Neuroprotective antioxidant effect of sex steroid hormones in traumatic brain injury

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Abstract: The aim of the present study was to evaluate the effect of different doses of sex steroid hormones on brain edema, BBB permeability, brain antioxidant enzyme activity, and MDA level after traumatic brain injury (TBI) in ovarectomized (OVX) rats. Female rats were divided into six (One sham and 5 TBI) groups including: vehicle, estrogen in physiologic (33.3 µg/kg) and pharmacologic (1mg/kg) doses, progesterone in physiologic (1.7 mg/kg) and pharmacological doses (8mg/kg). The results showed that compared to vehicle group, estrogen and progesterone groups showed significantly lower brain water content ($P<0.001$). Evans blue content was significantly lower in both estrogen doses and in progesterone physiologic dose ($P<0.001$). Evans blue content was significantly higher in progesterone pharmacologic dose ($P<0.001$).

Superoxide dismutase (SOD) activity was significantly higher in estrogen and progesterone pharmacologic doses ($P<0.001$). Glutathione peroxidase (GPx) activity was significantly lower in estrogen physiologic dose ($P<0.001$). It was concluded that the neuroprotective effect of different doses of sex steroid hormones after TBI, may be mediated by changes in oxidant agent activity.

Keywords: TBI, sex steroids, estrogen, progesterone, GPx, SOD, MDA, neuroprotection.

INTRODUCTION

Several million people, mostly children and young adults, are treated each year for severe head injury (Alexander 1992). Of these, thousands die and thousands will be permanently disabled. The most serious head injuries are caused by Road traffic crashes, and with the increasing use of vehicles in Asia and Africa, the global affliction of head injury can be expected to rise (Murray*et al*., 1997). Brain contusion is one of the complications produced by traumatic brain injury (TBI). In this situation the brain tissue is damaged (Katayama, Kawamata 2003). TBIs can be classified into primary and secondary, the former occurring immediately after trauma, and the latter appearing several hours or even days later (Ozdemir*et al*., 2005).

Brain edema which leads an expansion of brain volume has a crucial impact on TBI induced morbidity and mortality, and aggravates additional ischemic injuries (Unterberg*et al*., 2004).The blood–brain barrier (BBB) breakdown leads to many neurodegenerative diseases such as cerebral edema (Gilgun-Sherki*et al*., 2001). Although the precise mechanism of BBB breakdown is not known, it has been suggested that NO and free radicals cause an increase in BBB permeability (Heo*et al*., 2005).

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We concluded that the neuroprotective effect of different doses of sex steroids after TBI may be mediated by changes in the activity of oxidant agents. The sex steroid hormones secreted from ovaries serve as protective and trophic factors for nervous tissues in brain injuries (Stein*et al*., 2001). In a previous study in our lab, the role of sex hormones in reduction of brain edema after diffuse traumatic brain injury has been confirmed. The cause of most of these complications is vasogenic brain edema and that TBI initiates an early cascade of events leading to production of reactive oxygen species (ROS) (Dugan & Choi, 1994). ROS induce lipid and protein peroxidation and oxidation of nucleic acids, thereby causing further cell damage (Beni, Kohen, Reiter, Tan, & Shohami, 2004; Dugan & Choi, 1994; Lewen, Matz, Chan, & 2000).

Oxidative stress plays a major role in the development and aggravation of nerve cell death in a variety of human neurodegenerative disorders. In addition to this, it is well known that under certain conditions antioxidants such as vitamin E and vitamin C can develop strong preventive activities as shown recently (Zandi*et al*., 2004). β-Estradiol is a antioxidant similar to vitamin E, that has been suggested to act as a potential antioxidant in certain clonal and primary nerve cell cultures (Behl*et al*., 1995 and 1997). A role anti-oxidant suggested for progestins, which is mediated through a reduction in lipid peroxidation and improvement in cell membrane

stabilization (Kueblere*t al*., 2003, Roofe*t al*., 1997 and VanLandinghame*t al*., 2006).

The aim of the present study was to evaluate the effect of different doses of sex steroid hormones on brain edema, BBB permeability, and brain antioxidant enzyme activity such as superoxide dismutase (SOD), Glutathione Peroxides (GPx) levels, and malondialdehyde (MDA) level as an indicator of lipid peroxidation after diffuse TBI in ovarectomized (OVX) female rats.

MATERIALS AND METHODS

In this study 120 female Albino N-Mary rats (200-250g body weight) were used (each group includes two sub groups). The animals were kept in 20-22^{o}C and 12 hours dark/light conditions with free access to water and food in the animal house of Kerman Faculty of Medicine. This research was carried out under the approval of the Medical Ethics Committee of Kerman University of Medical Sciences Ref No: KA/86/65.

Bilateral ovarectomy

The animals were anesthetized by injection of 60 mg/kg thiopental (i.p.), the subabdominal part was shaved and an incision with a length of 2 cm was made, the skin, fascia and abdominal muscles were opened, and the fats and intestine were sheered of, until the uterus and its tubes were visible. Then the uterus tube and vascular base of ovaries were closed around proximal area using 4-0 catgut thread and were cut from distal area. Finally 1-2 ml of saline was poured in abdomen and the muscles and skin were pulled back and were stitched by catgut and 0-2 silk threads. The wound was washed with betadin solution. In order to avoid the interference of estrus cycle, all experimental animals were ovarectomized (OVX) two weeks before experiment (Wene*t al*., 2004).

Induction of TBI

The TBI was moderate and from diffuse kind, induced by Marmarou method (Marmarou*et al*., 1994). The Process of the TBI induction (device was made by Dept. of Physiology, Kerman University of Medical Sciences) was as follows: a 250g weight was dropped from a 2 meter height on the head of anesthetized rat [under 3% halothane in a mixture of 67%N2O /30%O2] with a metal disc (stainless steel) 10mm in diameter and 3mm thick, attached on the animal's skull. After inducing the trauma, the rat was immediately connected to animal respiratory pump (TSA animal respiratory compact, Germany). When spontaneous breathing and righting response were restored, endotracheal tube was removed. After recovery the rats were placed in individual cages (Marmarou*et al*., 1994 and Shahrokhi*et al*., 2010).

Experimental groups

The animals were divided into six groups, 10 rats in each. Brain injury was induced 2 weeks after ovarectomy and in all treatment groups, drugs (i.p injection) were administered 30 minutes after induction of brain trauma (O'Connor*et al*., 2005). The 6 groups of rats were as follows: 1) Sham group: the OVX rat underwent false brain trauma when they were anesthetized and did not receive vehicle or any drug. 2) Vehicle group: the OVX rat received equal volume of sesame oil (0.33 ml) injected intra-peritoneally (i.p) 30 minutes after brain injury (O'Connor*et al*., 2005). 3) Estrogen pharmacologic dose group: the OVX rats received 1mg/kg estrogen (Liu*et al*., 2005; O'Connor*et al*., 2005). 4) Estrogen physiologic dose group: the OVX rats received 33.3 µg/kg estrogen (O'Connor*et al*., 2005). 5) Progesterone pharmacologic dose group: the OVX rats received 8 mg/kg progesterone (O'Connor*et al*., 2005). 6) Progesterone physiologic dose group: the OVX rats received 1.7 mg/kg progesterone (O'Connor*et al*., 2005).

Drugs

β-estradiol, progesterone and their solvents were purchased from Abooreyhan Pharmaceutical Company (Iran).

Determination of brain water content

To measure the brain edema, the brain water content was determined, as the animal was anesthetized and the brain was extracted 24 hours after TBI. The weight of wet tissue was measured first and then incubated in 60^{o}C in an incubator (Memmert, Germany) for 72 hours to evaporate the tissue water and dry. The brain was weighed again after drying and water content was calculated by % using formula (O'Connor*et al*., 2005; Shahrokhi*et al*., 2010).

$$\%\ \text{water content} = \left[\frac{(\text{wet weight} - \text{dry weight})}{(\text{wet weight})}\right] \times 100$$

Determination of brain evans blue dye content

At 4 h after TBI, a dose of 2 ml/kg 2% Evans blue (EB) was injected intravenously through tail vein. Animals were anesthetized 5 h after TBI and perused using saline to remove intravascular EB dye. Animals were then decapitated, the brains removed and homogenized in phosphate buffered saline. Protein was precipitated by adding trichloroacetic acid, and the samples were cooled and centrifuged. The resulting supernatant was measured for absorbance of EB at 610 nm using a spectro-photometer (O'Connor*et al*., 2005).

Determination of SOD activity

24 h after TBI, using a SOD determination kit (sigma-Aldrich, England) with indirect method using nitroblue tetrazolium (NBT), SOD activity was determined. SOD Assay Kit-WST, SOD assaying by utilizing Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl(-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O2 is linearly related

to the activity of xanthine oxidase (XO), and is inhibited by SOD. Therefore, a colorimetric method can be used to determine 50% inhibition activity of SOD. Measuring the color development decrease at 440 nm using a spectrophotometer may quantify The SOD activity as an inhibition activity and the results were expressed as SOD U/µg protein (Sun, Oberley, & Li, 1988).

GPx activity Determination

Glutathione peroxidase (GPX) activity in tissue was determined 24 h after TBI using a Randox assay kit (according to the manufacturer's protocol). The enzyme activity was expressed as U/µg protein.

Determination of Malondialdehyde levels

The lipid peroxides as thiobarbituric acid reactive substance (TBARS) was assayed (24 h after TBI) according to the method of Esterbauer and Cheeseman. Briefly, serum was precipitated by 10 % trichloroacetic acid (TCA). The pink color resulting from Thiobarbituric acid's reaction with products of lipid peroxidation, mainly malondialdehyde (MDA), could be measured at 535 nm (Esterbauer & Cheeseman, 1990).

STATISTICAL ANALYSIS

Results are presented as the mean ± SEM. one-way analysis of variance (ANOVA) was performed to determine the differences among the groups followed by Tukey's post hoc test for differences between groups. $P<0.05$ was considered as statistically significant.

RESULTS

Effect on brain water content

Fig. 1 shows the brain water content in different groups of study. The amount of brain water content in vehicle (79.8 ± 0.23%) significantly increased compared to sham groups (76.6 ± 0.43%) ($p<0.001$). After physiologic and pharmacologic dose of estrogen injection the amount of brain water content significantly reduced to 76.58 ± 0.19% and 77.07 ± 0.2%, physiologic and pharmacologic dose of progesterone injection reduced to 77.42 ± 37% and 77.26 ± 0.15% in compared to vehicle group respectively ($p<0.001$).

Effect on BBB permeability

As shown in fig. 2, after physiologic and pharmacologic dose estrogen and physiologic dose progesterone injection, the content of Evans blue in brain tissue significantly reduced to 27.03 ± 0.32 µg/g, 26.78 ± 1 µg/g ($p<0.001$) and 28.65±0.18 µg/g ($p<.05$) compared to vehicle group (34.42 ± 0.47 µg/g) respectively. Whereas pharmacologic dose progesterone (46.5 ± 1) increased significantly compare to vehicle and sham (29.85±1.4 µg/g) groups ($p<0.001$). There was significant difference ($p<0.05$) between vehicle and sham groups.

Effect on Superoxide dismutase (SOD) activity

Fig. 3 shows Superoxide dismutase (SOD) activity in different groups of study. As shown in fig. 3, after pharmacologic dose estrogen and progesterone injection Superoxide dismutase (SOD) activity in brain homogenates increased significantly to 1 ± 0.2 units/µgprotein, 1.11 ± 0.11 units/µg protein in comparison with vehicle group (0.66 ± 0.06 units/µg protein) respectively ($p<0.05$).

Effect on Glutathione peroxides (GPx) activity

Fig. 4 shows Glutathione peroxides (GPx) activity in different groups of study. As shown in fig. 4, after physiologic dose estrogen injection, Glutathione

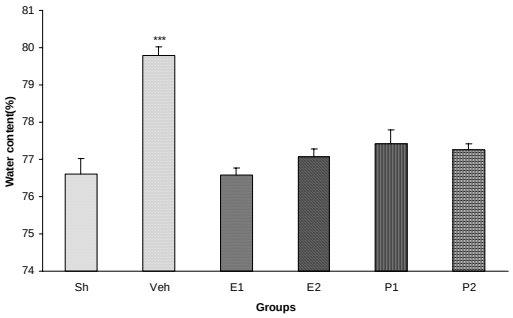

Fig. 1: Alterations in ovariectomized female rats' brain water content, following treatment of diffuse traumatic brain injury with either progesterone and estrogen pharmacologic and physiologic dose, or equal volume vehicle.

Sh = Sham ovarectomized; Veh = Vehicle; E1 = Estrogen physiologic dose, P1 = Progesterone physiologic dose, E2 = Estrogen pharmacologic dose; P2 progesterone pharmacologic dose. ***P< 0.001, Veh VS all groups.

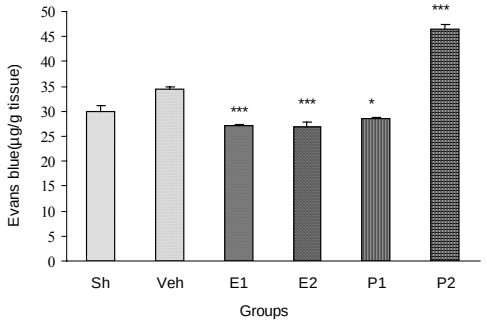

Fig. 2: Evans blue extravasation alterations in ovariectomized female rats, following treatment of diffuse traumatic brain injury with either progesterone and estrogen_pharmacologic and physiologic dose, or equal volume vehicle.

Veh = Vehicle; Sh = Sham ovarectomized; E1 = Estrogen physiologic dose; P1 = Progesterone physiologic dose, E2 = Estrogen pharmacologic dose. P2 progesterone pharmacologic dose *P<0.05 P1 vs.veh;***P< 0.001. E1, E2, and P1 vs. veh.

peroxides (GPx) activity in brain homogenates decreased significantly to 93.73 ± 3.86 units/µg protein in compared to vehicle group (143.17 ± 10.45 units/µg protein) P<0.01). The results of our research showed that vehicle (sesame oil) can cause significant increased Glutathione peroxides (GPx) activity compared to sham(103.3±9.3 units/µg protein) group (P<0.05).

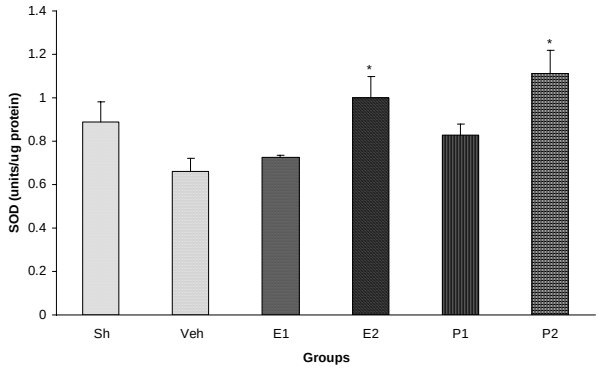

Fig. 3: Superoxide dismutase (SOD) activity in brain homogenates in ovariectomized female rats, following treatment of diffuse traumatic brain injury with either progesterone and estrogen physiologic and pharmacologic dose, or equal volume vehicle.

Sh = Sham ovarectomized; Veh = Vehicl; E1 = Estrogen physiologic dose; P1 = Progesterone physiologic dose. *P < 0.05, 2 or P2 vs. veh.

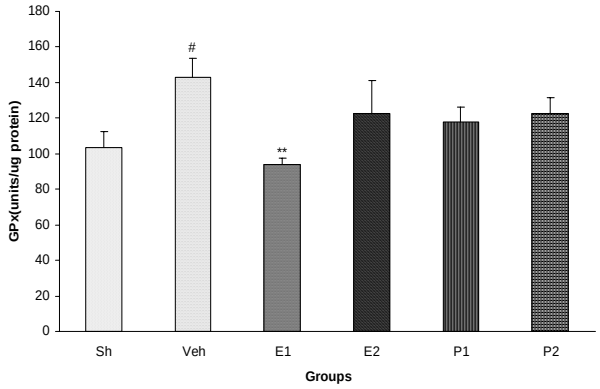

Fig. 4: Glutathione peroxides (GPx) activity in brain homogenates in ovariectomized female rats, following treatment of diffuse traumatic brain injury with either progesterone and estrogen physiologic and pharmacologic dose, or equal volume vehicle.

Sh = Sham ovarectomized; Veh = Vehicl; E1=estrogen physiologic dose; P1= progesterone physiologic dose E2=estrogen pharmacologic dose. ;P2 progesterone pharmacologic dose. #P < 0.05 veh vs. Sh;** P< 0.01. E1 Vs, Veh.

Effect on Malondialdehyde (MAD) levels

Fig. 5 shows Malondialdehyde (MAD) levels in different groups of study. The results of our research showed that the vehicle (sesame oil) (0.17 ± 0.05µl/µg protein) can cause significant decreased Malondialdehyde (MAD) levels compared to sham (0.56±0.09µl/µg protein) group (P<0.05).

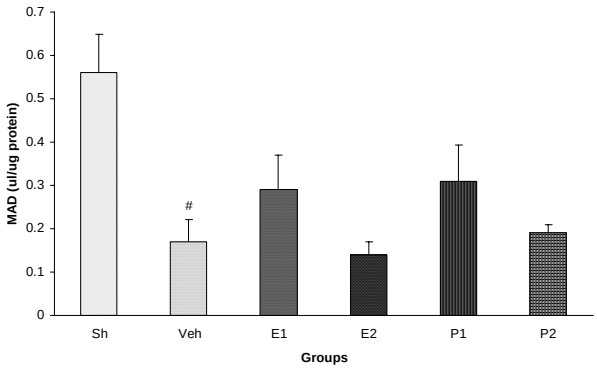

Fig. 5: Malondialdeide (MAD) levels in brain homogenates in ovariectomized female rats, following treatment of diffuse traumatic brain injury with either progesterone and estrogen physiologic and pharmacologic dose, or equal volume vehicle.

Sh = Sham ovarectomized; Veh = Vehicl; E1 = Estrogen physiologic dose; P1= progesterone physiologic dose E2 = Estrogen pharmacologic dose. P2 progesterone pharmacologic dose #P < 0.05 veh vs. Sh.

DISCUSSION

The protective effects of endogenous antioxidant enzymes against oxidative stress of nervous tissue are controlled by ovarian hormones. Some studies have reported these hormonal effects to be related to concentration and their antioxidant properties (Sun*et al*., 1988). Thus, the present study aimed at assessing the effects of female sex steroids in different doses, on brain edema, BBB permeability and activity of antioxidant enzymes including SOD, GPx and also MDA levels as an index for lipid peroxidation in ovariectomized female rats after TBI.

The results of the study showed that both physiologic and pharmacologic doses of estrogen or progesterone reduced water content, as brain edema index that the most reduction , three percent, was created by the physiologic dose of estrogen. Oconnor and others also showed that estrogen and progesterone cause the significant reduction in brain water content after TBI (Huang *et al*., 2010, Palomar-Morales, *et al*., 2010). It has been reported that steroids reduce the cerebral edema by reducing the inflammatory cytokines after TBI (He & Evans 2004).

In this study, it was found that pharmacological doses of progesterone only increased BBB permeability whereas estrogen and physiological dose of progesterone have decreased BBB permeability. Sundary and colleagues concluded that use of progesterone leads to induction of inflammatory factors and increases BBB permeability in ovariectomized rats through this action (Wise*et al*., 2001). The secondary inflammatory cascade may also increase BBB abnormalities after TBI (Beni*et al*., 2004). We showed pharmacologic dose of progesterone leads to increase in Evans blue content of brain as BBB permeability index.

It has also been indicated that BBB is strengthened after TBI induction by progesterone (Unterberg *et al*., 2004). In the other part of research, the Evans blue content was reduced by physiologic and pharmacologic doses of estrogen after the TBI induction. Estrogen reduced the BBB permeability of cerebrovascular endothelium by inhibiting the MMP2 and MMP9 after TBI induction (Paglia & Valentine, 1967). Furthermore, estrogen reduced the BBB permeability in ovariectomized rats that they had cerebrovascular inflammation (Wise*et al*., 2001).

Regarding the fact that one of the inherent and physiologic properties of the brain is its high lipid concentration and demand to energy they make prone the brain to free oxygen radicals damage (Pajovic *et al*., 2003). Since Estradiol is a nervous protective antioxidant, in the other section of our survey we showed pharmacologic doses of estrogen or progesterone lead to increasing of SOD levels in brain hemogenat. 17-beta estradiol is a phenol compound which has a high similarity with the famous anti-oxidant, alpha- tocopherol (vitamin E). Because the estradiol has a lipophilic structure, it has the ability of ROS detoxification (Behl 2002). Some results showed anti- oxidant action of estrogen is completely independent of estrogen receptor activity or expression (Behl 2002). However, some other studies stated that estrogen increase the SOD expression by estrogen receptors (Unfer *et al*., 2006) that most effect is done by ER-α 2008 and Traupe *et al*., 2007b). Research suggests that sexual steroids play a role in oxidative stress. The anti-oxidant enzymes activity varies during the menstrual cycle as sexual steroids concentration changes (Pajovic *et al*., 2003). Reduced SOD activity decreased in menopause cycle that this reduction was prevented by ovarian hormones consumption (Unfer *et al*., 2006). Brain anti-oxidant enzymes activity changes in estrus cycle (Pajovic *et al*., 2003). SOD levels are less in male rats than female ones (Huang *et al.,2010*). Female sexual hormones modulate the SOD activity and SOD activity decreases in ovarectomized rats 2010). The SOD activity is higher in females than in males because of high estradiol concentration (Kerksick *et al.,* 2008).

These results have shown that brain SOD activity increased 24 hours after TBI induction as a result of progesterone and pharmacologic dose of estrogen. Razmara and colleagues observed that estrogen caused suppression of oxidative stress by brain mitochondria (Traupe *et al*., 2007a). Dong and colleagues have demonstrated that fitoestrogen combination increases SOD activity and the survival of neurons in toxic hippocampus (Dugan & Choi 1994). A different study reported that SOD activity in the brain also increases during the proestrus phase. Kiray and colleagues reported that estrogen doesn't have any effect on brain SOD activity in ovariectomized rats under stress (Liu*et al*., 2005). Ozgonul and colleagues have also explained that estrogen had no effect on SOD activity in ovariectomized rats (Shahrokhi *et al*., 2010). Also, estrogen has a little effect on the tissue SOD activity after sport (Pajovic *et al*., 2003) and it doesn't have any effect on the SOD activity in pancreatic cells (Palomar-Morales *et al*.,2010). Thus, the anti- oxidant action of sexual steroids is dependent on consumed dose, tissue property and animal condition. Aggarwal and colleagues suggested that progesterone had an important effect on the activity of all antioxidants and exerts its protection effect by this function (Alexander, 1992). SOD activity is increased in human endometrial tissue by progesterone (Dhote & Balaraman 2007). Progesterone inhibits the ROS activity (Verma & Rana 2008). The SOD activity is exaggerated when a typical combination therapy consists of taking estrogen with progesterone is used (Unfer *et al*., 2006).

On the other hand, Pajovics and colleagues showed that SOD activity is reduced by progesterone in ovariectomized rats (Stein, 2001). Progesterone does not have any effect on the SOD activity in pancreatic cells (Palomar-Morales *et al*.,2010). This contradiction could probably be due to differences in conditions in which the study has been carried out and the consumed dose.

In addition, it has been reported that some compounds relating to sesame oil had anti lipoperoxidative and detoxification abilities (Sun*et al*., 1988). Prescription of olive oil as vehicle resulted in the increasing of GPx activity (Pajovic *et al*., 2003).

Our results showed that estrogen and progesterone solvent (sesame oil) have caused a significant increase in GPx activity compared to sham subjects. Therefore, the results of this study indicate that physiological doses of estrogen reduce the brain GPx activity within 24 hours after TBI induction. GPx activity in the brain also decreased during the proestrus phase (high concentration of E) (VanLandingham *et al*., 2006). Pajovic and colleagues confirmed that consummation of estrogen 2 hours after TBI induction could decrease GPx activity in testectomized rats (Sun *et al*., 1988), effects which were consistent with the results of this study. Moreover, Pajovis.SL and colleagues determined that the use of estradiol benzoate does not have any side effects on brain GPx activity (Stein, 2001). Lauren and colleagues (2007) which have carried out some research on the effects of estrogen in spatial memory, have indicated that estrogen could cause the brain to increase GPx activity (Alexander, 1992). Glutathione activity decrease is inhibited in ovarectomized rats by estradiol (Calegare *et al.,2010*).

The results showed that sesame oil could exert helpful effects on the improvement of brain damage after TBI. In this regard, some reports have suggested that various compounds in sesame oil may have anti lipoperoxidative (Sun*et al*., 1988). However, some studies reported

estradiol as decreasing brain MOA levels in OVX rats (Kasimay *et al*., 2009), or progesterone as decreasing MOA levels (Verma & Rana 2008).

In general, we can conclude that these hormones have prominent Neuroprotective roles in ovariectomized rats after TBI induction; these Neuroprotective effects seem to be caused by several mechanisms. One of the mechanisms by which ovarian steroids act through their neuroprotective actions drawn increased activity of anti-oxidant enzymes such as SOD. It is necessary to determine the estrogen receptor types and other mechanisms of estrogen and progesterone in future studies. Also, the pharmacologic anti-oxidant action of sexual steroids requires further research.

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