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Zeliha Kurtoglu*	Ahmet Hakan Ozturk†	Celal Bagdatoglu‡
Gurbuz Polat**	Mustafa Aktekin††	Deniz Uzmansel‡‡
Handan Camdeviren§	Ozlen Bagdatoglu¶	Mustafa Sargon

*Mersin University,

†Mersin University,

‡Mersin University,

**Mersin University,

††Mersin University,

‡‡Mersin University,

§Mersin University,

¶Mersin University,

||Hacettepe University,

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Zeliha Kurtoglu, Ahmet Hakan Ozturk, Celal Bagdatoglu, Gurbuz Polat, Mustafa Aktekin, Deniz Uzmannel, Handan Camdeviren, Ozlen Bagdatoglu, and Mustafa Sargon

Abstract

In this study, we evaluated the effects of trapidil on crush injury by monitoring nitric oxide, malondialdehyde and transforming growth factor-Beta2 levels and by transmission electron microscopy in the rat sciatic nerve. The sciatic nerve was compressed for 20 sec by using a jewelers forceps. Trapidil treatment groups were administrated a single dose of trapidil (8 mg/kg) intraperitoneally just after the injury. The crush and crush + trapidil treatment groups were evaluated on the 2nd, 7th, 15th, 30th and 45th days of the post-crush period. On the 7th and 15th days, damage in thin and thick myelinated axons, endoneural edema and mitochondrial swelling were less severe in the trapidil group histopathologically. These findings supported the idea that trapidil prevented cell damage and edema at the injury site. Day/group interaction with regard to serum nitric oxide, malondialdehyde and transforming growth factor-Beta2 levels did not show significant changes.

KEYWORDS: trapidil, crush injury, peripheral nerve, electron microscopy, nitric oxide

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Original Article

Effects of Trapidil after Crush Injury to a Peripheral Nerve

Zeliha Kurtoglu^{a*}, Ahmet Hakan Ozturk^a, Celal Bagdatoglu^b, Gurbuz Polat^c,
Mustafa Aktekin^a, Deniz Uzmannel^a, Handan Camdeviren^d,
Ozlen Bagdatoglu^c, and Mustafa Sargon^e

Departments of ^aAnatomy, ^bNeurosurgery, ^cBiochemistry, ^dBiostatistics,
Mersin University, Faculty of Medicine, Mersin, Turkey, and

^eDepartment of Anatomy, Hacettepe University, Faculty of Medicine, Ankara, Turkey

In this study, we evaluated the effects of trapidil on crush injury by monitoring nitric oxide, malondialdehyde and transforming growth factor- β 2 levels and by transmission electron microscopy in the rat sciatic nerve. The sciatic nerve was compressed for 20 sec by using a jeweler's forceps. Trapidil treatment groups were administrated a single dose of trapidil (8 mg/kg) intraperitoneally just after the injury. The crush and crush + trapidil treatment groups were evaluated on the 2nd, 7th, 15th, 30th and 45th days of the post-crush period. On the 7th and 15th days, damage in thin and thick myelinated axons, endoneural edema and mitochondrial swelling were less severe in the trapidil group histopathologically. These findings supported the idea that trapidil prevented cell damage and edema at the injury site. Day/group interaction with regard to serum nitric oxide, malondialdehyde and transforming growth factor- β 2 levels did not show significant changes.

Key words: trapidil, crush injury, peripheral nerve, electron microscopy, nitric oxide

It is well known that pathological events such as trauma, compression and crushing directly cause mechanical injury to nerve fibers and deteriorate neuronal functions by impeding the intraneural microvasculature [1, 2]. Morphological alterations occurring as consequences of compression of the peripheral nerve could include demyelination and remyelination, axonal degeneration and regeneration, focal, multifocal or diffuse nerve fiber loss and endoneural edema [3]. Additionally, direct mechanical injury or ischemia or both can cause acute endothelial injury that can result in endothelial edema, a granulocyte plug or microvascular thrombosis. These factors interrupt the reflow and can cause continuous fiber

injury. Moreover, endoneural edema may develop due to microvascular compression [2]. Toxic substances released from neutrophils and macrophages after injury can impair tissue protection in normal conditions and permit the accumulation of free oxygen radicals [3-9].

The protective effects of trapidil have been demonstrated in the peripheral nervous system in the treatment of ischemia and reperfusion injury. This effect of trapidil is related to its vasorelaxant effect, to the inhibition of inflammatory responses via macrophage inactivation and probably to the elevation of nitric oxide (NO) levels which neutralize free superoxide anion radicals [3].

Response to injury could be evaluated by measuring end products of NO, which play a role in tissue protection, malondialdehyde (MDA) level, which is a lipid peroxidation sign, and transforming growth factor beta-2 (TGF- β 2) level, which is thought to play a role in

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*Corresponding author. Phone:+90-324-341-0878; Fax:+90-324-341-2312
E-mail:zkurtoglu@mersin.edu.tr (Z. Kurtoglu)

nervous tissue recovery.

Our search of the literature revealed no previous study about the effects of trapidil on peripheral nerve crush injury. In this study, we evaluated the response to trapidil after a crush injury in the rat sciatic nerve histopathologically by transmission electron microscopy and biochemically by examining the end products of NO (nitrite and nitrate), MDA and TGF- β 2 levels.

Materials and Methods

The procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Mersin University Medical Faculty. In this study 81 female albino rats (10 weeks old and weighing 200–225 g) were used. Seven rats without a crush injury were used as the control group. The crush and crush + trapidil groups were each divided into 5 subgroups after the crush injury based on the regeneration period, on the 2nd, 7th, 15th, 30th and 45th days. Rats were anesthetized by ketamin HCl at a dose of 50 mg/kg intramuscularly. The sciatic nerve was exposed at the right gluteal region without any damage to the muscle tissue and crushed for 20 sec with a jeweler's forceps (no: 5) in sterile operative conditions [10]. Crush level was marked on the muscle by a 4/0 non-absorbable silk suture, and then the incision site was closed. Rats in the therapeutic groups were administrated a single dose of trapidil (8 mg/kg) (Rocornal; Rentschler Biotechnologie GmbH, Laupheim, Germany) intraperitoneally just after the injury. The dose of trapidil was chosen on the basis of the daily human dose and previous experiments that reported substantial benefits [11].

Histopathological Assessment. The sciatic nerves were harvested on the 2nd, 7th, 15th, 30th and 45th days after the crush injury. A 5-mm section including the crush site of the nerve tissue was dissected, fixed *in situ* with 2.5% gluteraldehyde in 0.1 mol/L phosphate buffer (pH 7.2) at 4 °C for 2 to 4 h, postfixed with 1% osmium tetroxide in phosphate buffer (pH 7.2) and dehydrated with serially increasing concentrations of alcohol. The tissues were then washed with propylene oxide and embedded in Araldite 6005 (Ciba-Geigy, Summit, NJ, USA). Semithin transverse sections of 1 to 2 μ m were cut with a glass knife in a LKB Nova ultramicrotome (LKB-Produkt AB, Bromma, Sweden), stained with toluidine blue and observed with a Nikon Optiphot light microscope by an observer blinded to which group the

sections came from (Nikon Co., Tokyo, Japan). The same ultratome was used to obtain thin transverse sections (60–90 nm thick), which were contrast-treated with uranyl acetate and lead citrate and observed with a JEOL JEM 1200 electron microscope (JEOL, Ltd., Tokyo, Japan).

Biochemical Assessment. NO Assay.

Serum levels of nitrite and nitrate were measured as oxidized end products of NO based on the Griess reaction [12].

Blood samples were obtained via continuous catheterization and immediately centrifuged at 4000 r.p.m. for 10 min. Serum samples were preserved at -70 °C until they were used for the assay. Equal volumes of serum and iso-osmotic potassium phosphate buffer were ultrafiltrated at 4000 r.p.m. for 45 min at room temperature. The ultrafiltrate was collected and used in the test. Nitrates were quantitatively converted to nitrites for analysis. Enzymatic reduction of nitrate to nitrite was carried out by using coenzymes (NADPH, FAD) in the presence of nitrate reductase in the incubation assay. N-1-(naphthyl) ethylenediamine dihydrochloride, sulphanilamide and incubation solutions were mixed at a ratio of 1:1:2 (v/v). These mixtures were incubated for 5 min at room temperature in dimmed light and measured at $\lambda = 540$ nm. Sodium nitrite (1.00 mM) was used as a standard to determine nitrite, and potassium nitrate (80 mM) was used as a standard to determine nitrate-nitric oxide colorimetric assay (Roche, Mannheim, Germany).

Lipid Peroxide Assay. Malondialdehyde levels indicating lipid peroxidation were determined by the thiobarbituric acid reaction. The principle of the method depends on measurement of the pink color produced by interaction of barbituric acid with malondialdehyde elaborated as a result of lipid peroxidation. The colored reaction 1, 1, 3, 3-tetraethoxy propane was used as the primary standard. The determination of MDA levels was performed by the method of Yagi [13]. All biochemical measurements were performed in a blinded fashion.

TGF- β 2 Assay. Immunoassay of TGF- β 2 was designed for the sensitive and specific detection of biologically active transforming growth factor β 2 (TGF- β 2) in an antibody sandwich format. In this format, flat-bottom plates were coated with TGF- β Coat mAb, which binds soluble TGF- β 2. A second antibody, anti-TGF- β 2 pAb, was added to complete the sandwich. After washing, antibody conjugate (horseradish peroxidase, TGF- β HRP) was added and bound the sandwich

complex. Finally, the chromogenic substrate 3, 3', 5, 5'-tetramethyl benzidine (TMB) was added. The amount of specifically bound TGF- β 2 in the sample was proportional to the color generated in the coupled oxidation-reduction reaction and was quantitated against a standard curve generated with known amounts of TGF- β 2. Using this assay, we quantitated biologically active TGF- β 2 in serum in the range of 32-1,000 pg/ml (TGF β 2 E_{max}[®] ImmunoAssay System, 143540, Promega Co., Madison, WI, USA).

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Statistical Method. Factorial analysis of variance with 2 factors was used for the statistical evaluation of the data. The Bonferoni post hoc test was used to determine the meaningful differences. Data were analyzed for day/group interaction. Descriptive statistics of the results are given in Table 1, Fig. 1-3. Type 1 error rate was accepted as 0.05 in statistical calculation. SPSS (ver. 11.5) was used for calculations.

Table 1 Descriptive statistics for serum NO, MDA and TGF- β 2

Day	Group	n	Serum nitrite/nitrate mean \pm SE	Serum MDA mean \pm SE	Serum TGF- β 2 mean \pm SE
	Control	7	16.612 \pm 1.104	6.024 \pm 0.624	3.553 \pm 0.462
2	Cr	8	22.325 \pm 2.088	7.756 \pm 0.631	5.220 \pm 0.701
	Cr + Tr	8	33.699 \pm 8.084	8.721 \pm 1.425	6.720 \pm 0.800
7	Cr	7	19.439 \pm 1.889	5.230 \pm 0.442	6.330 \pm 0.598
	Cr + Tr	8	18.005 \pm 1.589	4.081 \pm 0.162	7.999 \pm 1.064
15	Cr	8	23.231 \pm 2.346	6.574 \pm 0.163	8.426 \pm 1.191
	Cr + Tr	7	20.436 \pm 3.687	7.616 \pm 0.577	8.624 \pm 0.882
30	Cr	7	33.930 \pm 9.488	5.801 \pm 0.566	14.069 \pm 2.192
	Cr + Tr	7	28.553 \pm 3.505	5.797 \pm 0.352	10.591 \pm 1.361
45	Cr	8	78.069 \pm 13.767	7.801 \pm 0.801	10.096 \pm 1.170
	Cr + Tr	6	92.473 \pm 21.639	9.665 \pm 1.407	13.410 \pm 1.572

Cr, crush applied; SE, standard error; Tr, trapidil applied.

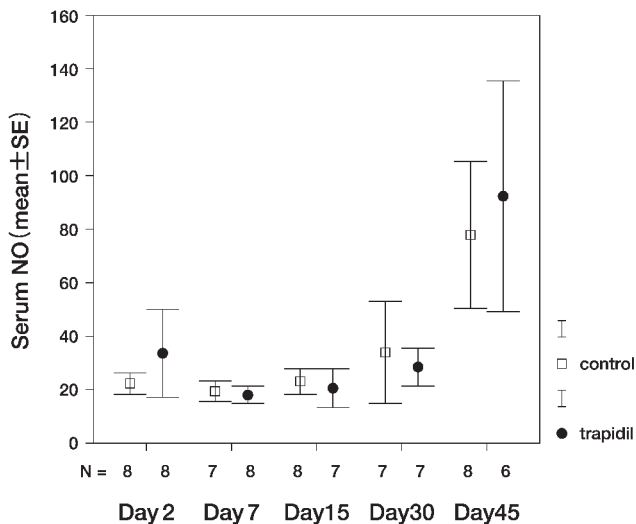


Fig. 1 The mean \pm SE for serum NO (μ mol/L) for each group on each day is shown.

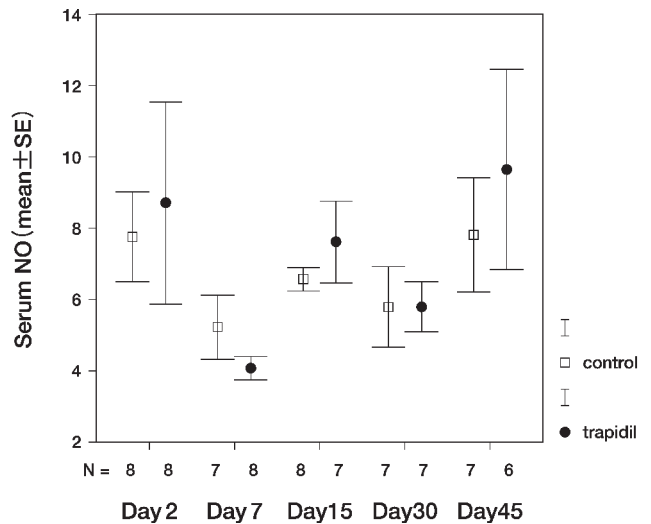


Fig. 2 The mean \pm SE for serum MDA (nmol/ml) for each group on each day is shown.

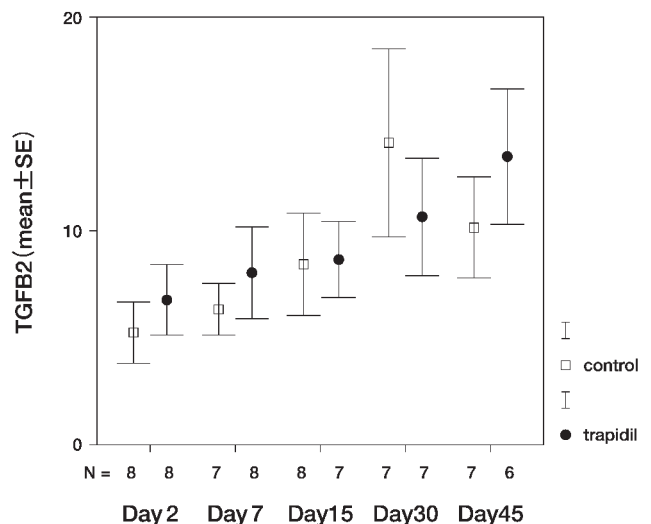


Fig. 3 The mean \pm SE for TGF- β 2 (pg/ml) for each group on each day is shown.

Results

Histological Assessments. Ultrathin sections were taken from the crush site of each sciatic nerve. The myelin sheaths of thin and thick myelinated axons, unmyelinated axons, endoneural edema and axonal mitochondria were evaluated for each group.

2nd day group: The ultrastructure of sciatic nerves of both the crush and treatment groups showed the greatest injury in this group. Injury seemed to be worse in thick myelinated axons in both groups. Myelin separation was the most prominent finding in thick myelinated axons. In thin myelinated axons myelin separation was less severe. Endoneural edema and mitochondrial swelling were observed in both groups. Unmyelinated axons seemed normal in all the specimens in both groups (Figs. 4a, 4b).

7th day group: When compared with both groups of the 2nd day and the crush group of the 7th day, damage in thin and thick myelinated axons was less severe in the treatment group. Swelling of the mitochondria and edema of the endoneurium were less severe in the treatment group. Unmyelinated axons were normal in both groups (Figs. 4c, 4d).

15th day group: There was mild myelin separation in the thin and thick myelinated axons of the treatment group. Edema of the endoneurium and swelling of the mitochondria were again mild compared with the treatment and crush groups of the 2nd and 7th days and the crush group of the 15th day. The crush group of the 15th day was similar in severity of injury to the crush group of the 7th day. Again unmyelinated axons were normal in appearance (Figs. 4e, 4f).

30th and 45th day groups: Both the treatment and the crush groups showed milder damage signs when compared with both groups of the 2nd, 7th and 15th days. There was no major difference in the appearance of the degeneration signs between the treatment and the crush groups for the 30th and 45th days. Myelin separation and edema were scarce in the treatment and crush groups in both groups. Unmyelinated axons were normal in appearance (Figs. 4g, 4h).

Biochemical Assessments. With regard to serum NO calculations, we found the day/group interaction to be insignificant statistically ($P = 0.700$). Because of this result, we did not examine the differences between the days and groups separately. There was a meaningful difference between the days ($P = 0.001$). In both groups the differences between the 2nd and 45th, 7th and 45th,

15th and 45th, and 30th and 45th days were observed to be statistically meaningful (Fig. 1, Table 1).

With regard to serum MDA levels, the day/group interaction was not found to be meaningful statistically ($P = 0.353$). Because of this result, we did not examine the differences between the days and groups separately. The difference between the days was meaningful, and in both groups the difference was meaningful between the 2nd and 7th, 2nd and 30th, 7th and 15th, 7th and 45th, and 30th and 45th days (Fig. 2, Table 1).

With regard to TGF- β 2 levels, the day/group interaction was not found to be meaningful statistically ($P = 0.086$). Because of this result, we did not examine the differences between the days and groups separately. The difference between the days was meaningful ($P = 0.0001$), and in both groups the differences were found meaningful only between the 2nd and 30th, 2nd and 45th, 7th and 30th, 7th and 45th, and 15th and 30th days (Fig. 3, Table 1).

Discussion

In the present study electron microscopic findings in trapidil-treated groups on the 7th and 15th days showed amelioration after a crush injury. On the other hand, levels of NO, MDA and TGF- β 2, which inform us about tissue damage and the healing process, were not found to be meaningful when compared statistically both within the groups and between the groups.

It has been reported that after a crush injury, endoneural edema in the peripheral nerve influenced the microenvironment by increasing the pressure, decreasing the blood flow or changing the electrolyte concentration in the endoneurium. If the restoration of adequate circulation is delayed, ischemia creates Wallerian-like axonal degeneration [14-18]. Degeneration in the myelin and axons was observed a week later in the lesion site and distal to it. It was also stated that in 3 weeks, most of the axons were regenerated and remyelinated, and functional recovery was completed in 4-5 weeks [14, 19]. Trapidil is well known as a vasorelaxant which prevents tissue damage by diminishing the vasospasm caused by a crush injury [20, 21]. In our study electron microscopic findings showed that myelin separation and endoneural and mitochondrial swelling were less prominent in the trapidil groups on the 7th and 15th days, which supported the notion of trapidil's tissue protective effects.

Adequate external pressure application to a peripheral

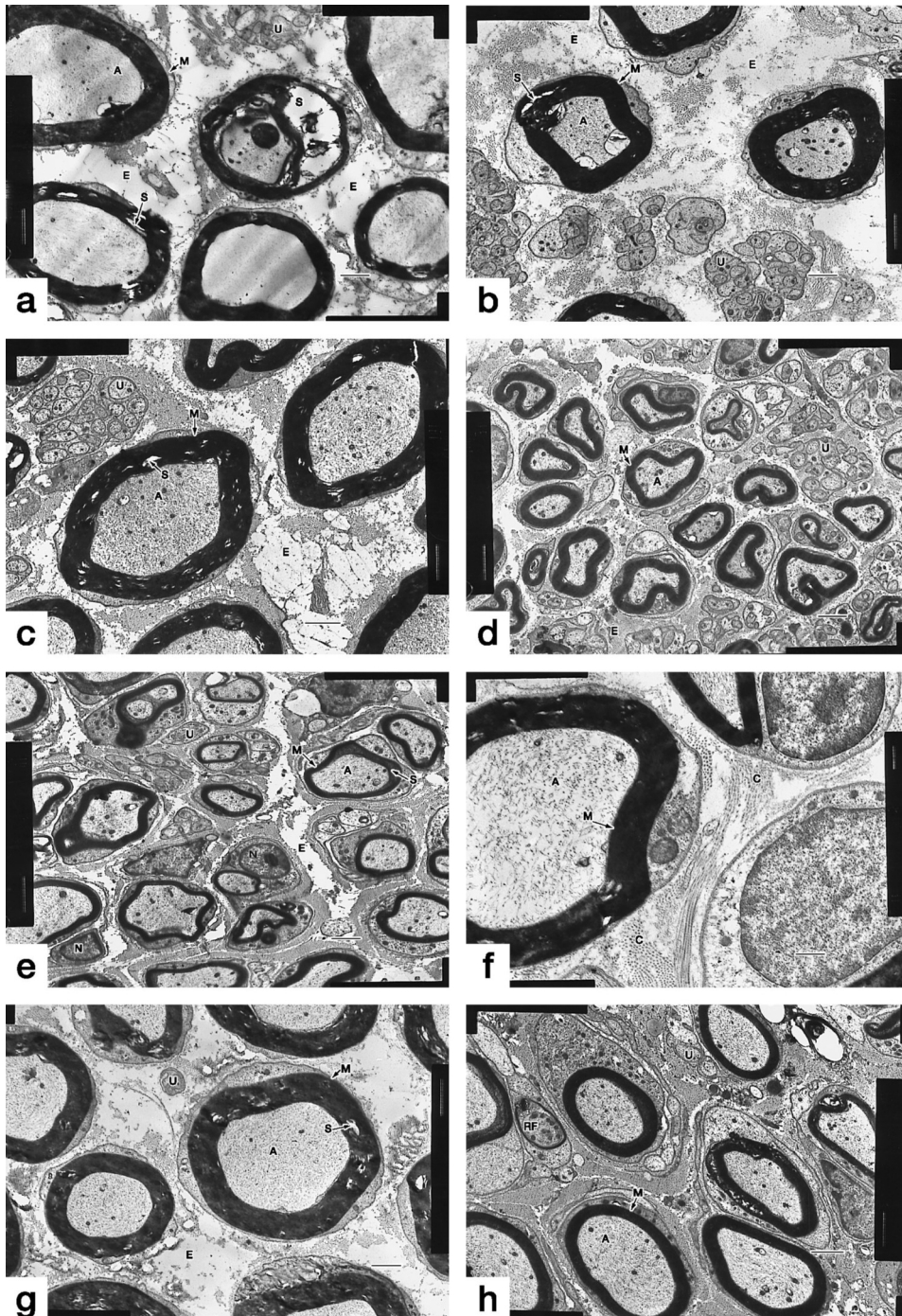


Fig. 4 a and b, electron micrographs of a transverse section of a rat sciatic nerve from the crush site of the crush group and the crush + trapidil group on the 2nd day illustrating myelin separation (arrow), myelinated axons (A), mitochondrial swelling (S), endoneurial edema (E) and normal unmyelinated axons (U) (bars indicate $1.8 \mu\text{m}$); c and d, electron micrographs of the crush and crush + trapidil groups on the 7th day illustrating the same findings, which are less severe in the latter group (bars indicate $1.8 \mu\text{m}$); e and f, electron micrographs of the crush and crush + trapidil groups from the 15th day, again illustrating the same findings of lesser severity in the crush + trapidil group (bars indicate $1.8 \mu\text{m}$ and $0.75 \mu\text{m}$ respectively), (N, Schwann cell nucleus; C, collagen fibrils); g and h, electron micrographs of the crush and crush + trapidil groups on the 45th day illustrating milder signs of myelin separation and endoneurial edema. Regenerating fibers (RF) are also seen in the crush + trapidil group (bars indicate $1.8 \mu\text{m}$).

nerve results in complete circulatory arrest [14]. In addition to direct mechanical injury, this condition also causes local ischemia and triggers biochemical reactions due to the microvascular endothelial damage [2]. The peripheral nerve responds to trauma by an inflammatory reaction with increased vascular permeability and intraneural edema. Epineural edema develops rapidly due to the early response of epineural blood vessels even in cases of mild trauma. In a serious trauma such as a crushing injury, a prominent increase in the endoneural fluid pressure causes a short period of total or subtotal localized ischemia [1]. This process ends with the release of endogenous chemical mediators, increased vascular permeability and an impaired nerve/blood barrier [1, 16, 22].

Biochemical and pathological changes develop as a result of oxidative stress and lipid peroxidation in the impaired nerve/blood barrier. Local ischemia in the tissue causes metabolic impairment, which in turn allows the production of the toxic oxygen metabolites such as superoxide anion, hydrogen peroxide and hydroxyl radicals by the polymorphonuclear leucocytes that infiltrated the lesion site. Free oxygen radicals and cytokines which are responsible for cell damage are released from neutrophils. Additionally, neutrophils, by causing adhesion, impair the flow mechanically by obstructing the capillaries. Thus endothelial function is impaired and vascular permeability increases [22]. We suggest that trapidil could attenuate those mechanisms by its prevention of neutrophil adhesion.

After the injury, NO accelerates cell migration, facilitates angiogenesis and wound healing, causes vasodilatation, has an antiatherogenic effect by platelet aggregation inhibition, provides endothelial leukocyte adhesion and leukocyte activation and is an endogenous inhibitor of chemotaxis [4, 6, 8, 9, 23-25]. It takes part in neurogenesis and protection from neuronal damage [3, 26].

It is reported that in mammals the axonal injury resulted in NOS upregulation dramatically in most of the central and peripheral neuron types. In particular, the inducible form of nitric oxide synthase (iNOS) is released in response to activation of cytokine and endotoxin, and increases NO synthesis to toxic levels in ischemia [26, 27]. Efron *et al.* reported that iNOS activity reached the maximum level on the 1st day and decreased on the 3rd and 5th days [25]. Increase of NO in serum on the 2nd day in both the treatment and crush groups, in contrast

to the control group in our study, is in accordance with their results (Table 1).

It is reported that in ischemia/reperfusion injuries, free superoxide anion radicals appeared in response to tissue damage, neutralized endogenous NO and moreover decreased iNOS activation. For this reason, end products of NO, nitrite and nitrate levels decreased after the peak level reached on the first day [3, 25]. Serum NO level decreased in both groups on the 7th and 15th days in our study, which is in accordance with these studies. On the other hand, trapidil's effect on serum NO levels in case of crush injury does not coincide with the results of Bagdatoglu *et al.*, who described ischemia/reperfusion injury in which trapidil increased the release of nitrite and nitrate levels [3]. One reason for this disparity in findings is that the endothelial area affected in a crush injury could be smaller than that in an ischemia/reperfusion injury, so the biochemical changes noted in the study by Bagdatoglu *et al.* were not meaningful statistically. The other reason may be that NO levels in their study were evaluated in the tissue. Furthermore, the mechanism of NO increase during the first days after trapidil administration is not well understood.

In tissue ischemia and injury, endothelial cells, tissue mast cells, monocytes, circulatory neutrophils and platelets produce eicosanoids. From these, tromboxane A₂, by inducing neutrophils increases the release of free oxygen radicals and by regulating CD11/CD18 activity provides diapedesis. Trapidil's cell damage and edema preventive effects at the injury site have been explained by some authors as the result of the inhibition of tromboxan A₂ by producing a membrane-stabilizing effect and the inhibition of an inflammatory response by macrophage inactivation [3, 23]. We interpret the histopathological signs on the 7th and 15th days, such as the milder degree of myelinated fiber damage, mitochondrial swelling and endoneural edema in the treatment group in our study, as trapidil's protective effects in nerve tissue. Mitochondrial changes in Schwann cells are thought to be caused by the induction of neutrophil-derived free oxygen radicals by tromboxane A₂, and trapidil as a tromboxane A₂ inhibitor might have decreased these effects.

In crush injuries, lipid radicals get oxygenated and produce lipid peroxide radicals which transforms them to malondialdehyde (MDA) [4, 9]. MDA is used as a lipid peroxidation indicator in tissue injuries. In nerve injuries, due to the excessive amount of lipids in nerve tissue, MDA increases easily [3, 28]. In one study, the

authors reported that serum MDA started to increase on the first day, but then decreased to normal levels at the end of the first week [3]. The higher levels of serum MDA on the second day of our study for both groups in contrast to the control group were consistent with this data (Table 1). Increasing MDA as well as NO levels on the 45th day were thought to be caused by the metabolic alterations occurred in the tissue healing process.

Cytokines take part in many biological activities such as inflammation, tissue regeneration, cell proliferation and wound healing [24]. Transforming growth factor- β (TGF- β) is a cytokine released from activated macrophages during tissue injury, and it increases in ischemic conditions. This is thought to be an adaptive response to ischemic injury [29]. TGF- β 2, a member of the TGF- β family, is reported to play a role in wound healing and repair, and it additionally has neuroprotective effects [24, 30]. Trapidil's effect on TGF- β 2 has not been described in the literature. In our study, a gradual increase of TGF- β 2 levels was observed in both groups after the second day as a predicted response to tissue healing (Fig. 3, Table 1). However, TGF- β 2 levels did not show any difference according to the days between the crush and treatment groups. Thus our study did not show any evidence that trapidil decreased TGF- β 2 levels.

In conclusion, our histological examination indicated that intraperitoneal administration of trapidil prevented cell damage and edema at the injury site. Because of the lack of harmony between the histological and biochemical results in this preliminary study, we hypothesize that the trapidil dose used in this study might have been below or just at the threshold of effect and higher doses or multiple injections might show detectable alterations in the NO, MDA and TGF- β 2 levels.

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