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Time- and Dose-Dependent Neuroprotective Effects of Sex Steroid Hormones on Inflammatory Cytokines after a Traumatic Brain Injury

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Abstract

Following a traumatic brain injury (TBI), excessive release of proinflammatory cytokines is the major cause of cerebral edema and neuronal loss. This study was designed to examine changes in concentrations of some proinflammatory cytokines—including interleukin-1 beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), and transforming growth factor-beta (TGF-β)—in a rat model of TBI in which the animals were treated with different doses of estrogen or progesterone 6 and 24 h after the TBI. Adult female rats were divided into 14 groups. Hormones or vehicle were given intraperitoneally 30 min after a moderate TBI was induced by the Marmarou method. The levels of proinflammatory cytokines in brain were measured at 6 and 24 h after the TBI. A high dose of estrogen (E2) or a low dose of progesterone (P1) increased brain levels of IL-1β 52.7% and 79.2% respectively at 6 h after the TBI. By 24h, IL-1β levels in the brain were 27.5% and 27% lower following administration of estrogen low dose (E1) or E2, respectively. High-dose administration of progesterone reduced brain levels of IL-6 to 45.9% at 6 h after the TBI, and P1 and E1 treatment significantly decreased IL-6 levels at 24 h. Brain levels of TNF-α were 72.5% lower at 6 h after the TBI following P2 treatment and 48.5% higher at 24 hrs following treatment with E2. The levels of TGF-β were also 3.37 times higher 24 h after the TBI following treatment with E1. Both doses of the hormones tested increases TGF-β levels 6 h after the TBI. Based on our findings, we conclude that progesterone and estrogen influence the levels of proinflammatory cytokines either at the primary or secondary stages after a TBI. Accordingly, this study suggests a mechanism by which hormones reduce cerebral edema.

Key words: traumatic brain injury; IL-1β; IL-6; TNF-α; TGF-β

Introduction

Although intracranial inflammatory responses induced by a traumatic brain injury (TBI) or brain ischemia play crucial roles in wound healing processes, they are the leading cause of secondary brain injuries following a TBI (Morganti-Kossmann et al., 2002; Rothwell and Hopkins, 1995). Brain contusion-induced inflammation contributes to these secondary brain injuries (Holmin and Mathiesen, 2000). Brain contusions induce functional disorders mostly through edema. Experimental studies have shown that uncontrolled brain edema is a major cause of many of the disabilities and mortalities that accompany TBIs (Stein DG, 2008).

Central nervous system events following a brain trauma occur during the acute or chronic recovery phases (Morrison et al., 1998). The acute phase is sub-divided into a primary and secondary step. In primary step, the injury results from the trauma itself, whereas in the secondary step, the injury is caused by release of chemical factors and cytokines. The onset of the chronic recovery phase depends on the development of the injury and the expression of important mediators (e.g., cytokines) for tissue reconstruction (McIntosh et al., 1998; Morrison et al., 1998). Brain edema formation, compression of the blood brain barrier, release of inflammatory cytokines and growth factors are among the late responses to a traumatic injury. A complex cytokine network forms around the traumatized region within the brain during both the primary and secondary (late) phases of recovery (Morganti-Kossmann et al., 2002).

Cytokines like interleukin-1 beta (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α) promote inflammatory responses, whereas cytokines such as interleukin 4 (IL-4) and

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interleukin 10 (IL-10) inhibit inflammatory responses in the central nervous system (Olsson, 1995). Elevated levels of IL-1β and TNF-α have been observed in the cerebrospinal fluid of brain parenchyma tissues following brain traumas (Ross et al., 1994). These increased levels of these proinflammatory cytokines can clearly intensify the brain lesions that occur during ischemia, trauma, or cytotoxic brain injuries (Lawrence et al., 1998). The production of proinflammatory cytokines after a TBI has been reported in animal models (Taupin et al., 1993; Woodroofe et al., 1991) and humans (Morganti-Kossman et al., 1997; Ross et al., 1994).

Central administration of IL-1β leads to brain edema and cell death (Holmin and Mathiesen, 2000). Therefore, the measurement of cytokines within the brain aids in the understanding of the inflammatory responses induced by TBI. Several studies have shown that IL-1β, IL-6, and TNF-α are among the main mediators of neural inflammation (Holmin and Hojeberg, 2004; Morganti-Kossmann et al., 2002; Rothwell and Hopkins, 1995). Up-regulation of transforming growth factor-beta (TGF-() after a TBI regulates activation of the brain vessel endothelium (Dore-Duffy et al., 1994; McIntosh et al., 1998), which in turn prevents the influx of leukocytes across the blood brain barrier (Fee et al., 2004; Hoffman et al., 2006; Hussein et al., 2004).

Recent studies indicated that estrogen and progesterone significantly decrease brain edema and increase functional recovery after a trauma. For example, edema was not found in female animals with hyper-progestinemia (Roof et al., 1993). An inverse correlation was observed between levels of progesterone in serum and brain edema following a TBI (Wright et al., 2001). Our previous study showed that progesterone and its metabolite allopregnanolone are effective in the reduction of brain edema (Ahmad molaie et al., 2007). The neuroprotective properties of estradiol on brain ischemia have been observed in female rats (Won et al., 2006). Estrogen and progesterone can decrease vasogenic edema after TBIs (Ahmad molaie et al., 2007; Hoffman, 2001). Estrogen and progesterone reduce brain edema, blood brain barrier permeability, and intracranial pressure after TBIs (Shahrokhi et al., 2010). Simultaneous use of estrogen and progesterone decreased brain edema and improved neurologic outcomes in an experimental brain trauma model (Soltani et al., 2009). In previous studies, that research group showed sex steroid hormones reduce neural inflammation. In the present study, to determine the mechanism underlying the anti-inflammation effects of these hormones, we measured the levels of proinflammatory cytokines (TGF-β, TNF-α, IL-6, and IL-1β) after brain trauma induced by the Marmarou method in ovariectomized rats. We evaluated the effects of estrogen and progesterone on the levels of brain cytokines at 6 and 24 hours after the TBI. We used these two time points because cytokine expression changes over the different phases of trauma (Morganti-Kossmann et al., 2002; Rothwell and Hopkins, 1995), the inhibitory effects of progesterone on the production of inflammatory cytokines is time dependent (He et al., 2004), and the inhibitory effects of estrogen on IL-1β production occurs over two steps (Das et al., 2002).

Methods

Animals

This study was conducted in accordance with the guidelines for animal experiments of Kerman University of Medical Sciences. The study protocols were approved by the ethical committee (No: EC/KNRC/87-19) of the University. Mature female Albino N-Mari rats (weighing 200–250 g) were housed in an air conditioned room at 22–25°C, with a 12-hour light/dark cycle. Animals had free access to food and water.

Bilateral ovariectomy procedures

The animals were anesthetized using 60 mg/kg thiopental intraperitonealy (i.p.). The sub-abdominal region was shaved and an incision of 2 centimeter was created. The skin, fascia, and abdominal muscles were opened, and fats and the intestine were sheared off until the uterus and its tubes became apparent. Then, the tube of the uterus and vascular base of the ovaries were twisted by a 4 Cat coot thread around the proximal area and were cut at the distal area. Finally, 1–2 ml of saline solution was poured into the abdomen and the muscles and skin were replaced and stitched by 0–2 aught silk suture. The wound was washed with Betadine solution. To avoid confounds from different effects on estrus cycles, all experimental animals were ovariectomized (OVX) two weeks before the experiments (Crandall et al., 2006).

Experimental protocols

Adult female rats were randomly divided into 7 groups consist of two subgroups. The experimental groups were as follows: 1) sham surgery group: received no vehicle or drug, 2) TBI group: OVX rats that underwent brain traumas, 3) vehicle- treated group: OVX rats that received equal-volume injections of estrogen or progesterone vehicle (sesame oil containing 1% benzyl alcohol) after the TBI, 4) E1-treated group: OVX rats that received physiologic doses of 17-β estradiol (33.3 μg/kg), 5) E2-treated group: OVX rats that received pharmacologic doses of 17-β estradiol (1 mg/kg), 6) P1-treated group: OVX rats that received physiologic doses of progesterone (1.7 mg/kg), 7) P2-treated group: OVX rats that received pharmacologic doses of progesterone (8 mg/kg). 17-β estradiol or progesterone were given as a single-dose i.p. 30 min after the TBI (Ahmad molaie et al., 2007; O'Connor et al., 2005). 17-β estradiol, progesterone, and their vehicles were purchased from Aburaihan pharmaceuticals (Tehran, Iran).

Brain IL-1β, IL-6, TNF-α, TGF-β, 17β-estradiol, and progesterone were measured in all groups at 6 or 24 hours after the TBI.

Diffuse TBI model

All animals were intubated before the TBI. The TBI was a moderate and diffuse injury based on the Marmarou method (O'Connor et al., 2005). The TBI induction device was made by the Department of Physiology of the Kerman University of Medical Sciences. The protocol was as follows: a 250g weight was dropped from 2 meters above the head of the anesthetized rat while a metal disc (stainless steel) 10mm in diameter and 3mm thick was attached on the animals' skull. Following induction of the TBI, all rats were immediately connected to a respiratory pump (TSA animal respiratory compact, Germany). Following restoration of spontaneous breathing, the intratracheal tube was removed. After recovery, each rat was housed in an individual cage.

Measurement of brain cytokines and hormones

Rats received an overdose of thiopental (50 mg/kg, i.p) 6 or 24 hours after a TBI (Dubal et al., 2001; Holmin and Hojeberg, 2004), and were perfused intracardially with phosphate buffered saline (pH = 7.4) for 1min to remove vesicular blood. Then, each brain was removed quickly and immediately frozen in liquid nitrogen. The brain was weighed and homogenized in T-PER^{TM} tissue protein extraction reagent with 0.5% Triton-x100, 150 mM NaCl, 50 mM tris, and a protease inhibitor cocktail (500 mg tissue per 2 ml of the reagent). Following homogenization, samples were shaken on a shaker (Behdad, Iran) for 90 min and then centrifuged (Rotina, Germany) at 4°C and 4000 × g for 15 min, and the supernatant was collected as a homogenate. The protein content of the supernatant

was estimated using a protein assay reagent kit to ensure an equal amount of protein from each sample was used for the assay (Taupin et al., 1993).

ELISA kits for IL-1β, IL-6, TNF-α, TGF-β, 17β-estradiol, and progesterone were purchased from American BMS and the assays were performed according to the manufacturer's guidelines. The concentrations of the cytokines were quantified as picograms or nanograms of antigen per milliliter of the supernatant.

Statistical analysis

All quantitative data are expressed as the mean ± the standard error of the mean (SEM). If a repeated measures two-way analysis of variance (ANOVA) revealed a significant interaction between the group and time factors, comparisons of changes between groups or across time were assessed separately by a parametric one-way ANOVA. One-way ANOVA was used for concentration analyses. Fisher's least square difference was employed for ANOVA post-hoc analysis. Comparisons of changes in cytokines levels at different times were analyzed by independent t-tests. All differences were considered significant at $P < 0.05$.

Results

Changes in brain IL-1β levels at 6 and 24 hours after TBI

Because two-way repeated measures ANOVA revealed a significant interaction between treatment group and time for IL-1β (across time $P < 0.01$, interaction between group and time $P < 0.001$, and between group $P < 0.001$), the results of statistical analysis by one-way ANOVA are reported here.

Figure 1 shows the effects of different doses of progesterone and estrogen on the levels of brain IL-1β 6 and 24 hours after TBI. The brain levels of IL-1β were significantly increased by a high dose of estrogen (272.7 ± 17.8 pg/ml, $P < 0.01$) or a low dose of progesterone (319.8 ± 27.3 pg/ml, $P < 0.001$) compared to vehicle (178.7 ± 22.7 pg/ml) at 6 hours after TBI. This level was significantly decreased by a high dose (223.6 ± 30.1 pg/ml) or low dose (224.6 ± 32.8 pg/ml) of estrogen compared to vehicle at 24 hours after TBI. A TBI decreased the levels of IL-1β 6 hours later, but conversely increased IL-1β levels 24 hours later compared to sham surgeries. Therefore, diffuse TBI decreased brain IL-1β levels in the early stage but increased these levels in the delayed stage of trauma ($P < 0.01$) (fig 2). Significant differences in the vehicle group were not found 6 and 24 hours after TBI.

Reductions in brain IL-6 levels by sex steroids

Because two-way repeated measures ANOVA revealed a significant interaction between treatment group and time for IL-1β (across time $P < 0.01$, interaction between group and time $P < 0.001$, and between group $P < 0.001$), the results of statistical analysis by one-way ANOVA are reported here.

Figure 3 shows the effects of different doses of estrogen or progesterone on brain IL-6 levels 6 and 24 hours after the TBI. A high dose of progesterone decreased brain IL-6 levels (340.3 ± 52.7 pg/ml) compared to vehicle (629.1 ± 28.4 pg/ml, $P < 0.01$) at 6 hours after the TBI. Brain IL-6 levels significantly differed following high or low dose progesterone treatments ($P < 0.05$). These levels decreased following a low dose of progesterone (852.4 ± 99.1 pg/ml, $P < 0.05$) or a low dose of estrogen (563 ± 32.7 pg/ml, $P < 0.001$) compared to vehicle (1073 ± 46.1 pg/ml) at 24 hours after the TBI. Therefore, estrogen decreased brain IL-6 levels during the delayed stage of trauma. TBI increased brain IL-6 levels at 6 hours after the TBI, but IL-6 levels were conversely lower at 24 hours after the TBI compared to sham surgeries ($P < 0.05$) (fig.2). There were no significant differences in the vehicle groups 6 and 24 hours after the TBI.

Changes in brain TNF-α levels 6 and 24 hours after the TBI

Because two-way repeated measures ANOVA revealed a significant interaction between treatment group and time for TNF-α (across time $P < 0.001$, interaction between group and time $P < 0.05$, and between group $P < 0.001$), the results of statistical analysis by one way ANOVA are reported here.

Fig. 4 shows the effects of different doses of estrogen or progesterone on TNF-α levels in the brain 6 and 24 hours after the TBI. A high dose of progesterone decreased brain TNF-α levels (80.4 ± 0.09 pg/ml) compared to vehicle (287.2 ± 42.6 pg/ml), at 6 hours after the TBI ($p < 0.01$). A high dose of estrogen increased brain TNF-α levels (902.1 ± 248.5 pg/ml) compared to vehicle

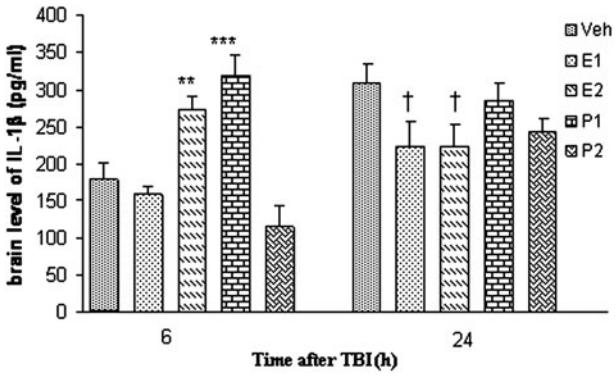

FIG. 1. Effects of estrogen and progesterone on brain level of IL-1β following TBI in OVX female rats (n = 7/ group). IL-1β was measured at 6 and 24 h post injury. ***$P < 0.001$as compared to veh at 6 h post injury. **$P < 0.01$ as compared to veh at 6 h post injury.†$P < 0.05$ as compared to veh at 24 h post injury. Values represent mean ± SEM. Veh: vehicle; E1: physiological dose of estrogen; E2: pharmacological dose of estrogen; P1 : physiological dose of progesterone ; P2 :pharmacological dose of progesterone.

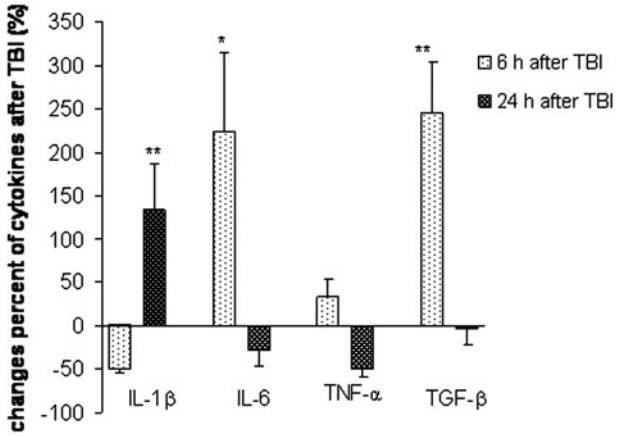

FIG. 2. Changes percent of IL-1β, IL-6, TNF-α, and TGF-β following TBI in OVX female rats compared to sham (n = 7/ group). Cytokines were measured at 6 and 24 h post injury. **$P < 0.01$ as compared cytokines groups, at 6 and 24 h post injury. *$P < 0.05$ as compared cytokines groups, at 6 and 24 h post injury. Values represent mean ± SEM.

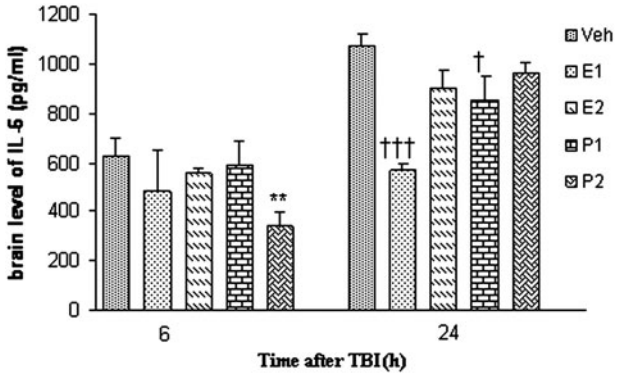

FIG. 3. Effects of estrogen and progesterone on brain level of IL-6 following TBI in OVX female rats (n = 7/group). IL-6 was measured at 6 and 24 h post injury. **P < 0.01 as compared to veh at 6 h post injury. †††P < 0.001 as compared to veh at 24 h post injury. †P < 0.05 as compared to veh at 24 h post injury. Values represent mean ± SEM. Veh: vehicle; E1: physiological dose of estrogen; E2: pharmacological dose of estrogen; P1 : physiological dose of progesterone ;P2 : pharmacological dose of progesterone.

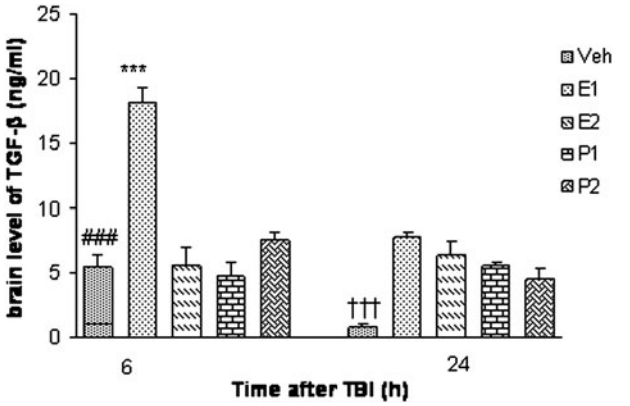

FIG. 5. Effects of estrogen and progesterone on brain level of TGF-β following TBI in OVX female rats (n = 7/group). TGF-β was measured at 6 and 24 h post injury. †††P < 0.001 as compared to all groups at 24 h post injury. ***P < 0.001 as compared to all groups at 6 h post injury. $^{\#\#\#}$P < 0.001 as compared vehicle groups, at 6 and 24 h post injury. Values represent mean ± SEM. Veh: vehicle; E1: physiological dose of estrogen; E2: pharmacological dose of estrogen; P1: physiological dose of progesterone; P2: pharmacological dose of progesterone.

(607.2 ± 46.9 pg/ml, P < 0.05), at 24 hours after the TBI. Brain TNF-α levels were higher at 6 hours and lower at 24 hours after the TBI compared to sham surgeries (fig2). After the TBI, the levels of TNF-α in were higher in the vehicle group at 24 hours compared to at 6 hours (p < 0.001).

Sex steroids increased brain TGF-β levels after the TBI

Because two-way repeated measures ANOVA revealed a significant interaction between treatment group and time for TGF-β (across time P < 0.001, interaction between group and time P < 0.001, and between group P < 0.001), the results of statistical analysis by one way ANOVA are reported here.

Fig. 5 shows the effects of different doses of sex steroids on brain TGF-β levels at different times. A low dose of estrogen increased brain TGF-β levels (18.2 ± 1.1 ng/ml) compared to vehicle (P < 0.001) at 6 hours after the TBI, and there was a significant difference between the group that received a low dose of estrogen and other groups (P < 0.001). Different doses of estrogen and progesterone increased brain TGF-β levels compared to vehicle across time (P < 0.001). These levels were higher at 6 hours but lower at 24 hours after the TBI compared to sham surgeries (P < 0.01)(fig2). Moreover, these levels decreased in the vehicle group over time (P < 0.001).

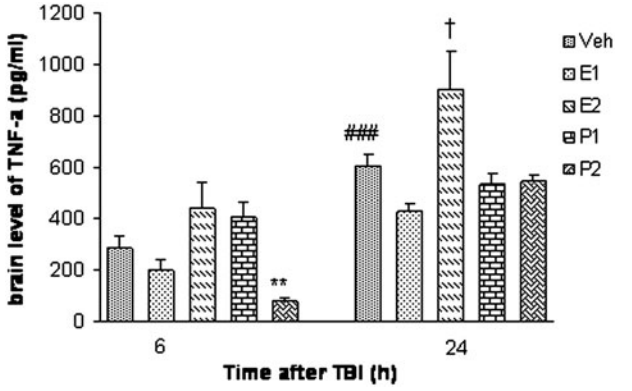

FIG. 4. Effects of estrogen and progesterone on brain level of TNF-α following TBI in OVX female rats (n = 7/group). TNF-α was measured at 6 and 24 h post injury. **P < 0.01 as compared to veh at 6 h post injury. $^{\#\#\#}$P < 0.001 as compared vehicle groups, at 6 and 24 h post injury. †P < 0.05 as compared to veh at 24 h post injury. Values represent mean ± SEM. Veh: vehicle; E1: physiological dose of estrogen; E2: pharmacological dose of estrogen; P1: physiological dose of progesterone; P2: pharmacological dose of progesterone.

Changes in brain estrogen and progesterone levels

Because two-way repeated measures ANOVA revealed a significant interaction between treatment group and time for estradiol and progesterone (across time P < 0.01, interaction between group and time P < 0.05, and between group P < 0.001 for estradiol; and across time P < 0.001, interaction between group and time P < 0.01, and between group P < 0.001 for progesterone), the results of statistical analysis by one way ANOVA are reported here.

Fig. 6 and 7 show changes in 17β-estradiol and progesterone across the different groups in this study 6 and 24 hours after the TBI. There were no significant differences in the brain levels of 17β-estradiol at the different time points after the TBI. The highest 17β-estradiol level was observed following high dose of estrogen at 6 hours (1619.7 ± 484.9 pg/ml) and at 24 hours (608.7 ± 403 pg/ml) after the TBI (P < 0.001). The brain 17β-estradiol levels decreased after injection of estrogen over time (P < 0.001). The levels of progesterone in the brain significantly decreased over time and were 27.9 ± 2.5 ng/ml at 6 hours and 12.3 ± 0.51 ng/ml at 24 hours after the TBI (P < 0.001) in the vehicle groups. A high dose of progesterone increased the levels of progesterone in the brain at both 6 hours (43.6 ± 104 ng/ml) and 24 hours (27.7 ± 306 ng/ml) after the TBI (P < 0.01).

Discussion

Studies have suggested that sex steroid hormones inhibit the inflammatory edemas caused by TBI, and provide neuroprotection

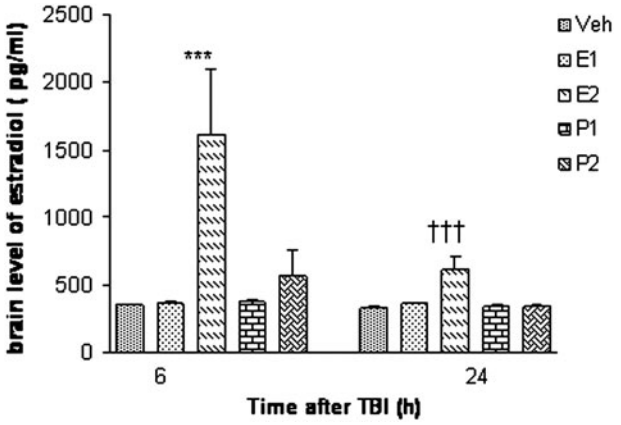

FIG. 6. Effects of estrogen administration on brain level of β-estradiol following TBI in OVX female rats (n = 7/group). β-estradiol was measured at 6 and 24 h post injury. ***$P < 0.001$ as compared to veh at 6 h post injury. $^{\dagger\dagger\dagger}P < 0.001$ as compared to veh at 24 h post injury. Values represent mean ± SEM. Veh: vehicle; E1: physiological dose of estrogen; E2: pharmacological dose of estrogen; P1: physiological dose of progesterone; P2: pharmacological dose of progesterone.

after a TBI. We examined the hypothesis that these sex steroids inhibit inflammation by influencing cytokine concentrations.

Brain IL-1β levels after the TBI

Our results show that high doses of estrogen and low doses of progesterone increase brain IL-1β levels in the primary stage of the acute phase of the injury (i.e., 6 hours after the TBI). Twenty four hours after the TBI (secondary step), these hormones produced opposite effects on brain IL-1β levels. These data suggest that the effects of estrogen and progesterone on the levels of IL-1β are time and dose dependent. TBIs lead to a 51% decrease in IL-1β during the primary step of the acute phase of the injury, whereas there

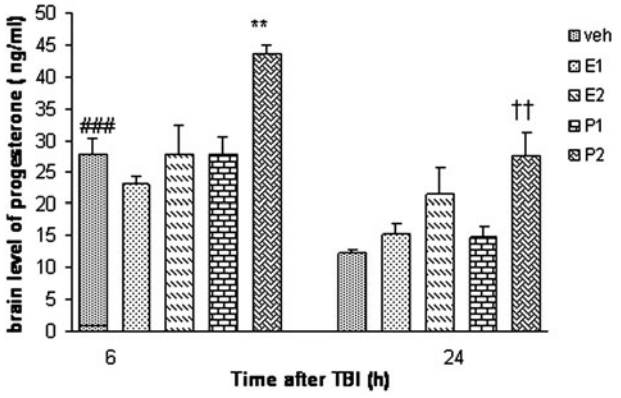

FIG. 7. Effect of progesterone administration on brain level of progesterone following TBI in OVX female rats (n = 7/ group). Progesterone was measured at 6 and 24 h post injury. ###$P < 0.001$ as compared vehicle groups, at 6 and 24 h post injury. **$P < 0.01$ as compared to veh at 6 h post injury. $^{\dagger\dagger}P < 0.01$ as compared to veh at 24 h post injury. Values represent mean ± SEM. Veh: vehicle; E1: physiological dose of estrogen; E2: pharmacological dose of estrogen; P1: physiological dose of progesterone; P2: pharmacological dose of progesterone.

is 1.3 fold increase in IL-1β in the secondary step of the acute phase of the injury.

Alterations in the levels of cytokines in different models of injury—such as frontal brain ischemia (Taupin et al., 1993), injuries from fluid-percussion (Fan et al., 1996; Taupin et al., 1993), spinal cord injury (Streit et al., 1998), and experimental TBIs (McIntosh et al., 1998; Morrison et al., 1998)—have been reported. The changes in cytokines level usually start 30 minutes after a brain injury and continue for at least 24 hours after the trauma. In this study, we showed decreased levels of IL-1β 6 hours after the TBI in rats, which is in agreement with the observations of LaMarca et.al. (2007) in mice after brain ischemia (LaMarca et al., 2007), of Minami et.al. (1992) in rats after transitional ischemia, and with those made after brain injuries and TBIs (Bartfai et al., 2007; Jones et al., 2005a; Konsman et al., 2007). One possible mechanism for elevated IL-1β levels in the presence of estrogen or progesterone at 6 hours after the TBI could be that we used ineffective dose of these hormones in this model (Gibson et al., 2005; Holmin and Hojeberg, 2004; Jones et al., 2005a; Lacut et al., 2003). Alternatively, it may derive from the regulating effects of these hormones on inflammatory responses (Cutolo et al., 2006). Several studies have reported that estrogen (Das et al., 2002; Houdeau et al., 2007) or progesterone (Chen et al., 2008; Gibson et al., 2005; He et al., 2004) decrease IL-1β levels after a brain injury. The probable reasons for the discrepancies with the presently reported results may be the type and severity of the injury, the dose and the treatment regimen of the hormones, and the type of vehicle used.

On the other hand, decreased production of IL-1β in the presence of estrogen at 24 hours after trauma occurred concurrently with induction of its mRNA expression and was followed in turn by the maximum production of this cytokine (Hans et al., 1999). Whereas the concentration of IL-1β in patients with higher ICP is more than those with lower ICP (Shiozaki et al., 2005), inhibition of IL-1β production prevents the neuronal injury caused by TBI (Lu et al., 2007). Administration of an IL-1β receptor antagonist following TBI decreases neuronal inflammation (Jones et al., 2005b). As such, we assumed that the altered levels of IL-1β by estrogen at either dose tested were the mechanism for the anti-inflammatory action of estrogen and its other neuroprotective effects. The decreased levels of IL-1β following estrogen treatment in our model are consistent with other studies (Das et al., 2002; Houdeau et al., 2007; Schaefer et al., 2005), and the ineffectiveness of progesterone on the levels of IL-1β has been confirmed by a number of previous researchers (Jones et al., 2005a). Progesterone presumably exerts its anti-edema effects through other mechanisms (Gibson et al., 2005; Hoffman et al., 2006; Lenzlinger et al., 2001). Some studies have indeed proposed that decreasing effects of progesterone on the levels of IL-1β after a TBI, which is opposite to the findings of the present study (Chen et al., 2008; Gibson et al., 2005; He et al., 2004). Moreover, it has been reported that estrogen in the primary step of the acute phase of the injury increases but in the next step of the acute phase decreases the levels of IL-1β, whereas progesterone has only unitary effects (Das et al., 2002). Therefore, according to the results of our work, as only estrogen has inhibitory effects on IL-1β, reduced IL-1β levels is likely the mechanism for the neuroprotective effects of estrogen either in the primary or secondary steps of an injury, whereas progesterone is only neuroprotective in the primary step of the acute phase of recovery after a TBI.

The high concentration of brain β-estradiol in the groups treated with a high dose of estrogen probably led to the effectiveness of a high dose of estrogen 6 h after the TBI. The effectiveness of both

doses of the hormones at 24h after the TBI is probably related to brain physiologic concentrations of estrogen, as with progesterone.

Brain IL-6 levels *after a TBI*

Progesterone reduced IL-6 levels in both the primary step and secondary step of the acute phase. However, this effect depends on the dose of progesterone, as only the low dose of estrogen at 24 hours after the TBI decreased the levels of this cytokine. TBI increased the levels of IL-6 2.2 fold in the primary step of the acute phase of the injury, whereas 24 hours after the TBI, the concentration of this cytokine decreased to about 0.25 fold. Because progesterone in both the primary and secondary step decreased the levels of IL-6, it seems that this cytokine has more important role in the mediation of the anti-inflammatory actions of progesterone compared to estrogen. IL-6 induces loss of neurons, disturbances in BBB function, and vascular insufficiency accompanied by severe neurological disorders (Lenzlinger et al., 2001). The effects of progesterone in the different phases after a TBI depend on the dose used. This finding is consistent with the results of other researchers (Chen et al., 2008; Cutler et al., 2007). Moreover, other studies have also shown the decreasing effect of estrogen on the level of IL-6 (Hrekova et al., 2002; Jain et al., 2004).

The high concentration of brain progesterone in the group treated with a high dose of progesterone probably is because it reduced IL-6 levels 6 h after the TBI. In addition, the effectiveness of low doses of the hormones at 24 after TBI is probably related to brain physiologic concentration of brain hormones. A number of studies have reported opposite effects of estrogen (Crandall et al., 2006; Jonsson, 2007; Lacut et al., 2003) and progesterone (Crandall et al., 2006; Lacut et al., 2003) on IL-6 levels. The reasons for these different results may the type and severity of the injury, the regimen used for the hormones, and the type of vehicle used.

Brain TNF-α levels after a TBI

We showed that a high dose of progesterone in primary step of acute phase reduced TNF-α levels. In contrast, during the secondary step of the acute phase, progesterone lost its effectiveness and a high dose of estrogen increased the levels of TNF-α. Because it has been reported that rising TNF-α levels during the acute phase of recovery is harmful (Sullivan et al., 1999), the decreasing effects of progesterone on this cytokine may prevent the deleterious effects of TNF-α on the BBB, brain edema, white blood cell infiltration, and necrosis (Olsson, 1995; Ross et al., 1994). On the other hand, it has been reported that higher TNF-α levels in the secondary step after a TBI are beneficial because it increases NGF (Lenzlinger et al., 2001; Scherbel et al., 1999), thus estrogen probably has protective effects in the secondary step mediated by increased levels of TNF-α.

The TBI we induced caused a 30% increase in TNF-α levels 6 h later and a 50% decrease of this cytokine 24 h later. There were no significant differences in the levels of this cytokine at the different times after the TBI. Increasing TNF-α following a TBI has been reported to be a brain parenchyma response to the injury (Rostworowski et al., 1997).

It can be proposed that a high dose of progesterone in the acute primary step of the injury decrease TNF-α levels by decreasing IL-6 levels. The decreasing effects of progesterone on TNF-α in the primary step (Das et al., 2002) and increasing effects of estrogen and ineffectiveness of progesterone in the secondary step of the injury have been confirmed by other studies (Gibson et al., 2005; Houdeau et al., 2007; Jones et al., 2005a). Nevertheless, there are some differences between the results of our work and that of other researchers (Houdeau et al., 2007; Hrekova et al., 2002; Jain et al., 2004; Yuan et al., 2002).

Brain TGF-β levels after a TBI

A low dose of estrogen in the primary step of the acute phase of the injury caused elevated TGF-β levels. In the secondary step, both doses of the hormones increased TGF-β levels, but the potency of estrogen was greater than that of progesterone. Despite increasing TGF-β levels in the acute phase of the TBI, estrogen caused an additional enhancement of TGF-β levels. Since the anti-inflammatory effects of TGF-β are predominant over its inflammatory effect, one the anti- inflammatory mechanisms of sex steroids may be increasing TGF-β levels. A number of studies have described the effects of estrogen and progesterone on TGF-β levels (Gibson et al., 2005; Hatthachote and Gillespie, 1999). These hormones possibly increase TGF-β levels via an IL-1β pathway (Hoffman et al., 2006) and cytokines released from the brain and blood cells to prevent BBB damage (Lenzlinger et al., 2001). TBI increases TGF-β by nearly 250% within 6 h of the injury, but this effect has dissipated by 24 h after a TBI. Higher TGF-(levels occur during the first day of injury, (Jonsson, 2007; Shifren et al., 2008). TGF-β inhibits the production of IL-1β, TNF-α, and oxygen free radicals, which possibly exerts anti-inflammatory effects (Chiaretti et al., 2008). A biphasic pattern of TGF-β following trauma has been reported by Rimaniol et. al. (Rimaniol et al., 1995). There is also a report that shows an increase in TGF-β levels during the chronic phase of brain trauma (McIntosh et al., 1998).

Overall, the present study showed that at 6 h after TBI estrogen and progesterone increased IL-1β and TGF-β (only estrogen) levels, whereas progesterone decreased IL-6 and TNF-α levels. This may provide a mechanism by which evidence that treatment with these hormones should be conducted after a brain injury. It was also shown that sex steroids are effective in the primary step, and estrogen itself in the secondary step decreased IL-1β and IL-6 levels and increased TNF-α and TGF-β levels. Progesterone in the secondary step also decreased IL-6 and increased TGF-β levels. It seems that estrogen, mostly in the primary step, and progesterone, in the secondary step, regulate brain cytokine expression. Furthermore, these effects depend on the dose of the hormone used. Moreover, our results suggest that reduced levels of proinflammatory cytokines is one mechanism for the possible anti-inflammatory effects of sex hormones. However, other mechanisms may be involved in the neuroprotective effects of these hormones. In future studies we will further investigate the mechanisms by which sex steroids and proinflammatory cytokines interact in TBI.

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References

Ahmad molaie, L., Khaksari, M., Sepehri, G., Dabiri, S., Asadikaram, G., Mahmodi, M., and Shahrokhi, N. (2007). Comparison of the effects of progesterone, allopregnanolone and gender on supressing edema formation after traumatic brain injury. J. kerman university of Medical sciences 15, 47–59.

Bartfai, T., Sanchez-Alavez, M., Andell-Jonsson, S., Schultzberg, M., Vezzani, A., Danielsson, E., and Conti, B. (2007). Interleukin-1 system in CNS stress: seizures, fever and neurotrauma. Ann. N. Y. Acad. Sci. 1113, 173–17.

Chen, G., Shi, J., Jin, W., Wang, L., Xie, W., Sun, J., and Hang, C. (2008). Progesterone administration modulates TLRs/NF-kappaB signaling pathway in rat brain after cortical contusion. Ann. Clin. Lab. Sci. 38, 65–74.

Chiaretti, A., Antonelli, A., Riccardi, R., Genovese, O., Pezzotti, P., Di Rocco, C., Tortorolo, L., and Piedimonte, G. (2008). Nerve growth factor expression correlates with severity and outcome of traumatic brain injury in children. Eur. J. Paediatr. Neurol. 12, 195–204.

Crandall, C., Palla, S., Reboussin, B., Hu, P., Barrett-Connor, E., Reuben, D., and Greendale, G. (2006). Cross-sectional association between markers of inflammation and serum sex steroid levels in the postmenopausal estrogen/progestin interventions trial. J. Womens Health 15, 14–23.

Cutler, S. M., Cekic, M., Miller, D. M., Wali, B., VanLandingham, J. W., and Stein, D. G. (2007). Progesterone improves acute recovery after traumatic brain injury in the aged rat. J. Neurotrauma 24, 1475–1486.

Cutolo, M., Capellino, S., Sulli, A., Serioli, B., Secchi, M. E., Villaggio, B., and Straub, R. H. (2006). Estrogens and autoimmune diseases. Ann. N. Y. Acad. Sci. 1089, 538–547.

Das, C., Kumar, V. S., Gupta, S., and Kumar, S. (2002). Network of cytokines, integrins and hormones in human trophoblast cells. J. Reprod. Immunol. 53, 257–268.

Dore-Duffy, P., Balabanov, R., Washington, R., and Swanborg, R. H. (1994). Transforming growth factor beta 1 inhibits cytokine-induced CNS endothelial cell activation. Mol. Chem. Neuropathol. 22, 161–175

Dubal, D. B., Zhu, H., Yu, J., Rau, S. W., Shughrue, P. J., Merchenthaler, I., Kindy, M. S., and Wise, P. M. (2001). Estrogen receptor alpha, not beta, is a critical link in estradiol-mediated protection against brain injury. Proc. Natl. Acad. Sci. U. S. A. 98, 1952–1957.

Fan, L., Young, P. R., Barone, F. C., Feuerstein, G. Z., Smith, D. H., and McIntosh, T. K. (1996). Experimental brain injury induces differential expression of tumor necrosis factor-alpha mRNA in the CNS. Brain Res. Mol. Brain. Res. 36, 287–291.

Fee, D. B., Sewell, D. L., Andresen, K., Jacques, T. J., Piaskowski, S., Barger, B. A., Hart, M. N., and Fabry, Z. (2004). Traumatic brain injury increases TGF beta RII expression on endothelial cells. Brain Res. 1012, 52–59.

Gibson, C. L., Constantin, D., Prior, M. J., Bath, P. M., and Murphy, S. P. (2005). Progesterone suppresses the inflammatory response and nitric oxide synthase-2 expression following cerebral ischemia. Exp. Neurol.193, 522–530.

Hans, V. H., Kossmann, T., Joller, H., Otto, V., and Morganti-Kossmann, M. C. (1999). Interleukin-6 and its soluble receptor in serum and cerebrospinal fluid after cerebral trauma. Neuroreport 10, 409–412.

Hatthachote, P., and Gillespie, J. I. (1999). Complex interactions between sex steroids and cytokines in the human pregnant myometrium: evidence for an autocrine signaling system at term. Endocrinology 140, 2533–2540.

He, J., Evans, C. O., Hoffman, S. W., Oyesiku, N. M., and Stein, D. G. (2004). Progesterone and allopregnanolone reduce inflammatory cytokines after traumatic brain injury. Exp. Neurol. 189, 404–412.

Hoffman, G. E., Merchenthaler, I., and Zup, S. L. (2006). Neuroprotection by ovarian hormones in animal models of neurological disease. Endocrine 29, 217–231.

Hoffman, S. W., He, J., and Stein, D.G. (2001). Allopregnanolone promotes functional recovery from TBI by reducing cerebral edema and neuronal death. Acad. Emerg. Med. 8, 496–497.

Holmin, S., and Hojeberg, B. (2004). In situ detection of intracerebral cytokine expression after human brain contusion. Neurosci. Lett. 369, 108–114.

Holmin, S., and Mathiesen, T. (2000). Intracerebral administration of interleukin-1beta and induction of inflammation, apoptosis, and vasogenic edema. J. Neurosurg. 92, 108–120.

Houdeau, E., Moriez, R., Leveque, M., Salvador-Cartier, C., Waget, A., Leng, L., Bueno, L., Bucala, R., and Fioramonti, J. (2007). Sex steroid regulation of macrophage migration inhibitory factor in normal and inflamed colon in the female rat. Gastroenterology 132, 982–993.

Hrekova, S. P., Vodianyk, M. O., & Chernyshov, V. P. (2002). Effect of progesterone and 17beta-estradiol on proinflammatory cytokine costimulatory proliferative activity. Fiziol. Zh. 48, 63–69.

Hussein, M. Z., Al Fikky, A., Abdel Bar, I., and Attia, O. (2004). Serum IL-6 and IL-12 levels in breast cancer patients. Egypt. J. Immunol. 11, 165–170.

Jain, S. K., Kannan, K., Prouty, L., and Jain, S. K. (2004). Progesterone, but not 17beta-estradiol, increases TNF-alpha secretion in U937 monocytes. Cytokine 26, 102–105.

Jones, N. C., Constantin, D., Prior, M. J., Morris, P. G., Marsden, C. A., and Murphy, S. (2005). The neuroprotective effect of progesterone after traumatic brain injury in male mice is independent of both the inflammatory response and growth factor expression. Eur. J. Neurosci. 21, 1547–1554.

Jones, N. C., Prior, M. J., Burden-Teh, E., Marsden, C. A., Morris, P. G., and Murphy, S. (2005). Antagonism of the interleukin-1 receptor following traumatic brain injury in the mouse reduces the number of nitric oxide synthase-2-positive cells and improves anatomical and functional outcomes. Eur. J. Neurosci. 22, 72–78.

Jonsson, D. (2007). The biological role of the female sex hormone estrogen in the periodontium–studies on human periodontal ligament cells. Swed. Dent. J. Suppl. 11–54.

Konsman, J. P., Drukarch, B., and Van Dam, A. M. (2007). (Peri)vascular production and action of pro-inflammatory cytokines in brain pathology. Clin. Sci. 112, 1–25.

Lacut, K., Oger, E., Le Gal, G., Blouch, M. T., Abgrall, J. F., Kerlan, V., Scarabin, P. Y., and Mottier, D. (2003). Differential effects of oral and transdermal postmenopausal estrogen replacement therapies on C-reactive protein. Thromb. Haemost. 90, 124–131.

LaMarca, B. D., Chandler, D. L., Grubbs, L., Bain, J., McLemore, G. R., Jr., Granger, J. P., and Ryan, M. J. (2007) Role of sex steroids in modulating tumor necrosis factor alpha-induced changes in vascular function and blood pressure. Am. J. Hypertens. 20, 1216–1221.

Lawrence, C. B., Allan, S. M., and Rothwell, N. J. (1998). Interleukin-1beta and the interleukin-1 receptor antagonist act in the striatum to modify excitotoxic brain damage in the rat. Eur. J. Neurosci. 10, 1188–1195.

Lenzlinger, P. M., Morganti-Kossmann, M. C., Laurer, H. L., and McIntosh, T. K. (2001). The duality of the inflammatory response to traumatic brain injury. Mol. Neurobiol. 24, 169–181.

Lu, K. T., Wu, C. Y., Yen, H. H., Peng, J. H., Wang, C. L., and Yang, Y. L. (2007). Bumetanide administration attenuated traumatic brain injury through IL-1 overexpression. Neurol. Res. 29, 404–409.

McIntosh, T. K., Juhler, M., and Wieloch, T. (1998). Novel pharmacologic strategies in the treatment of experimental traumatic brain injury. J. Neurotrauma 15, 731–769.

Morganti-Kossman, M. C., Lenzlinger, P. M., Hans, V., Stahel, P., Csuka, E., Ammann, E., Stocker, R., Trentz, O., and Kossmann, T. (1997). Production of cytokines following brain injury: beneficial and deleterious for the damaged tissue. Mol. Psychiatry. 2, 133–136.

Morganti-Kossmann, M. C., Rancan, M., Stahel, P. F., and Kossmann, T. (2002). Inflammatory response in acute traumatic brain injury: a double-edged sword. Curr. Opin. Crit. Care 8, 101–105.

Morrison, B., 3rd, Saatman, K. E., Meaney, D. F., and McIntosh, T. K. (1998). In vitro central nervous system models of mechanically induced trauma: a review. J. Neurotrauma 15, 911–928.

O'Connor, C. A., Cernak, I., and Vink, R. (2005). Both estrogen and progesterone attenuate edema formation following diffuse traumatic brain injury in rats. Brain Res. 1062, 171–174.

Olsson, T. (1995). Critical influences of the cytokine orchestration on the outcome of myelin antigen-specific T-cell autoimmunity in experimental autoimmune encephalomyelitis and multiple sclerosis. Immunol. Rev. 144, 245–268.

Rimaniol, A. C., Lekieffre, D., Serrano, A., Masson, A., Benavides, J., and Zavala, F. (1995). Biphasic transforming growth factor-beta production flanking the pro-inflammatory cytokine response in cerebral trauma. Neuroreport 7, 133–136.

Roof, R. L., Duvdevani, R., and Stein, D. G. (1993). Gender influences outcome of brain injury: progesterone plays a protective role. Brain Res, 607, 333–336.

Ross, S. A., Halliday, M. I., Campbell, G. C., Byrnes, D. P., and Rowlands, B. J. (1994). The presence of tumour necrosis factor in CSF and plasma after severe head injury. Br. J. Neurosurg. 8, 419–425.

Rostworowski, M., Balasingam, V., Chabot, S., Owens, T., and Yong, V. W. (1997). Astrogliosis in the neonatal and adult murine brain post-trauma: elevation of inflammatory cytokines and the lack of requirement for endogenous interferon-gamma. J. Neurosci. 17, 3664–3674

Rothwell, N. J., and Hopkins, S. J. (1995). Cytokines and the nervous system II: Actions and mechanisms of action. Trends Neurosci. 18, 130–136.

Schaefer, T. M., Wright, J. A., Pioli, P. A., and Wira, C. R. (2005). IL-1beta-mediated proinflammatory responses are inhibited by estradiol via

down-regulation of IL-1 receptor type I in uterine epithelial cells. J. Immunol. 175, 6509–6516.
Scherbel, U., Raghupathi, R., Nakamura, M., Saatman, K. E., Trojanowski, J. Q., Neugebauer, E., Marino, M. W., and McIntosh, T. K. (1999). Differential acute and chronic responses of tumor necrosis factor-deficient mice to experimental brain injury. Proc. Natl. Acad. Sci. U. S. A. 96, 8721–8726.
Shahrokhi, N., Khaksari, M., Soltani, Z., Mahmoodi, M., and Nakhaee, N. (2010). effect of sex steroid hormones on brain edema, intracranial pressure and neurologic outcome after traumatic brain injury. Can. J. Physiol. Pharmacol. 88, 414–421.
Shifren, J. L., Rifai, N., Desindes, S., McIlwain, M., Doros, G., and Mazer, N. A. (2008). A comparison of the short-term effects of oral conjugated equine estrogens versus transdermal estradiol on C-reactive protein, other serum markers of inflammation, and other hepatic proteins in naturally menopausal women. J. Clin. Endocrinol. Metab. 93, 1702–171.
Shiozaki, T., Hayakata, T., Tasaki, O., Hosotubo, H., Fuijita, K., Mouri, T., Tajima, G., Kajino, K., Nakae, H., Tanaka, H., Shimazu, T., and Sugimoto, H. (2005). Cerebrospinal fluid concentrations of anti-inflammatory mediators in early-phase severe traumatic brain injury. Shock 23, 406–410.
Soltani, Z., Khaksari, M., Shahrokhi, N., Nakhaeei, N., and Shaibani, V. (2009). Effect of Combined Administration of Estrogen and Progesterone on Brain Edema and Neurological Outcome after Traumatic Brain Injury in Female Rats. Iranian Journal of Endocrinology and Metabolism 10, 629–638.
Stein, D.G., Wright, D. W., and Kellermann, A.L. (2008). Does progesterone have neuroprotective roperties? Ann. Emerg. Med. 51, 164–172.
Streit, W. J., Semple-Rowland, S. L., Hurley, S. D., Miller, R. C., Popovich, P. G., and Stokes, B. T. (1998). Cytokine mRNA profiles in contused spinal cord and axotomized facial nucleus suggest a beneficial role for inflammation and gliosis. Exp. Neurol. 152, 74–87.
Sullivan, P. G., Bruce-Keller, A. J., Rabchevsky, A. G., Christakos, S., Clair, D. K., Mattson, M. P., and Scheff, S. W. (1999). Exacerbation of damage and altered NF-kappaB activation in mice lacking tumor necrosis factor receptors after traumatic brain injury. J. Neurosci. 19, 6248–6256.
Taupin, V., Toulmond, S., Serrano, A., Benavides, J., and Zavala, F. (1993). Increase in IL-6, IL-1 and TNF levels in rat brain following traumatic lesion. Influence of pre- and post-traumatic treatment with Ro5 4864, a peripheral-type (p site) benzodiazepine ligand. J. Neuroimmunol. 42, 177–185.
Won, C. K., Kim, M. O., and Koh, P. O. (2006). Estrogen modulates Bcl-2 family proteins in ischemic brain injury. J. Vet. Med. Sci. 68, 277–280.
Woodroofe, M. N., Sarna, G. S., Wadhwa, M., Hayes, G. M., Loughlin, A J., Tinker, A., and Cuzner, M. L. (1991). Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: evidence of a role for microglia in cytokine production. J. Neuroimmunol. 33, 227–236.
Wright, D. W., Bauer, M. E., Hoffman, S. W., and Stein, D. G. (2001). Serum progesterone levels correlate with decreased cerebral edema after traumatic brain injury in male rats. J. Neurotrauma 18, 901–909.
Yuan, K., Wing, L. Y., and Lin, M. T. (2002). Pathogenetic roles of angiogenic factors in pyogenic granulomas in pregnancy are modulated by female sex hormones. J. Periodontol. 73, 701–708.

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