

## Inhibitory Effect of Progesterone on Inflammatory Factors After Experimental Traumatic Brain Injury

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**Objective** Traumatic brain injury (TBI) is one of the leading causes of morbidity and mortality in young people. Inflammatory cytokines play an important part in the pathophysiology of TBI. Recent studies demonstrate that progesterone significantly reduces cerebral edema and enhances functional recovery from TBI and stroke in several animal models. This study was designed to investigate the inhibitory effect of progesterone on inflammatory response after traumatic brain injury. **Methods** Progesterone was injected intraperitoneally using rats as a model of traumatic brain injury, and Western blot technique was applied to detect the expression of three inflammation-related factors: nuclear factor kappa B p65 (NFκB p65), glial fibrillary acidic protein (GFAP), and tumor necrosis factor-α (TNF-α). The water content of injured brain was also examined. A neurological severity score was recorded to evaluate the effect of progesterone on neurodeficit recovery. **Results** NFκB p65, GFAP, and TNF-α were increased in all injured animals. In rats treated with progesterone, the expression level of NFκB p65 and TNF-α were reduced significantly in comparison with vehicle-treated rats. However, progesterone did not alter the expression of GFAP in the injured rats. Progesterone also reduced the water content of injured brain and the lesion volume. In addition, progesterone-treated injured rats showed significant improvements in the Neurological Severity Score test, compared with vehicle-treated ones. **Conclusions** Progesterone inhibits the inflammatory response after experimental traumatic brain injury and mitigates the severity of brain damage.

**Key words:** Traumatic brain injury; Progesterone; Inflammation

### INTRODUCTION

Traumatic brain injury (TBI) is one of the leading causes of morbidity and mortality in young people, for which there is still no effective clinical treatment to counteract the secondary neurodegenerative processes. Recent studies have strongly suggested a crucial for inflammatory agents in acute post-traumatic neuronal degeneration<sup>[1]</sup>. During the early stage after the trauma, the release of pro-inflammatory cytokines stimulates immune cells to become phagocytic. Activation of immune cells, in turn, triggers the production of free radicals and additional pro-inflammatory compounds, such as cytokines, prostaglandins, extracellular matrix proteases, complement factors, cell adhesion molecules, and inducible nitric oxide synthase<sup>[2]</sup>. The expression of pro-inflammatory factors therefore attracts immune cells to cerebrovascular surfaces, where they increase vasopermeability, immune cell invasion, and further cytokines in the CNS. The inhibition of inflammation

may reduce cell death, gliosis, and edema.

Researches have shown that after contusion injury of the cerebral cortex, posttraumatic treatment with progesterone relieves edema and improves the recovery of cognitive performance<sup>[3]</sup>. Progesterone also reduces neuronal loss<sup>[4]</sup>, blood-brain barrier compromise<sup>[5]</sup>, and the mediators of inflammation<sup>[6]</sup>. While these studies indicate the neuroprotective properties of the steroid, the mechanism by which progesterone achieves these benefits is still unclear.

There might be quite a few ways by which progesterone produces its neuroprotective result. Progesterone can bind to some cellular receptors and alter their activity. For example, progesterone acts both as a sigma-1 receptor antagonist and as a GABA<sub>A</sub> receptor agonist. Both these actions reduce neuronal excitatory tone and down-regulate excitotoxicity after brain injuries. Though not a free radical scavenger, progesterone can inhibit oxidative damage in the CNS<sup>[5]</sup>. Excitotoxic by-products of oxidative insults trigger an inflammatory immune

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response by releasing cytokines and other inflammatory factors that contribute to brain edema and neuronal loss. As an anti-inflammatory agent, progesterone has been shown to inhibit the activity of the pro-inflammatory transcription factor, nuclear factor kappa B (NF $\kappa$ B)<sup>[6]</sup>. NF $\kappa$ B is an upstream regulator of inflammation which activates TBI-induced inflammatory cytokines, such as tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-1 beta (IL-1 $\beta$ ), C3, and glial fibrillary acidic protein (GFAP)<sup>[7]</sup>. Considering the increasing evidence that inflammatory factors TNF $\alpha$ , IL-1 $\beta$ , and NF $\kappa$ B all contribute to brain injury pathology<sup>[2]</sup>, the interaction of progesterone with the cascade of inflammation is worth studying further.

In this study, we focused on some of the cellular mechanisms mediating the neuroprotective effects of progesterone, aiming to understand how functional recovery occurs after this neurosteroid is injected.

## MATERIALS AND METHODS

### *Animal Models*

Sixty-eight male Sprague-Dawley (SD) rats, weighing 250-300 g, were obtained from Animal Center of Zhejiang University, Hangzhou, China. The rats were housed in temperature- and humidity-controlled animal quarters with a 12 h light/dark cycle, room temperature at 23°C $\pm$ 1°C and access to food and water ad lib. All procedures were approved by the Institutional Animal Care Committee, and were in accordance with the guidelines of the National Institutes of Health on the care and use of animals.

Each rat was anesthetized with sodium pentobarbital (50 mg/kg), and mounted in a stereotaxic device with its head in a horizontal position. Body core temperature (37°C) was maintained with a homeothermic heating blanket system. Under aseptic conditions, a midline incision was made in the scalp, and the cranium was exposed. At the right side, a 3-mm diameter craniotomy was performed 3 mm posterior and 2.5 mm lateral to the bregma. For rats receiving cortical contusion, a standardized parietal contusion was produced by letting a steel rod weighing 30 g with a flat end diameter of 1.8 mm fall onto the intact dura from a height of 30 cm (modified after the method of Feeney *et al.*)<sup>[8]</sup>. The rod was allowed to compress the tissue with a maximal depth of 3 mm, resulting in a contusion injury of the brain. After the impact, the fascia and scalp were sutured. The sham controls were also anesthetized and the cranium was exposed without craniotomy and contusion.

### *Drug Administration*

Progesterone (10 mg/kg) was dissolved in 25% 2-hydroxypropyl- $\beta$ -cyclodextrin (HBC, Roquette,

France)<sup>[9]</sup>. The experimental animals were divided randomly into four groups: sham+HBC (SH,  $n=11$ ), sham+progesterone (SP,  $n=11$ ), injury+HBC (IH,  $n=19$ ), and injury+progesterone (IP,  $n=19$ ). The animals received the first dose of either progesterone or an equal volume of HBC intraperitoneally 30 min after the injury. Subsequent injections were given at 6 h, 12 h, and 24 h after injury.

### *Hematoxylin and Eosin (HE) Staining and Morphological Analysis*

48 h after the surgery, 2 rats from each group were given an overdose of pentobarbital and decapitated. The whole brain was extracted and fixed in 4% formaldehyde for 24-48 h and then embedded in paraffin. Coronary sections (6  $\mu$ m) were made. The sections that encompassed the contused areas in the injured rats and the equivalent areas in the sham-operates were stained with hematoxylin and eosin (HE).

### *Western Blot*

Western blot analyses were performed on samples that encompassed the contused areas in the injured rats and the equivalent areas in the sham-operates. 48 h after the impact, 5 rats of each group were given an overdose of pentobarbital and decapitated. Brains were extracted, divided at bregma, and then sagittally bisected. The dorsal half of the right posterior quarter, which included the injured area, was used for Western blot analysis. After being weighed, brain tissues were homogenized at 4°C in lysis buffer (20 mmol/L Tris-HCl pH 7.0, 1 mmol/L EDTA, 1% Triton X-100, and 0.5% PMSF). After centrifuge, the supernatant was collected and assayed for protein concentration using BCA protein assay kit (Pierce, 23227). An SDS Laemmli sample buffer was then added to sample aliquots and incubated at 90°C for 10 min. Samples containing 30  $\mu$ g of protein were then loaded on a 10% SDS-PAGE gel and run at 200 V for 2 h. Gels were rinsed in Western transfer buffer for 10 min and loaded against a PVDF membrane prepared for Western transfer. Western transfer was made on a BioRad Criterion apparatus at 100 V for 30 min under cooling conditions. The PVDF membrane was then rinsed in TBS/Tween and incubated in skim milk for 3 h on a shaker at room temperature.

The proteins of interest were labeled with the following antibodies: NF $\kappa$ B p65 (Santa Cruz, sc-372, 1:500), GFAP (Santa Cruz, sc-9065, 1:2 000), and TNF- $\alpha$  (Santa Cruz, sc-1350, 1:500). Primary antibody dilution (in TBS/Tween with 5% skim milk) was applied to the membrane and incubated on a shaker overnight at 4°C. Membranes were thoroughly

rinsed in PBS/Tween and then incubated in appropriate HRP-conjugated secondary antibody (Santa Cruz; 1:5000) for 1 h at room temperature followed by thorough rinse in PBS/Tween and detection *via* chemiluminescent agents (Pierce). Chemiluminescent bands were detected on a Kodak Medical X-Ray Film (Blue Sensitive). After being washed in stripping buffer (2% SDS, 62.5 mmol/L Tris-HCl, 100 mmol/L  $\beta$ -mercaptoethanol) for 30 minutes at 50°C, the PVDF membrane was rinsed with TBS/Tween, and exposed again to  $\beta$ -actin antibody (1:2000, Santa Cruz) as an internal standards control. The bands on the films were scanned and analyzed with ScionImage software (Frederick, USA). The ratio between the target proteins and  $\beta$ -actin band densities was used for quantitative evaluation of the concentrations of the interested proteins.

#### Lesion Volume

Five rats from the IH and IP group were sacrificed 48 h after surgery, and brains were rapidly removed and cut into 1-mm slices. To quantify lesion volume, brain slices were incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) in phosphate buffer for 30 min at room temperature in the dark, and stored at 4°C in neutral 10% formalin for up to 7 days prior to analysis. Digital images of each slice were taken, and lesion volume, defined as the area of unstained tissue, calculated using ScionImage software (Frederick, USA).

#### Brain Water Determination

Four rats of each group were sacrificed by cervical dislocation 48 h after injury, the brains excised, and a 4-mm coronal slice encompassing the entire area of damaged tissue was dissected and sectioned into hemispheric volumes. Brain samples were placed in pre-weighed glass vials and weighed on a five-point decimal scale (wet weight). Lids were removed and the vials were placed in an oven at 90°C for 2 days, and then reweighed (dry weight). The

percentage of water in each sample was then calculated from the formula: %water=100× (wet weight-dry weight)/wet weight.

#### Neurological Severity Score

In all rats, a Modified Neurological Severity Score (mNSS) test<sup>[10]</sup> was performed at 24 h, 7, 14, and 21 d after the impact by an investigator who was blinded to the experimental groups. Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18). The mNSS is a composite of motor, sensory, reflex, and balance tests. In the severity scores of injury, 1 score point is awarded for the inability to perform the test or for the lack of a tested reflex; thus, the higher the score, the more severe is the injury.

#### Statistics

All statistical tests were conducted using SPSS software (version 10.0 for PC) and a *P* value less than 0.05 was taken as significant. Data were first tested for normality and homogeneity of variance. For the comparisons of lesion volume and mNSS score among different groups, the Student's *t* test was applied. For the expression levels of proteins and the brain water content, data were analyzed by one-way ANOVA and SNK post-hoc test.

## RESULTS

#### HE Staining and Morphological Analysis

In HE staining, normal morphology was observed in the brain sections of sham-operates (SH and SP group). The sections of injured rats receiving vehicle (IH group) showed that neurons and glial were shrunken, and extensive neuronal loss and neutrophil infiltration were evident 48 h after traumatic brain injury. In the sections of injured rats treated with progesterone (IP group), the neural damage was to a certain extent attenuated, compared with that in IH group (Fig. 1).

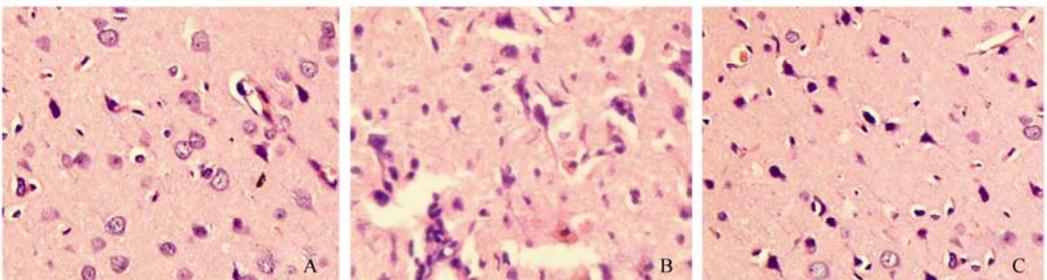
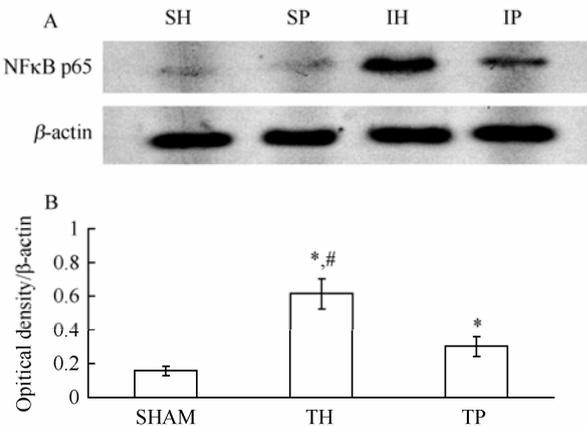


FIG. 1. Representative HE staining sections of contused or equivalent areas. Normal morphology is observed in sham-operated animals (A), while extensive neuronal loss and neutrophil infiltration are evident in IH animals (B). The level of neural damage in IP animals was to a certain extent attenuated, compared to those of IH group (C). Magnification: 20×.

Western Blot

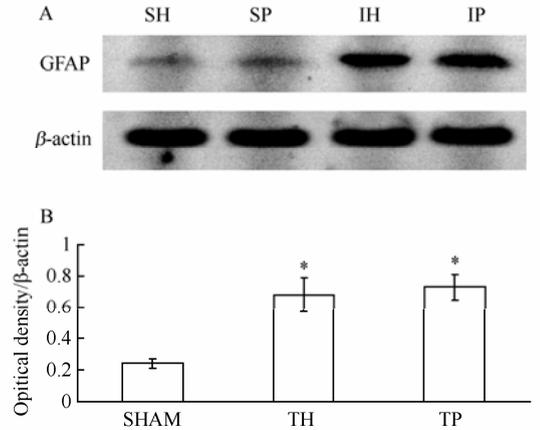
For all three proteins analyzed, no significant difference was found between the sham-operates receiving vehicle (SH) and the sham-operates receiving progesterone (SP). Therefore, the SH and SP groups were incorporated into one group (Sham) in the subsequent statistical analysis for the expression level of interested proteins.

One-way ANOVA of the NFκB p65 isomer expression showed differences among the groups ( $F=32.17, P<0.05$ ). In post-hoc analysis, the values in sham group were significantly lower than those in injured rats treated with either vehicle or progesterone. Further analyses showed that densitometric values in the progesterone-treated injured animals (IP group) were significantly lower than those in vehicle-treated injured rats (IH group) ( $P<0.05$ ) (Fig. 2). One-way ANOVA of GFAP expression levels showed differences among the groups ( $F=18.85, P<0.05$ ). The values in sham group were significantly lower than those in either IH or IP group. Post-hoc analysis did not show any difference between the IH and IP groups ( $P>0.05$ , Fig. 3). One-way ANOVA of the TNF-α exhibited differences among the groups ( $F=44.05, P<0.05$ ). The values in sham group were significantly lower than those in either IH or IP group. Further analyses showed that densitometric values in the IP group animals were significantly lower than those in IH group ( $P<0.05$ ) (Fig. 4).



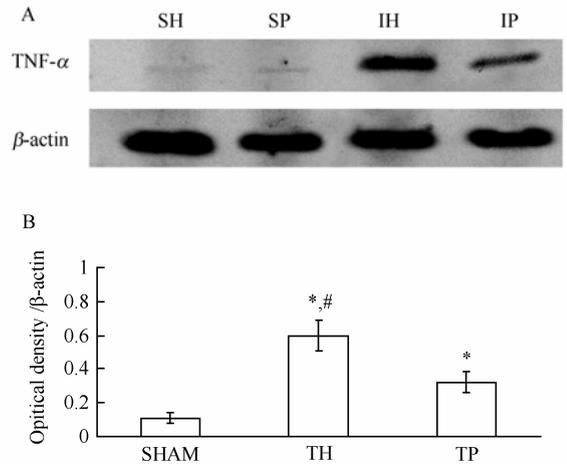
Note. \*Significantly different from SHAM group ( $P<0.05$ ); # significantly different from TP group ( $P<0.05$ ).

FIG. 2. Expression of NFκB p65 in TH, TP and SHAM groups. (A) A representative Western blot illustrating the difference in the expression level of this protein. (B) Relative NFκB p65 optical density, compared with β-actin band in the same sample.



Note. \*Significantly different from SHAM group ( $P<0.05$ ).

FIG. 3. Expression of GFAP in TH, TP and SHAM groups. (A) A representative Western blot illustrating the expression level of this protein. (B) Relative GFAP optical density, compared with β-actin band in the same sample.



Note. \*Significantly different from SHAM group ( $P<0.05$ ); # significantly different from TP group ( $P<0.05$ ).

FIG. 4. Expression of TNF-α in TH, TP and SHAM groups. (A) A representative Western blot illustrating the difference in the expression level of this protein. (B) Relative TNF-α optical density, compared with β-actin band in the same sample.

Lesion Volume

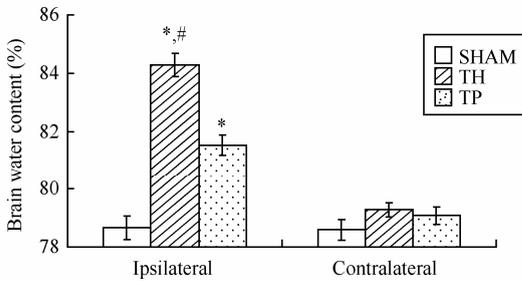
Controlled impact injury induced a region of necrotic tissue in the right parietal cortex, as visualized by an absence of TTC staining. Injury volume was expressed as the percentage of the area of the unstained region over the total area of contralateral (uninjured) hemisphere. IP animals displayed a significantly smaller lesion volume (5.71%) than the

## DISCUSSIONS

IH controls (7.11%) ( $t=3.817$ ,  $P<0.05$ ).

### Brain Water Content

Using the wet and dry weight determination, no difference in the percentage of brain water between contralateral hemispheres of injured and sham groups was observed ( $F=0.641$ ,  $P>0.05$ ). Injured brain hemispheres displayed an increased percentage of brain water than did ipsilateral hemispheres of sham-injured animals ( $F=14.14$ ,  $P<0.05$ ). In post-hoc analysis, the values in sham group were significantly lower than those in either IH or IP group. The densitometric values in the IP animals were significantly lower than those in the IH rats ( $P<0.05$ ) (Fig. 5).

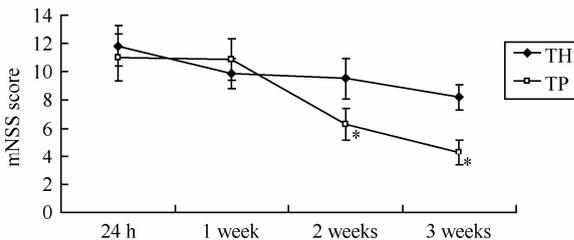


Note. \*Significantly different from SHAM group ( $P<0.05$ ); # significantly different from TP group ( $P<0.05$ ).

FIG. 5. The effect of progesterone on brain water content after injury

### Neurological Severity Score

In the sham groups, the mNSS values were normal (0 point) for both the progesterone-injected ( $n=2$ ) and the vehicle-treated ( $n=2$ ) groups. In the injured groups, no differences between the progesterone-treated injured group and the vehicle-treated group was observed ( $P>0.05$ ) at 24 h or 1 week after the impact, whereas at 2 weeks and 3 weeks after the impact, the mNSS value in the IP group ( $n=5$ ) became significantly lower than that in the IH group ( $n=5$ ) ( $P<0.05$ ) (Fig. 6).



Note. \*Significantly different from TH group ( $P<0.05$ ) at a given time point group ( $P<0.05$ ).

FIG. 6. The effect of progesterone on neurodeficit recovery based on a mNSS test.

Inflammatory reaction plays a key role in the pathophysiology of traumatic brain injury. Some crucial contributors to these processes are the various immune factors. They induce a series of events including expression of adhesion molecules, cellular infiltration, and extra secretion of inflammatory cytokines, resulting in either regeneration or cell death. However, recent studies on the role of inflammatory cytokines after acute brain injury have yielded contradictory results about whether their action contributes to repair mechanisms or exacerbates the pathophysiology of trauma<sup>[2]</sup>. A commonly accepted theory is that inflammatory cytokines may produce opposing effects at different times after the brain injury happens. That is to say, the inflammatory cytokines may produce a neurotoxic function at the early stage (1-2 d) after TBI, while it can produce neuroprotective function in the later posttraumatic phase (2-4 weeks)<sup>[2]</sup>. In view of the dual and opposite effect of inflammatory cytokines, it is reasonable to assume that an effective inhibition of inflammatory response at the acute period is beneficial to the recovery from TBI. Thus we began drug administration within 30 min after the injury in this study, and the following injections were confined to 24 h after the injury.

Research seeking new treatments for TBI has shown that progesterone improves neuronal survival and functional recovery after brain injury in rodent models<sup>[4]</sup>. Furthermore, deleterious inflammatory cytokines including tumor necrosis factor (TNF- $\alpha$ )<sup>[11]</sup> and interleukin-1 $\beta$  (IL-1 $\beta$ )<sup>[11]</sup> are reduced following treatment with progesterone after frontal cortex injury. Serum levels of progesterone are inversely correlated with the degree of cerebral edema following TBI. Progesterone diminishes brain water content, compared with untreated TBI controls, reflecting a decrease in brain edema<sup>[3]</sup>. Cytotoxic edema is caused by accumulation of fluid primarily within the astrocytes. Progesterone treatment results in a significant decrease in the accumulation of reactive astrocytes in the proximity of the cortical wound after penetrating and cortical impact head injury<sup>[4]</sup>.

NF $\kappa$ B activity is increased following brain injury and remains detectable for up to 1 year after injury. Its inhibition can decrease expression of TNF- $\alpha$ , IL1 $\beta$ , ICAM, and cyclooxygenase-2<sup>[12]</sup>. Neurosteroids are known regulators of NF $\kappa$ B activity which down-regulate cyclooxygenase-2 *via* NF $\kappa$ B inhibition<sup>[13]</sup>. Progesterone has been reported to inhibit NF $\kappa$ B-mediated transcription in two ways. The better-characterized route is promoting the

production of the endogenous inhibitor of NF $\kappa$ B (IkB)<sup>[7]</sup>. Recent studies show that, when progesterone is present, the progesterone receptor forms a heterodimer with NF $\kappa$ B p65 and in turn inhibits binding of the transcription factor to its binding site<sup>[6]</sup>. Progesterone, which decreases concentration of p65 monomer, decreases the assembly of p65-p50 heterodimer and favors assembly of the inactive p50-p50 homodimer, thus decreasing NF $\kappa$ B-mediated inflammatory transcription<sup>[14]</sup>.

Increased TNF has been detected in various experimental models of TBI, such as fluid percussion injury and closed head injury. TNF levels have been associated with the neurological deficit in rodents subjected to experimental TBI<sup>[11]</sup>. *In vivo* experiments based on the neutralization of TNF action after TBI using specific inhibitors have corroborated the hypothesis of the potential toxicity of this cytokine, because the animals showed a better neurological outcome and decreased neuronal death, and blood-brain barrier dysfunction<sup>[11]</sup>.

In the present study, the expression of TNF- $\alpha$  and NF $\kappa$ B p65 proteins increased significantly 48 h after the trauma, which could be inhibited by intraperitoneal progesterone injection. The down-regulation of these two inflammatory cytokines might contribute to the overall suppression of inflammatory reaction and thus attenuate the unfavorable effects after TBI. TNF- $\alpha$  and IL-1 $\beta$  stimulate the synthesis and release of C3, NF $\kappa$ B, and GFAP, while C3 and NF $\kappa$ B in turn promote the release of TNF- $\alpha$ <sup>[7]</sup>. In such a self-enhancing course, inhibition of one or more of the cytokines may restrain the whole inflammatory process. However, the question still remains about the relationship between the two cytokines in the presence of progesterone.

Increased GFAP is an indicator of reactive gliosis associated with inflammation and cytotoxic edema. In contrast to a penetrating brain injury model<sup>[15]</sup>, where progesterone was shown to decrease reactive astrogliosis, our current results do not show an effect of progesterone on the expression level of GFAP at 48 h post-TBI. This disagreement can be explained by the severity of the injury, dose of progesterone, and the method of drug delivery. However, the results reported here are in agreement with another recent study, which showed that systemically administered progesterone did not affect the number of GFAP-positive astrocytes<sup>[16]</sup>. Our results suggest that the increase in GFAP expression in reactive astrocytes after brain injury is caused by factors which are not influenced by progesterone treatment.

Brain edema, defined as the increase of brain water content caused by breakdown of the blood-brain barrier and the infiltration of

inflammatory factors, is a serious complication of TBI. It is documented that progesterone can reduce brain edema in different animal models of injury<sup>[17]</sup>. Roof *et al.* reported that progesterone can reduce edema at doses that are equivalent to those associated with pregnancy in the rat<sup>[5]</sup>. In the present study, injury-induced increases in brain tissue water content, indicating edema formation in the contusion area at 48 h after TBI, were substantially reduced by progesterone treatment. Since a causal factor in brain edema is the breakdown of the blood-brain barrier, Roof *et al.* examined the effect of progesterone injections on the integrity of the blood-brain barrier and found evidence that progesterone treatment after injury may result in a more intact barrier. In addition, they reported that progesterone could reduce membrane breakdown by interfering with free radical-induced lipid peroxidation<sup>[5]</sup>. Based on the results of the present study, the inhibition of inflammatory factors may be another crucial mechanism by which progesterone reduces the extent of traumatic brain edema.

Our study also suggested that progesterone administration could reduce the lesion volume after TBI. The unstained necrotic area in progesterone-treated animals was about 20% less than that in the vehicle-injected controls. This may be a result of neuroprotective effect of progesterone, mediated by inhibition of posttraumatic inflammation. In addition, compared with the vehicle-treated ones, progesterone-injected animals showed a better recovery in the mNSS test, especially at 2 weeks or more after the trauma. That seems to suggest that the beneficial effect of progesterone may remain for a long time even the administration of this drug is confined to a relative short duration (24 h in this case).

The present work supports previous studies demonstrating a therapeutic, anti-inflammatory role of progesterone following TBI. Inflammation is a complex process and may involve differential regulation and response at different post-TBI times. While cytokines and inflammatory regulators may play a beneficial role in the later periods of recovery and rehabilitation<sup>[2]</sup>, such factors are certainly detrimental to patient's outcome in the acute stages of head injury. Treatments as progesterone which are capable of down-regulating inflammation and edema without provoking adverse effects are optimal candidates for the treatment of TBI pathology.

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