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Progesterone Improves Acute Recovery after Traumatic Brain Injury in the Aged Rat

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ABSTRACT

Recent evidence has demonstrated that treatment with progesterone can attenuate many of the pathophysiological events following traumatic brain injury (TBI) in young adult rats, but this effect has not been investigated in aged animals. In this study, 20-month-old male Fischer 344 rats with bilateral contusions of the frontal cortex ($n = 4$ per group) or sham operations received 8, 16, or 32 mg/kg of progesterone or vehicle. Locomotor activity was measured at 72 h to assess behavioral recovery. Brain tissue was harvested at 24, 48, and 72 h, and Western blotting was performed for inflammatory and apoptotic factors. Edema was assessed at 48 h by measuring brain water content. Injured animals treated with 8 and 16 mg/kg progesterone showed decreased expression of COX-2, IL-6, and NFκB at all time points, indicating a reduction in the acute inflammatory process compared to vehicle. The 16 mg/kg group also showed reduced apoptosis at all time points as well as decreased edema and improved locomotor outcomes. Thus, in aged male rats, treatment with 16 mg/kg progesterone improves short-term motor recovery and attenuates edema, secondary inflammation, and cell death after TBI.

Key words: aging; frontal cortex; inflammation; progesterone; recovery; traumatic brain injury

INTRODUCTION

PROGESTERONE ADMINISTRATION after traumatic brain injury (TBI) improves short- and long-term behavioral recovery and reduces inflammation, apoptosis, lesion volume, and edema in laboratory animals (Asbury et al., 1998; Attella et al., 1987; Chang et al., 1999; Galani et al., 2001; Grossman et al., 2004; Kumon et al., 2000; Lowery, 2002; Roof, 1994, 1997). These results are not limited to experimental models. A recent Phase IIa clinical trial (Wright et al., 2007) reports that 4 days of post-TBI intravenous progesterone reduces mortality by more than 50% in moderately to severely injured human patients and enhances functional outcomes at 30 days for the moderately injured. This is the first clinical trial to show pharmacological protection in TBI patients, indicating that progesterone may be an effective clinical treatment in humans.

Most studies attempting to develop treatments for TBI focus on otherwise healthy young adults despite the fact that the past decade has seen a 21% increase in TBI events in individuals over the age of 65 (Adekoya et al., 2002). Further, the mortality rate resulting from TBI and its complications in the elderly is more than twice that of young

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and adult victims (Mosenthal et al., 2002). Age has also been found to be an independent predictor of mortality and early outcome in the geriatric human population with TBI (Mosenthal et al., 2002), indicating the importance of the problem in this demographic.

Increased mortality and morbidity after injury in the elderly are likely due to several factors. First, endogenous levels of most circulating hormones are lower than in younger groups, potentially decreasing the intrinsic ability of these subjects to respond to severe injury (Alkayed et al., 2000; Gangula et al., 2002; Bounds et al., 2003). Second, the aged are more likely to have complicating health factors such as altered metabolism or derangement of cardiovascular, hepatic, renal, and immune systems, leading to increased frailty (Laumer et al., 1992; Mosenthal et al., 2002; Lipsitz, 2004). Specific to the nervous system and its ability to recover from injury are the documented age-related loss of blood–brain barrier (BBB) function (Campbell et al., 2007), decrease in CYP enzyme activity (Meng et al., 2007), alteration in intracellular Ca^{2+} homeostasis (Mattson and Magnus, 2006; Raza et al., 2007), neuroinflammatory changes (Griffin et al., 2006; Maher et al., 2005; Godbout and Johnson, 2004; Kovacs, 2005), increased oxidative stress (Siqueira et al., 2005), altered neurotrophin metabolism and signaling (Williams et al., 2006), and alterations in basal forebrain cholinergic system function (Niewiadomska et al., 2002; Geula et al., 2003). All of these play key roles in TBI pathophysiology, and most have been implicated as mechanisms of potential neuroprotection in the development of treatments for TBI. Age-related alteration in precisely the systems that control the development of injury therefore suggests a potential confound in the applicability of data obtained for normal adult subjects to the aged population, especially if these are directed at a specific system.

Following lateral fluid percussion brain injury, for example, aged animals are impaired in Morris water maze performance and show disruptions in the beam walk and beam balance, tasks in which younger animals display fewer or no deficits (Hamm et al., 1992). Since recovery parameters and system homeostasis are clearly altered in the aging organism, it is possible that treatments such as progesterone that have been shown to be effective in younger counterparts may not work in this population or may require different dosing and treatment regimens. This is not a trivial issue as it has important consequences for the human population, and any potential TBI treatment must therefore be demonstrated to work across different age groups if it is to be considered successful.

Very little literature has specifically addressed the effects of progesterone treatment for CNS injury in aged animals, but the few studies that do show it to be generally beneficial. In a model of stroke, Alkayed et al. (2000) demonstrated that the loss of intrinsic neuroprotection due to estropause in aged females, as measured by increased infarct size after ischemic injury, is attenuated by replacement of both estrogen and progesterone. Ibanez et al. (2004) looked at remyelination in young and old rats with progesterone treatment after damage to brain-stem white matter and found that, while the latency of repair was increased in aged rats regardless of treatment, progesterone doubled the expression of myelin compared to elderly controls. Azcoitia et al. (2003) and Ibanez et al. (2003) also reported that supplementary progesterone promotes the expression of myelin proteins in the damaged sciatic nerves of young and 22–24-month-old male rats with peripheral nerve crush injuries. In addition to these nervous system-specific effects, neurosteroid administration also appears to confer general systemic benefits and has been shown to reverse the increased hypertension morbidity documented in postmenopausal female rats (Gangula et al., 2002).

Given the severity of the problem of TBI in the aged and the fact that progesterone has proven to be effective in a number of studies involving young adult animals and human patients (Stein, 2003, 2005; Wright et al., 2007), we asked whether progesterone could have significant beneficial effects in senescent TBI subjects as well, especially in the acute phase of injury. Brain trauma is generally followed by a stereotyped molecular response that can be investigated through expression levels of proteins such as TNFα, IL-6, and the inflammation-associated transcription factor NFκB and its inhibitor, IκB. Since much of the damage that occurs after TBI is secondary to the initial insult (Royo et al., 2003), acute phase reactants play a very important role in the evolution of the injury, and the benefit of a treatment can be measured partly by the effect it has on these compounds (Bazan et al., 2005; Bramlett and Dietrich, 2004; Kovacs, 2005; Stamatovic et al., 2006).

The development of edema is an important factor in the generation of secondary injury, and is frequently the proximal cause of death after TBI (Galani et al., 2001). The p-glycoprotein (PGP) efflux pump, a molecular marker of BBB function, is able to reduce cytotoxic and vasogenic edema by integrating into membranes and helping to maintain cellular homeostasis (Dazert et al., 2006). An effective treatment for TBI would therefore be expected to reduce edema, as measured by brain water content, and to do so through molecular mechanisms such as the upregulation of PGP expression in the lesion area.

To determine the neuroprotective effects of progesterone during the initial evolution of TBI in the aged rat, we examined edema, levels of inflammatory cytokines, and apoptotic cell death at several time points in the acute

phase of damage. To confirm that reduced inflammation and cell death are associated with improved behavioral recovery, we also tested the spontaneous locomotor behavior of our old animals during the same period. Based on the beneficial effects of post-TBI progesterone administration in adult animals and the positive response of aged animals to progesterone after stroke and nerve crush injuries, we hypothesized that old male rats would also exhibit decreased expression of inflammatory cytokines and improved behavioral outcomes following progesterone treatment.

METHODS

Subjects

Eighty-five aged (20-month-old) male Fischer 344 rats weighing 450–550 g at the time of injury were used in this experiment. Food and water were provided *ad libitum* before and after surgery. The animal housing facility was maintained on a reverse 12:12 light-dark cycle, at 22°C (SD = ±1) with appropriate humidity levels. The air within the colony was continually cycled via an air filtration system complying with government legal standards for animal research. Rats were handled for a minimum of two months following their arrival in the housing facility and prior to surgery. This study was conducted in a facility approved by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) in accordance with NIH guidelines. All experimental animal procedures were approved by the Emory University Institutional Animal Care and Use Committee (IACUC; protocol 146-2005).

Surgery and Contusion Injury

Rats were anesthetized using isoflurane gas (5% induction, 1.5% maintenance, 700 mmHg N_2O, 500 mmHg O_2) and then mounted in a Kopf stereotaxic device. The incision area was shaved and sterilized with Betadine® antiseptic and 70% isopropanol. Core body temperature (~37°C) was maintained with a homeothermic heating blanket system (Harvard Apparatus, Holliston, MA). Physiological parameters were monitored with SurgiVet™ (model V3304) pulse oximetry: heart rate was maintained above ~300 beats per minute and SpO_2 kept above 90%. Using aseptic techniques, a midline incision was made into the skin and fascia covering the skull. After appropriate time under anesthesia, the animals assigned to the sham group had their incision sutured closed and were placed into heated recovery boxes. In the experimental groups, medial, lateral, and dorsal stereotaxic coordinates were determined at bregma after incision, and a 6-mm-diameter mid-sagittal bilateral craniotomy was performed 3 mm anterior to bregma. Cortical contusion injury (CCI) to the medial frontal cortex (MFC) was induced with a pneumatic cortical contusion device (5-mm-diameter) to a depth of 2.5 mm at a pressure of 1.7 psi, impact time of 50 msec, and velocity of 2.25 m/sec. Sutures were used to close the incision after bleeding ceased. Animals were then placed into heated recovery boxes and allowed to recover from the anesthetic before being returned to their home cages (Hoffman et al., 1994). Animals dehydrated due to blood loss were given 10 mL of lactated Ringer's solution subcutaneously within 6 h of injury.

Progesterone Administration

Lesion animals ($n = 4$ animals per group per experimental condition) were randomly assigned to one of four treatment groups, henceforth referred to with the following acronyms: P8 (8 mg/kg progesterone), P16 (16 mg/kg progesterone), 32 mg/kg progesterone, and V (vehicle [22.5% 2-hydroxypropyl-β-cyclodextrin]). Treatments were administered intraperitoneally at 1 h post-injury, and then subcutaneously at 6 h post-injury and every 24 h thereafter until the brains were harvested. Serving as intact, or normal, baselines, sham groups (S) received no injury or injections. Animals were killed at 24, 48, and 72 h following injury with 1 mL Nembutal, decapitated, and their brains prepared for protein analysis.

Activity Testing

Testing for activity was done under red light in a quiet environment 1 day prior to injury, then again at 72 h post-injury. The purpose of testing prior to injury was to obtain a baseline for each animal that could be used to calculate percent change at 72 h post-injury. For each trial, up to four animals were tested simultaneously in individual boxes using the Digiscan Activity Monitoring System (AccuScan Instruments Inc., Columbus, OH), with a total of three trials per test day per squad. Rats were placed in the furthest left corner of the Digiscan Activity Box with the recording apparatus on. Exactly 5 min later, the computer stopped recording movements, ensuring that all tests were the same length regardless of start time. Animals were returned to their home cages at the end of testing. The activity boxes were cleaned with 70% ethanol and dried between trials.

Tissue Preparation

Brains were processed for protein analysis by taking tissue samples from the penumbral region of the contusion and the corresponding area in sham brains. The tissue was snap-frozen in 2-methylbutane, chilled on dry

ice, and homogenized in T-per (78510; Pierce, Rockford, IL) and 10 μL/mL protease inhibitor cocktail (P8340; Sigma, St. Louis, MO). Resulting homogenates were centrifuged for 20 min at 10,000*g*. A Coomassie plus protein assay (Pierce, 1856210) was performed to ensure that all samples contained equivalent amounts of protein. Reducing sample buffer was prepared as 0.625 M Tris, 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.001% Bromophenol Blue. Samples consisting of homogenate, dH2O, and sample buffer were prepared at a 2 μg/μL protein concentration, incubated at 90°C for 10 min, and stored at −20°C.

Western Blot Analysis

Fifteen microliters of each sample (30 μg protein) was added to individual wells of 4–20% Tris-HCL acrylamide Criterion gel (BioRad, Hercules, CA). The gel was run at 200 V for approximately 1 h. Proteins were transferred to a polyvinylidene difluoride (PVDF) nitrocellulose membrane at 100 V for 30 min, and then incubated overnight in KPL milk diluent blocker (50-82-00 KPL, Gaithersburg, MD) in a 1:5 dilution at 4°C.

Blots were incubated with a polyclonal goat or rabbit primary antibody for COX-2 (ab15191; Abcam, Cambridge, MA), NFκB p65 (3034; Cell Signaling Inc., Danvers, MA), IκB (9248; Cell Signaling), IL-6 (AB1839; Chemicon Inc., Temecula, CA), TNFa (AB1441; Chemicon), PGP (ab3364; Abcam), and cleaved caspase-3 (Asp175, 9661S; Cell Signaling) in KPL milk diluent: phosphate-buffered saline (PBS, pH 7.4; 1:20) and agitated overnight at 4°C. Membranes were rinsed in PBS/Tween and incubated with secondary antibody donkey anti-goat IgG-HRP or goat anti-rabbit IgG-HRP (1:1000) in KPL diluent for 2 h at room temperature. The blots were then incubated in chemiluminescent Super-Signal West Dura substrate (Pierce, 34076) for 5 min. Bands were detected on a Kodak Image station 440CF scanner (Rochester, NY) and analyzed with the accompanying Kodak 1D densitometry image analysis software. Band intensity was compared between treatment groups run on the same blots. β-Actin was used as a loading control on all samples.

Edema Analysis

At 48 h post-injury, brains from animals assigned to the edema study ($n = 3$ per condition) were extracted and dissected into anterior and posterior sections; the anterior section contained the entire lesion area. Each section was placed in a pre-labeled and pre-weighed tube that was immediately capped. Each tube was reweighed to the nearest 0.01 mg, and then opened and placed in a 60°C oven with 15 mmHg vacuum pressure for 48 h. Samples were reweighed after drying, and the percent water content was calculated by [(wet wt − dry wt)/(wet wt)]*100. The percent difference in water content between the anterior peri-contusional and the posterior distal section was calculated for each sample by: [(anterior H_2O% − posterior H_2O%)/(posterior H_2O%)]*100.

Statistical Analysis

All results were expressed as the mean ± the standard error of the mean (SEM). Statistical significance was set at $p < 0.05$, and data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer *post hoc* tests.

RESULTS

Pre- and Post-Surgical Complications in Aged Rats

Of a total of 85 old rats that entered the facility, 9.2% died prior to surgery due to various causes including tumors, 4.6% died during the surgical procedure from anesthesia or blood loss, and 10.7% died after surgery was completed but before the scheduled euthanasia. In contrast, young adult animals undergoing the same procedure had an attrition rate of approximately 1–2%. We also observed more blood loss during surgery in the old animals, as well as increased sensitivity to anesthesia and more extensive cranial hematomas on brain extraction compared to young adults. Old sham animals also showed significant bleeding during fascial clearing, something not generally observed in younger animals.

32 mg/kg Dosage

At 48 h, the first time point investigated, we administered a 32 mg/kg progesterone dose to our animals in addition to 8 and 16 mg/kg. Consistent with the results of the dose response study conducted in young adult animals by Goss et al. (2003), the 32 mg/kg treatment exhibited fewer beneficial cellular/morphological and behavioral effects compared to the 8 and 16 mg/kg doses at 48 h (data not shown). Accordingly, we narrowed our dose response study at 24 h and 72 h to 8 and 16 mg/kg progesterone and vehicle.

24 Hours

Figure 1 shows the inflammatory cytokine response at 24 h. One-way ANOVAs demonstrated significant group differences for IL-6 ($p < 0.05$, $F = 4.75$; Fig. 1A), IκB ($p < 0.05$, $F = 10.97$, Fig. 1B), and COX-2

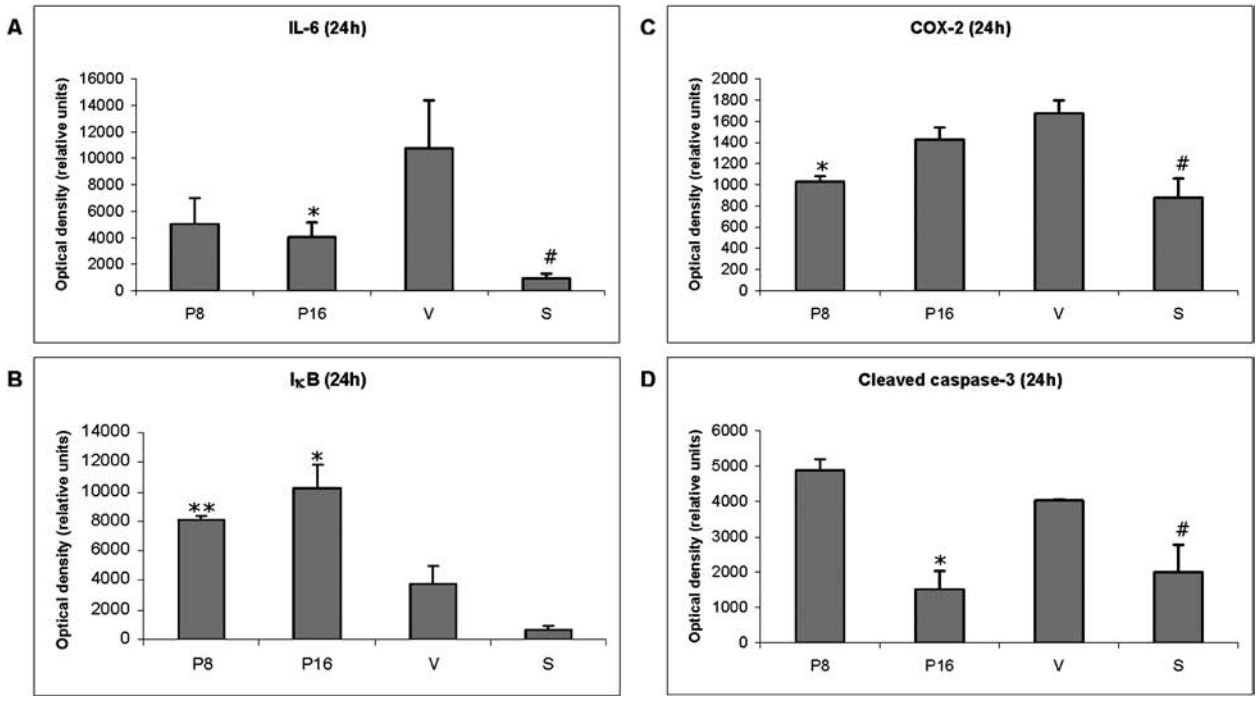

FIG. 1. (**A–C**) Inflammatory response at 24 h. Levels of both IL-6 (*, A) and IκB (*, B) are positively affected by 16 mg/kg progesterone (P16) versus vehicle-treated animals (V) at 24 h. IκB inhibits the activity of NFκB, and increased levels lead to reduced inflammation. 8 mg/kg progesterone (P8) also increases levels of IκB (**). COX-2 levels (C) were significantly reduced by 8 mg/kg (*), although they remain at vehicle levels with 16 mg/kg. Sham levels are lower for IL-6 (#) and COX-2 (#), but not for IκB. (**D**) Cell death at 24 h. Levels of active caspase-3 are decreased to sham (#) levels with 16 mg/kg progesterone treatment (*), indicating reduced apoptosis compared to vehicle. Caspase-3 levels remain comparable to vehicle for the 8 mg/kg progesterone dose.

($p < 0.05$, $F = 6.04$; Fig. 1C). Post-hoc analysis showed that 16 mg/kg (P16) progesterone produced a decrease in IL-6 expression ($p = 0.038$) and an increase in IκB expression ($p = 0.004$) over vehicle (V). IκB prevents translocation of NFκB into the nucleus and therefore acts as an anti-inflammatory agent. Treatment with 8 mg/kg (P8) progesterone significantly increased IκB levels as well ($p = 0.026$). Eight mg/kg also decreased levels of COX-2 ($p = 0.032$) compared to vehicle, although 16 mg/kg appeared to have no effect. Sham (S) expression levels were significantly lower for IL-6 ($p = 0.007$) and COX-2 ($p = 0.032$), indicating an injury effect. IκB levels were unaffected by the lesion. All Western blot results are shown in relative units of optical density.

Figure 1D shows Western blotting for cleaved caspase-3, the "gatekeeper" molecule in the extrinsic apoptosis pathway (Budihardjo et al., 1999) and a marker of apoptotic cell death. One-way ANOVA showed a significant treatment effect ($p < 0.05$, $F = 9.05$), with levels of cleaved caspase-3 comparable to sham levels with 16 mg/kg treatment ($p = 0.019$ vs. vehicle), but no different than vehicle with 8 mg/kg. As expected, sham group levels were also significantly lower than vehicle ($p = 0.044$).

48 Hours

Figure 2 shows inflammatory cytokine analysis for the four treatment groups at 48 h. One-way ANOVA showed a significant effect of progesterone treatment on NFκB ($p < 0.05$, $F = 9.59$; Fig. 2A), IκB ($p < 0.05$, $F = 15.17$, Fig. 2B), and COX-2 ($p < 0.05$, $F = 5.67$; Fig. 2C). Both COX-2 ($p = 0.019$) and NFκB p65 ($p = 0.030$) were decreased by 16 mg/kg progesterone, but not by 8 mg/kg, compared to the vehicle lesion group. Only 8 mg/kg treatment increased IκB over lesion vehicle ($p = 0.002$). Sham animals showed significantly lower expression for NFκB ($p = 0.002$) and COX-2 ($p = 0.009$) than lesion animals. Once again, IκB did not show a lesion effect.

Figure 2D shows cleaved caspase-3 levels at 48 h. Both 8 mg/kg ($p = 0.011$) and 16 mg/kg ($p = 0.010$) progesterone treatments provided a significant decrease in apoptotic cell death (one-way ANOVA, $p < 0.05$, $F = 6.59$)

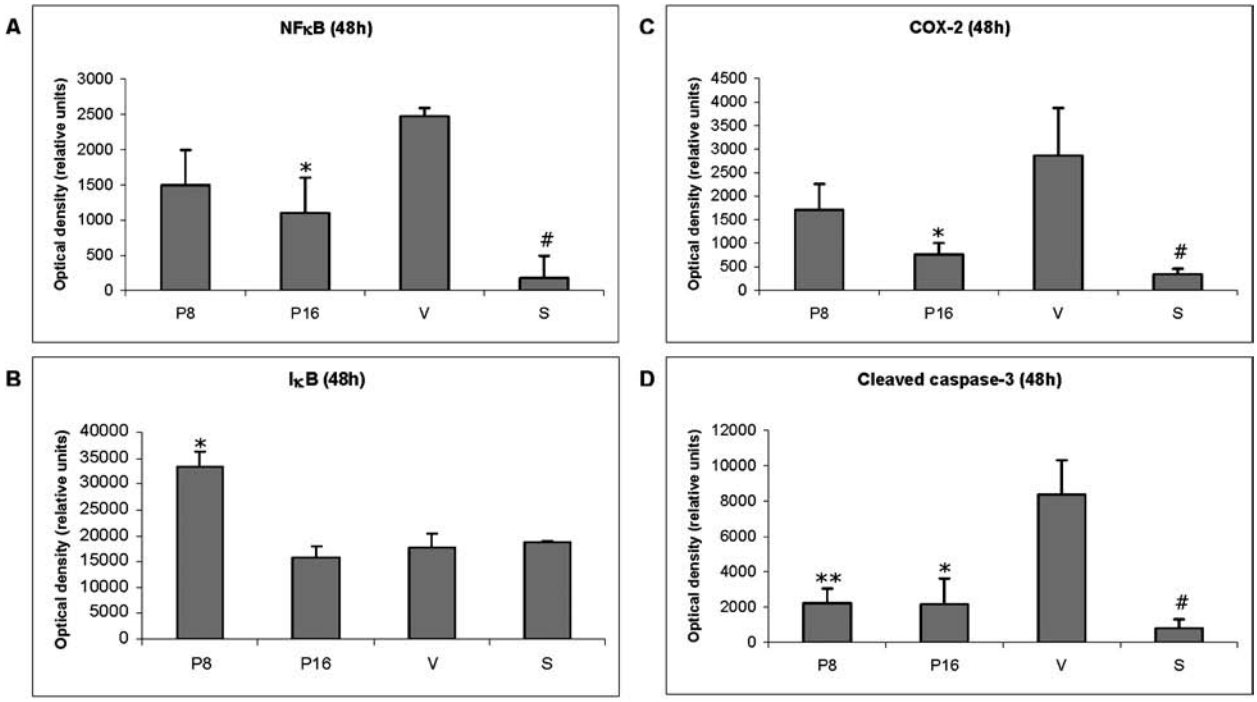

FIG. 2. (**A–C**) Inflammatory response at 48 h. Levels of both NFκB p65 (A) and COX-2 (C) are significantly reduced by 16 mg/kg progesterone at 48 h (*), while IκB (B) is increased by 8 mg/kg progesterone (*). Sham animals are significantly different from vehicle for NFκB and COX-2 but not IκB (#). (**D**) Cell death at 48 h. Levels of cleaved caspase-3 are significantly decreased by both 8 mg/kg (**) and 16 mg/kg (*) progesterone, as they are in the sham group (#), indicating reduced apoptosis compared to vehicle at 48 h.

at this time-point. Sham levels were also significantly lower than vehicle ($p = 0.004$).

Edema at 48 Hours

As seen in Figure 3A, edema was significantly reduced at 48 h by treatment ($p < 0.001$, $F = 33.51$) with 16 mg/kg ($p = 0.005$) but not with 8 mg/kg progesterone. As expected, sham animals also demonstrated lower levels of edema than vehicle-treated ($p < 0.001$) or progesterone-treated ($p_{8,16} = 0.001$, 0.011) lesion animals.

Figure 3B is generally consistent with the data in Figure 3A. One-way ANOVA shows that PGP protein levels, hypothesized to be inversely proportional with edema, are increased for progesterone-treated rats ($p < 0.05$, $F = 25.42$). Increased PGP is seen with both 8 mg/kg ($p = 0.012$) and 16 mg/kg ($p = 0.007$) progesterone treatment compared to vehicle, indicating improved BBB integrity at this dose. The 8 mg/kg dose shows significantly increased PGP expression, but only a trend regarding edema (Fig. 3A), suggesting that there are additional factors involved in the attenuation of edema seen at 16 mg/kg. There appears to be a clear lesion effect, since sham animals are also significantly different from all other groups, but in the other direction ($p_{P8,P16} < 0.001$, $p_V = 0.008$).

72 Hours

Treatment results at 72 h are shown in Figure 4. Progesterone showed a reduction in IL-6 levels ($p < 0.05$, $F = 5.61$; Fig. 4A) at both the 8 mg/kg ($p = 0.008$) and 16 mg/kg ($p = 0.008$) doses compared to vehicle. The same was the case for TNFα ($p < 0.05$, $F = 8.94$; Fig. 4B), also at both 8 mg/kg ($p = 0.034$) and 16 mg/kg ($p = 0.035$) doses. No differences in COX-2 levels were seen with any treatment or shams ($p = 0.125$, $F = 2.60$; Fig. 4C). Sham responses were significantly lower for both IL-6 ($p = 0.009$) and TNFα ($p = 0.001$) compared to lesioned animals.

Figure 4D shows an overall treatment effect on active caspase-3 at 72 h ($p < 0.05$, $F = 4.45$). This was evident with 16 mg/kg ($p = 0.014$), but not 8 mg/kg progesterone compared to vehicle. Sham animals were, as expected, also significantly lower than vehicle ($p = 0.048$).

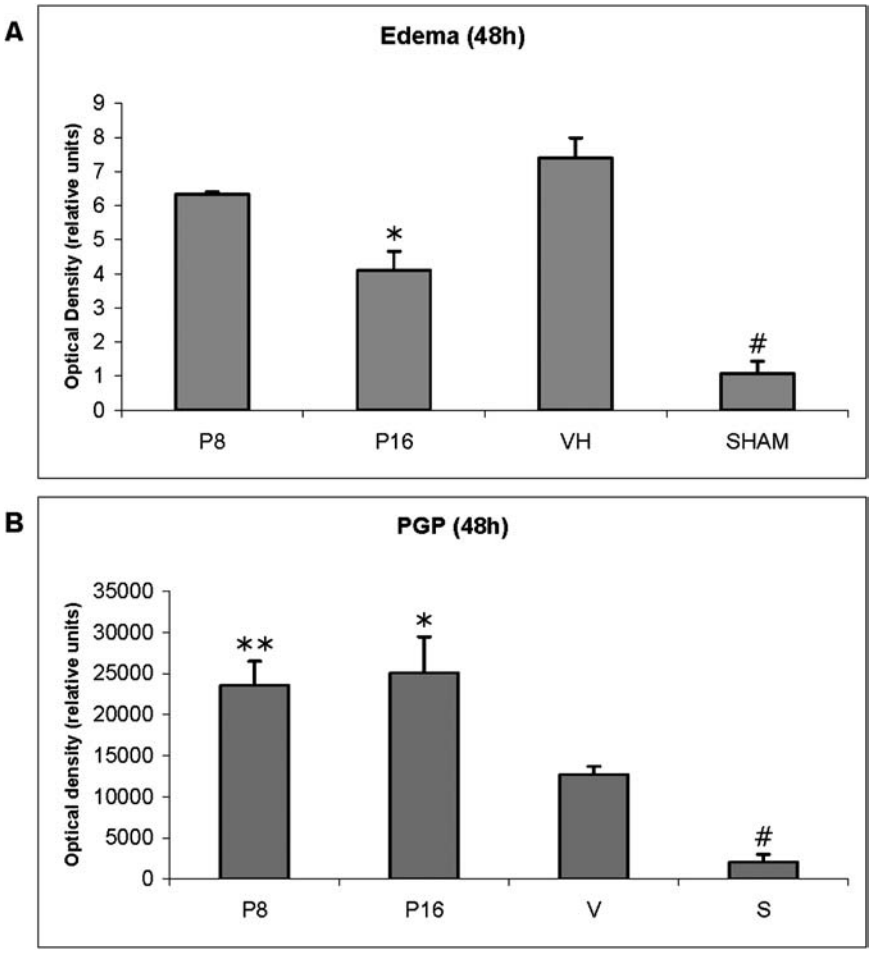

FIG. 3. (**A**) Edema at 48 h. Animals treated with 16 mg/kg progesterone (*), but not 8 mg/kg progesterone, show significantly less brain edema compared to vehicle, as do sham animals (#). (**B**) Blood–brain barrier integrity at 48 h. Animals treated with 8 mg/kg (**) and 16 mg/kg (**) progesterone express significantly more PGP, a marker of blood–brain barrier integrity, than those administered vehicle. Sham results (#) show a clear lesion effect on PGP expression.

Locomotor Activity at 72 Hours

Figure 5 shows activity data as a ratio of testing performed 72 h post-injury to that done before surgery. One-way ANOVA results show an overall treatment effect on locomotor behavior ($p < 0.05$, $F = 5.66$). The 16 mg/kg treatment group displayed an increase in total distance traveled compared to both 8 mg/kg ($p = 0.029$) and vehicle ($p = 0.012$), indicating increased spontaneous activity (Fig. 5A). The 8 mg/kg dose did promote a significant anxiolytic effect, however, as evidenced by increased center time compared to vehicle in Figure 5B ($p = 0.005$; overall ANOVA, $p < 0.05$, $F = 21.27$). While the 16 mg/kg dose showed an increasing trend over vehicle on this metric, this difference was not significant. Sham animals were different from vehicle on both distance ($p = 0.019$) and center time ($p = 0.001$).

DISCUSSION

In this study, we show that our model of bilateral frontal CCI can be successfully applied to old animals. In addition, progesterone treatment is shown to have beneficial molecular and behavioral effects similar to those seen in younger animals after TBI. Despite many similarities between the current results in the aged and our previously published data with young adults (Goss et al., 2003; Pettus et al., 2005), we observed a number of important differences. These include greater mortality in the old rats, both pre-and post-TBI, increased (3–4×) bleeding, and enhanced sensitivity to anesthesia. Because of these findings, our TBI model was slightly adjusted for the elderly animals: (1) they were handled for a much longer period prior to surgery (2 months vs. 1 week for young animals) in order to reduce stress through greater

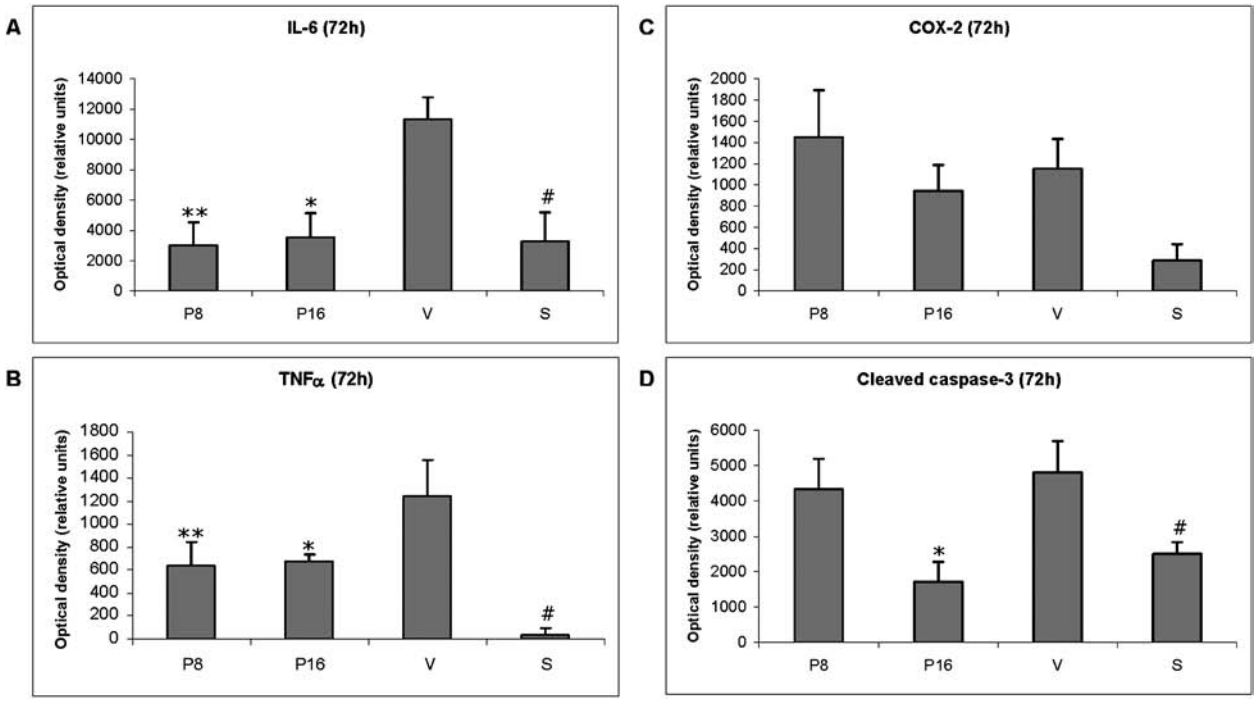

FIG. 4. (**A–C**) Inflammatory response at 72 h. IL-6 (A) and TNFα (B) are decreased compared to vehicle for both 8 mg/kg (**) and 16 mg/kg (**) progesterone. COX-2 (C) levels are not significant. Panel (**D**) Cell death at 72 h. 16 mg/kg (*) progesterone treatment decreases active caspase-3 to near sham levels at 72 h, showing significantly reduced perilesional apoptosis with treatment. As at 24 h, 8 mg/kg is no different than vehicle. Sham animals are also lower than vehicle (#).

contact; (2) anesthesia was maintained at a higher O_2% with a lower overall isoflurane percentage during surgery; and (3) the aged animals were given subcutaneous lactated ringers solution post-surgery to replace fluids lost through increased bleeding, a procedure not required in younger conspecifics.

We had initially planned a dose-response study with three progesterone doses (8, 16, and 32 mg/kg) at three time points (24, 48, and 72 h). After analyzing inflammatory factor expression at 48 h, however, we noted that 32 mg/kg was consistently less beneficial than the low and median doses of 8 and 16 mg/kg. These results were comparable to previous outcomes seen in young animals (Goss et al., 2003; Vink, 2007), so only the 8 and 16 mg/kg doses were used for the entirety of the study.

A number of well-established molecular markers associated with secondary injury and subsequent recovery after TBI were used as benchmarks in this study. NFκB is a dimeric inflammatory transcription factor that requires the p65-p50 isoform for translocation to the nucleus (Ghosh and Karin, 2002). Thus, an increase in the p65 subunit of NFκB can be taken to indicate inflammatory NFκB activity, as the p50-p50 dimer is transcriptionally inactive. The IκB inhibitor protein also acts to contain NFκB in the cytosol, rendering it inactive (Wissink et al., 1998). We found that 16 mg/kg progesterone decreased levels of the p65 NFκB subunit and increased IκB at various time points after injury, suggesting a direct attenuation of inflammation. IL-6, a cytokine secreted by T-cells and macrophages that stimulates inflammation after trauma (Lenzlinger et al., 2001), was also reduced by both 8 and 16 mg/kg progesterone at 24 and 72 h. COX-2 levels, known to be elevated in activated macrophages at an injury site (Cernak et al., 2001), were reduced early after TBI showing suppression at 24 and 48 h, and levels of TNFα, a ubiquitous acute phase inflammatory factor, were also reduced at 72 h. Consistent with our previous results in younger animals (Grossman et al., 2004), these data suggest that progesterone reduces acute inflammation through its effects on various components of the inflammatory cascade in aged rats. The reduction in inflammation observed with progesterone treatment in the elderly is an especially interesting result, since increased levels of IL-6 and other inflammatory factors have been found to be associated with normal aging (Johnson, 2006) in addition to traumatic injury (Ruppel et al., 2002), and could therefore serve as confounding or exacerbating factors in determining treatment efficacy.

Reduced inflammation is associated with a reduction in secondary apoptotic cell death and is therefore an im-

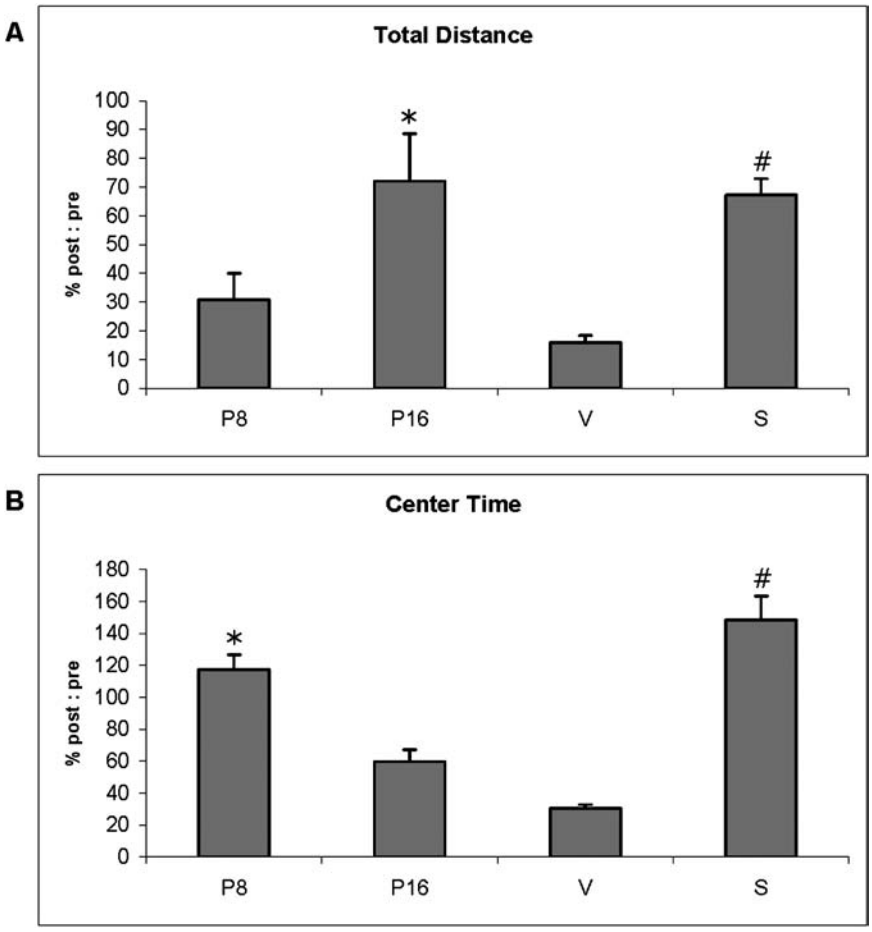

FIG. 5. Locomotor activity at 72 h. Animals treated with 16 mg/kg (*) progesterone show increased total distance traveled (**A**) compared to those given 8 mg/kg and vehicle, with a near-sham level of spontaneous activity. There is no significant difference between the 8 mg/kg and vehicle groups. 8 mg/kg (*) also induces a significant anxiolytic effect compared to vehicle, as demonstrated by increased center time (**B**).

portant aspect of neuroprotection in the early phase of the injury response (Verma, 2000). We assayed cell death by measuring levels of activated caspase-3, the final effector molecule in the extrinsic apoptotic pathway and an important marker of tissue loss. Our data analysis showed decreased cleaved caspase-3 at all time points with 16 mg/kg progesterone, implying significantly reduced cell death at this dose. Significantly, we also observed marked improvement in locomotor activity at 72 h with the 16 mg/kg dose, suggesting a relationship between better behavioral outcome and decreased levels of inflammatory cytokines and reduced cell loss. This effect was not observed with 8 mg/kg treatment.

Another important factor in damage secondary to brain trauma is the rapid development of cytotoxic and vasogenic edema, both of which are known to be attenuated by the expression of PGP. Present on neurons and on endothelial cells of capillaries throughout the brain, PGP is a membrane-bound protein that works as an efflux pump to remove low molecular weight toxins from cells and as such is a key molecular marker of BBB integrity (Karssen et al., 2004). We found a reduction in edema 48 h after TBI with 16 mg/kg progesterone treatment that correlated with increased expression levels of PGP. This observation can be taken to suggest that progesterone confers protection against TBI-induced edema, at least partially by increasing the expression of PGP and maintaining BBB function (Mima et al., 1999). These results suggest the presence of additional factors. Thus, while PGP levels were elevated in both the 8 mg/kg and 16 mg/kg treated animals, edema was significantly reduced only by 16 mg/kg progesterone, although the 8 mg/kg dose did show a trend in the same direction. This suggests that the extent of edema is not wholly dependent on PGP expression and that a reduction inflammation also contributes to reduced swelling. The larger implication is that

while progesterone can affect a variety of systems, its efficacy is likely due to pleiotropic action on multiple interacting systems and not individual factors.

Of the two dosages studied, 16 mg/kg progesterone produced the most consistent beneficial effects on all measures over the time points we examined. These benefits, as compared to the 8 mg/kg dose, are summarized in Table 1. A reduction in inflammatory response was also seen in the 8 mg/kg group, and the outcomes trended in the same direction as those seen with 16 mg/kg in many cases where they were not significant. However, overall, the results were more variable and there was less observable behavioral improvement compared to those animals receiving a 16 mg/mL dose of progesterone. The 16 mg/kg dose also decreased brain swelling by more than 50% compared to vehicle-treated and 8 mg/kg treated groups, a result similar to that repeatedly observed in younger rats with TBI (Goss et al., 2003). Given the complex nature of the injury and the potentially multivalent effects of administered progesterone there may be other molecular modes of action that we have not assayed here. These data suggest that it is important to consider multiple effects and interactions when evaluating treatment efficacy and that behavioral and other measures such as edema are important in confirming the molecular results. Because 16 mg/kg was found to be optimally protective in both young and old animals, both on a molecular and physiological scale, we suggest that this is the "best dose" for treating brain injury in mature rats.

Overall, our results indicate that progesterone treatment at 16 mg/kg decreases inflammation, reduces cell death, and improves BBB integrity in the acute phase of injury in aged rats with TBI. These molecular events are correlated with reduced edema and improved measures of functional activity, and are consistent with data obtained in younger conspecifics, although further studies on recovery in the chronic post-injury period still need to be performed. The optimal doses and duration of treatment were also similar to those found in younger animals, suggesting that progesterone and its metabolites may be effective as a treatment for TBI across the developmental spectrum. Given the rapidly increasing significance of brain injury in the aging human population, and the promising outcomes of progesterone treatment in early clinical trials (Wright et al., 2007), these results could have a significant impact on the clinical management of TBI.

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Table 1. Dosage Outcomes Compared to Vehicle

	Time	*16 mg/kg PROG*	*8 mg/kg PROG*
IL-6	24 h	++	←
IκB		++	++
COX-2		→	++
Caspase-3		++	0
NFκB	48 h	++	←
IκB		0	++
COX-2		++	←
Caspase-3		++	++
PGP		++	++
Edema		++	←
IL-6	72 h	++	++
TNFα		++	++
COX-2		→	0
Caspase-3		++	0
Total distance		++	0
Center time		++	0
Total positive outcomes		13	7

++, Significant positive difference from vehicle.

←, →, Positive trend from vehicle.

0, No difference from vehicle.

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