# The Effects of Two Different Concentrations of Levobupivacaine on Free Radicals in Blood, Brain, and Heart Tissues of Adult Rats

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## *Abstract*

**Objective:** Levobupivacaine is considered one of the less toxic local anesthetics. In this study, we aimed to investigate the effects of two different concentrations of levobupivacaine on the levels of free oxygen radicals in blood, heart, and brain tissues of rats.

**Methods:** Thirty-four rats were divided into three groups. Group 1 received 2.5 $mg.kg^{-1}$ IV levobupivacaine (n=12), group 2 received 5 $mg.kg^{-1}$ IV levobupivacaine (n=12), and group C (control) received IV 0.9% sodium chloride solution (n=10). Every group was evaluated in two separate subgroups where samples were taken at 2nd hour (A) and 24$^{th}$ hour of the injection (B) after sacrification. Malondialdehyde, superoxide dismutase, glutathione, and nitric oxide levels were determined in the blood, heart, and brain tissues of rats biochemically.

**Results:** After administration of levobupivacaine, the levels of malondialdehyde increased while the levels of superoxide dismutase, glutathione, and nitric oxide decreased in the tissues.

**Conclusion:** Administration of levobupivacaine may cause an oxidative stress response.

**Keywords:** Levobupivacaine, reactive oxygen species

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## *Levobupivakain'in Farklı İki Konsantrasyonunun Erişkin Ratlarda Kan, Kalp ve Beyin Serbest Oksijen Radikallerine Etkileri*

## *Öz*

**Amaç:** Levobupivakain toksik etkileri az olduğu düşünülen lokal anesteziklerden biridir. Bu çalışmada farklı iki dozda uygulanan levobupivakainin, ratlarda kan, kalp ve beyin dokusundaki serbest oksijen radikali düzeyleri üzerine etkisini incelemeyi amaçladık.

**Yöntemler:** Otuz dört rat, group 1 levobupivakain 2.5$mg.kg^{-1}$ IV (n=12), group 2 levobupivakain 5$mg.kg^{-1}$ IV (n=12) ve 0.9% sodyum klorid solüsyonu verilen group C kontrol (n=10) olarak üç gruba ayrıldı. Daha sonra her grup kendi arasında sakrifikasyon sonrası 2.saatte (A) ve 24. saatte (B) olacak şekilde iki bölümde değerlendirildi. Ratların kan, kalp ve beyin dokusu örneklerinde biyokimyasal olarak, malondialdehit, süperoksit dismutaz, glutatyon ve nitrik oksit düzeyleri incelendi.

**Bulgular:** Levobupivakain verildikten sonra, dokularda malondialdehit artarken, süperoksit dismutaz, glutatyon ve nitrik oksit düzeyleri azaldı.

**Sonuç:** Levobupivakain oksidatif stres yanıta neden olabilmektedir.

**Anahtar Sözcükler:** Levobupivakain, serbest oksijen radikalleri

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Bupivacaine is a long-acting local anesthetic that has been preferred over short-acting agents like lidocaine on neural blockage for years. However, a long-acting agent also carries a higher risk of toxicity [1, 2]. In many studies, toxic effects of bupivacaine have been reported and the underlying mechanism has been examined. Unami et al. [3] used apoptosis to explain the molecular mechanism of neurotoxicity and myotoxicity of bupivacaine. Levobupivacaine hydrochloride is the S-enantiomer of bupivacaine and

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recent studies have shown that levobupivacaine has less neurotoxic and cardiotoxic effects compared to bupivacaine [3, 4].

We aimed to evaluate the effects of intravenous (IV) injection of levobupivacaine on free oxygen radicals at the tissue level.

## Material and Methods

This study was performed after obtaining the approval from Istanbul University Cerrahpaşa School of Medicine Experimental Animal's Ethics Committee, in compliance with the "Guide for Use and Care of Laboratory Animals."

Male Wistar albino rats were permitted ad libitum access to standard laboratory chow and water. Rats were purchased from the Research Institute for Experimental Medicine (Istanbul University, Istanbul, Turkey).

Animal model: Thirty-four Sprague-Dawley adult rats (between 8 and 10 weeks) weighing 200–300 g were divided into three groups. Group 1 received 2.5 $mg.kg^{-1}$ IV levobupivacaine (n=12), group 2 received 5 $mg.kg^{-1}$ IV levobupivacaine (n=12), and group 3 was kept as the control group (n=10). Every group was evaluated in two separate subgroups where samples were taken at the 2nd hour (A) and 24th hour (B) after the injection. The procedures that the rats will undergo were determined using a computer that appoints randomly produced numbers. These numbers were recorded to the tails of the rats with a permanent pen. For anesthesia, 50 $mg.kg^{-1}$ intraperitoneal ketamine hydrochloride (Ketalar, Eczacibasi, Turkey) was used.

The temperatures of the animals were kept stable by using heaters. The concentration of the levobupivacaine solution (Chirocaine, Abbott Laboratories, Turkey) was 2 $mg.mL^{-1}$. The tail vein was chosen for the IV administration. Tepid water was used to visualize the veins and the pre-determined dose of levobupivacaine was given in 3 min. The control group (n=10) received IV 0.25 $mL.kg^{-1}$ 0.9% sodium chloride solution. The administrator was aware of the group of the rat prior to the injection. The random numbers designated to the rats were also recorded on the tissue samples and the person who did the biochemical study was blinded. Breathing, heartbeats (by palpation), and the behaviors of the rats were observed. Seizures, the irregularity of heart, and respiration of the rats, and the number of dead rats, if any, were recorded.

Collection of tissue samples: Two hours after the injection, under ketamine anesthesia, six rats were chosen from each group that has been administered levobupivacaine and five from the control group randomly. From each rat, 1 mL of blood was acquired by full thickness cut of the tails, and brain and heart tissues were removed rapidly while oxygen was administered through a specially designed mask. The same procedure was repeated under anesthesia to the remaining rats.

Biochemical analysis: Blood samples were collected in heparinized vacutainer tubes and immediately transported to the laboratory in a cooler with ice. Upon arrival, plasma was separated by centrifugation (+4°C, 3 000 r/min, 10 min), divided into 0.5–1.0 mL aliquots, placed in cryovials, and stored at –70°C until being analyzed. Erythrocytes were washed three times in 5 mL 0.9% sodium chloride solution and hemolyzed by diluting 4-fold with water, and glutathione (GSH) was studied in erythrocytes on the same day. The tissues were weighed, washed in 0.9% sodium chloride solution, and homogenized in ice-cold 0.15 M KCl 100 $g.L^{-1}$·omogenates of 20% were obtained and sonicated twice at 30 s intervals at 4°C. Homogenates were centrifuged at >10.000 *g* for 15 min at 4°C. All biochemical parameters in homogenates were studied on the same day.

Determination of lipid peroxidation: The lipid peroxidation (as malondialdehyde, MDA) level was measured using the method of Buege and Aust [5]. Briefly, the plasma and tissue homogenates were mixed with TCA–TBA–HCl [15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid, and 0.25 N hydrochloric acid] solution, BHT (2,6,-di-tert-butyl-4-methylphenol), and DETAPAC (diethylenetriamine pentaaetic acid). The mixture was heated for 60 min in a boiling water bath. After centrifugation, the absorbance was recorded at 532 nm by using 1,1,3,3 tetraethoxypropane as standard. The total protein concentration was measured by the method of Lowry et al. [6].

Determination of nitric oxide (NO): Plasma and tissue concentrations of NO were measured through its stable metabolites nitrate and nitrite. Nitrate was first reduced by nitrate reductase to nitrite and then nitrite was determined spectrophotometrically by the Griess reaction [7]. The Griess reagent, a mixture (1:1) of 0.2% N-(1-naphthyl)-ethylene-diamine and sulfanilamide in 5% phosphoric acid, gave a red-violet diazo dye with nitrite, which was measured in the visible range at 540 nm.

Determination of Cu-Zn-superoxide dismutase (SOD): Plasma and tissue Cu-Zn-SOD activities were determined by the method of Sun et al. [8] by inhibition of nitro blue tetrazolium reduction with xanthine/xanthine oxidase used as a superoxide generator. One unit of SOD was defined as the amount of protein that inhibits the rate of nitro blue tetrazolium reduction by 50%.

Determination of glutathione: Erythrocyte and tissue glutathione (GSH) concentrations were determined according to the method of Beutler et al. [9] using meta-

phosphoric acid for protein precipitation and 5′-5′-dithiobis-2-nitrobenzoic acid for color development.

Data presentation and statistical analysis: All data are expressed as means and standard deviations (mean±SD) and 95% confidence intervals. In statistical evaluation, the differences between the groups are determined by Kruskal–Wallis variance analysis. The comparisons among the groups were performed by Mann–Whitney U test. Values of $p \leq 0.05$ were considered statistically significant. Statistical Package for the Social Sciences version 11 (SPSS Inc.; Chicago, IL, USA) statistics program was used for statistical analysis.

## Results

No systemic toxic effects were observed in the first group of 12 rats. In the second group, generalized convulsions were observed in four out of 12 rats and one rat experienced cardiac arrest, did not respond to CPR, and was accepted to be exitus. This rat was excluded from the study and was replaced with a new rat that has undergone the same specific procedure. No toxic effects were seen in this new rat or seven other rats.

MDA: MDA levels in blood, brain, and heart tissue increased significantly parallel to an increase in levobupivacaine doses in all groups. MDA levels increased in the levobupivacaine administered groups compared to the control group. Although 5 mg levobupivacaine administration caused higher MDA levels compared to 2.5 mg levobupivacaine, the difference was not significant. Higher MDA levels were also observed in the group where the tissues were taken after 24 h. There were no important effects on the heart tissue of group 2 (5 mg.kg$^{-1}$ levobupivacaine) (Table 1, 2; Figure 1).

SOD: SOD levels decreased significantly in all groups after administration of levobupivacaine except for the 2$^{nd}$ hour's samples of brain and heart. A decrease in SOD levels was observed upon the administration of levobupivacaine compared to the control group. This was only significant in the 2$^{nd}$ hour in the 5 mg levobupivacaine group, whereas it was significantly decreased for all the groups in the 24$^{th}$ hour. Administration of different doses of 2.5 mg and 5 mg of levobupivacaine had no significant effect on the SOD levels statistically. Although SOD levels in the samples of the 24$^{th}$ hour were found lower compared to the 2$^{nd}$ hour, this difference was only significant in the tissue samples of blood and brain of the group that received 2.5 mg levobupivacaine (Table 1, 2; Figure 2).

GSH: GSH levels decreased with levobupivacaine administration in all groups. GSH alterations were not statistically significant in the heart samples taken from the groups 1A, 2A, and 2B compared to the control group. A significant decrease was determined in the plasma and brain. When the different dose administra-

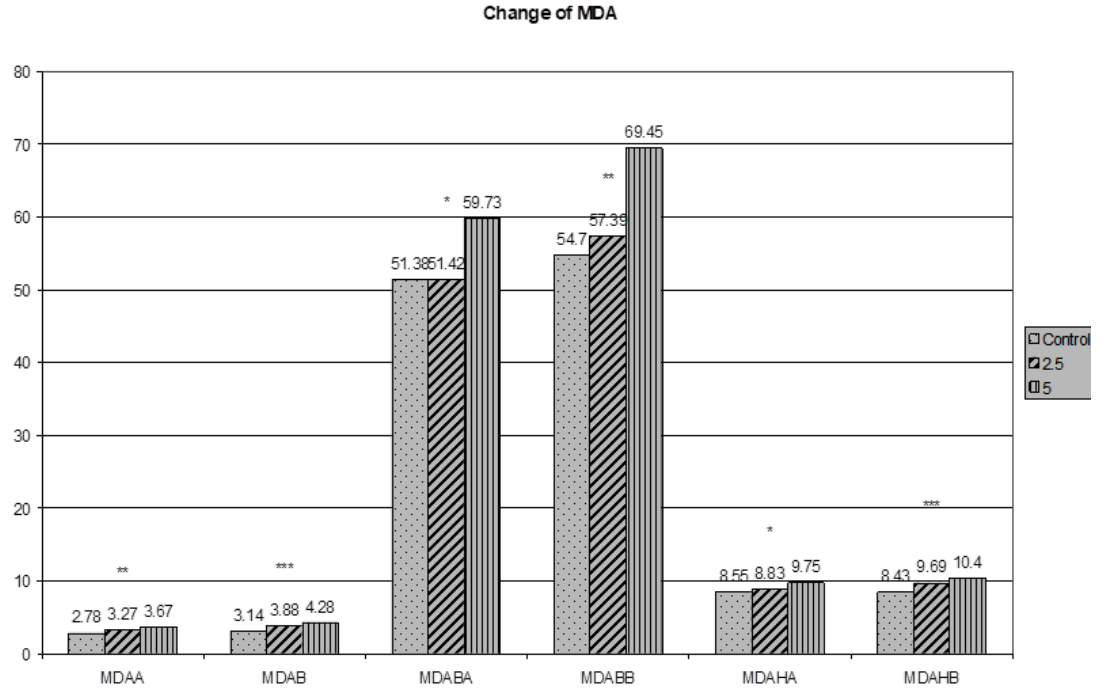


**Figure 1.** Change of MDA

MDAA: Plasma malondialdehyde at the 2$^{nd}$ hour; MDAB: Plasma malondialdehyde at the 24$^{th}$ hour, MDABA: brain malondialdehyde at the 2$^{nd}$ hour; MDABB: brain malondialdehyde at the 24$^{th}$ hour, MDAHA: heart malondialdehyde at the 2$^{nd}$ hour; MDAHB: heart malondialdehyde at the 24$^{th}$ hour

**Table 1.** Results of 2nd hour samples

| Plasma/tissue concentrations Mean±SD | 1A | 2A | CA | p |
|---|---|---|---|---|
| MDA nmol/mL | 3.27±0.28 | 3.67±0.29 | 2.78±0.34 | 0.008** |
| SOD U/mL | 22.45±2.30 | 19.80±1.87 | 24.90±1.96 | 0.018* |
| NO µmol/L | 21.70±2.25 | 19.07±1.60 | 21.58±2.13 | 0.065 |
| GSH mg/g Hb | 3.73±0.21 | 3.45±0.29 | 4.10±0.22 | 0.009** |
| MDAB nmol/g | 51.42±2.92 | 59.73±3.61 | 51.38±3.22 | 0.010* |
| SODB U/mg | 0.43±0.04 | 0.39±0.03 | 0.44±0.04 | 0.218 |
| NOB µmol/mg | 0.43±0.01 | 0.41±0.01 | 0.44±0.01 | 0.024* |
| GSHB nmol/mg | 10.48±0.78 | 9.57±0.52 | 13.05±0,65 | 0.004* |
| MDAH nmol/g | 8.83±0.70 | 9.75±0.72 | 8.55±0.40 | 0.044* |
| SODH U/mg | 0.05±0.004 | 0.05±.05 | 0.06±0.04 | 0.165 |
| NOH nmol/g | 14.37±2.18 | 10±1.16 | 16.60±1.49 | 0.005** |
| GSHH nmol/mg | 9.67±0.53 | 9.57±1.19 | 9.05±1.99 | 0.795 |

1A: 2.5 mg $kg^{-1}$ levobupivacaine 2nd hour group; 2A: 5 mg $kg^{-1}$ levobupivacaine 2nd hour group; CA: control 2nd hour group; MDA: plasma concentrations of malondialdehyde; SOD: plasma concentrations of Cu–Zn-superoxide dismutase; NO: plasma concentrations of nitric oxide; GSH: erythrocyte concentrations of glutathione; MDAB: brain concentrations of malondialdehyde; SODB: brain concentrations of Cu–Zn-superoxide dismutase; NOB: brain concentrations of nitric oxide; GSHB: brain concentrations of glutathione; MDAH: heart concentrations of malondialdehyde; SODH: heart concentrations of Cu–Zn-superoxide dismutase; NOH: heart concentrations of nitric oxide; GSHH: heart concentrations of glutathione; SD: standard deviation

**Table 2.** Results of 24th hour samples

| Plasma/tissue concentrations Mean±SD | 1B | 2B | CB | p |
|---|---|---|---|---|
| MDA nmol/mL | 3.88±0.25 | 4.28±0.37 | 3.14±0.21 | 0.001*** |
| SOD U/mL | 19.89±1.72 | 18.42±1.51 | 24.17±1.71 | 0.001*** |
| NO µmol/L | 18.95±2.02 | 17.90±1.97 | 21.69±2.08 | 0.018* |
| GSH mg/g Hb | 3.5±0.28 | 3.12±0.20 | 4.06±0.31 | 0.001*** |
| MDAB nmol/g | 57.39±4.46 | 69.45±4.58 | 54.7±5.18 | 0.002** |
| SODB U/mg | 0.39±0.02 | 0.39±0.02 | 0.49±0.07 | 0.028* |
| NOB µmol/mg | 0.41±0.01 | 0.4±0.02 | 0.43±0.01 | 0.012* |
| GSHB nmol/mg | 9.92±0.43 | 9.45±0.62 | 12.50±0.40 | 0.001*** |
| MDAH nmol/g | 9.69±0.62 | 10.4±0.41 | 8.43±0.51 | 0.001*** |
| SODH U/mg | 0.05±0.005 | 0.05±0.005 | 0.06±0.005 | 0.008** |
| NOH nmol/g | 11.76±1.31 | 8.50±1.15 | 16.56±2.01 | 0.000*** |
| GSHH nmol/mg | 8.7±1.09 | 8.52±1.45 | 9.74±0.49 | 0.098 |

1B: 2.5 mg $kg^{-1}$ levobupivacaine 24th hour group; 2B: 5 mg $kg^{-1}$ levobupivacaine 24th hour group; CB: control group 24th hour group; MDA: plasma concentrations of malondialdehyde; SOD: plasma concentrations of Cu–Zn-superoxide dismutase; NO: plasma concentrations of nitric oxide; GSH: erythrocyte concentrations of glutathione; MDAB: brain concentrations of malondialdehyde; SODB: brain concentrations of Cu–Zn-superoxide dismutase; NOB: brain concentrations of nitric oxide; GSHB: brain concentrations of glutathione; MDAH: heart concentrations of malondialdehyde; SODH: heart concentrations of Cu–Zn-superoxide dismutase; NOH: heart concentrations of nitric oxide; GSHH: heart concentrations of glutathione; SD: standard deviation

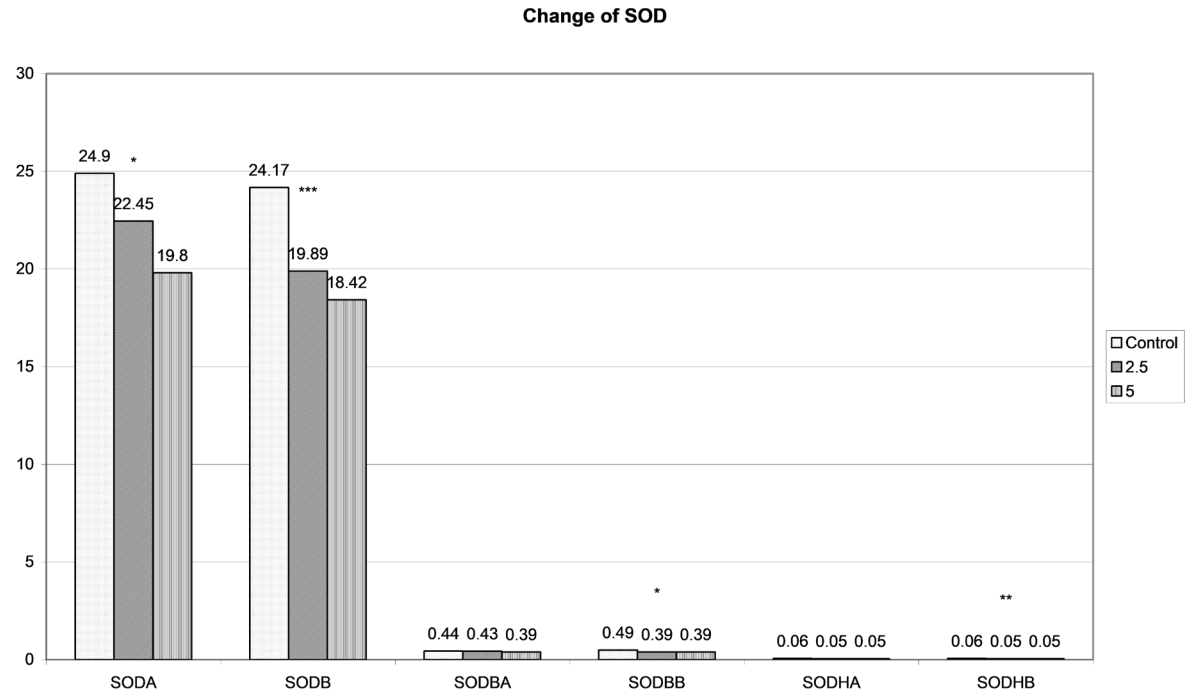


**Figure 2.** Change of SOD

SODA: Plasma superoxide dismutase at the 2nd hour; SODB: Plasma superoxide dismutase at the 24th hour; SODBA: brain superoxide dismutase at the 2nd hour; SODBB: brain superoxide dismutase at the 24th hour; SODHA: heart superoxide dismutase at the 2nd hour; SODHB: heart superoxide dismutase at the 24th hour

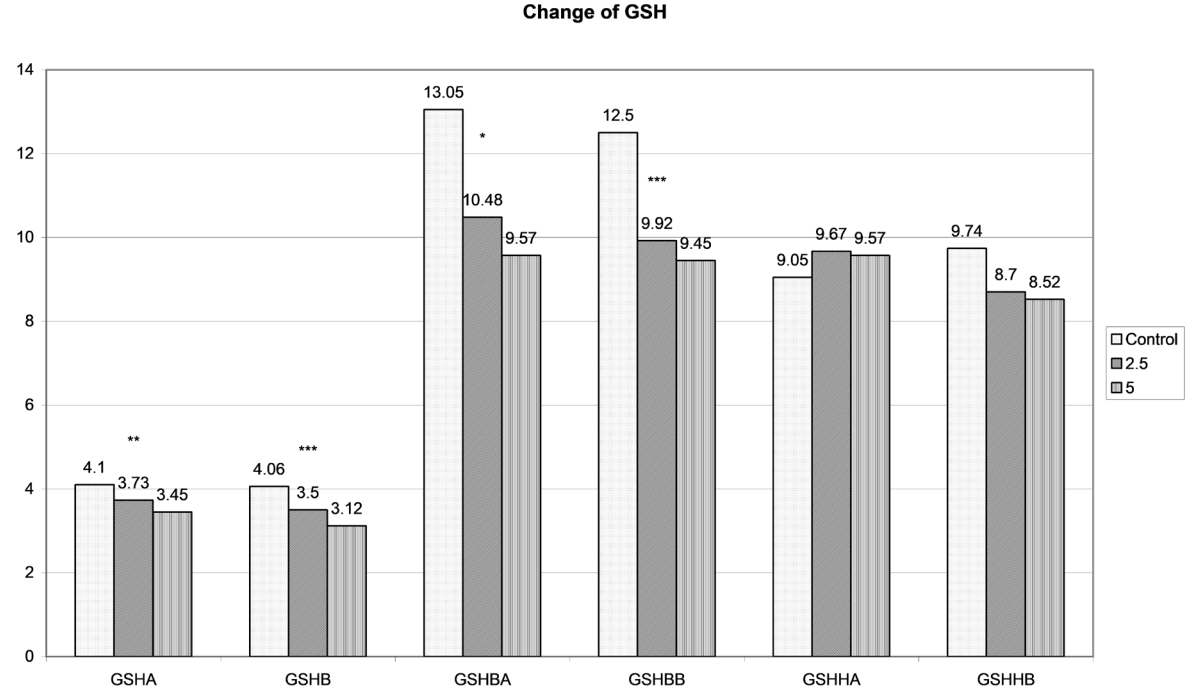


**Figure 3.** Change of GSH

GSHA: Plasma glutathione at the 2nd hour; GSHB: Plasma glutathione at the 24th hour; GSHBA: brain glutathione at the 2nd hour; GSHBB: brain glutathione at the 24th hour; GSHHA: heart glutathione at the 2nd hour; GSHHB: heart glutathione at the 24th hour

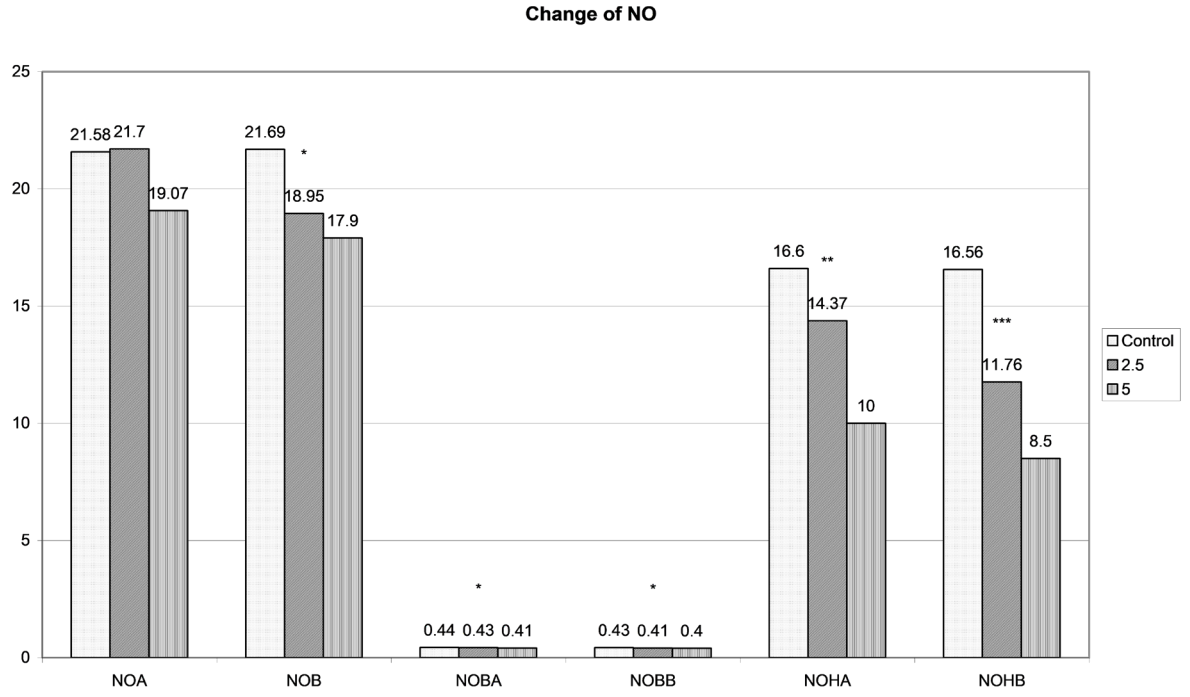


**Figure 4.** Change of NO

NOA: Plasma nitric oxide at the 2nd hour; NOB: Plasma nitric oxide at the 24th hour; NOBA: brain nitric oxide at the 2nd hour; NOBB: brain nitric oxide at the 24th hour; NOHA: heart nitric oxide at the 2nd hour; NOHB: heart nitric oxide at the 24th hour

*: p<0.05, **: p<0.01 , ***: p<0.001

tions were compared, the decreases in the GSH levels were found significant only in the brain tissue in 2nd hour and in blood in 24th hour (Table 1, 2; Figure 3).

NO: NO levels decreased significantly with levobupivacaine administration. This decrease was especially evident in the heart tissues. When the different dose administrations were compared, high dose of levobupivacaine was found to cause a significant decrease in NO levels especially in the heart tissue (Table 1, 2; Figure 4).

## Discussion

The studies on the local anesthetics concentrate mainly on predisposing factors, the treatment of the toxic effects, the mechanism of the toxicity, and their stereoselective actions [10]. We have planned our study to determine the effects of levobupivacaine on the free oxygen radicals, which have been stated as a mechanism for the toxicity of the long-acting local anesthetics. In one of their review, Heavner et al. [10] evaluated the cardiotoxicity of local anesthetics on their intact isolated heart model. The authors stated that especially the cardiotoxicity of the local anesthetics increases with their lipid solubility and the potency of nerve blockage and that S(-) isomer is less cardiotoxic than R(+) isomer of bupivacaine. They have also stated that the cardiotoxicity decreases in the order of prilocaine < mepivacaine < ropivacaine < levobupivacaine < rasemic bupivacaine < R(+) bupivacaine < etidocaine < tetracaine. There are also studies reporting that oxidative stress response might be responsible for the toxicity of the local anesthetics [3, 4, 11]. To explain the mechanism of the toxic effects of bupivacaine, it is claimed that it is a strong uncoupler for mitochondrial oxidative phosphorylation and induces apoptosis [3]. Unami et al. [3] have investigated the bupivacaine-induced apoptosis with biochemical and microarray analysis methods and stated that this apoptosis comes about by the receptor and mitochondria-mediated activations. The researchers state that the dysfunction of the mitochondria is a result of major biochemical incidents like the changes in mitochondrial membrane potentials, release of cytochrome C, and the dysregulation of $Ca^{++}$ and that the activators of proteases released from the mitochondria trigger the apoptosis. The central component of apoptosis is a cascade of proteolytic enzymes named as caspases. According to the researches, all of the caspases are activated by substances to be apoptotic bodies in similar doses. It is thought that many genes are responsible for the bupivacaine-induced apoptosis altogether.

Park et al. [4] stated that bupivacaine may cause apoptosis in Schwann Cell Line by reactive oxygen species. Tan et al. [12] reported tetracaine causes apoptosis.

Johnson et al. [13] tried to explain the neurotoxicity of lidocaine by mitochondrial dysfunction, which is a result of apoptotic activation. Collectively, these data indicate that neurotoxicity of lidocaine involves mitochondrial dysfunction with activation of apoptotic pathways.

Results of our study indicate that the administration of levobupivacaine causes an increase in the levels of MDA, which is a byproduct of lipid peroxidation in blood, heart, and brain tissue when compared with the control group. The levels of antioxidant enzyme SOD decreased in both blood and brain significantly parallel to the administration of levobupivacaine. The decrease in the heart tissue was less compared to the other tissues. The level of GSH, which is another antioxidant enzyme, decreased significantly in blood and brain tissues with the administration of levobupivacaine, whereas the decrease was not significant statistically in the heart tissues taken at the 2nd hour. In our search of the literature, we could not encounter any studies that deal with the effects of levobupivacaine on free oxygen radicals. Our results agree with the study of Unami et al. [3] on bupivacaine that levobupivacaine toxicity may cause apoptosis.

Another factor that is blamed for the toxicity of local anesthetics is NO [14-16]. NO is an endothelium-derived relaxing factor and an important neurotransmitter that plays a considerable role in the regulation of the cardiovascular and cerebral functions. The role of NO has been investigated on the potency of constitution of seizures activity and cardiac arrhythmias of many different agents including anesthetic agents. The inhibition of the enzyme NO synthase (NOS) that enables the production of NO from L-arginine modifies the toxicity of the local anesthetics like bupivacaine or cocaine [14, 15]. Heavner et al. [16] stated that inhibition of nitric oxide production in rats markedly enhanced the cardiovascular toxicity of lidocaine and tetracaine. Shi et al. [17] showed that the selective inhibition of neuronal NOS by 7-nitroindazole does not modify the bupivacaine-induced seizure and arrhythmias whereas the nonselective NOS inhibition by L-NAME (N(omega)-nitro-L-arginine methyl ester) does not alter the bupivacaine-induced seizure threshold. It does increase sensitivity of the formations of cardiac arrhythmias very significantly (six-fold) for both the dose of bupivacaine that produced arrhythmia and the myocardial bupivacaine concentrations at the onset of arrhythmia after L-NAME pretreatment. As a result, they have concluded that NO does not play a role in bupivacaine-induced seizure activity and endothelial NO is a moderate cardioprotective factor for the bupivacaine-induced arrhythmias. They have also stated that neuronal NO does not play a significant role in arrhythmogenic effects of bupivacaine.

In our study, the levels of NO decreased in all of the samples upon administration of levobupivacaine. These results show that NO may be a factor in the cardiotoxicity of levobupivacaine just as it was stated for the other local anesthetics in the studies discussed above.

As a conclusion, in our study we have observed that the IV administration of levobupivacaine in adult rats causes an oxidative stress response. It was also shown that the administration of 5 $mg.kg^{-1}$ of levobupivacaine increases the MDA levels especially in the brain tissues and decreases NO levels in the heart when compared with the group that was administered 2.5 $mg.kg^{-1}$ levobupivacaine. We believe the increase in MDA in brain and decrease in NO levels in the heart may be responsible for the neurotoxicity and cardiotoxicity of levobupivacaine, respectively.

**Ethics Committee Approval:** Ethics committee approval was received for this study from the Ethics Committee of Istanbul University-Cerrahpaşa, Cerrahpaşa School of Medicine Experimental Animal.

**Informed Consent:** N/A.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept - Y.T., G.K.; Design - Y.T., G.K.; Supervision - Y.T., G.K.; Resource - Y.T., Materials - Y.T., A.K.G., G.K.; Data Collection and/or Processing - Y.T., A.K.G.; Analysis and/or Interpretation - Y.T., H.U., A.K.G., E.F.A., Ö.K.D., G.K.; Literature Search - Y.T., H.U., A.K.G., E.F.A., Ö.K.D.; Writing - Y.T., H.U., A.K.G., E.F.A., Ö.K.D.; Critical Reviews - Y.T.,G.K.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

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