

# EFFECTS OF ALL-TRANS RETINOIC ACID ON REDUCING HYPEROXIA-INDUCED OXIDATIVE STRESS IN MICE BRAIN

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## ABSTRACT

**Objective:** Brain is a very sensitive organ to oxidative stress due to its large amount of polyunsaturated fatty acid content and low antioxidant activity. In this study, effect of exogenous all-trans-retinoic acid (RA) on brain antioxidant activity was investigated in mice under hyperoxia-induced oxidative stress.

**Material and Method:** Adult C57BL/6J mice were divided into 4 groups. Two groups were given daily either peanut oil/dimethylsulfoxide (PoDMSO) mixture or 50 mg/kg RA dissolved in PoDMSO (RA-PoDMSO). The remaining two groups were treated with PoDMSO or RAPoDMSO as described above, following hyperoxia (100% oxygen) for 72 h. The treatments were given daily 50 µl intraperitoneal injections for 12 days, with a 2 day interruption on days 6 and 7. Lipid peroxidation (LPO) and glutathione (GSH) levels as well as activities of several antioxidant enzymes

were assayed in brain tissue to evaluate oxidative stress, biochemically.

**Results:** Elevated LPO levels, increased catalase, superoxide dismutase, glutathione peroxidase and glutathione-S-transferase activities, and decreased GSH levels were observed in hyperoxic mice. RA administration to hyperoxic mice improved these biochemical alterations in favour of reduced oxidative stress.

**Conclusion:** Exogenous RA, a small lipophilic molecule, is effective in reducing oxidative stress in the brain which can receive only small lipophilic molecules due to the presence of the blood-brain barrier. Also, RA exhibits an antioxidant effect by inhibiting the GSH depletion and increased LPO levels in brain of hyperoxic mice, directly.

**Key Words:** Hyperoxia, oxidative stress, all-trans-retinoic acid, glutathione, brain *Nobel Med* 2013; 9(3): 22-26

## FARE BEYNİNDE HİPEROKSİ İLE UYARILAN OKSİDATİF STRESİN AZALTILMASINDA TÜM-TRANS RETİNOİK ASİDİN ETKİLERİ

### ÖZET

**Amaç:** Beyin, büyük miktarda çoklu doymamış yağ asidi içermesi ve düşük antioksidan aktivite göstermesi nedeniyle oksidatif strese karşı çok duyarlı bir organdır. Bu çalışmada, hiperoksi ile uyarılan oksidatif stres altındaki farelerde tüm-trans-retinoik asit (RA)'in beyinin antioksidan aktivitesi üzerindeki etkisi araştırıldı.

**Materyal ve Metod:** Erişkin C57BL/6J fareler 4 gruba ayrıldı. İki gruba fıstık yağı/dimetilsülfoksit karışımı (FYDMSO) veya FYDMSO'da çözünmüş 50 mg/kg RA günlük olarak verildi. Diğer iki gruba 72 saatlik hiperoksiyi (%100 oksijen) takiben yukarıda belirtildiği gibi FYDMSO veya RA-FYDMSO verildi. Tüm enjeksiyonlar 50 ml'lik hacimlerde intraperitoneal yoldan 12 gün süresince yapıldı. Enjeksiyona 6. ve 7. günlerde 2 gün ara verildi. Oksidatif stresi belirlemek

için beyin dokusunda birkaç antioksidan enzim aktivitesiyle birlikte lipid peroksidasyon (LPO) ve glutatyon (GSH) seviyeleri biyokimyasal olarak ölçüldü.

**Bulgular:** Hiperoksik farelerin beyni LPO seviyesinde yükselme, katalaz, süperoksit dismutaz, glutatyon peroksidaz, glutatyon-S-transferaz enzimlerinin aktivitelerinde artış ve glutatyon seviyesinde azalma görüldü. Hiperoksik farelere RA uygulaması erişkin beynindeki oksidatif stresi azaltma yönünde bu biyokimyasal değişiklikleri iyileştirdi.

**Sonuç:** Küçük lipofilik bir molekül olan eksojen RA, kan-beyin bariyeri nedeniyle sadece lipofilik moleküllerin geçişine izin veren beyinde oksidatif stresin azaltılmasında etkilidir. Ayrıca RA, GSH azalmasını ve LPO yükselmesini doğrudan durdurarak hiperoksik farelerin beyinde antioksidan etki gösterir.

**Anahtar Kelimeler:** Hiperoksi, oksidatif stres, tüm-trans-retinoik asit, glutatyon, beyin *Nobel Med 2013; 9(3): 22-26*

### INTRODUCTION

Oxidative stress is known as an imbalance between the production and manifestation of reactive oxygen species (ROS). An excessive increase in tissue ROS levels results in significant damage to cell structures, such as cellular membrane and DNA/RNA and cause the oxidation of polyunsaturated fatty acids in lipids (lipid peroxidation, LPO) and amino acids in proteins, and the inactivation of specific enzymes by oxidation of cofactors.<sup>1</sup>

Hyperoxia causes cell injury due to increased endogenous ROS production. Brain consumes up 20% of oxygen used by the whole body. Hyperoxic conditions increase the percentage of oxygen consumption of brain. Central nervous system cells can produce excessive ROS and reactive nitrogen species under hyperoxic conditions. Since the brain contains a large amount of polyunsaturated fatty acids and shows poor antioxidant activity, it is especially sensitive to oxidative stress. Higher amounts of ROS play a role in the neurodegenerative complications as well as in a number of brain damages.<sup>2-4</sup> Several reports point out that exposure of immature rodents to 80% oxygen over a period of 24 h induced diffuse neuronal cell and oligodendrocyte death associated with oxidative and nitrosive stress.<sup>2,3</sup> It is stated that hyperoxia triggered apoptosis in N9 microglial cells, leading to massive production of ROS, interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$ .<sup>4</sup> Recently it has been demonstrated that early postnatal hyperoxia exposure

induced apoptotic neuronal cell death in rat pups.<sup>5</sup> The neurons are more susceptible to oxidative stress than other brain cell types. Moreover, the accumulating evidence indicated that microglia can become deleterious and damage neurons. The overactivated microglia release ROS that cause neuronal damage in neurodegenerative diseases.<sup>6,7</sup> Neurodegenerative diseases including Parkinson's disease, Alzheimer's disease and Huntington's disease are characterized by progressive loss of neurons accompanied with increased ROS and/or reactive nitrogen species and mitochondrial dysfunction.<sup>6,7</sup>

Vitamin A is a radical scavenger and an effective chain-breaking antioxidant. Also, it prevents the initiation of LPO.<sup>8</sup> Retinoids, a group of compounds derived from vitamin A, perform various functions in the central nervous system. Using in vitro peroxidation system, antioxidant activities of retinoids have been ranked in a declining order as retinol > retinal > retinyl palmitate > retinoic acid.<sup>9</sup> All-trans-retinoic acid (RA), a retinoid, is a lipophilic molecule with low molecular weight. Since oxidative stress and dedifferentiation of neurons appear to be common pathological findings of certain neurodegenerative disorders, Lee and colleagues suggested that RA may offer therapeutic promise.<sup>10</sup>

The aim of this study was to determine the effect of RA on antioxidant activity of mice brain under hyperoxia-induced oxidative stress and to test RA as a →

neuroprotective agent in excessive oxidative stress in brain tissue.

## MATERIAL and METHOD

### Animals

All experimental procedures were approved by Istanbul University Animal Care and Use Committee (Number:43/25.02.2010). Forty adult male C57BL/6J mice (8-10-weeks-old) were housed in humidity- and temperature-controlled rooms on a 12-h light-dark cycle and were given food and water ad libitum. The animals were fasted for 15 h prior to treatments, but were given free access to water.

### Experiment

Hyperoxia was used to induce oxidative stress in mice brain. Mice were divided into the following experimental groups (n=10 each group): Group 1- control mice administered with peanut oil/dimethylsulfoxide (PoDMSO, 1:1); Group 2- mice treated with RA (50 mg/kg) dissolved in PoDMSO (RA-PoDMSO); Group 3- mice treated with PoDMSO following hyperoxia (100% oxygen); Group 4- mice administered with RA-PoDMSO following hyperoxia. Mice in groups 1 and 2 were kept at the room air conditions throughout the experiment. Those in groups 3 and 4 were kept in hyperoxic conditions for 72 h and subsequently maintained at the room air conditions for 12 days. The treatments were given daily as a 50 µl intraperitoneal injection for 12 days, with a 2 day interruption on days 6 and 7. Continuous exposure to a 100% oxygen was achieved by a flow-through system in a 0.28 m<sup>3</sup> chamber at 1 atmosphere pressure for 72 h. The oxygen level was monitored using an oxygen sensor. The CO<sub>2</sub> was removed by absorption into sodalime. Mice were given free access to food and water during hyperoxia. At the end of the experiment, mice were killed by intraperitoneal injection of a 1:1 mixture of 40 mg/kg ketamin and 10 mg/kg xylazin before harvesting the brain for analysis.

### Biochemical analysis

Whole brain tissues were homogenized in cold 0.9% NaCl with glass equipment in order to obtain a 10% homogenate (w/v). The homogenates were centrifuged at 10,000 g for 10 minutes. The clear supernatants were used for protein, reduced glutathione (GSH), LPO and antioxidant enzyme analysis. GSH levels were determined according to Beutler's method using Ellman's reagent.<sup>11</sup> LPO levels in brain homogenates were estimated by Ledwozyw's method.<sup>12</sup> Catalase (CAT) and superoxide dismutase (SOD) activities

were assayed in brain tissues by the method of Aebi and Mylroie's method, respectively.<sup>13,14</sup> Glutathione peroxidase (GPx) activity was determined by the method described by Paglia and Valentine and modified by Wendel.<sup>15,16</sup> Glutathione-S-transferase (GST) activity was determined using Habig's method.<sup>17</sup> The protein content in the supernatants was estimated by the method of Lowry using bovine serum albumin as standard.<sup>18</sup>

### Statistical analysis

Biochemical results were evaluated using an unpaired *t*-test and ANOVA variance analysis using the NCSS statistical computer package. The values were expressed as mean±SD. Analysis between control and experimental groups was performed using the Mann-Whitney test. *p*< 0.05 was considered as significant.

## RESULTS

Brain GSH and LPO levels are presented in Table 1. The brain GSH levels were significantly decreased in the hyperoxic mice compared to the remaining groups ( $p_{ANOVA}=0.015$ ). Brain GSH level was found to be significantly decreased in hyperoxic mice compared to the control group (*p*<0.05). Exogenous RA increased the GSH levels significantly in brain of hyperoxic mice (*p*<0.05). The brain LPO levels were increased in hyperoxic mice as compared to the remaining groups ( $p_{ANOVA}=0.027$ ). Brain LPO levels were increased in hyperoxic mice as well, compared to controls (*p*<0.05). Exogenous RA significantly decreased the LPO level in the brain of hyperoxic mice (*p*<0.05).

Brain CAT and SOD activities were presented in Table 1. Brain CAT and SOD activities were significantly increased in the hyperoxic mice as compared to the remaining mice. ( $p_{ANOVA}=0.0001$ ). The activities of CAT and SOD in the brain of these mice were significantly higher than the controls as well (*p*<0.0001). The activities of these enzymes were decreased in the hyperoxic mice which received RA compared with hyperoxic mice untreated with RA (*p*<0.0001).

Brain GPx and GST activities were significantly increased in the hyperoxic mice in comparison to the other groups ( $p_{ANOVA}=0.0001$ , Table 1). GPx and GST activities were found significantly elevated in the hyperoxic mice (*p*<0.0001). RA administration to hyperoxic mice decreased the GPx and GST activities in the brain (*p*<0.001; *p*<0.0001).

## DISCUSSION

Hydroxyl radicals, which are one of ROS, result in LPO referred as oxidative degradation of lipids and →

organic radical formation which induces cell damage.<sup>19</sup> They most often affect polyunsaturated fatty acids, because they contain multiple double bonds between methylene-CH<sub>2</sub>-groups which possess especially reactive hydrogen. Brain is a very sensitive target for the LPO-induced damage because of a high level of polyunsaturated lipids in neuronal cell membranes. It is reported that hyperoxia-induced oxidative stress resulted in brain damages accompanied with neuronal and glial cell death.<sup>2,3,5</sup> ROS and subsequent LPO products are effective on neuronal death and pathogenesis of neurodegenerative disorders.<sup>20</sup> Onodera and colleagues determined that oxidative stress and increased LPO, stimulated a decline in the cognitive function of rat cerebral cortex and hippocampus.<sup>21</sup> Malondialdehyde (MDA), end-product of LPO, was used as a marker for LPO in the present study. The brain of hyperoxic mice have still exhibited overexpressed MDA levels despite of increased antioxidant enzyme activities even after 12 days of room air conditions following hyperoxia. We think that endogenous antioxidant mechanisms of adult mice brain were not enough to reduce hyperoxia-induced LPO. Our data clearly demonstrated that exogenous RA has attenuated LPO levels for 12 days of room air conditions following hyperoxia in the brain of hyperoxic mice.

The glutathione system includes GSH, GPx, glutathione reductase, and GST. It is well known that they scavenge superoxide and eliminate hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as well as lipid peroxides or their harmful secondary metabolites. Brain GSH concentration is lower than those of the liver, kidney, spleen, or small intestine.<sup>22</sup> Decreased intracellular GSH weakens the tissue resistance to oxidants and worsens oxidative damage in the experimental spinal cord injury of mice.<sup>23</sup> Oxygen-induced cell death is associated with reduced GSH, increased oxidized GSH levels and increased LPO at 12-48 h of hyperoxia in the developing brain.<sup>24</sup> Brain GSH depletion leads to increased production of superoxide and hydroxyl radicals, and H<sub>2</sub>O<sub>2</sub>.<sup>25</sup> Similarly, the present study reported GSH depletion in addition to high LPO levels as well as elevated SOD, CAT, GPx and GST activities in brain of mice subjected to hyperoxia. We suggest that the drop in GSH levels after hyperoxia might probably be due to its consumption during oxidative stress. Also, the fact that increased antioxidant enzyme activity could not ameliorate oxidative stress in hyperoxic mice brain may emphasize the importance of GSH level, which acts directly and indirectly (non-enzymatic pathway) on the elimination of hyperoxia-induced superoxide anion, H<sub>2</sub>O<sub>2</sub> and MDA formations in mice brain. This was confirmed by the biochemical measurement done in our study. Our findings show

that RA administration to hyperoxic mice regressed oxidative stress in brain, by inhibiting GSH depletion and increase in the MDA level. A similar protective effect was obtained in another study.<sup>26</sup> This study reported that RA protected the primary cultures of embryonic neurons from oxidative damage and apoptosis by inhibiting GSH depletion.<sup>26</sup> Likewise, RA applied at a physiological concentration significantly decreased hyperglycemia-induced oxidative stress by inhibiting the decrease in GSH levels and SOD activity in addition to ameliorating elevated LPO levels and total thiol levels, and it protected neurons from oxidative stress in developing cortical neurons isolated from 16-day-old rat embryos.<sup>27</sup> Additionally, several reports have shown that RA increased GSH synthesis via cysteine uptake in neurons.<sup>28,29</sup> Our study revealed that RA treatments applied to hyperoxic mice hindered excessive LPO production in brain, resulting in higher GSH level than basal GSH levels in control mice.

It is well known that SOD activity, as well as CAT and GPx activities can give information about superoxide production and H<sub>2</sub>O<sub>2</sub> turnover, respectively. In neonate brain of transgenic mice at 7 days of hyperoxia, overexpression of SOD played an important role in significant protection against hyperoxia-induced brain damage.<sup>30</sup> The exposure to hyperoxia induces the upregulation of antioxidant enzymes, such as SOD, CAT and GPx in brain tissue.<sup>31,32</sup> In the present study, we declare that hyperoxia stimulated SOD activity in mice brain maintained at room air condition for 12 days following hyperoxia. In addition, the increase in CAT and GPx activities may be associated with the increase of SOD activity and, in turn H<sub>2</sub>O<sub>2</sub> generation in the brain of hyperoxic mice. Moreover, CAT and GPx activities increased up to 5- and 2-fold, respectively, in brain of hyperoxic mice compared to that of the controls. We emphasized that increase in CAT activity was very important to eliminate H<sub>2</sub>O<sub>2</sub> in the brain of hyperoxic mice, because the brain of these mice exhibited depressed GSH, a substrate for GPx. Researches have shown that RA reduce vulnerability to oxidative stress in embryonic neurons by increasing of SOD, CAT, and GPx activities in brain in addition to rat sertoli cells and chondrocytes.<sup>24,33,34</sup> However, RA treatments to hyperoxic mice resulted in regressed CAT, SOD and GPx activities in adult brain maintained at room air conditions for 12 days following hyperoxia. We suggested that RA administration has contributed to the endogenous antioxidant mechanisms of adult mice brain for 12 days room air following hyperoxia, by inducing hyperoxia-induced LPO and ROS elimination. Thus, RA treatments would offer a better environment for brain against oxidative stress. →

In conclusion, exogenous RA administration can be suggested as an antioxidant agent for regression of pathological circumstances originated from elevated oxidative stress via the inhibition of GSH depletion and contribution to endogenous antioxidant mechanisms

in adult mice brain. The poor antioxidant system and non-regenerative character of adult brain make it more sensitive against tissue damage under oxidative stress. Consequently, exogenous RA can protect the brain from hazardous effects of oxidizing species.



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