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Effect of Dexamethasone in Mediating Oxidative Stress Induced by Sodium Nitroprusside on Frog Sciatic Nerve Action Potentials

Deksametazonun Sodyum Nitroprussid ile Oluşturulan Oksidatif Hasarda Kurbağa Siyatik Sinir Aksiyon Potansiyelleri Üzerine Etkisi

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ABSTRACT

Objective: High concentrations of nitric oxide cause a neurotoxic effect on nerve action potentials. Although glucocorticoids can decrease that effect, the degree of mediation is not known. This study determined the effect of dexamethasone on frog sciatic nerve axon fibers subjected to in vitro oxidative stress.

Methods: Frog sciatic nerves were isolated into Groups: -Control Group-incubation in Ringer's solution; SNP Group- incubation in 10^{-2} M sodium nitroprusside solution; SNP+DEX Group-incubation in 10^{-2} M sodium nitroprusside solution followed by incubation in 10^{-3} M dexamethasone solution; DEX group- incubation in 10^{-3} M dexamethasone solution.

Results: In the SNP group, significant changes were observed in the action potential velocity of propagation ($p<0.01$), the action potential maximum amplitude ($p<0.01$), the slope of the emerging phase ($p<0.01$), and the area under the signal curve ($p<0.05$). Considering electrophysiological parameters, conduction velocity; maximum amplitude; and signal area values increased above normal by dexamethasone incubation after sodium nitroprusside exposure. In biochemical parameters, the group that received sodium nitroprusside increased the thiobarbituric acid reactive substances (TBARS) concentration ($p<0.001$) and decreased superoxide dismutase (SOD) activity ($p<0.01$).

Conclusion: Findings supported our hypothesis that dexamethasone reverses damage and decreases oxidative damage to nerve action potential caused by exposure to sodium nitroprusside.

Key Words: Dexamethasone, glucocorticoids, oxidative damage, nerve action potentials, nitric oxide

ÖZET

Amaç: Nitrik oksit yüksek konsantrasyonlarda sinir aksiyon potansiyeli üzerinde nörotoksik etkiye neden olur. Glukokortikoidler bu etkiyi azaltmasına rağmen, derecesi bilinmemektedir. Bu çalışmada in vitro oksidatif strese maruz bırakılan kurbağa siyatik sinir akson lifleri üzerinde deksametazonun etkisi araştırıldı.

Yöntemler: Kurbağa siyatik sinirleri; kontrol (Ringer solüsyonunda inkübasyon); SNP (10^{-2} M sodium nitroprusside solüsyonun inkübasyonu); SNP+DEX (10^{-2} M sodium nitroprusside solüsyon inkübasyonunu takiben 10^{-3} M dexamethasone solüsyonunda inkübasyon); DEX (10^{-3} M dexamethasone solüsyonda inkübasyon) olacak şekilde gruplara ayrıldı.

Bulgular: SNP grubunda, aksiyon potansiyel yayılma hızı ($p<0,01$), maksimum genliği ($p<0,05$), yükselen fazın eğimi ($p<0,001$) ve sinyal altı alanda ($p<0,05$) anlamlı azalma görülmüştür. Deksametazon uygulamasıyla bu değişikliklerde anlamlı artma gözlenmiştir ($p<0,001$; SNP grubuna göre). Biyokimyasal parametrelerde, SNP grubunda tiyobarbitürik asit reaksiyona giren maddelerin (TBARS) düzeyinde ($p<0,001$) artma süperoksit dismutaz (SOD) aktivitesinde ($p<0,001$) ise azalma görülmüştür. Deksametazon uygulamasıyla artan TBARS düzeyi azalmış, SOD aktivitesi ise artmıştır ($p<0,05$; SNP grubuna göre).

Sonuç: Bulgularımız deksametazonun, oksidatif stresin neden olduğu sinir hasarındaki iyileştirici etkisini antioksidan savunma sistemi üzerinden yaptığı görülmüştür.

Anahtar Sözcükler: Deksametazon, glukokortikoid, oksidatif hasar, sinir aksiyon potansiyeli, nitrik oksit

Introduction

In the nervous system, information is transmitted along axons by rapid changes in the cells' membrane potential. During these changes, the cell membrane loses polarity, activating voltage dependent Na^+ channels, which draws sodium (Na^{+})

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into the cell until the action potential reaches maximum. This process ends the first or depolarization phase. Depolarization of the cell triggers voltage dependent potassium (K^+) channel to open, which quickly discharges K^+ ions into the extracellular fluid. When K^+ ions exit, Na^+ channels progressively lose activity, halting Na^+ flow into the cell until the membrane repolarizes (1). Amplitude, time, and conduction velocity of action potentials are cellular attributes that may give information about Na^+ and K^+ transport. Amplitude, the area under the curve, and conduction velocity of the compound action potential are directly proportional to Na^+ transport. Additionally, the area under the curve and the amplitude of the action potential that is lost can be used to predict the number of nerve fibers stimulated (2).

There are many studies about the efficiency of nitric oxide (NO) in the regulation of the functions of ion channels (3-5). When Chen and Schofield (6) investigated the effect of NO donors on superior cervical ganglion neurons, they observed that NO increased the Ca^{++} flow through the mechanism pathway dependent on cyclic guanosine monophosphate (cGMP). NO is an unstable radical that converts to nitrite/nitrate anions, which are more stable. Clearly, high amounts of NO produce a cytotoxic effect (7, 8). NO and reactive oxygen species interact to give off the peroxynitrite anion ($ONOO^-$). This anion quickly releases nitrogen dioxide and the hydroxyl anion, both of which are very reactive and toxic (9). Damage from ONOO, a nitric oxide derivative, can be illustrated by measuring the circulating levels of this anion (7, 8).

A major effect of oxidative stress is nerve damage. Several studies have demonstrated the benefits of glucocorticoids in treating this injury (10-13). However, the total effect of dexamethasone, a synthetic glucocorticoid, on the oxidative stress continuum for the nervous system is not completely understood and its effect on the sciatic nerve axon has not yet to be studied. This study investigates the effect of dexamethasone on frog sciatic nerve axon fibers subjected to *in vitro* oxidative stress.

Methods

All experiments in this study were performed in accordance both with the Declaration of Helsinki and the "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised 1985)."

Tissue Preparation

Twenty-four male Rana ridibunda frogs weighing 30-40 g were anesthetized. After anesthesia, the spinal cords were destroyed, and the sciatic nerves were quickly excised. The isolated nerve fibers were buffered in Ringer's solution (120 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $NaHCO_3$, 15 mM HEPES) for 15 minutes at a pH of 7.2, a suitable physiologic level for frogs. All measurements were recorded with the preparations equilibrated at room temperature (14).

Drugs

Dexamethasone and sodium nitroprusside used in the experiments were obtained from Sigma-Aldrich, Germany. Each concentration of dexamethasone and sodium nitroprusside was prepared by adding and then incubating the resulting composite mixture in Ringer's solution. In the last stage, nerve fibers were divided into four Groups according to the process outlined below.

Experimental Groups

Group I (Control Group): Incubated in Ringer's solution for 90 minutes.

Group II (SNP Group): Incubated in a 10^{-2} M Sodium nitroprusside (SNP=NO donor) solution for 90 minutes.

Group III (SNP+DEX Group): Incubated for 45 minutes in a 10^{-2} M SNP solution, followed by incubation in a 10^{-3} M Dexamethasone (DEX) solution for 45 min.

Group IV (DEX Group): Incubated for 45 minutes in a 10^{-3} M Dexamethasone solution.

Electrophysiological Techniques

All measurements were performed *in vitro*, using extracellular recording techniques. After 15 minutes of stabilization in Ringer's solution, nerve segments were placed in a nerve chamber containing Ag/AgCl electrodes with a 0.5 mm distance separating each electrode. These electrodes were used for either stimulating nerves or recording electrical changes. An electrophysiological recording device was used for bipolar measurements. In this system, two electrodes were connected to a stimulator and two to a PowerLab, 8sp recorder. The final electrode was used for grounding purposes. The signal data produced by this device was saved in a computer through an analog-to-digital converter.

Initially, the threshold potentials (~0.08 V) of tissues were determined for electrophysiological evaluations. Maximal compound action potentials were formed by generating supramaximal dose impulses with respect to the values obtained from the previous step. Single square pulses of supramaximal strength at 0.5 ms in duration were used. Action potential conduction velocity, action potential maximum value (amplitude), slopes of the action potential rising phase, and the area under the curve were calculated by use of Chart 5.0 software. The value of conduction velocity for the compound action potential was estimated by dividing the calculated distance between the active record electrode and the simulating electrode by the time calculated to have passed between the stimulus and maximum value of the signal.

Biochemical Techniques

The tissue samples were dissected and washed with ice cold 0.9% NaCl solution, weighed and homogenized (1/10, w/v). The homogenization process for the tissues used a tissue grinder fitted with a Teflon pestle and a buffering solution of ice cold sucrose medium (1 mM EDTA, 0.2 M Tris-HCl, 0.32

M sucrose) at pH 7.4. The homogenate was sonicated with an MSE sonicator twice at 30 s intervals at a temperature of 0°C and a power output of 38 W. The sonicated homogenates were centrifuged at 4°C and 2000 rpm for 10 min for the analysis of thiobarbituric acid-reacting substances (TBARS) and at 4°C and 15000 rpm for 15 min for the measurement of superoxide dismutase (SOD) activity. The by-product of the centrifuge, homogenate supernatants, were used for the biochemical assays.

Measurement of the TBARS Levels

Tissue TBARS levels, the lipid peroxidation markers, were determined with the spectrophotometric method (15). Homogenate supernatants samples were heated in a water bath for 20 min and after cooling were centrifuged at 2000×*g* for 15min. At the conclusion of this process, an interaction between one molecule of TBARS and two molecules of thiobarbituric acid created a pink color that was measured at 560 nm spectrophotometrically (1208 UV-Shimadzu Spectrophotometer, Japan) and TBARS concentration was calculated using 1.56×105 M^{-1} cm^{-1} as molar extinction coefficient.

Measurement of the SOD Activity

Tissue SOD activity was measured using a method modified from Sun et al. (16). During the experiment, this activity was measured in an alkaline medium titrated to pH: 10.2. This assay for superoxide dismutase (SOD, EC 1.15.1.1) involves inhibition of nitro blue tetrazolium (NBT) reduction with xanthine–xanthine oxidase used as a superoxide generator. One unit SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. The reaction mixture consisted of 40 mL of 0.3 mmol/L xanthine solution, 20 mL of 0.6 mmol/L EDTA solution, 20 mL of 150 umol/L NBT solutions, 12 mL of 400 mmol/L Na_2CO_3 solution and 6 mL of bovine serum albumin. This admixture yielded 167 U/L as the final concentration of xanthine oxidase and formazan, which was determined spectrophotometrically at 560 nm.

Statistical analysis

Graphs were plotted with the percentage values of data from electrophysiological measurements, with the exception of conduction velocity data, which used real data. For biochemical data, real values were used to plot all graphs. All data was plotted as mean±SEM. After distribution of Groups was tested, an ANOVA comparison test and a Tukey test were performed post-hoc. Values of $p<0.05$ were accepted as statistically significant. For statistical analysis, Instat Statistical Package Program (Instat Graphad Software v 5.0, San Diego, CA, USA) was used.

Results

Compound Action Potential Related Findings

When the conduction velocities of action potentials were compared, the SNP Group was decreased with respect to

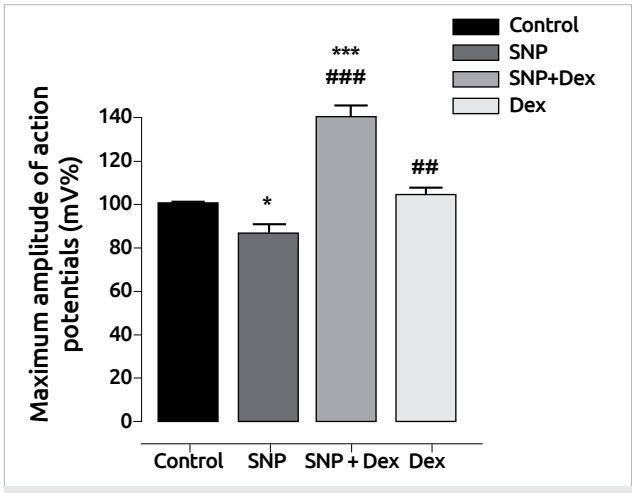

Figure 2. Comparison of action potential maximum amplitudes. **p<0.01,***p<0.001; with respect to the control Group. ##p<0.01, ###p<0.001; with respect to the SNP Group

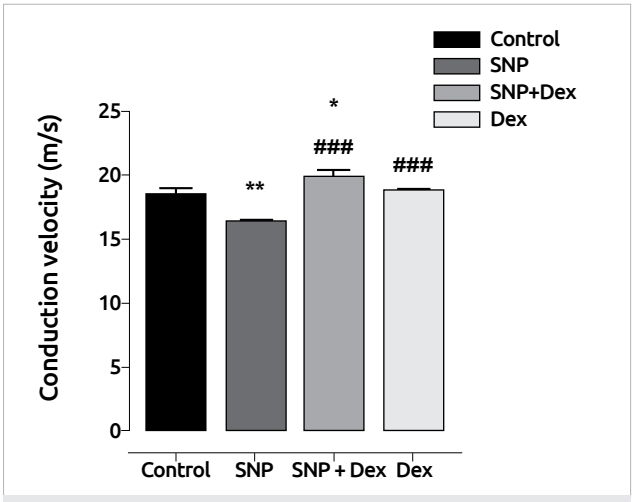

Figure 1. Comparison of action potential conduction velocity of all Groups. *p<0.05,**p<0.01; With respect to the control Group. ###p<0.001; with respect to the SNP Group

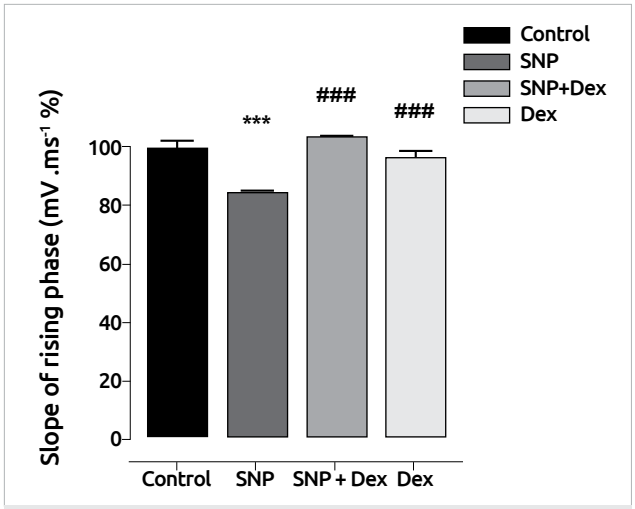

Figure 3. Graph of the change of rising phase slope. **p<0.01; with respect to the control Group. ###p<0.001; with respect to the SNP Group

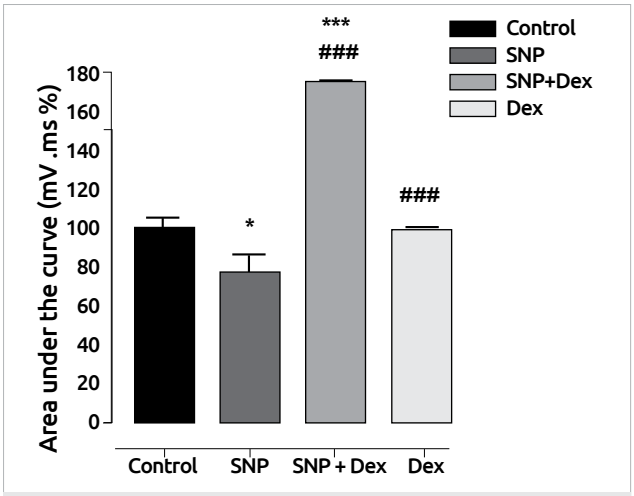

Figure 4. Distribution of areas under the curve, estimated by the integration of signals, with respect to Groups. *p<0.05, ***p<0.001; with respect to control Group. ###p<0.001; with respect to the SNP Group

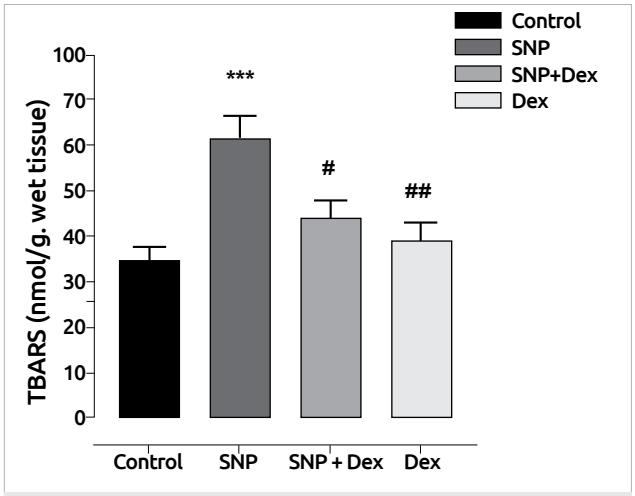

Figure 5. Distribution of TBARS levels with respect to Groups. ***p<0.001; with respect to the control Group. ##p<0.01,###p<0.001; with respect to the SNP Group

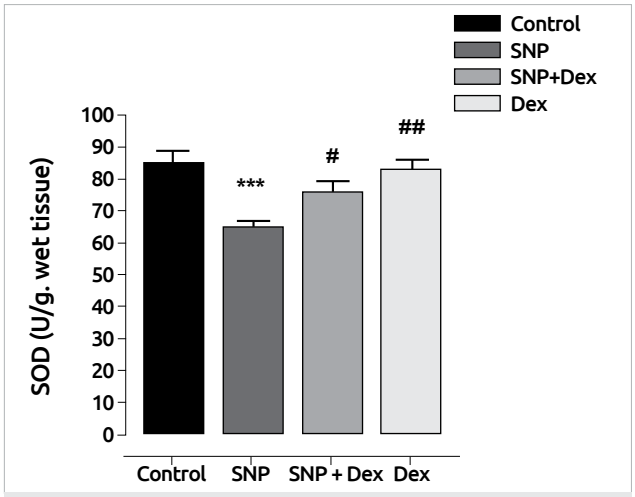

Figure 6. Superoxide dismutase activity values for Groups. ***p<0.001; with respect to the control Group. #p<0.05,##p<0.01; with respect to the SNP Group

the control Group: 18.5±0.5 m/s vs. 16.4±0.2 m/s; p<0.01 (11%); whereas, the conduction velocity for the SNP+DEX Group was increased with respect to the control Group: 20.0±0.4 m/s; p<0.05 (8%). There was no statistically significant difference observed for DEX Group 18.8±0.1 m/s; p>0.05 (Figure 1).

The maximum amplitude values of action potentials decreased 13% (100.0±0.9 vs. 86.8±3.4; p<0.05) in the SNP Group with respect to the control; whereas a 40% (140.4±5.3; p<0.001) increase was observed in the SNP+DEX Group with respect to the control Group. There was no statistically significant difference observed for the Group that only received dexamethasone (104.7±2.4; p>0.05) (Figure 2).

A rising phase slope of compound action potentials was estimated. According to the results, a 16% (100.0±1.9 vs. 84.6±1.1; p<0.001) decrease was observed in the SNP Group with respect to the control Group. Additionally, an insignificant 3% (103.7±0.6; p>0.05) increase was observed in the SNP+DEX Group with respect to the control Group. On the other hand, no statistically significant difference was observed for the Group that only received dexamethasone (96.6±1.8; p>0.05) (Figure 3).

According to the comparison in terms of area under the curve for action potentials, a 22% (100.0±5.6 vs. 78.1±8.4; p<0.05) a decrease was observed in the SNP Group with respect to the control Group. This decrease was not observed for dexamethasone incubation, which yielded a 75% (175.2±1.8; p<0.001) increase with respect to the control Group. There was no statistically significant difference observed for the Group that only received dexamethasone (99.3±1.1; p>0.05) (Figure 4).

Oxidative Stress Related Findings

Biochemical results were correlated with the electrophysiological results. For the SNP Group, TBARS- an indicator for lipid peroxidation-increased significantly (p<0.001). This increase was not observed in the SNP+DEX Group (vs. control; p>0.05). Moreover, there was no change observed for the that received only dexamethasone (p>0.05). TBARS levels for Group I, II, III, IV were, on average, 34.3±2.8, 61.4±4.8, 44.0±3.5, 39.0±3.4 (nmol/g. wet tissue), respectively.

Superoxide dismutase activity decreased with SNP usage (p<0.05). For SOD values, no statistically significant difference was observed either in the SNP+DEX Group (vs. control; p>0.05) or in the group that only received dexamethasone (vs. control; p>0.05). The measured values were recorded as 85.1±3.8, 64.7±2.3, 76.5±2.9, 83.5±2.3 (U/g, wet tissue), respectively (Figure 5, 6).

Discussion

Although the frog myelinated sciatic nerve is not a mammalian nerve, the tissue has physiologic and morphologic prop-

erties much like mammalian sciatic nerves (17, 18). Due to this characteristic, many experimental models can be applied to frog nerve fibers (19, 20). In this study, dexamethasone reversed electrophysiological and biochemical changes to nerve action potential in the frog sciatic nerve fibers caused by exposure to *in vitro* SNP oxidative stress. Dexamethasone was the synthetic glucocorticoid used to investigate these changes.

With the aging of the human population worldwide, the need to resolve tissue damage associated with oxidative stress has taken on a higher urgency. Oxidative stress and lipid peroxidation are observed in the case of most neurodegenerative illnesses (21-23). Oxidative stress is the change of equilibrium between pro-oxidant and antioxidant defense system components in favor of the oxidant compound (24). Aldehydes, oxidant compounds, are the last product of lipid peroxidation (25). Lipid peroxidation increases with a free radical chain reaction mechanism and produces lipid hydroperoxides. Malondialdehyde (MDA) is the most abundant single aldehyde of lipid aldehydes. The determination of MDA with TBARS is the method used most frequently in lipid peroxidation studies (26). In addition, the reaction of NO with a superoxide radical yields peroxinitrites, which cause the cellular damage and, in this way, NO participates in oxidative stress (8).

Superoxide dismutase is a cellular antioxidant that saves cells from oxidative stress. SOD is known to decrease in many illnesses, which allows it to be viewed as a marker for MDA lipid peroxidation (27). This study confirmed an important finding: low SOD activity was observed in the SNP Group when compared to the control Group during acute oxidative stress. Low SOD activity demonstrated that endogenous SOD was consumed, causing an oxidative defensive response. Moreover, the corresponding increase in TBARS level within that same group strengthened the damaging effect of oxidative stress at the cellular membrane level of tissues.

In the nervous system, information is transmitted along axons through a cyclic process called the action potential. During this cycle, the cell membrane loses polarity, activating voltage dependent Na^+ channels, which pull Na^+ into the cell until reaching the maximum limit. This process ends the depolarization phase. Depolarization of the cell triggers voltage dependent K^+ channels to open, which quickly discharge K^+ ions into the extracellular fluid. When K^+ ions exit the cell, Na^+ channels progressively lose activity halting Na^+ flow into the cell until the membrane repolarizes (1). The action potential conduction velocity, amplitude, slope of the rising phase, and the area under the curve were estimated using the recorded extracellular performance data. Research has shown that the area under the compound action potentials curve is proportional to the nerve fibers when the nerve fibers are activated (28, 29). As a result of this experiment, the researchers conclude that oxidative stress interferes with impulse conduction along the sciatic axon; and thus, prolongs the action potential in at least some nerve fibers.

A large body of evidence demonstrates that ion currents are affected by free radicals, which affect pumps (30-36). However, little is known about the effect of oxidative stress on the full spectrum of nerve electrophysiological properties along the axon. This is the first scientific study that addresses the effect of oxidative stress on axonal propagation. Therefore, our study expands the current body of knowledge on this aspect of oxidative stress. Several studies and models depict the effect of oxidative stress on sodium channels (34, 37). For example, in two separate studies, Matsuura and Shattock (30), then Jabr and Cole (31) found an acceleration in the Na^+/Ca^{++} exchanger in the case of oxidative stress. Conversely, Shattock and Matsuura (32) observed Na^+ channel constraint as an effect of oxidative stress. With respect to Na^+ channels only, our study concurs with Shattock and Matsuura (32) that sodium channels are inhibited as a result of the prolongation of the action potential.

The experimental results of this study were focused on determining whether glucocorticoids mediated or ameliorated the effects of oxidative stress. Our results coincided with the one published study, which found that dexamethasone alone had no effect on axonal conduction (38). However, many positive changes were observed for the DEX group. Most importantly, we hypothesize that a new pathway was generated by SNP group for dexamethasone. We offer this finding as a new area for investigation.

Additionally, the *in vivo* and *in vitro* studies show that glucocorticoids affect redox equilibrium in the direction of either pro-oxidant or antioxidant. In other words, some studies demonstrate the beneficial effect of glucocorticoids as having a strengthening effect on the immune system and a mediating effect on oxidative damage (10-13). This study considered the antioxidant strengthening effect of dexamethasone. Similarly, we found that glucocorticoids affect the SOD synthesis in the degree of gene expression (39). Particularly, the application of dexamethasone after SNP caused electrophysiological results greater than the results for the control group; showing that dexamethasone should be controlled.

Conclusion

The primary aim of this study was to determine the direct effect of oxidative stress on sciatic nerve action potentials along the axon. In the process, in addition to observing a change in compound action potentials, we also observed an imbalance in redox equilibrium in the direction of either pro-oxidant or antioxidant. Through this experiment, we detected the disruption of the electrophysiological properties along the sciatic nerve axon and an acute defensive response by the endogenous defense system. We also hypothesize that a new pathway was generated by the SNP Group for dexamethasone. As a limitation of this study, SOD expression could be evaluated by means of western blot technique. Hence deep perspective could be added to the present suggestion.

Dexamethasone application decreased the oxidative stress effect of SNP by strengthening SOD level, which is indicative of a strengthening of cellular defenses. Hence, we argue for further studies to identify the contributions that glucocorticoids (like dexamethasone) can make to health and science beyond neuroprotection. Our research strongly suggests the need to further investigate the use of glucocorticoids as a possible treatment for such problems as peripheral nerve damage caused by oxidative stress.

Conflict of Interest: No conflict of interest was declared by the authors.

Peer-review: Externally peer-reviewed.

Ethics Committee Approval: Ethics committee approval was not received due to lack of ethic committee during the experimentation in 2003.

Author Contributions: Concept - U.A.; Supervision - U.A.; Materials - U.A., P.A.; Data Collection and/or Processing - U.A., P.A.; Analysis and/or Interpretation - U.A. C.D. D.T. Writing - U.A., A.B.

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