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Post-Stroke Infections Exacerbate Ischemic Brain Injury in Middle-Aged Rats: Immunomodulation and Neuroprotection by Progesterone

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Abstract

We investigated the effect of delayed, prolonged systemic inflammation on stroke outcomes and progesterone (P4) neuroprotection in middle-aged rats. After transient middle cerebral artery occlusion/reperfusion (MCAO) surgery, rats received P4 (8 or 16 mg/kg) or vehicle injections at 2h, 6h and every 24h until day 7 post-occlusion. At 24h post-injury systemic inflammation was induced by giving 3 doses of lipopolysaccharide (LPS; 50 mg/kg, at 4h intervals) to model post-stroke infections. We measured serum brain-derived neurotrophic factor (BDNF), pro-inflammatory cytokines, and behavioral parameters at multiple times. Serum BDNF levels decreased more in the vehicle+LPS group compared to vehicle-alone at 3 and 7 days post-injury ($P<0.05$). Vehicle-alone showed a significant increase in IL-1 β , IL-6, and TNF α levels at different times following stroke and these levels were further elevated in the vehicle+LPS group. P4 at both doses produced a significant ($P<0.05$) decline in cytokine levels compared to vehicle and vehicle +LPS. P4 restored BDNF levels at 3 and 7 days post-stroke ($P<0.05$). Behavioral assessment (rotarod, grip strength, sensory neglect and locomotor activity tests) at 3, 5 and 7 days post-stroke revealed that the vehicle group had significant ($P<0.05$) deficits in all tests compared to intact controls, and performance was worse in the vehicle+LPS group. P4 at both doses produced significant functional improvement on all tests. Systemic inflammation did not show an additive effect on infarct volume but P4 at both doses showed significant infarct reduction. We suggest that post-stroke infection exacerbates stroke outcomes and P4 exerts neuroprotective/modulatory effects through its systemic anti-inflammatory and BDNF regulatory actions.

Keywords

BDNF; Immunodepression; LPS; Post-stroke infection; Progesterone

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DISCLOSURE/CONFLICT OF INTEREST

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1. INTRODUCTION

Stroke is a complex systemic disease causing severe long-term disability and death worldwide (Mathers et al., 2009). Stroke prognosis is highly variable, depending not only on stroke severity and the patient's age, but also on stroke-associated complications. Post-stroke infection (such as pneumonia and urinary tract infections) is one of the major complications during the acute phase and accounts for one-third of all in-hospital deaths and the worsening of long-term outcomes in ischemic stroke patients (Grau et al., 1999; Emsley and Hopkins, 2008; Koennecke et al., 2011). Preventive antibiotic treatments to reduce infection rate and improve stroke outcome have not been very successful. According to a recent meta-analysis, prophylactic antibiotic treatment in acute-stroke patients is effective in preventing infections but does not reduce mortality (Westendorp et al., 2012). Importantly, none of the clinical trials in this meta-analysis were sufficiently powered to support an analysis of the impact of a preventive antibiotic treatment on stroke outcome. Moreover, a major problem with preventive antibacterial therapy is that it can promote antibiotic resistance in common bacteria with very serious consequences for the patient's well-being and recovery (Meisel et al., 2012).

Stroke disturbs the normally well-balanced interplay between the nervous and immune systems by compromising sympathetic and parasympathetic neural connections with lymphoid organs including the hypothalamo-pituitary-adrenal (HPA) axis (Woiciechowsky et al., 1998; Meisel et al., 2005). The sympathetic nervous system (SNS) has been reported to play a key role in impaired anti-bacterial immune response following stroke in a mouse model of focal cerebral ischemia (Prass et al., 2003, 2006). Clinical studies suggest that stress-mediated immunodepression driven by the SNS and HPA axis is an essential facilitating factor in the onset of post-stroke infections (Chamorro et al., 2007; Harms et al., 2008, 2011). In this scenario, immediately after stroke onset, massive release of pro-inflammatory cytokines activates the HPA axis and parasympathetic and sympathetic nervous systems. The activated HPA axis and SNS release noradrenaline, glucocorticoids and acetylcholine, mounting a "systemic anti-inflammatory response" that negatively affects the function of the innate and adaptive immune systems and contributes to morbidity, infection and death in stroke patients (Emsley et al., 2003; Smith et al., 2004; Prass et al., 2003; Meisel et al., 2005). Some investigators have proposed that selective immunomodulation to prevent post-stroke infections may be an alternative to preventive antibiotic treatment (Meisel and Meisel, 2011).

In the past two decades, only one drug has been shown to be clinically effective for stroke treatment, with genetically engineered tissue plasminogen activators (tPAs) still the only FDA-approved agents to treat stroke. Given the high risk-to-benefit ratio and very narrow therapeutic time window for tPA, development of an agent which has no negative side effects, and can efficiently prevent secondary ischemic brain damage, inhibit HPA axis-associated immunodepression, and enhance neuronal repair would be a major breakthrough. We propose that the neurosteroid progesterone (P4) might be a suitable candidate. P4 is a pleiotropic drug targeting several perpetrators of stroke injury. P4's safety and efficacy have already been demonstrated in two independent phase II clinical trials for traumatic brain injury (TBI) (Wright et al., 2007; Xiao et al., 2008). Two independent Phase III, NIH- and industry-sponsored, multi-center trials of P4 treatment for moderate to severe TBI are now underway and in interim analysis (<http://clinicaltrials.gov/ct2/show/record/NCT00822900> and <http://www.synapse-trial.com/>). If successful, these trials could stimulate further interest in testing P4 in patients with adult ischemic stroke (Stein, 2012). Our laboratory has recently published a number of studies demonstrating substantial neuroprotection by P4 in several animal stroke models (Sayeed et al., 2006; Sayeed et al., 2007; Sayeed and Stein, 2009; Ishrat et al., 2009, 2010, 2012). P4 treatment not only improves functional/behavioral

outcomes in several injury models, but also reduces inflammatory cytokines, brain tissue necrosis, apoptosis, and cerebral edema (Stein, 2008).

In this study, we tested the hypothesis that P4, as a potent anti-inflammatory agent, would inhibit post-stroke systemic infection and inflammation-induced cellular damage, and improve functional recovery. To mimic the systemic effect of post-stroke infections, we administered multiple doses of lipopolysaccharide (LPS) to maintain prolonged systemic inflammation (Langdon et al., 2010). Serum pro-inflammatory cytokines (interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor α (TNF α)) and brain-derived neurotrophic factor (BDNF) were measured at multiple time points. We also investigated whether the systemic inflammation increased brain infarction and worsened functional outcomes, and whether P4 treatment would reduce infarct volume and improve functional recovery.

2. EXPERIMENTAL PROCEDURES

All behavioral, biochemical and histological assays were performed independently and were double-blinded.

2.1. Animals, treatment regimen and induced systemic inflammation

Middle-aged male Sprague-Dawley rats (450–500 grams; 13 months of age at the beginning of the experiments; Charles River Laboratories, Wilmington, MA) were used according to procedures approved by the Institutional Animal Care and Use Committee, Emory University (Protocol # 200–1517). The rats were quarantined for 7 days before the experiment and housed in individual cages in a room maintained at 21–25° C, 45–50% humidity, a 12-h light/dark cycle and free access to food and water. Rats were randomized to the treatment conditions and then the identity of the groups was coded to avoid experimenter bias. The animals were formed into 5 groups (n = 6/group): (1) Sham+vehicle (Sham); (2) MCAO+vehicle (Veh); (3) MCAO+vehicle+LPS (Veh+LPS); (4) MCAO+LPS+P4 8 mg/kg (LPS+P1); (5) MCAO+LPS+P4 16 mg/kg (LPS+P2). P4 (P-0130; Sigma-Aldrich Co., St. Louis, MO) was dissolved in 22.5% 2-hydroxypropyl- β cyclodextrin and administered by intraperitoneal (i.p.) injection 2 h post-occlusion, and then subcutaneously (s.c.) at 6 h, and then every 24 h until day 7 post-occlusion. To model the systemic effect of post-stroke infections, LPS (50 μ g/kg; i.p; 3 separate doses at 4-h intervals) were given at 24, 28 and 32 h post-occlusion (Langdon et al., 2010). On day 7, animals were killed and their brains removed after transcardial perfusion. For each outcome measure, we calculated the starting sample sizes and power needed to reject the null hypothesis with a p-value of 0.05. The number of rats per group at these criteria was determined to be 4 to reject the null hypothesis (H_0) at $P < 0.05$ with a power of 0.80 (effect size $f = 0.25$).

2.2. Transient middle cerebral artery occlusion (MCAO)

Prior to MCAO surgery, isoflurane anesthesia was induced at 5% and then maintained at 1.5–2% during surgery in 2:1 nitrous oxide and oxygen. Focal cerebral ischemia was induced by occlusion of the right middle cerebral artery as previously described (Longa et al., 1989). A midline incision was made on the ventral surface of the neck, and the right common carotid arteries were isolated and ligated with 6.0 silk suture. The internal carotid artery and the pterygopalatine artery were temporarily occluded with a microvascular clip. A 4-0 Doccol filament (Doccol Corporation, Redlands, CA) was introduced into the internal carotid artery through the incision in the external carotid artery. The filament was advanced approximately 20 mm distal to the carotid bifurcation. Relative cerebral blood flow (CBF) was monitored by laser Doppler (LD) for the entire 2 h of occlusion. Drug treatment was randomly assigned 5 min before onset of reperfusion. After 2 h of MCAO, the occluding

filament was withdrawn back into the common carotid artery to allow for reperfusion. Relative CBF was then monitored for 5 min before the wound was sutured and the rats were then permitted to recover from anesthesia. Pulse oximetry (SurgiVet™ V3304; Waukesha, WI) was used to maintain heart rate at approximately 350 bpm, with blood oxygen saturation (SpO₂) levels N95%. Anesthesia duration was the same for all groups.

2.3. Temperature monitoring

Body temperature was monitored throughout surgery (by rectal probe) and maintained at ~37.0° C using an automated heat lamp (Harvard Apparatus, South Natick, MA). We monitored post-ischemic core body temperature hourly from 1–4 h post-injury (before LPS injections) and then 24–35 h post-injury (after LPS injections).

2.4. Serum cytokines and BDNF assay

For serum isolation, blood was collected from the tail vein by the tail nick method at 6, 24, 48, 72 and 168 h post-injury and allowed to coagulate for 30 min at room temperature followed by centrifugation for 5 min at 1000×g. Using sandwich ELISA kits, serum IL-1β (BMS630, eBioscience, San Diego, CA), IL-6 (BMS625, eBioscience) and TNFα (BMS622, eBioscience) were measured at 6, 24, 48, and 72 h post-stroke. Total BDNF (CYT306, Millipore, Billerica, MA) levels were measured at 1, 3 and 7 days post-stroke according to the manufacturer's instructions.

2.5. Behavioral testing

2.5.1. Motor Coordination--Accelerating Rotarod—Motor impairment was assessed with the accelerating rotarod (Ishrat et al., 2009). Rats were given 3 training sessions 5 min apart before surgery. The animals were habituated to the stationary rod, and then placed on the rotating rod. The rod was started at 2 rpm and then accelerated linearly to 20 rpm within 300 sec. Latency to fall off the rotarod was determined before ischemia (pre-surgery) and at 3, 5 and 7 days post-surgery.

2.5.2. Grip strength—Forelimb grip strength was measured pre-surgery and again at 3, 5 and 7 days post-surgery with a grip-strength meter (Columbus Instruments, Columbus, OH). A digital reading (in Newtons) of 3 successive trials was obtained for each rat, and then averaged for analysis.

2.5.3. Sticky-tape removal test—To assess somatosensory dysfunction after tMCAO, we used a modified adhesive removal (sticky-tape) test. A removable sticky label was placed on the underside of the animal's paw contralateral to the stroke, and the time taken for the rats to sense (contact latency) and remove (removal latency) the label was recorded during a 180-sec observation period. Two trials per animal were averaged for analysis before ischemia (pre-surgery) and at 3, 5 and 7 days post-surgery.

2.5.4. Spontaneous locomotor activity—Digiscan™ activity-monitoring boxes were used to assay spontaneous motor activity pre-surgery and then at 3, 5, and 7 days post-surgery. Each session lasted 5 min and was conducted under red-light conditions.

2.6. Analysis of infarct volume

Cerebral infarct size was evaluated using previously applied methods (Ishrat et al., 2010). On post-ischemia day 7, animals were deeply anesthetized using isoflurane. After transcardial perfusion with cold saline followed by 10% buffered formalin, brains were extracted, fixed in gradient sucrose solution and cut coronally into 20-μm sections for histological analysis. Brain sections were stained in 0.1% cresyl violet solution for 10 min at

45° C, and then rinsed in distilled water. Stained sections were fixed by serial dehydration in alcohol and xylene and mounted with xylene-based cyto seal. Fixed sections were coded to hide group identity and then scanned. The infarct areas, defined as areas showing reduced Nissl staining under light microscopy, were traced and quantified with an image-analysis system. The infarct area was measured on each brain section using NIH imaging software (Image-J, version 1.38, NIH, Rockville, MD). Infarct size was then calculated by multiplying the infarct area on each section by the distance between sections and represented as a percentage of the size of the contralateral hemispheric side $\pm$ SEM.

2.7. Spleen and thymic atrophy

On the day of euthanasia (day 7 post-stroke), spleen and thymus were isolated from different groups to determine the systemic effect of stroke on the peripheral immune system and modulatory effects of P4. We compared the organ weights in different groups as a marker of post-stroke atrophy.

2.8. Statistical analysis of data

With sample sizes determined by power analysis, repeated measures one-way analysis of variance (RM-ANOVA) was used for behavioral experiments followed by LSD and Tukey's tests for independent comparisons. Significance was set at $P<0.05$. Data are presented as mean $\pm$ standard error of the mean (SEM). For brain infarction data, we used unpaired t-tests (two-tailed).

3. RESULTS

3.1. Cerebral blood flow measurements and body temperature

Cortical CBF was reduced by at least 70% of pre-ischemic values in all rats subjected to MCAO. There were no significant differences in cortical CBF after occlusion among rats given vehicle, P4, LPS or combination treatment. There were no significant differences in the increase in relative CBF in drug-treated compared to vehicle-treated rats after reperfusion (data not shown). We recorded each animal's post-ischemic core temperature from 1–4 h post-injury (before LPS injections) and then 24–35 h post-injury (after LPS injections). We did not observe any significant increase in core body temperature on the day of surgery in any group. Administration of LPS at 24, 28, and 32 h post-injury slightly increased core temperature (by $\sim 0.4^{\circ}\text{C}$) for only a 5-h interval (28–33 h post-injury) in all the rats subjected to MCAO surgery (data not shown).

3.2. Effect of P4 on spleen and thymic atrophy

We compared the organ weight of spleen (Fig. 1A) and thymus (Fig. 1B) in the different experimental groups. One-way ANOVA revealed a significant group effect in spleen ($F_{(4, 25)} = 3.433$; $P<0.023$) and thymus ($F_{(4, 25)} = 4.281$; $P<0.009$) weights. A significant ($P<0.05$) reduction in spleen ($P<0.006$) and thymus weights ($P<0.001$) was observed in the Veh+LPS group compared to their sham counterparts. The Veh-alone group showed no difference in spleen weight but had a significant ($P<0.01$) reduction in thymus weight compared to shams. P4 at both 8 and 16 mg doses showed a significant recovery in spleen weight ($P<0.01$; $P<0.009$ respectively) and thymus weight ($P<0.02$; $P<0.03$ respectively) compared to the Veh+LPS group.

3.3. P4 modulates inflammatory cytokine levels

3.3.1. Interleukin-1 β (IL-1 β)—Repeated measures ANOVA revealed a significant group effect ($F_{(4, 25)} = 153.60$; $P<0.001$). Post-hoc analysis showed that serum IL-1 β levels were significantly ($P<0.05$) higher in the Veh group at all time points compared to sham values

(Fig. 2A). Delayed multiple LPS injections starting 24 h post-injury significantly ($P<0.05$) enhanced IL-1 β release at 48 and 72 h compared to the Veh group. P4 at both doses significantly decreased IL-1 β levels at all time points compared to the Veh and Veh+LPS groups. There was no significant difference between the two doses of P4.

3.3.2. Interleukin-6 (IL-6)—Repeated measures ANOVA revealed a significant group effect ($F_{(4,25)} = 179.56$; $P<0.001$). Post-hoc analysis showed that serum IL-6 levels were significantly ($P<0.05$) higher in the Veh group at all time points compared to sham values (Fig. 2B). Delayed multiple LPS injections (starting 24 h post-injury) significantly ($P<0.05$) enhanced IL-6 release at 48 and 72 h in the Veh+LPS group compared to Veh values. P4 at both doses significantly decreased IL-6 levels at all time points compared to the Veh or Veh+LPS groups. There was no significant difference between the two doses of P4.

3.3.3. Tumor necrosis factor- α (TNF- α)—Repeated measures ANOVA revealed a significant group effect ($F_{(4,25)} = 58.94$; $P<0.001$). Post-hoc analysis showed that serum TNF- α levels were significantly ($P<0.05$) higher in the Veh and Veh+LPS groups at 24, 48 and 72 h post-injury compared to sham values (Fig. 2C). Delayed multiple LPS injections (starting 24 h post-injury) significantly ($P<0.05$) enhanced TNF- α release at 48 and 72 h compared to the Veh group. P4 at both doses significantly decreased TNF- α levels at 24, 48 and 72 h post-injury compared to Veh or Veh+LPS groups. There was no significant difference between the two doses of P4.

3.4. P4 provides trophic support to ischemic brain by modulating BDNF

Repeated measures ANOVA revealed a significant group effect ($F_{(4,25)} = 24.838$; $P<0.001$). Post-hoc analysis showed that serum BDNF levels were significantly ($P<0.001$) decreased in the Veh and Veh+LPS groups at days 3 and 7 post-injury compared to sham-operated rats (Fig. 3). This effect was more pronounced in the Veh+LPS group at days 3 and 7 post-injury ($P<0.01$ and $P<0.001$ respectively) compared to Veh. At day 1 post-injury, no difference in serum BDNF levels was observed in any groups. P4 treatment at both doses showed a significant increase ($P<0.001$) in BDNF levels compared to the Veh and Veh+LPS groups at 3 and 7 days post-injury. The highest levels of BDNF were observed at day 7 post-injury in P4-treated groups, suggesting direct involvement of BDNF in P4's neurotrophic action. There was no difference in the two doses of P4 on BDNF release.

3.5. Delayed, systemic inflammation exacerbates post-stroke functional outcomes and P4 treatment reduces the ensuing behavioral deficits

3.5.1. Rotarod—Repeated measures ANOVA revealed a significant group effect ($F_{(4,26)} = 33.059$, $P<0.001$) in rotarod activity. Vehicle-treated rats showed a significantly ($P<0.001$) impaired ability to remain on the moving rotarod compared to shams at days 3, 5 and 7 post-injury (Fig 4A). Delayed LPS exposure in vehicle++LPS-treated rats significantly ($P<0.01$, 0.04) worsened rotarod performance compared to the Veh group at days 3 and 7 post-injury respectively. This detrimental effect of LPS was highly significant ($P<0.001$) compared to sham values. *Post-hoc* analyses showed that P4 treatment at both 8 and 16 mg/kg significantly ($P<0.001$) reduced this motor deficit at days 3, 5 and 7 post-stroke compared to the Veh+LPS group. On day 7, there were no significant differences in motor recovery between the two doses of P4.

3.5.2. Grip strength—A significant group effect ($F_{(4,26)} = 42.263$, $P<0.001$) was shown by repeated measures ANOVA (Fig 4B). A significant ($P<0.001$) reduction in muscle strength in the Veh animals was observed at 3, 5 and 7 days post-injury compared to shams. *Post-hoc* analyses showed a significant ($P<0.01$, 0.05, 0.01) decrease in grip strength in the Veh+LPS group compared to the Veh alone group. P4 at both doses significantly ($P<0.001$)

improved grip strength at 3, 5 and 7 days post-stroke compared to the Veh+LPS group. There was no significant difference in motor recovery between the two doses of P4 at day 7 post-injury.

3.5.3. Sticky-tape removal—Repeated measures ANOVA showed significant group effects in the sticky-tape contact ($F_{(4, 26)} = 65.973$, $P < 0.001$) and removal ($F_{(4, 26)} = 150.712$, $P < 0.001$) latencies. *Post-hoc* analyses showed a significant ($P < 0.001$) increase in contact and removal latency to remove the sticker from the contralateral forepaw in the Veh group compared to sham values at 3, 5 and 7 days post-injury (Fig. 4C). Systemic inflammation significantly increased contact latency ($P < 0.001$, 0.01 at days 3 and 5 respectively) and removal latency ($P < 0.002$, 0.01, 0.001 at days 3, 5 and 7 respectively) in the Veh+LPS group compared to Veh animals. P4 treatment showed a highly significant ($P < 0.001$) decrease in both contact and removal latencies at all time points compared to the Veh+LPS group. No significant differences in contact and removal latency were observed between the two doses of P4 at day 7 post-injury.

3.5.4. Locomotor Activity—Repeated measures ANOVA showed significant group effects ($F_{(4, 25)} = 63.423$, $P < 0.001$). *Post-hoc* analyses showed a significant ($P < 0.05$) decrease in distance travelled in the Veh group at all time points compared to shams and this effect was worse in the Veh+LPS animals (Fig. 4D). P4 treatment at both doses equally produced a significant ($P < 0.001$) recovery in locomotor activity at days 3, 5 and 7 compared to the Veh and Veh+LPS groups.

3.5. Infarct volume: effect of systemic inflammation and P4 treatment

On day 7 post-injury, infarct volume in the Veh group was not significantly different from that in the Veh+LPS group (Fig. 5). However, P4 at both 8 and 16 mg significantly ($P < 0.05$) reduced infarct volume compared to either of the vehicle groups.

4. DISCUSSION

Our findings demonstrate that: (1) delayed and prolonged systemic inflammation exacerbates stroke outcome, and (2) P4 treatment enhances behavioral recovery and reduces stroke infarct volume in middle-aged rats with an induced systemic infection. We also show that P4 treatment inhibits the inflammatory response to ischemic injury and enhances neurotrophic BDNF levels with BDNF having been shown to play a critical role in neuronal recovery/repair (Greenberg et al., 2009; Meyer et al., 2012; Su et al., 2012).

4.1. Progesterone and the systemic inflammatory response to ischemic stroke

Both CNS-specific and systemic inflammation can play a critical role in the outcome of brain injury, but the systemic component of the disease is infrequently given attention in animal experiments on brain injury and its molecular and functional consequences (see Stein, 2012 for review). However, a few laboratories have demonstrated the effects of acute systemic inflammation induced by a single bolus dose of LPS either immediately before or after ischemia to model the systemic effects of an infection (Spencer et al., 2007; McColl et al., 2007, 2008; Marsh et al., 2009). In our project, we adopted a delayed prolonged systemic inflammation approach to post-stroke infection because we think that model is more clinically relevant (Langdon et al., 2010). Since delayed low doses of LPS do not produce the acute inflammation or febrile response that may occur at higher doses (Spencer et al., 2007), our model produced more prolonged inflammatory conditions to mimic what might occur in human stroke patients.

Infection is the most common cause of fever in stroke victims (Kumar et al., 2010). In the acute stage of the disease, a post-stroke rise in body temperature has been independently associated with an increased risk of poor outcome in patients (Polderman, 2008), so we believe it is clinically important to differentiate whether the injury and associated functional deficits result from the stroke itself or from post-stroke pyrexia. In our study we recorded each animal's post-ischemic core temperature at several time points over 48 h. Post-stroke injection of LPS at 24, 28, and 32 h slightly increased core temperature (by $\sim 0.40^{\circ}\text{C}$) only over a 5-h period (28 to 33 h post-injury), an increase not sufficient to worsen functional deficits or brain infarction, given the finding that a post-ischemic brain temperature of 39°C showed no effects on stroke outcomes (Kim et al., 1996). Unlike our study with its prolonged post-injury time points, previous reports on hyperthermia and stroke observed brain temperature only during or shortly after stroke (MacLellan et al., 2006). Further, the comparatively low doses of LPS may not have been sufficient to significantly raise body temperature. This finding is consistent with others (Langdon, 2010). Taking all the data together we propose that the observed deficits in short-term stroke outcomes are due in mainly to systemic inflammation induced by LPS.

Stroke is a multi-organ systemic disease which induces a biphasic effect on the peripheral immune system characterized by an early activation of peripheral leukocytes followed by a delayed severe immunosuppression and atrophy of the spleen and thymus (Dziennis et al., 2011). Offner et al. (2006) reported the drastic effects of stroke on the gross morphology and cell numbers in spleen and thymus, and on splenocyte function and cellular distribution by 96 h after stroke. Our findings are in agreement with these reports. We observed a reduction in spleen and thymus weights in the Veh+LPS group which was significantly restored after P4 treatment. These findings suggest that P4 not only provides neuroprotection to the ischemic brain but may also modulate post-stroke immunodepression. Since spleen and thymus are the major organs which regulate peripheral immune cells under both healthy and pathological conditions, it would be interesting to explore the specific cellular/molecular effects of P4 on splenic/thymic atrophy following stroke. We are currently investigating these mechanisms in another series of experiments.

We observed that MCAO significantly increases serum levels of pro-inflammatory cytokines IL-1 β and IL-6 starting at 6 h and for up to 72 h post-injury. Serum TNF α level was significantly higher at 24, 48 and 72 h, but no significant difference was observed at 6 h among the treatment groups. It is possible that some TNF α derives from activated astrocytes and that may be why we did not observe higher serum/systemic TNF α levels at 6 h. Although TNF α has been reported to be up-regulated in ischemic brain within 6 h post-injury (Jiang et al., 2011), serum cytokines may have a different response from those of brain cytokines. Our findings are in agreement with Langdon et al. (2010), who also observed a delayed response for TNF α and fast response for IL-1 β and IL-6. These cytokine levels were further elevated by LPS injections administered at 24 h post-injury. The cause of this increase could be that microglia and macrophages recognize LPS and respond by secreting pro-inflammatory cytokines such as TNF α and IL-6 (Boje and Arora, 1992; Bhat et al., 1998). Our findings are in agreement with previous reports also showing elevated levels of serum IL-1 β , IL-6 and TNF α after prolonged LPS injection at various time points after injury (Langdon et al., 2010). A single low dose of LPS given immediately after global ischemia has been reported to elevate IL-6 and TNF α (Spencer et al., 2007). McColl et al. (2007) for the first time demonstrated that acute systemic inflammation induced by LPS injection is detrimental to stroke outcome, and that the resulting brain damage and neurological deficits were IL-1 dependent in stroke in mice. Systemic inflammation has been reported to impair stroke outcome by increasing brain inflammation, blood-brain barrier injury and brain edema formation in mice after experimental stroke (Denes et al., 2011). We observed that P4 treatment at both 8 and 16 mg doses significantly reduced

serum levels of IL-1 β , IL-6 and TNF α compared to the Veh+LPS group. There are other studies showing that P4 is a potential anti-inflammatory agent following acute stroke or TBI (Gibson et al., 2005; Cekic et al., 2011; Hua et al., 2011; Wang et al., 2011). P4 is also known to reduce LPS and IFN- γ -stimulated nitrite production in a dose-dependent manner (Miller et al., 1996; Robert and Spitzer, 1997).

4.2. Progesterone and BDNF: the neurotrophic response to injury

P4 not only protects ischemic brain from inflammatory damage, it also provides trophic support to the injured brain by up-regulating neurotrophic factors that promote functional recovery. Neurotrophic factors are known to exert beneficial effects on cellular and behavioral recovery following brain injury (Sofroniew et al., 2001; Althaus et al., 2008; Su et al., 2012; Morali et al., 2012). The best-understood trophic factors in the contexts of TBI and stroke are NGF and BDNF. BDNF is mainly synthesized by neurons and found in substantial amounts in brain (Hu and Russek, 2008; Meyer et al., 2012), where it plays a crucial role in neuronal survival and plasticity through the p75NTR and TrkB receptors (Chen et al., 2005; Greenberg et al., 2009). BDNF is also present in the blood, where it is at a higher concentration in serum than in plasma (Fujimura et al., 2002). Although the cellular source of the BDNF present in plasma is not known, there are several reports suggesting that BDNF may be secreted into plasma by endothelial or circulating immune cells (Kerschensteiner et al., 1999; Nakahashi et al., 2000; Bayas et al., 2002; Wang et al., 2006). The brain has been hypothesized to be an additional source of the BDNF present in plasma based on the evidence of parallel changes in serum and cortical brain BDNF during postnatal development in rats (Karege et al., 2002). BDNF is one of the neurotrophic factors whose expression is affected by cerebral ischemia (Abe and Hayashi, 1997) and appears to protect against cerebral ischemia-induced neuronal loss (Mattson et al., 2004). Direct intracerebral, intraventricular, and intravenous administration of BDNF has been shown to reduce infarct volume in several stroke models (Mizuno et al., 2000; Shi et al., 2009).

We measured serum BDNF levels at 1, 3 and 7 days post-injury to determine whether BDNF plays a role in P4's beneficial effects in post-stroke systemic inflammation and functional recovery. We found that ischemic injury induces a significant decrease in serum BDNF levels at 3 and 7 days but not at 24 h post-injury. We also observed a significant increase in serum BDNF levels in our P4-treated group at 3 and 7 days post-injury. BDNF expression is thought to be modulated by P4 in several brain injury models (Coughlan et al., 2009; Jodha et al., 2009) and is involved in P4's mechanisms of action in the CNS (González et al., 2004; Kaur et al., 2007; Swiatek-De Lange et al., 2007). We previously reported that brain BDNF levels remained unchanged at 24 h post ischemia in a permanent rat model of stroke but decreased significantly at 3 and 14 days post-stroke. P4 treatment increased BDNF levels and was associated with better functional outcomes (Ishrat et al., 2012).

4.2.1. Do serum and brain levels of BDNF have to match?—It remains unclear whether serum BDNF levels match brain BDNF levels following stroke. There is both positive and negative evidence. Some researchers report that both serum and brain BDNF levels complement each other in response to stroke (Di Lazzaro et al., 2007; Jimenez et al., 2009; Yang et al., 2011; Zhou et al., 2011). In contrast, others argue that changes in regional brain BDNF levels are not associated with changes in plasma or serum (Elfving et al., 2010; Luo et al., 2010). One recent study suggests that circulating BDNF levels do not mirror brain BDNF levels after permanent stroke, and severe stroke is associated with high plasma BDNF in the very acute stage (Bejot et al., 2011). However, this study has the limitation that the stroke model used lacked reperfusion after the initial blockage, so the data may apply only to patients or experimental subjects lacking reperfusion. These findings suggest that

reperfusion may be associated with the passage of BDNF from the brain into the blood. In the present study, we did not measure and compare brain BDNF with serum BDNF levels at different time points, but it is an intriguing question needing further investigation to confirm whether brain BDNF levels mirror systemic BDNF levels following stroke, and whether the BDNF expression profile is time-dependent and varies in different stroke models. Further studies are also needed to explore whether systemic BDNF over-expression is simply a consequence of improved health of the P4-treated animals or a mechanism underlying P4 neuroprotection].

4.2.2. How BDNF and inflammation are related—In addition to providing trophic support, BDNF also plays a critical role in modulating the inflammatory process under different pathologic conditions. It inhibits microglial apoptosis and promotes microglial proliferation and phagocytic activity *in vitro* (Zhang et al., 2003). BDNF has also been reported to decrease TNF α expression while up-regulating the expression of IL-10, an anti-inflammatory cytokine, in a model of multiple sclerosis (Makar et al., 2009). BDNF activates NF κ B and protects cells from serum starvation and glutamate toxicity *in vitro* (Yeiser et al., 2004; Kajiya et al., 2009). Activated NF κ B not only inhibits apoptosis but also promotes neuronal survival, whereas its inhibition aggravates ischemic injury (Hill et al., 2001; Valerio et al., 2009). In an experimental stroke model, intranasal BDNF has been reported to modulate local inflammation and protects against ischemic brain by up-regulating anti-inflammatory cytokine IL-10, down-regulating the pro-inflammatory cytokine TNF α , and increasing the DNA-binding activity of NF κ B (Jiang et al., 2011). These findings support our data on systemic BDNF levels and cytokine expression. We speculate that high systemic BDNF levels may contribute to the observed anti-inflammatory effects of P4 and provide trophic support for the repair of damaged neurons in the injured brain. If true, this is one of the reasons P4 can be understood as a pleiotropic hormone (Stein, 2008; 2012).

4.3. Does LPS make a stroke infarct worse? It depends

We observed that animals with MCAO treated with either vehicle or multiple doses of LPS developed similar infarct size by day 7. Langdon et al. (2010) reported that young male rats subjected to 24-h delayed LPS treatment showed larger infarct volumes than animals treated with vehicle. Conversely, we observed no effect of LPS treatment on infarct volume in middle-aged rats. The difference may be due to the age factor. It has been reported that because of their attenuated inflammatory response to stroke, aged animals tend to develop smaller infarcts than their younger counterparts (Sieber et al., 2011). We speculate that because middle-aged rats have smaller infarcts, the low LPS dose we used may not have been sufficient to affect infarct volume. We observed that P4 treatment at both 8 mg and 16 mg/kg reduced infarct volume by 43.25% and 48.19% respectively compared to the Veh +LPS group. We and others have previously reported beneficial effects of P4 in different stroke models (Gibson and Murphy, 2004; Gibson et al., 2005; Sayeed et al., 2006, 2007; Ishrat et al., 2009).

In our study ischemia/reperfusion injury significantly impaired locomotion, grip strength, sensory neglect and motor activity in middle-aged rats over 7 days post-injury. Prolonged, systemic inflammation induced by LPS injections beginning 24 h after the stroke worsened the functional deficits compared to vehicle-treated animals. Systemic inflammation has been reported to impair behavioral functions in rats, and our findings are in agreement with those studies (McColl et al., 2007; Langdon et al., 2010). Treatment with P4 at both doses attenuated functional deficits on various behavioral tests (rotarod, grip strength, locomotion and sensory neglect) at different time points over the 7 days of post-injury treatment. These

findings are consistent with previous reports (Gibson and Murphy, 2004; Gibson et al., 2005; Sayeed et al., 2006, 2007; Ishrat et al., 2009; Gibson et al., 2011).

5.0. CONCLUSION

In this study, we investigated the short-term effects of post-stroke infection and the neuroprotective effects of the hormone P4 in middle-aged rats. Our findings indicate that post-stroke systemic inflammation worsens stroke outcomes as assessed by multiple outcome measures including expression of pro-inflammatory cytokines, infarct volume and behavioral testing. P4 treatment at both 8 and 16 mg/kg showed equally beneficial effects. Our data can be interpreted to indicate that BDNF can play a key role in modulating the detrimental effects of post-stroke systemic inflammation and providing trophic support essential for brain repair. At this point, we do not know all of the exact mechanisms or signaling pathways by which P4 modulates post-stroke systemic inflammation—this needs to be investigated in future studies. We will also need to consider the long-term and systemic effects of post-stroke infection on functional outcomes and the role of P4 treatment in keeping infection at bay. In most animal studies of stroke and TBI, mechanisms (and treatments) that can affect and alter systemic shock and sepsis are rarely given consideration in determining the biomarkers of injury and repair. This area of investigation needs much more attention if we are to understand and treat the complex factors that contribute to stroke and brain injury. Overall, our preliminary findings suggest that P4 treatment may be beneficial not only in the treatment of ischemic stroke itself but also in fighting post-stroke infections.

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ABBREVIATIONS

ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
CNS	central nervous system
FDA	food and drug administration
HPA	hypothalamo-pituitary adrenal axis
IL-1β	interleukin-1 β
IL-6	interleukin-6
LPS	lipopolysaccharide
MCAO	middle cerebral artery occlusion
P4	progesterone
SEM	standard error of the mean
SNS	sympathetic nervous system
TBI	traumatic brain injury
TNFα	tumor necrosis factor alpha
tPA	tissue plasminogen activator

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HIGHLIGHTS

- Prolonged systemic post-stroke inflammation worsens stroke outcomes in middle-aged rats.
- Systemic inflammation worsens behavioral deficits in stroke animals.
- Progesterone treatment reduces infarct volume after transient stroke.
- P4 reduced functional deficits by modulating pro-inflammatory cytokines and BDNF.

Fig. 1. Effect of P4 on stroke-induced spleen (A) and thymic (B) atrophy 7 days post-injury. Values are expressed as means $\pm$ SEM. Significant difference # P <0.05 compared to sham; * P <0.05 compared to vehicle+LPS (Veh+LPS).

Fig. 2.

Effect of post-stroke systemic inflammation on inflammatory markers (A) IL-1 β , (B) IL-6 and (C) TNF α at 6, 24, 48 and 72 h post-injury and modulation by P4. Values are expressed as means $\pm$ SEM. Significant difference [#] P <0.05 compared to sham; ^{*} P <0.05 compared to vehicle (Veh) and vehicle+LPS (Veh+LPS); [§] P <0.05 compared to Veh.

Fig. 3. Effect of P4 on serum BDNF levels at 1, 3, and 7 days post-injury. Values are expressed as means $\pm$ SEM. Significant difference [#] $P < 0.05$ compared to sham; ^{*} $P < 0.05$ compared to vehicle (Veh) and vehicle+LPS (Veh+LPS); [§] $P < 0.05$ compared to Veh.

Fig. 4. Effect of post-stroke systemic inflammation on functional outcomes at 3, 5 and 7 days post-injury using rotarod (A), grip strength (B), sticky tape removal (C), and locomotor activity (D) tests. Values are expressed as means $\pm$ SEM. Significant difference $^{\#}P < 0.05$ compared to sham; $^*P < 0.05$ compared to vehicle (Veh) and vehicle+LPS (Veh+LPS); $^{\$}P < 0.05$ compared to Veh. CL, contact latency; RL, removal latency.

Fig. 5.

Effect of post-stroke systemic inflammation and P4 treatment on brain infarction. Representative photographs of cresyl violet-stained sections are shown. Values are expressed as means $\pm$ SEM. Significant difference * $P < 0.05$ compared to Veh and Veh+LPS groups. Data were analyzed using a two-tailed unpaired t-test. Delayed systemic inflammation did not increase infarct size. P4 showed a similar reduction in infarct size at both doses.