reconstructive and Aeshetic Surgery, İstanbul University Cerrahpaşa Faculty of Medicine, İstanbul-*Turkey*
[2]Department of Plastic, Reconstructive and Aesthetic Surgery, Private Office Physician, Gaziantep-*Turkey*
[3]Department of Plastic, Reconstructive end Aesthetic Surgery, Private Antakya Yaşam Surgical Medical Center, Hatay-*Turkey*

ABSTRACT

BACKGROUND: Reversed vein grafting exposes the vessel to a period of ischemia, reperfusion and subsequent reactive oxygen species, which may damage endothelial tissue, smooth muscle cell proliferation and later the development of intimal hyperplasia. Tempol is a free radical scavenger that permeates biological membranes. This study investigates the effects of a free radical scavenger (Tempol) on intimal hyperplasia of femoral vein grafts and the level of reactive oxygen species in rats.

METHODS: Arterial defects created in the femoral artery of rats were repaired with ipsilateral femoral vein grafts. Tempol was administered to group T and saline to group C on a daily basis for a period of 28 days. Blood samples were measured.

RESULTS: The veins were stained with H&E and Verhoeff's elastic stains. Binary comparison revealed a statistically significant difference for intimal and medial thicknesses ($p<0.01$).

CONCLUSION: This study found that a free radical scavenger (Tempol) prevents the early development of intimal hyperplasia, most probably by inhibiting the infiltration of polymorph nuclear monocytes (PNM), with evidence of increased levels of antioxidant products and decreased levels of free oxygen radicals.

Keywords: Intimal hyperplasia; microsurgery; reactive oxygen species; Tempol; vein graft.

INTRODUCTION

Autogenous vein graft remains one of the most commonly used graft materials for the treatment of occlusive vascular disease or closing defects in the artery.[1–3] However, these grafts are prone to failure due to thrombosis, which occurs in as many as 20% of cases within the first week after surgery. Intermediate graft failure (from 30 days to 2 years after surgery) and late graft failure (≥2 years after surgery) occurs in 20%–50% cases within five years after surgery.[1,4] The main cause of intermediate and late vein graft failure following surgery is intimal and medial hyperplasia.[5]

Although the etiology of the intimal hyperplasia of the vein graft is not exactly known, many factors, such as graft manipulation, compliance with mismatch of artery, and vein graft, increase wall shear stresses and reperfusion can be responsible for triggering the mechanism of intimal hyperplasia. Endothelium also plays a pivotal role in the development of intimal hyperplasia.[6–8]

Reactive oxygen species (ROS), which are superoxide anions, hydroxyl radicals, hydrogen peroxide (H_2O_2), and peroxynitrite are produced in metabolic and physiological processes. They may damage cells by causing peroxidation of membrane lipids, denaturation of proteins, including enzymes and ion channels, and strand breaks in DNA. However, ROS have also recently been shown to stimulate vascular smooth muscle cell growth and proto oncogen expression that cause intimal hyperplasia.[9] Superoxide regulates redox-sensitive signaling pathways and acts as a direct vascular smooth muscle cell mitogen.[10] It also modulates vessel remodeling by activating

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Address for correspondence: Arif Türkmen, M.D.
İstanbul Üniversitesi Cerrahpaşa Tıp Fakültesi, Plastik Cerrahi Kliniği, İstanbul, Turkey
Tel: +90 212 - 414 30 00 E-mail: turkmenarif@yahoo.com

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matrix metalloproteinase[8,11] and influences vascular smooth muscle cell migration and apoptosis.[10,12] ROS can be removed by enzymatic or non-enzymatic antioxidative mechanisms in organisms, and their harmful reactions can be prevented or inhibited by antioxidant molecules.

Paraoxonase (PON1) is an ester hydrolase, which has both arylesterase and paraoxonase activities. Recently, PON1 has been studied for its antiatherogenic and antioxidant effects in a number of processes, including lipid and lipoprotein metabolism. Oxidative stress, which is the result of an increased number of lipid and protein oxidation products and a decreased number of antioxidant enzymes and vitamins, has been reportedly affects the expression and activities of PON1. Furthermore, oxidative stress has been shown to decrease PON1 activity and to down-regulate the serum expression of PON1.[13]

Tempol (4-hydroxy-2,2,6,6-tetramethyl-4-piperidinyloxyle) is a superoxide dismutase-mimetic compound, chemically stable, cell-permeable, and non-toxic, that has been shown to possess powerful anti-superoxide activity and prevent the formation of hazardous peroxyl radicals.[9,10,12,14] There are high levels of evidence from preclinical studies that Tempol reduces the degree of local and systemic inflammation and associated tissue injury. It has been used for the treatment of pancreatitis, pleurisy, arthritis, colitis, and uveoretinitis.[15]

The aim of this study was to investigate the effect of Tempol, which is a membrane-permeable, free radical scavenger, on the intimal hyperplasia of the vein graft for surgically created femoral artery defects in rats and its correlation with the level of the total antioxidant capacity (TAC), total oxidant status (TOS), and Paraoxanase-1 (Pon) level in blood.

MATERIALS AND METHODS

The study included 20 healthy adult, male Wistar Albino rats who were 105–120 days old and weighed 250–300 g. Subjects were randomly divided into two groups of ten subjects. Their care complied with the Principle of Laboratory Animal Care and the Guide for Care and Use of Laboratory Animals' (NIH Publication No. 80–23, revised 1985).

The rats were anesthetized with an intra peritoneal (ip) injection of ketamine (50 mg/kg) (Ketalar, Eczacıbaşı, Turkey), followed by another injection of xylazine (10 mg/kg), (Rompun, Bayer, Turkey) into the subcutaneous tissue of the right groin. The right femoral artery and vein were exposed. There was no obvious discrepancy between the femoral artery and vein diameters. A 2-cm femoral artery was removed, and the gap between arteries was then measured. A femoral vein graft was harvested from the right femoral vein, as needed, at the same time. The femoral vein graft was washed with a heparin–saline solution (5 IU/ml), and the arterial defect was repaired with the reversed interposition femoral vein graft in an end to-end manner using interrupted 10/0 nylon suture (Ethilon; Ethicon Inc.) under magnification (Fig. 1). The ischemia times of the veins were 43–49 min. The wounds were closed primarily.

The rats were randomized into two groups. The first group was the control (Group C, n=10) and the other was Tempol (Group T, n=10). Tempol granules were dissolved in sterile saline 10 mg/cc and then injected (30 mg/kg/day) intraperitoneally to Group T, while 0.8 cc saline was injected to Group C daily to obtain same stress conditions during the 28 days. Blood samples from the rats were obtained just before sacrifice to measure TAC, total oxidation status, and Proxanase–1 levels. All animals were sacrificed using sodium pentobarbital (100 mg/kg i.v) (Penthotal Sodium, Abbott Laboratuvarı A.Ş.). A catheter was inserted into the aorta and isotonic saline solution was infused until clear fluid was seen passing through the right femoral vein graft. The vein graft was removed including 5 mm of the distal and proximal parts of the anastomoses and was fixed in a buffered formaldehyde solution (10% phosphate-buffered saline, PBS) at room temperature.

Histological and Morphometric Studies

Cross-sections from the proximal third, middle third, and distal third of each vein graft were taken. Four-µm thick sections were cut from the middle of these segments, and six coated glass slides were prepared from each vein sample, stained with Verhoeff's elastic tissue stain and H&E. The intimal and medial layers were identified by a demarcation between the criss-cross orientation of the intimal hyperplasic smooth muscle cells and circular smooth muscle cells of the media. The outer limit of media was defined by the interface between the circular smooth muscle cells of the media and the connective tissue of the adventitia. The specimens were examined using a Nikon Eclips E600 microscope and imaged

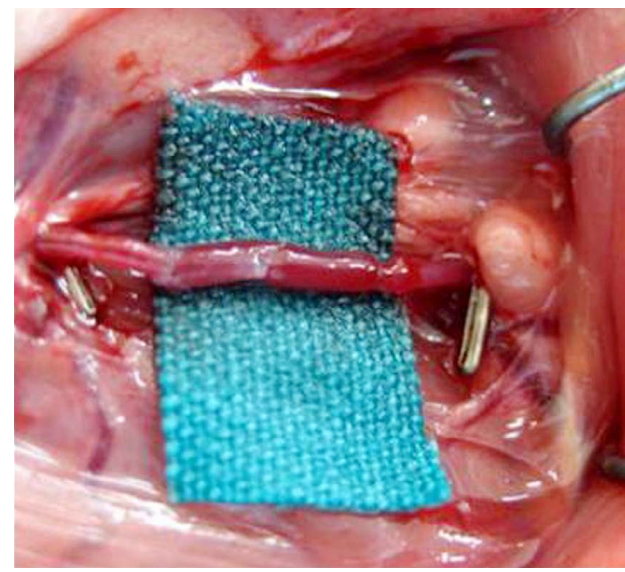

Figure 1. The reversed interpositional femoral vein graft in the arterial defect.

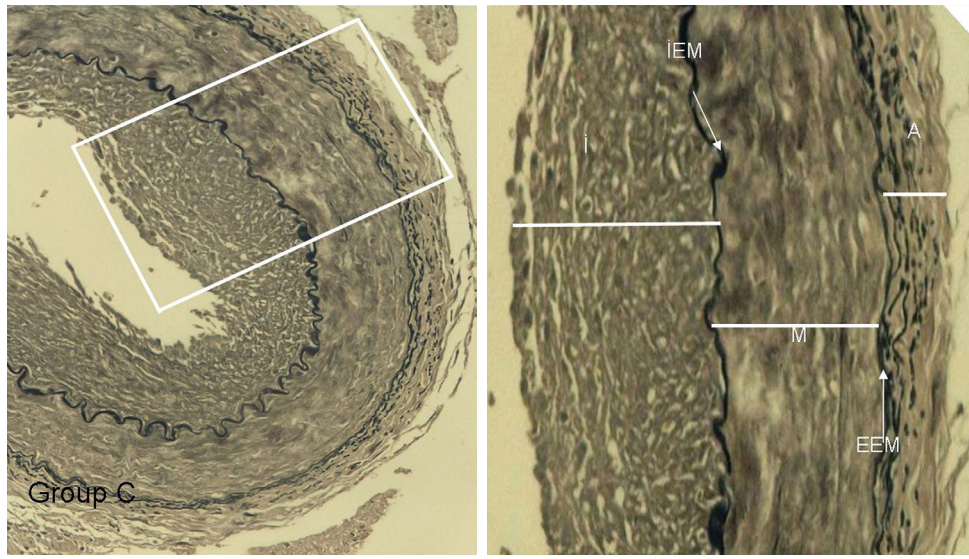

Figure 2. The light microscopic appearance of the intimal hyperplasia of the vein grafts.

with a Nikon Digital Sight DS-L1 photomicroscope (Fig. 2). A ratio of the intimal and medial areas was also calculated. The images were analyzed with a computer Analysis System Program.

Biochemical Analysis

Measurement of Total Antioxidant Capacity (TAC)

Plasma TAC levels were determined using a novel automated measurement method developed by Erel.[16] In this method, hydroxyl radical, which is the most potent radical, was produced via Fenton reaction. In this assay, the antioxidative effect of the sample against the potent free radical reactions (initiated by the produced hydroxyl radical) was measured. The results are expressed as mmol Trolox equivalent/L.

Measurement of Total Oxidant Status (TOS)

Plasma TOS levels were determined using a novel automated measurement method developed by Erel.[17] In this method, oxidants present in the sample oxidize the ferrous ion–o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The assay is calibrated with hydrogen peroxide, and the results are expressed in terms of μm hydrogen peroxide equivalent/L.

Oxidative Stress Index (OSI)

The percent ratio of TOS to TAS gave the oxidative stress index (OSI), which is an indicator of the degree of oxidative stress.[16,17] To perform the calculation, the result unit of TAS (mmol Trolox equivalent/L) was converted to mol equivalent/l and the OSI value was calculated as follows; OSI = [(TOS, mol/l) / (TAS, mmol Trolox equivalent/ l) × 100].

Measurement of Paraoxonase-1 Level

The basal activity of paraoxonase was measured using paraoxon. The rate of paraoxon hydrolysis (diethyl-p-nitrophenylphosphate) was measured by monitoring an increase in absorbance at 412 nm at 37°C on an autoanalyzer (Beckman Coulter, Fullerton, CA, and U.S). Paraoxonase activity was expressed as U/L of serum.[13]

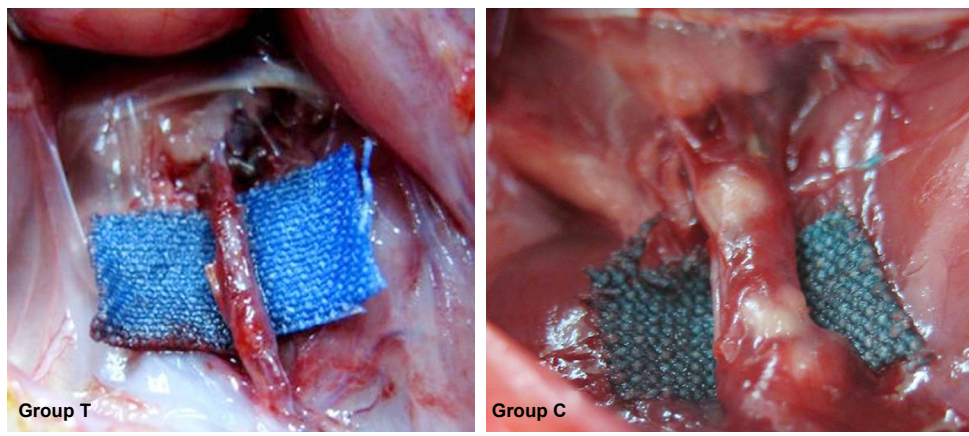

Figure 3. Scar tissue surrounding the graft at macroscopic appearance.

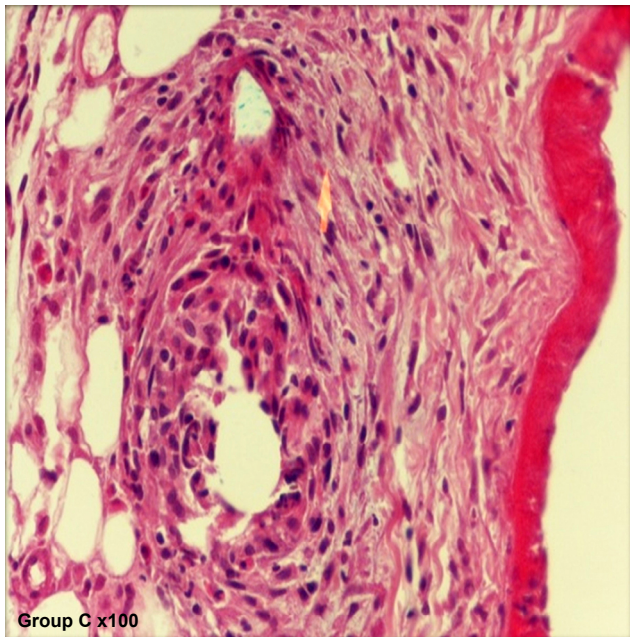

Figure 4. Intimal and media hyperplasia with proliferation of smooth muscle cells, and infiltration of polymorph nuclear cells.

Statistical Analysis

Statistical analysis was performed using SPSS (The Statistical Package for Social Sciences) software for Windows, release 14.0. Results are expressed as the mean±standard deviation (SD). Statistical analysis was performed using the Mann–Whitney U test and Paired t-test. Statistical significance was assumed at a level of $p<0.01$.

RESULTS

The study was completed 28 days following the surgery. In the post-operative follow-up period, infection was observed in only one subject of Group C and that study subject was excluded from the study and substituted with a new one. No complications were encountered during the experiment.

Histological Findings

There was a good blood flow within the vein grafts in both the groups. However, there was more scar tissue surrounding the vein grafts in the control group at observation, and dissection of the vein graft was more difficult (Fig. 3). The light microscopic appearance of the vein grafts in Group C demonstrated a profound degree of intimal and medial hyperplasia with the proliferation of smooth muscle cells and the infiltration of polymorph nuclear cells. The adventitial collagenous tissue had also increased (Figs. 4, 5).

The thickness of the intimal and medial layers of the vein graft as well as the ratio of the intima to media in Groups C and T, respectively, are shown in Table 1. There were statistically significant differences in the ratio of the intimal and medial layer thickness of the vein grafts between the two Groups ($p<0.01$). However, there were no statistically significant differences between the ratio of the intima and media of the two groups ($p\geq0.01$) (Table 1).

Biochemical Findings

Mean and SD values of TAC, TOS, OSI, and PON-1 levels are presented in Table 2. The results of TAC and PON-1 were significantly higher in Group T than in Group C ($p<0.01$). However, the result of TOS and OSI were significantly lower in Group T than in Group C ($p<0.01$) (Figs. 6–8).

DISCUSSION

The major findings of this study are that Tempol increases the level of TAC and PON-1, which are antioxidant products, at a significant level ($p<0.01$) and decreases the level of TOS and OSI, also at a statistically significant level. Furthermore,

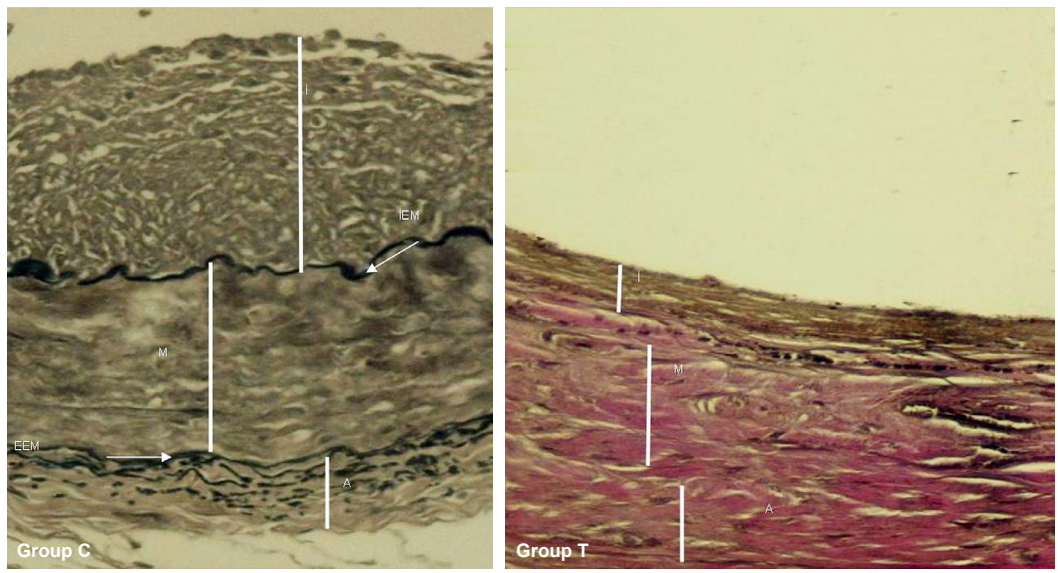

Figure 5. Compression of the vessel layers stained with Verhoeff's elastic tissue stain.

Table 1. The mean thickness of intimal and medial layers of the vessel and the mean ratio of the intima to media

Groups	The thickness of the intima (µm)	The thickness of the media (µm)	Intima/Media ratio
Group 1 (Group C)	15.530±3.438	21.830±3.176	0.706±0.095
Group 2 (Group T)	8.540±0.544	13.300±0.997	0.646±0.073

Table 2. The comparison of the level of total antioxidant capacity (TAC), total oxidation level (TOS), Proxanase–1(PON-1), and oxidative stress index (OSI)

Groups	TAC	TOS	OSI	PON-1
Group 1 (Group C)	1.231±0.188	7.482±2.008	0.625±0.217	85.645±36.482
Group 2 (Group T)	1.590±0.115	2.443±1.096	0.154±0.072	281.298±16.770

TAC: Total Antioxidant Capacity µmol Trolox Eqv./L; TOS: Total Oxidation Level µmol H2O2 Eqv./L; OSI: oxydative stress index TOS/TAS; PON-1: Proxanase–1; HDL-Associated enzyme.

the thickness of both intimal and medial layers of the vein was significantly thinner in Group T than Group C ($p<0.01$). Not surprisingly, there was no statistical difference in the intima to media ratio between Group T and Group C ($p\geq0.01$) because both intimal and medial layers increased in the control group and decreased in Tempol group; therefore, this ratio was constant.

The first interposition vein graft for arterial defect was used in the beginning of the nineteenth century. Intimal hyperplasia was also first described at that time.[18] Vein grafts undergo structural changes following insertion in to the arterial circulation with the development of intimal hyperplasia.[19] The internal pressure of the vein is up to 10 mmHg. Following vein graft repair, the vein is immediately subjected to an arterial pressure of 100 mmHg as well as an immediate increase in flow, longitudinal wall (shear) stress, circumferential stress, radial deformation, radial stress, pulsatile deformation, and pulsatile stress.[20] Hemodynamic forces have been postulated as promoters of intimal hyperplasia.[21] These are all associated with an increased expression of growth factors, adhesion molecule expression, and proliferation. Intimal hyperplasia is a universal response of a vein graft repair to an artery that results from both the migration of smooth muscle cells out of the media into the intima and proliferation of these smooth muscle cells.[22] Later, smooth muscle cells deposit a complex extracellular matrix.[23]

In general, intimal hyperplasia is a self-limiting process, which does not produce luminal compromise, and usually becomes quiescent within 2 years of graft insertion. However, in fo-

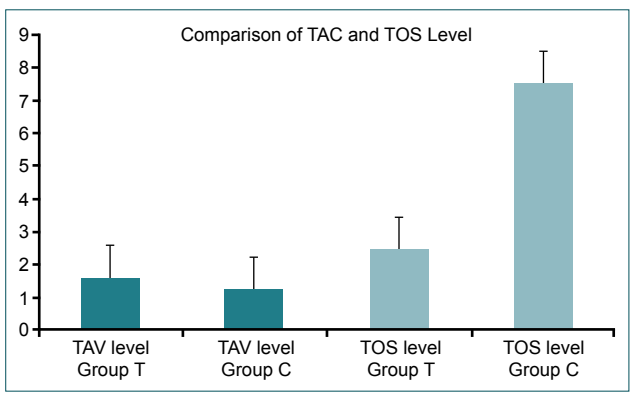

Figure 6. Comparison of the total antioxidant capacity (TAC) and total oxidant status (TOS).

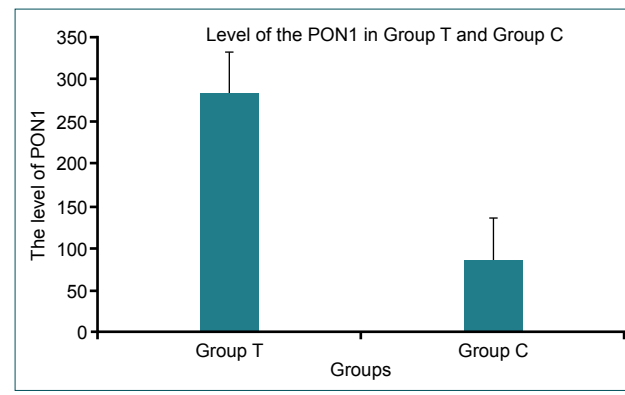

Figure 7. Paraoxonase-1 levels.

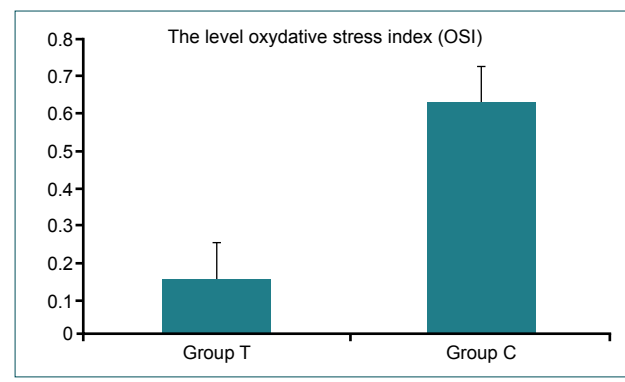

Figure 8. The oxidative stress index.

cal areas, the intimal hyperplasia can proceed to significant stenosis.[24–26] Many agents, which successfully reduce the formation of intimal hyperplasia in experimental vein grafts, can also affect the smooth muscle cell physiological phenotypes identified by alterations in vein graft vasomotor contractile function.[14,24,27] The process that leads to intimal hyperplasia and changes in endothelial and smooth muscle cell physiology begins early after grafting into the arterial circulation.[19] Recent studies suggest that the infiltration of PMNs and oxygen free radical injury are associated with the early development of intimal hyperplasia.[25,28,29]

Previous studies have shown that chronic in vivo administration of either aspirin with dipyridamole, heparin, verapamile, or captopril reduces the development of intimal hyperplasia in vein bypass graft.[22,30–37] Harvesting a vein has a period of ischemia due to loss of continuity of the vasa vasorum. A study showed that a pig saphenous vein becomes rapidly hypoxic after excision and remains so after implantation for at least a month. As the vein graft thickens rapidly, the graft is likely subject to an increase in oxygen demand, which may also increase hypoxia.[38] Hypoxia of the vein graft promotes superoxide anion (O2−) formation via activation of nicotinamide adenine dinucleotide phosphate oxidize, xanthine oxidise, and mitochondrial respiratory chain. Prolonged hypoxia regulates the expression of many proteins that can cause cell injury and are responsible for the vein graft failure,[30,39,40] Another study showed that H_2O_2 induced DNA synthesis and proto oncogen c-myc, which encodes a transcription factor that regulates cell proliferation, growth, and apoptosis and c-fos mRNA expression in vascular smooth muscle.

The administration of exogenous superoxide dismutase (SOD) is known to inhibit the injuries caused by oxygen-derived free radicals. However, it has an extremely short life span (6 min) and large molecular weight (30000 kd), making it unable to cross the cell membrane. This prevents its intracellular availability where free radicals are generated.[41–43] Tempol permeates biological membranes and accumulates in the cytosol. It is capable of scavenging superoxide and hydroxyl radicals at the membrane as well as both intracellular and extracellular. Although it's precise mechanism of action is still not clearly understood, many studies showed that Tempol attenuates the effects of superoxide anions. Not only does Tempol reduce the formation of hydroxyl radicals but it also prevents the injury/death caused by H_2O_2 in rat cardiac myoblasts by preventing the effects of hydroxyl radicals. ROS and peroxynitrite can cause DNA strand breaks, and this effect is abolished by Tempol.[15]

Systemic administration of Tempol causes a systemic vasodilatation and decreases the heart rate. These effects may be due to the formation of metabolite hydroxylamine and the enhanced bioavailability of nitric oxide (NO).[42] Vasodilatator effect of Tempol may also reduce the development of the intimal hyperplasia of the graft.

Tempol increases NO availability by inhibiting its destruction and other superoxide-derived ROS[12,31] and prevents the formation of hazardous peroxyl radicals. It protects against oxidant stress-induced endothelial dysfunction.[42] Our study showed that Tempol reduced scar formation around the vein graft, as well as the local inflammatory response within the intimal and medial layers of the vein graft.

Other free radical scavengers, such as vitamin E and lazaroid, have been used to reduce intimal hyperplasia of the vein graft. However, in contrast to the effect of Tempol, they scavenge hydroxyl radicals only in the membrane.[19,44] Moreover, there is very limited evidence that long-term exposure of cells to Tempol may cause cell injury.

Conclusion

We have established a link between ROS and intimal hyperplasia of the vein graft. Treatment with Tempol, which is a free radical scavenger, can decrease the early development of intimal hyperplasia, possibly by inhibiting the infiltration of PNM with evidence of increasing the level of antioxidant products and decreasing the level of free oxygen radicals, which are mediated injury to the endothelium of the vein grafts.

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DENEYSEL ÇALIŞMA - *ÖZET*

Serbest radikal tutucu Tempol'ün sıçanlarda ven grefti intimal hiperplazisine etkisi

Dr. Arif Türkmen,[1] Dr. Mehmet Doğan,[2] Dr. Metin Temel[3]

[1]İstanbul Üniversitesi Cerrahpaşa Tıp Fakültesi, Plastik, Rekonstrüktif ve Estetik Cerrahi Anabilim Dalı, İstanbul
[2]Serbest Muayenehane Hekimi, Plastik, Rekonstrüktif ve Estetik Cerrahi Uzmanı, Gaziantep
[3]Özel Antakya Yaşam Cerrahi Tıp Merkezi, Plastik, Rekonstrüktif ve Estetik Cerrahi Kliniği, Hatay

AMAÇ: Vasküler defektlerin onarımı sonrasında kullanılan ven greftinin iskemi ve reperfüzyon hasarı sonrası reaktif oksijen radikalleri oluşarak endotelyal dokuya, vasküler düz kaslara etki ederek intimal hiperplaziye neden olmaktadır. Tempol serbest radikalleri tutarak biyolojik memeranlarda stabilizasyon sağlamaktadır. Bu çalışmada femoral arter defektlerinin ven grefti ile onarıldığı sıçanlarda Tempol'ün intimal hiperplazi ve serbest oksijen radikallerinin düzeyleri değerlendirildi.

GEREÇ VE YÖNTEM: Sıçanların femoral arterlerinde bir defekt oluşturularak oluşan defekt aynı taraftan ven grefti alınarak onarıldı. Sıçanlar iki gruba ayrıldı. Yirmi sekiz gün boyunca hergün intraperitoneal olarak Tempol verilenler grup T ve serum fizyolojik verilenler grup C olarak adlandırıldı. Deney sonunda kan ve vasküler doku örnekleri alındı.

BULGULAR: Ven greftleri hematoksilen-eozin ve Verhoeff'in elastin boyası ile değerlendirildi. Gruplar intimal hiperplazi ve media tabakasındaki kalınlıkları bakımından karşılaştırıldığında istatistiksel olarak belirgin fark olduğu tespit edildi ($p<0.01$).

TARTIŞMA: Bu çalışmada serbest radikal tutucu Tempol'ün erken dönemde gelişen intimal hiperplaziyi engellediği gösterildi. Tempol'ün bu etkiyi media tabakası ve ven endoteline zararlı serbest oksijen radikallerinin düzeyini azaltarak ve anti-oksidan ürünlerin seviyesini arttırarak ve muhtemelen bu etkiler sonucu polimorf nükleer lökositlerin infiltrasyonunu engelleyerek gerçekleştirdiği düşünülmektedir.

Anahtar sözcükler: Intimal hiperplazi; mikrocerrahi; reaktif oksijen türevleri; Tempol; ven grefti.

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