

## Changes in Lipid Peroxidation and Antioxidant Environment of Spinal Fluid with The Use of Bupivacaine for Spinal Anesthesia

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### Summary

The objective of this study was to determine if bupivacaine has any effects on the antioxidant defence system and in lipid peroxidation as thiobarbituric acid substance (TBARS) of spinal fluid. The experiment was carried out on 15 male clinically healthy, 3-4 years old, male Tuj ram weighing an average  $56 \pm 5$  kg. Eight ml of 0.5% bupivacaine was used for the induction of spinal anesthesia (SA). To measure the levels of lipid peroxidation (LPO), glutathione (GSH), activity of glutathione peroxidase (GSHPx; EC: 1.11.1.9) and the levels of vitamine E in spinal fluid, samples were taken from the subarachnoid (intrathecal) space before anaesthesia (0. min) and at 5, 15, and 60 min. after anaesthesia. The levels of TBARS slightly increased at 5 min. after spinal injection of bupivacaine and this increase continued throughout anesthesia ( $P < 0.001$ ). Vitamin E levels were decreased at 5 min. after anesthesia ( $P < 0.001$ ) and this level recovered to control levels at 30 min. after bupivacaine injection. The activity of GSHPx and the levels of GSH in spinal fluid increased continually 5 min. after administration of bupivacaine ( $P < 0.001$ ). In conclusion, we found that while spinal injection of bupivacaine increased free radical levels in spinal fluid may be supported antioxidant environment of spinal fluid during anesthesia.

**Keywords:** *Bupivacaine, Spinal anesthesia, Spinal fluid, Antioxidants, Lipid peroxidation, Ram*

## Bupivacain ile Yapılan Spinal Anestezi ile Spinal Sıvı Lipid Peroksidasyon Düzeyi ve Antioksidan Sisteminde Meydana Gelen Değişiklikler

### Özet

Bu çalışmada, bupivacain ile yapılan spinal anestezinin antioksidan savunma sistemi ve lipid peroksidasyonunun bir indikatörü olan tiyobarbiturik asit substratları (TBARS) düzeyleri, üzerine etkisini belirlemek amaçlanmıştır. Bunun için 15 klinik olarak sağlıklı 3-4 yaşlarında ve ortalama  $56 \pm 5$  kg ağırlığında Tuj koçları kullanılmıştır. Spinal anestezi oluşturmak amacıyla 8 ml %0.5'lik bupivacain kullanıldı. Bupivacain ile spinal anestezi yapılmadan önce (0. dakika) ve anesteziden 5, 30 ve 60 dakika sonra subarachnoid (intrathecal) bölgeden alınan spinal sıvı numuneleri tiyobarbiturik asit substans (TBARS), E vitamini ve glutatyon (GSH) düzeyleri ile glutatyon peroxidaz (GSHPx, EC: 1.11.1.9) aktivitelerini belirlemek için kullanıldı. Bu çalışmada, TBARS düzeylerinin anesteziden 5 dak. sonra istatistiksel olarak artmaya başladığı ( $P < 0.001$ ) ve bu artışın anestezi boyunca sürdüğü gözlenirken, E vitamini değerlerinin anestezinin 5 dak.'sında düşerken bundan sonraki zaman dilimlerinde başlangıç seviyelerine döndüğü tespit edildi. Eritrosit GSHPx aktivitesi ile GSH düzeylerinin anestezi süresince istatistiksel olarak yükseldiği gözlemlendi ( $P < 0.001$ ). Sonuç olarak, bupivacain ile oluşturulan spinal anestezi ile spinal sıvının serbest radikal miktarı yükselmekle birlikte, bupivacain anestezi süresince spinal sıvının antioksidan ortamını desteklemektedir.

**Anahtar sözcükler:** *Bupivacain, Spinal anestezi, Spinal sıvı, Antioksidanlar, Lipid peroksidasyonu, Koç*



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## INTRODUCTION

Because of its simplicity spinal anesthesia is an easy regional technique to use in clinical practice. Providing the spinal anesthesia, among local anesthetics, intrathecal bupivacaine is used extensively to obtain potent and intense motor blockade<sup>1,2</sup>. However, it is showed *in vivo*<sup>3</sup> and *in vitro*<sup>4</sup> that local anesthetics modulate the inflammatory response. Also it is known that bupivacaine have an antioxidant properties aimed against lipid peroxidation<sup>5,6</sup>. Nevertheless, no reports addressed the effects of bupivacaine on the antioxidant system of spinal fluid including antioxidant enzymes as glutathione and glutathione peroxydase and vitamin E. Body has developed an antioxidant defense system which enzymatic, metal chelating and free radical scavenging properties<sup>7</sup>. The radicals formed by autooxidation react with proteins, enzymes and vitamins which then lose their biological function. GSH has a protective mechanism against the cell injury. Earlier findings also suggest that the presence of GSH in cells and tissues are associated to attenuate the oxidative stress<sup>8</sup>. GSH-P<sub>x</sub> is responsible for the neutralization of both organic and inorganic hydroperoxides<sup>9</sup>. Endogen free radicals such as peroxides damage the structure and function of the cells and antioxidant systems of cells such as glutathione and glutathione peroxydase bring about decomposition reactions<sup>10,11</sup>. Thus, it is essential to consider the concentration of antioxidant in the spinal cerebrospinal fluid (CSF) when investigating the relationship between oxidative stress and anesthetics used for spinal anesthesia. On the other hand, Pitkanen et al.<sup>12</sup> suggested that the clinical significance of aspirating spinal CSF before and after attempting spinal anesthesia with bupivacaine was found to be small.

While the relation between free radical reaction and defense mechanisms in CSF against CSF related illness was being in consideration<sup>13-15</sup>, there is no report that about the change in lipid peroxidation and antioxidant enzymes levels in spinal CSF with spinal injection of anesthetics in particular bupivacain. So, the purpose of this study is that show the action of spinally injected bupivacaine on LPO and antioxidant enzymes in the spinal CSF.

## MATERIAL and METHODS

### *Animals and Treatments*

The experiment was carried out on 15 male clinically healthy Tuj sheep, weighing average approximately 57 kg (50-65) and aged 3-4 years old. The animals were restricted in lateral recumbency on an operating table.

For the purpose of preventing possible respiratory and circulatory problems, necessary support was provided so as to hold the animals' neck and thorax in an upward position. The lumbosacral area was prepared for an aseptic injection. Prior to subarachnoid (intrathecal) injection, local infiltrative anaesthesia of the subcutaneous tissues and interspinous ligament was achieved. SA was induced by the administration of 8 ml of 0.5% bupivacaine (Heavy Marcain, Astra Södertälje, Sweden) through the lumbosacral space with an 18-gauge, 1.25×90 mm spinal needle. The spinal needle was inserted into the lumbosacral space and moved slowly forward into the subarachnoidal space. After aspiration of a quantity of cerebro-spinal fluid equivalent to the amount of local anaesthetic agent, bupivacaine was injected slowly. No reaction was noted during subarachnoidal injection in the animals. The desired quality anaesthesia was achieved within 20 to 60 sec. of the injection of the local anaesthetic agent. The duration of the efficacy of the anaesthesia was determined to be between 3.5 and 5 h. Additionally, in order to prevent the occurrence of any respiratory problems due to the expansion of bupivacaine in a cranial direction, the animals were kept inclined at approximately 30°C during anaesthesia.

### *Collection of Samples*

To measure the levels of lipid peroxidation (LPO), glutathione (GSH) and activity of glutathione peroxidase (GSHPx; EC: 1.11.1.9) 1,5 ml of spinal CSF samples were taken from the subarachnoid (intrathecal) space before anaesthesia (0. min), and at 5, 30 and 60 minute after anaesthesia. Immediately on removal the spinal fluid samples were centrifuged to remove any contaminates, pipetted into virgin polyethylene tubes and placed in a -20°C freezer until further analysis. Samples were thawed at room temperature and analyzed.

### *Analytical Procedures*

The end product of polyunsaturated fatty acid peroxidation, malondialdehyde (MDA), reacting with thio-barbituric acid (TBA) in samples was determined by Placer et al.<sup>16</sup> method modified by Matkovichs et al.<sup>17</sup>. The values of MDA reactive material were expressed in terms of the malondialdehyde (MDA) content (nmol/ml plasma), which served as a standard of 1,1,3,3-tetraethoxypropane (Sigma, Chemical Company St. Louis, MO, USA).

The GSH levels of spinal fluid were measured spectrophotometrically using Ellman's reagent<sup>18</sup>. GSHPx activity was determined using cumene hydroperoxide and reduced GSH as co-substrates and the loss of GSH following enzymic reaction was measured spectrophotometrically with Ellman's reagent at 37°C and 412

nm according to Lawrence and Burk<sup>19</sup> and Matkovic et al.<sup>17</sup>. The protein content in the tissue homogenate was measured by the method of Lowry et al.<sup>20</sup> with bovine serum albumin as the Standard.

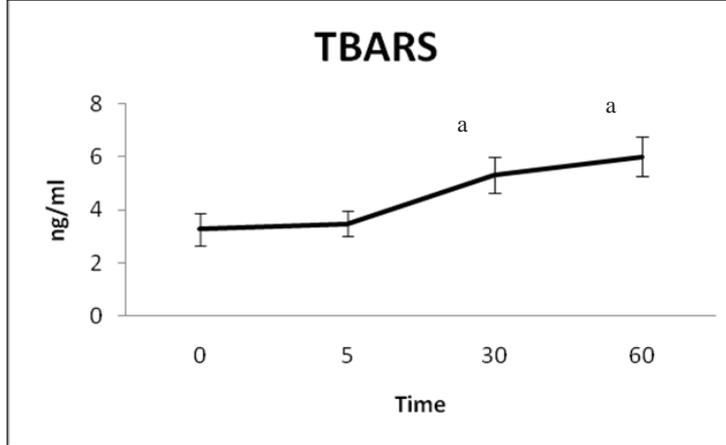
The vitamin E ( $\alpha$ -tocopherol) levels of plasma were determined in the frozen serum samples by the method described by McMurray et al.<sup>21</sup>. The relevant wavelengths for vitamin E detection were 292 and 330 nm. Calibration was performed using a standard solution of  $\alpha$ -tocopherol in methanol.

### Statistical Analysis

Statistical analysis (Anova Test and Post Hoc. Tukey) was performed using the SPSS software program (16.0). All results were expressed as the mean  $\pm$  standard deviation (SD). A P value  $<0.05$  was considered to be statistically significant.

## RESULTS

Mean values of investigated parameters and differences in spinal fluid between before and after anesthetiation times are presented with Figures.



**Fig 2.** Line graph showing the effects of spinal injection of bupivacaine on the levels of GSH in spinal fluid. a:  $P < 0.001$  in comparison to the 0th point of the time. Values are expressed as mean  $\pm$  SD

**Şekil 2.** Grafik intratekal bupivacaine enjeksiyonu ile spinal sıvı GSH düzeylerinde zamana göre belirlenen değişiklikleri göstermektedir. a:  $P < 0.001$  (0. dakikaya göre istatistiksel önemliliği göstermektedir. Değerler Ortalama  $\pm$  Standart Sapma olarak verilmiştir)

Effects of spinal injection of bupivacaine on the levels of TBARS in spinal fluid indicated in Fig. 1. The levels of TBARS slightly increased at 5 min after spinal injection of bupivacaine and this increase continued throughout anesthesia ( $P < 0.001$ )

As shown in Fig. 2 the levels of GSH in spinal fluid regularly increased throughout anesthesia ( $P < 0.001$ ).

Effects of spinal injection of bupivacaine on the activity of GSHPx in spinal fluid introduced in Fig. 3. The activity of GSHPx in spinal fluid statistically increased ( $P < 0.001$ ) during anesthetiation times.

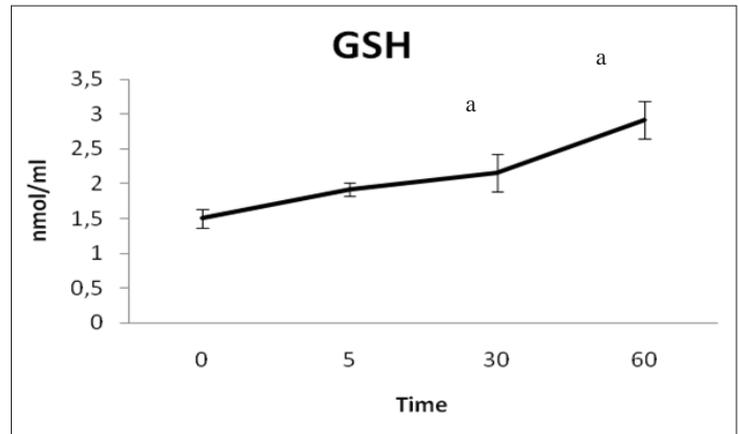
Effect of intraspinal bupivacaine injection on vitamin E levels was also exhibited in Fig. 4. Vitamin E levels were slightly decreased at 5 min after anesthesia ( $P < 0.001$ ) and this level recovered to control levels at 30 min after bupivacaine injection and approximately remain this levels after that time.

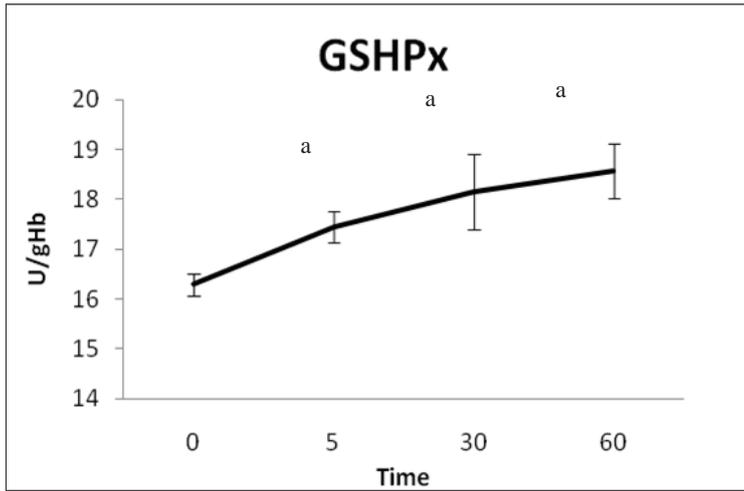
## DISCUSSION

The results of this study indicate that spinally injected bupivacaine has an effect on change the lipid

**Fig 1.** Line graph showing the effects of spinal injection of bupivacaine on the levels of TBARS in spinal fluid. a:  $P < 0.001$  in comparison to the 0th point of the time. Values are expressed as mean  $\pm$  SD

**Şekil 1.** Grafik intratekal bupivacaine enjeksiyonu ile spinal sıvı TBARS düzeylerinde zamana göre belirlenen değişiklikleri göstermektedir. a:  $P < 0.001$  (0. dakikaya göre istatistiksel önemliliği göstermektedir. Değerler Ortalama  $\pm$  Standart Sapma olarak verilmiştir)



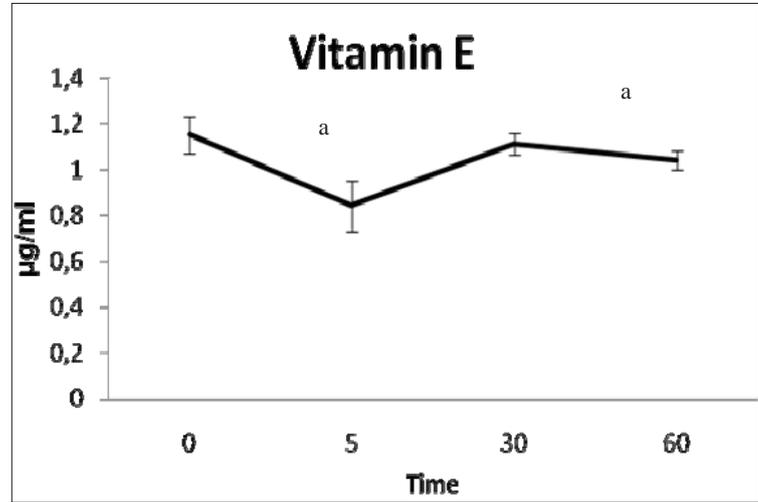


**Fig 3.** Line graph showing the effects of spinal injection of bupivacaine on the activity of GSHPx in spinal fluid. a:  $P < 0.001$  in comparison to the 0th point of the time. Values are expressed as mean  $\pm$ SD

**Şekil 3.** Grafik intratekal bupivacaine enjeksiyonu ile spinal sıvı GSHPx aktivitelerinde zamana göre belirlenen değişiklikleri göstermektedir. a:  $P < 0.001$  (0. dakikaya göre istatistiksel önemliliği göstermektedir. Değerler Ortalama  $\pm$  Standart Sapma olarak verilmiştir

**Fig 4.** Effects of spinal injection of bupivacaine on the levels of vitamin E in spinal fluid. a:  $P < 0.001$  in comparison to the 0th point of the time. Values are expressed as mean  $\pm$ SD

**Şekil 4.** Grafik intratekal bupivacaine enjeksiyonu ile spinal sıvı E vitamini düzeylerinde zamana göre belirlenen değişiklikleri göstermektedir. a:  $P < 0.001$  (0. dakikaya göre istatistiksel önemliliği göstermektedir. Değerler Ortalama  $\pm$  Standart Sapma olarak verilmiştir)



peroxidation and antioxidant enzymes in the spinal CSF of rams. LPO is one of the prominent manifestations of oxidative stress. Reactive oxygen species are induced oxidation and peroxidation of membrane phospholipids, thereby causing damage to the phospholipid molecule as well as to other molecules in the cells<sup>7</sup>. Unsaturated phospholipids, glycolipids and cholesterol in cell membranes are particularly susceptible to lipid peroxidation, because they exist in high concentrations and contain an abundant source of peroxidation substrates, particularly Polyunsaturated Fatty Acids (PUFA)<sup>22</sup>. PUFA are found in abundance in mammalian membrane lipids and are the most likely targets of Reactive Oxygen Species (ROS). As the nervous system is enriched with PUFA, it is particularly vulnerable to such stress. Increased levels of lipid hydroperoxides and hydroxyphospholipids have been associated with oxidative stress and membrane injury that occur in pathological conditions such as spinal cord injury<sup>23</sup>. The main product of lipid peroxyl radical transformation is lipid hydroperoxide. Lipid peroxides cause membrane instability and degradation and a significant decrease in membrane fluidity<sup>23</sup>. When

membrane phospholipids are released from damaged membrane, causing elevation in arachidonic acid metabolism that contribute to inflammation. It is likely that the increased levels of lipid peroxides in the spinal CSF may cause lipid peroxidation. Some disease<sup>24-26</sup> and some drugs used to treat illness<sup>13,27</sup> causes alteration on the free radical defence mechanisms and lipid peroxidation products in CSF. So, it is suggested that the oxidation of CSF lipoproteins play a role in the pathogenesis of neurodegenerative diseases. Nevertheless, Rosen et al.<sup>14</sup> showed that large volume of local anesthetics include bupivacaine, injected for spinal anesthesia, was not neurotoxic when injected into subarachnoid space of sheep, and also these anesthetic solutions have not been produced significant abnormalities in sheep spinal CSF composition. On the other hand, local anesthetic agents include bupivacaine have membrane stabilizing properties which may induce changes in membrane polarization<sup>28</sup>. This mechanism may account for the protective effect of bupivacaine against oxidative stress. So, Lenfant et al.<sup>29</sup> found that bupivacaine provide a protective effect against induced free radical increase

with 2,2-azobis dihydrochloride. Also, De luliis et al.<sup>30</sup> have been showed that bupivacaine have an antioxidant effect on LPO of rat liver microsomes. However, there are no clinical studies that point out the importance of alterations in the lipid peroxidation status of CSF with spinally injected bupivacaine. In the current study we showed that the lipid peroxidation in CSF changed during spinal anesthesia with bupivacaine. This results may be due to bupivacaine induced inflammatory response which cause rise of LPO in spinal fluid.

Free radical-mediated damage occurs as a consequence of GSH depletion, depressed antioxidant enzyme activities and enhanced LPO. On the other hand, marked increase in oxidative stress may have been potentiated by increased antioxidant enzyme activities. Nevertheless, antioxidants can scavenge ROS before they can cause damage to various biomolecules or prevent oxidative damage from spreading out by interrupting the radical chain reaction of LPO. The disappearance of the endogenous antioxidants in plasma was measured in relation to the formation of lipid hydroperoxides formed from endogenous lipids. GSH is a major non-protein thiol in mammals and is essential for structural and metabolic integrity of cells.<sup>8</sup> GSH plays an important role in maintaining the stability of the membrane as a direct free radical scavenger<sup>11</sup> and in the protection of intracellular components such as sulphhydryl enzymes against oxidative denaturation. Also, it has been demonstrated to scavenge superoxide radical in a dose dependent manner<sup>31</sup>. Further, the key feature of this reaction is that it does not produce H<sub>2</sub>O<sub>2</sub> in the system<sup>10</sup>. It is known that H<sub>2</sub>O<sub>2</sub> plays a critical role in the regulation and expression of antioxidant enzymes in various cellular systems. Catalase (CAT) and glutathione peroxidase (GSHPx) are two different enzymes present in the cellular system that also neutralize H<sub>2</sub>O<sub>2</sub>. GSHPx which is GSH dependent antioxidant factor is a cytosolic protein that associates readily with membranes and reduces both organic and inorganic phospholipid hydroperoxides to alcohols. GSHPx changes the hydroperoxide group to the much less toxic hydroxy moiety. This enzyme not only allows the removal of the toxic ROOH moiety but also permits the regeneration of a membrane lipid molecule through reacylation. GSHPx system is particularly important in preventing free radical initiation in membranes since it is a very effective scavenger of H<sub>2</sub>O<sub>2</sub><sup>8,31,32</sup>. H<sub>2</sub>O<sub>2</sub> catabolism by GSHPx suppresses hydroperoxide-dependent lipid peroxidation and prevents  $\alpha$ -tocopherol oxidation, whereas peroxyl radical scavenging by  $\alpha$ -tocopherol prevents radical-induced GSHPx inactivation<sup>33</sup>. The nervous system is vulnerable to the damaging effects of highly reactive free radicals for several reasons. Antioxidant defenses

are critically important to protect the neural tissues from oxidative damage. Indeed, numerous pathophysiological conditions have been associated with increased levels of oxidative stress<sup>27</sup>. Intrathecal concentration of antioxidants might be of relevance due to the antioxidant protection against oxidative initiation of degenerative processes. After increase of LPO due to action of many used drugs, a pronounced protective effect of antioxidants was suggested by researchers<sup>11,13,26,34</sup>. In the present study, we observed a relationship between the concentration of free radical reactions as lipid peroxides and the biological defense mechanisms as the activity of GSHPx and the levels of GSH in the spinal fluid. These results showed that increased LPO causes a rise in antioxidant levels and activity during anesthesia.

Antioxidant enzymes protect the cellular structures against lipid peroxidation initiated by active species of oxygen and that antioxidant micromolecules such as vitamin E is also responsible for the inactivation of lipid peroxides<sup>32,35</sup>. Vitamin E is a most abundant lipid-soluble antioxidant present in biological membranes and lipoproteins that inhibits peroxidation of polyunsaturated fatty acids (PUFA) by scavenging free radicals<sup>33</sup>. In the nervous system, specific mechanisms transfer nutrients and other essential substances from blood into spinal fluid. These mechanisms are also involved in the concentration of the substance within the central nervous system<sup>36</sup>. The neuroprotective effects of vitamin E are wholly mediated by its antioxidant property. Mostly on the basis of symptoms of primary vitamin E deficiency, it has been demonstrated that vitamin E has a central role in maintaining neurological structure and function<sup>34</sup>. So, orally supplemented vitamin E reaches the CSF and brain<sup>37</sup>. In the present study it was observed that intra-spinal injection of bupivacaine has a protective effect on the levels of vitamin E in spinal fluid. Also, low levels of vitamin E in spinal CSF may be due to plasma levels of vitamin E have been present below the reference values. This result may be due to either antioxidant activity of bupivacaine or the antioxidant synergism between  $\alpha$ -tocopherol and GSH and GSHPx that mediated via the action of redox cycles.

In conclusion, we found that while spinal injection of bupivacaine increased free radical levels in spinal fluid, it may be supported by an antioxidant environment of spinal fluid during anesthesia.

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