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Progesterone in the Treatment of Transient Ischemic Stroke: A Dose-Response Study

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Abstract

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Stroke is the third leading cause of death in America and accounts for 6% of the total U. S. health care budget. In spite of the devastating effects stroke exerts on society, only one treatment has ever been developed that has passed clinical trials: tissue plasminogen activator (tPA). Unfortunately, tPA use is limited to less than 5% of stroke victims, leaving 95% without viable treatment. Studies conducted in numerous neural insult models have identified progesterone (PROG) as a potent, pleiotropic neuroprotective steroid. Preliminary studies conducted using PROG in the treatment of murine ischemic stroke models have demonstrated that PROG may also be neuroprotective in stroke. The aim of this study was to determine the best dose of PROG for the treatment of transient ischemic stroke in middle-aged rats. An intraluminal filament and suture method was used to induce a transient (2 h) occlusion in the right middle cerebral artery. Three doses of PROG were examined: 8 mg/kg, 16 mg/kg, and 32 mg/kg. Treatment was administered two and six hours post-ischemia, then once daily for seven consecutive days; the last two doses were tapered in order to prevent PROG withdrawal syndrome. Cognitive and behavioral outcomes were evaluated using accelerating rotarod and Morris water maze paradigms. Twenty-two days post-occlusion, rats were perfused and infarct volume quantified. Results, in conjunction with previously reported findings, suggest that 8 mg/kg of PROG is within the ideal therapeutic range for treating ischemic stroke. Further studies should confirm these findings in other models of stroke and in models of populations that are at a higher risk for developing stroke, such as diabetic and hypertensive populations.

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Introduction

Stroke

Stroke is the third leading cause of death in America, following heart disease and cancer ("Heart disease", 2010; Heron *et al.*, 2006). Someone dies as the result of a stroke approximately every three minutes (Carmichael, 2005), and those who survive consume approximately 6% of the total U.S. health care budget (Durukan & Tatlisumak, 2007). A stroke is a serious brain injury that occurs when there is a disturbance in blood supply to any part of the brain, and can be the result of either ischemia (lack of blood supply to a region due to a cerebrovascular occlusion) or hemorrhaging (blood permeating the brain) (Stein & Hurn, 2009); approximately 87% of strokes are ischemic while the remaining 13% are hemorrhagic ("Heart disease", 2010). It is important to realize that a stroke is not an isolated incident within the brain. The primary injury to neural tissue is typically the beginning of a complex and devastating series of events that lasts over hours, days and even months; these events include cytotoxic and vasogenic cerebral edema and secondary pro-inflammatory, excitotoxic, oxidative stress, necrotic and apoptotic cascades that lead to progressive brain damage (Stein & Hurn, 2009). While the regions primarily injured by the stroke are often irreparable, the penumbra (region surrounding the primary infarct) may be saved by intervention.

In humans, it has been reported than 80% of ischemic strokes occur in the middle cerebral artery (MCA) ("Heart disease", 2010; Slater, Curtin, Johns & Schmidt *et al.*, 2011). Occlusion in this region can result in extensive tissue damage to the neocortex, striatum and hippocampus. Observed deficits are dependent upon the brain regions damaged and the severity of the injury. MCA stroke patients typically experience some degree of somatosensory, motor and memory impairment (Slater *et al.*, 2011). In addition to these

general stroke deficits, those with a lesion in the left hemisphere are more likely to experience dystaxia and dysphasia, while those with a lesion in the right hemisphere are more likely to experience hemispatial neglect.

In spite of the predominance of stroke incidence in the United States, only one pharmacological treatment has been developed that has passed clinical trials, tissue plasminogen activator (tPA). tPA is an endogenous fibrin specific activator for the conversion of plasminogen to plasmin and can stimulate thrombolysis, and is thereby capable of restoring blood flow to embolism-induced ischemic tissue (Cronin, 2010; Lapchak, Chapman & Ziyin, 2000). However, it can only be given under very stringent conditions; it must be given to someone who has a confirmed ischemic stroke with an intact cerebral occlusion within 3-4.5 hours after stroke onset; therefore a patient is not eligible for treatment if they do not know with certainty when the stroke began. While clinical studies have demonstrated that tPA can be beneficial when given to ischemic stroke patients under these strict guidelines, tPA has also been associated with increased risk of intracerebral hemorrhage (Hacke *et al.*, 2004; Hemmen, Rapp, Emond, Rama & Lyden, 2010) and is capable of inducing increased vascular permeability and infarct volume following cerebral stroke (Lapchak *et al.*, 2000).

These devastating side effects are attributable to the neurotoxic cascades tPA is able to initiate after gaining access to extracellular neuronal space in patients that already have a compromised blood brain barrier. tPA has been shown to stimulate other proteolytic enzymes, such as matrix metalloproteinase-9, which has been implicated in facilitating the break down of the blood brain barrier (Romanic, White, Arleth, Ohlstein & Barone, 1998; Sayeed, Hoffman & Stein, 2006); stimulate the production of caspase-3 (Kaur, Zhao, Klein, Lo & Buchan, 2004), promoting apoptosis; cleave the N-Methyl-D-aspartate (NMDA) NR1 subunit and amplify

intracellular calcium conductance, increasing excitotoxicity (Baron *et al.*, 2010; Kaur *et al.*, 2004). All of these may perpetuate the secondary effects of the stroke following focal ischemia.

Interestingly, endogenous tPA is expressed predominantly in neural tissues that are most susceptible to ischemic damage, including the hippocampus (Lee *et al.*, 2007). Lee *et al.* (2007) demonstrated that tPA knockout mice exhibit less neuronal loss in the hippocampus following transient global ischemic stroke than wild type, and express less MMP-9 and caspase-3 than wild type mice, suggesting that tPA plays a role in the hippocampus's greater susceptibility to ischemia than other neuronal tissues.

Contrary to Lee *et al.*'s (2007) findings, Echeverry, Wu, Haile, Guzman & Yepes (2010) demonstrated that tPA presence in the hippocampus may actually be neuroprotective. The CA1 region of the hippocampus is the most susceptible to ischemic injury, but interestingly is the region of the hippocampus that does not express basal levels of tPA (Salles *et al.*, 2002). The expression of tPA in CA1 neurons was induced via ischemic preconditioning in mice. Successive exposure to lethal levels of hypoxia resulted in a decrease in cell death in CA1 neurons. Echeverry *et al.* (2010) was also able to demonstrate that the tPA neuroprotective mechanism was a result of a non-proteolytic interaction between tPA and member of the LDL receptor family. This finding may reconcile the opposing findings between Echeverry *et al.* (2010) and Lee *et al.* (2007). Further studies have supported the hypothesis that tPA may be neuroprotective via its non-proteolytic mechanisms. It has been shown to be critically involved in neurite outgrowth and path finding during brain development and synaptic plasticity (Sappino *et al.*, 1993; Seeds, Williams & Bickford, 1995; Zhang, Kanaho, Frohman & Tsirka, 2005). Moreover, it has been demonstrated to attenuate zinc induced neuronal toxicity in cortical cultures, and, when injected into cerebrospinal fluid, also reduced kainate seizure-induced

hippocampal neuronal death in adult rats (Kim, Park, Hong & Koh, 1999). In spite of the apparent dual nature of tPA, the excitotoxic and hemorrhagic induced side-effects have yet to be overcome by the neuroprotective properties tPA may exhibit. As a result of its deleterious effects, less than 5% of patients that experience a stroke are eligible to receive tPA or any other kind of treatment besides rehabilitation, illustrating the strong need for a broader, more innocuous treatment for stroke (Hemmen *et al.*, 2010).

Progesterone and Traumatic Brain Injury

Because of the numerous and diverse effects acute brain damage has on brain pathology, cognitive ability, memory, behavior and motor skills and on inflammatory cascades throughout the body, it is understandable why the past several decades have yielded few safe, effective pharmacotheraputic treatments (Beauchamp, Mutlak, Smith, Shohami & Stahel, 2008, 2008; Cronin, 2010; Stein & Hurn, 2009; Temkin *et al.*, 2007). However, recent clinical trials have demonstrated the neurosteroid progesterone (PROG) to be effective at reducing mortality and increasing both behavioral and cognitive recovery in traumatic brain injury (TBI) (Wright *et al.*, 2007). Similar to stroke in many regards (Leker & Shohami, 2002), TBI is a heterogeneous and multisystem disorder that results from a primary neural insult, which leads to the initiation of similar secondary pro-inflammatory cascades, oxidative stress, cerebral edema and apoptosis (Stein & Hurn, 2009; Stein, Wright & Kellermann, 2008). Now in phase III clinical trials, PROG has increased survival by 60% and improved behavioral and cognitive outcomes four-fold in enrolled, moderate to severe TBI patients at 30 days and six months following injury (Wright *et al.*, 2007; Xiao, Wei, Yan, Wang & Lu, 2008). Furthermore, it has a low side-effect profile in adults and is extremely accessible and inexpensive. Recent clinical trials of other

once promising treatments, such as corticosteroids (once the mainstay in TBI treatment), magnesium sulfate and hypothermia resulted in higher mortality rates in treatment groups compared with controls (Beauchamp *et al.*, 2008; Stein & Hurn, 2009; Temkin *et al.*, 2007). Due to the similarity in secondary cascades and the need for neuroprotection, it is hoped that PROG as a treatment for stroke will one day prove as effective as it has for TBI.

Progesterone

Despite mounting evidence, the clinical efficacy and use of PROG, what some consider to be a mere female hormone, is still debated. PROG as a treatment for neurological insult was discovered as a result of a clinical observation that females recovered better from TBI than males, a finding that has been replicated in both rats and humans (Cutler, Pettus, Hoffman & Stein, 2005; Stein & Hurn, 2009; Stein & Wright, 2010). PROG, a neurosteroid that is produced in equal amounts in both male and female brains, is pleiotropic and naturally involved in the modulation of the immune system, aquaporin and proteolytic enzyme expression, and GABAergic transmission, all of which are altered by traumatic brain injury (Stein & Hurn, 2009). Thus far, studies have indicated that PROG plays a role in attenuating cytogenic and vasogenic cerebral edema, excitotoxicity and apoptosis as well as promoting remyelinization in numerous neural insult models (Stein, 2008; Stein & Hurn, 2009). Any one (or combination) of these effects could account for the neuroprotective effect of PROG.

It was originally thought that PROG was solely involved in pro-gestational development; circulating PROG levels are 9-10 times higher in pregnant compared with non-pregnant women, and in pseudo-pregnant but not in non-pregnant rats (Stein & Hurn, 2009). These findings suggest that PROG might have evolved neuroprotective effects to safeguard the fetus. Such

observations support the hypothesis that after brain injury, many processes involved in CNS repair are thought to recapitulate normal brain development (Eidelberg & Stein, 1974)

Progesterone and Stroke

In fact, studies have shown that women who are in their third trimester of pregnancy are significantly less likely to experience either a hemorrhagic or ischemic stroke compared with women who are close to or have just delivered (Salonen, Lichtenstein, Bellocco, Petersson & Cnattingius, 2001). This is likely the dramatic drop in PROG near and following birth (Eidelberg & Stein, 1974). Another study, conducted by Savitz, Schlaug, Caplan & Selim (2005), reported that women treated with tPA had significantly better functional outcomes at 90 days survival compared with me. All other contributing factors accounted for, such as early recanalization, it is possible that the observed sex differences are potentially attributable to circulating levels of estrogen or progesterone; the latter is more likely considering the mounting evidence supporting progesterone as neuroprotective (Stein & Hurn, 2009).

Furthermore, studies conducted in murine models of stroke have confirmed PROG's ability to reduce infarct size and improve behavioral outcomes in both transient (Sayeed *et al.*, 2006) and permanent (Ishrat, Sayeed, Atif, Hua & Stein *et al.*, 2010) middle cerebral artery occlusion models of stroke. PROG treatment doses and regiments were based on previous TBI data, which suggest that 8 mg/kg of PROG is the best therapeutic dose. Furthermore, studies conducted in TBI rat models have demonstrated the differential ability of PROG to improve outcome when given in non-therapeutic doses (Goss, Hoffman & Stein, 2003). This emphasizes the importance of obtaining specific preliminary data regarding the

effect of dose and duration of treatment of PROG for the treatment of stroke in order to optimize cognitive and behavioral outcomes (Cutler *et al.*, 2005; Goss *et al.*, 2003). A dose-response study has yet to be conducted in a stroke model of acute brain injury and is the primary aim of this study

Method

Subjects

50 male Sprague-Dawley middle-aged rats, (500-550 g) were examined in this dose-response study. Middle-aged rats were used in order to model age as a risk factor for stroke ("Heart disease", 2010); these animals may represent a form of pathology that is more typical of what might be seen in older individuals, making them a more appropriate model of what is seen in a clinical setting. Animals were housed in individual plastic cages on a 12/12h light/dark cycle. They were ordered from Harlan Laboratories, housed for one month prior to surgery and handled daily beginning one week prior to surgery. The experiment was conducted in five cohorts, each consisting of approximately 15 rats. The objective was to have a sample size of 8-10 animals from each treatment group survive and successfully complete behavioral and histological analysis; 12 sham, 8 vehicle, 10 P8, 10 P16 and 10 P32 rats met these criteria. However, not all animals were included in statistical analysis, including those that did not meet laser doppler flow inclusion criteria, those that were unable to complete the behavioral tasks, and those in which technical difficulties were encountered during data collection.

Injury

A transient ischemic stroke was induced in rats in the middle cerebral artery (middle cerebral artery occlusion: MCAO). Isoflurane anesthesia was used and a SurgiVet pulse oximeter monitored and maintained SpO2 at levels ≥ 90%. Body core temperature was maintained at 37.0 ± 0.5 ºC using a rectal thermometer and heat lamp system. Rats were mounted in a stereotaxic frame and a dorsal, midline incision made in the scalp. A right, unilateral craniotomy was made to allow for the insertion of a small laser doppler in order to measure blood flow in the right frontal cortex. The rat was then turned over and a medial incision made in its throat. The right pterygopalatine artery, which branches from the internal carotid artery, was isolated and temporarily occluded in order to ensure consistent occlusion of the MCA. The right carotid artery was then isolated from surrounding connective tissue. The external carotid was permanently cauterized and a small incision made for the insertion of a 4-0 monofilament occluder. This was threaded 20 mm through the external carotid, into the internal carotid and terminated in the distal MCA. After insertion of the filament, blood flow through the pterygopalatine artery was restored. It is unlikely that observed deficits following this stroke surgery are attributable to the temporary occlusion of the pterygopalatine artery (Chen, Ito, Takai & Saito, 2008), but it is necessary for generating a specific, highly replicable infarct in the MCA. Reduced blood flow was measured using the laser doppler; occlusion was defined as a 50% or more reduction in blood flow. The occluder remained in place for 120 minutes. Bleeding was monitored and wounds covered in cotton soaked in saline solution. Five minutes before reperfusion, the first treatment injection was given intraperitoneally. After reperfusion, which was defined as a return of blood follow to 75 % or more of baseline levels, the rat was monitored for an additional five minutes and then the wounds cleaned, sutured and

the animal allowed to recover from anesthesia. Animals which did not meet reperfusion criteria were excluded from further behavioral analysis. Sham rats were anesthetized, mounted in the stereotaxic frame and had their scalps and necks cut and sutured but were not subject to a craniotomy nor to any artery cauterization. Surgery procedures were adapted from a similar study conducted by Smrcka *et al*. (2001).

Progesterone Treatment

Treatments were prepared by dissolving PROG in vehicle, 22.5% 2-hydroxypropyl-β-cyclodextrin vehicle solution. There were five treatment groups, including a sham injected with vehicle (sham), a tMCAO plus vehicle group (vehicle), and three tMCAO plus PROG groups. Treatments consisted of 8 mg/kg, 16 mg/kg and 32 mg/kg PROG doses (P8, P16, and P32 respectively). All treatment groups were included in each cohort and researchers were blinded to treatment. The first treatment was administered five minutes before reperfusion by intraperitoneal injection with subsequent treatments administered subcutaneously at 6 h and then at 24 h intervals for seven days. The PROG dose was halved the sixth day and then halved again the seventh day in order to prevent PROG withdrawal syndrome (Cutler *et al.*, 2005).

Behavioral testing

Animals began training for the accelerating rotarod test six days before surgery, and baseline measures taken the day before surgery. Rotarod tests were subsequently performed on days 2, 6, 9 and 21 days post-surgery. Animals were trained and tested for spatial navigation learning in the Morris water maze on days 13-21 post-surgery. Throughout injections, behavioral testing and histological analysis, experimenters were blinded to animal

treatment groups.

Rotarod. Locomotor function was evaluated using the accelerating rotarod test. Rats were trained for five days prior to surgery on the accelerating rotarod apparatus. For training, rats were required to walk on the accelerating rotarod for a period of five minutes per day. The first day of training, the rod only accelerated to 1 rpm. The second day of training the rod accelerated up to 3 RPM, the third day of training up to 4 rpm and fourth and fifth days up to 5 rpm. Rate of acceleration was 0.1 RPM per second. For testing, rats were placed on the rotarod, which began at 1 rpm and accelerated up to 5 rpm over a period of five minutes. Latency to fall of the rotarod was measured one day before surgery (baseline), and on days 2, 6, 9, and 21 post-surgery.

Spatial Navigation testing in the Morris water maze. Cognitive deficits, including deficits in short- and long-term memory acquisition and maintenance, were measured using the Morris water maze (MWM) behavioral test. The maze was a circular pool of water (115 cm diameter), made opaque by white nontoxic paint. A white platform (11 cm x 11 cm) was hidden 2 cm beneath the water's surface. The room was lit and contained various visuospatial cues, including symbols on the walls, shelves, and a white curtain comprising one blank wall. Black permanent marker was applied to the dorsal part of each rat's head and neck area, avoiding the sutures. Rats received two trials per day over eight consecutive days of testing. The platform remained in the same position relative to both the maze and the room throughout the testing. A single trial consisted of placing a rat in the water from one of two starting locations and allowing it to swim for 90 seconds or until the rat reached the platform for at

least three seconds. Rats were allowed to remain on the platform and examine the surrounding area in order to identify visuospatial cues that they may use to recognize the location of the hidden platform for 20 seconds. If the rat failed to reach the platform, the researcher guided the rat to the platform, and the rat was given 20 s to examine the surrounding area. Data was collected using Top Scan Lite 2.0 software, which detected and tracked the black head in contrast with the opaque water. Path length and latency to reach the platform were measured. A five-minute inter-trial interval was allowed during which each rat was warmed and dried with a heater, which also provided a low, constant background noise. After completing testing for the day, animals were again warmed and dried using the heater and returned to their home cages. On the 9th consecutive day of testing, a probe trial was conducted in which the platform was removed from the arena. The MWM was digitally divided into four quadrants and the amount of time the rat spent in the quadrant where the platform had been located was measured during the first 30 s of the trial.

Histological Analysis

On day 22, animals were perfused and brains excised and preserved in 10% buffered formalin for 24 hours. Tissue was then dehydrated in 10, 20 and 30 % sucrose in 0.1 M Phosphate Buffer Solution for 24 hours each. One fully dehydrated, the brains were embedded in O.T.C. (optimum cutting temperature) compound and frozen at -80 °C until sectioning. Tissue was sectioned at -25 °C into 20 μm sections and five sections every millimeter were mounted and stained with cresyl violet stain (Figure 7). A reduction in staining has been correlated with a decrease in neuron density; this was defined as the region of infarct. Infarct volume was quantified as a percentage of contralateral hemisphere staining intensity using

Image J software.

Statistical Analyses

Latency to fall from the accelerating rotarod, and latency and distance traveled to reach the platform in the MWM were analyzed using a Repeated measures Analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post-hoc analysis. Time spent in the probe quadrant of the MWM and infarct size was analyzed using a One-way ANOVA, followed by LSD post-hoc analysis. Significance was defined by $p \leq 0.05$.

Results

Rotarod

Time spent on the rotarod was expressed as a percentage of baseline performance (Figure 1). Repeated measures ANOVA, followed by LSD post-hoc analysis on latency to fall off the accelerating rotarod demonstrated significant ($p \leq 0.049$) group effects. Significant motor deficits ($p \leq 0.005$) were observed in vehicle treated (96.30 ± 14.71, 48.40 ± 13.32, 86.79 ± 11.03, 102.34 ± 10.37) compared with sham rats (102.55 ± 7.89, 96.30 ± 5.29, 92.69 ± 8.47, 82.25 ± 6.94) at 2, 6, 9 and 21 days post-surgery respectively. Furthermore, analysis showed that P8, P16 and P32 treated rats were all significantly different from those given vehicle only (P8: 78.11 ± 19.02, 86.79 ± 15.87, 71.52 ± 12.24, and 90.70 ± 14.40, $p \leq 0.011$; P16: 77.05 ± 17.40, 78.83 ± 17.67, 95.89 ± 12.83, and 85.04 ± 9.91, $p \leq 0.032$; P32: 62.94 ± 14.47, 102.34 ± 12.78, 111.97 ± 20.92, and 98.34 ± 13.44, $p \leq 0.004$) at 2, 6, 9 and 21 days post-surgery respectively. The most substantial deficit (50% compared with sham) in motor ability was observed in the vehicle-treated rats at 6 days post-surgery; all groups reached 100% recovery compared with

sham animals by day 21 (P8: 110%, P16: 103% and P32: 120%), except those given vehicle only, which reached just 67% of sham performance by post-surgery day 21.

Morris water maze

Learning Paradigm. For measures of mean path length and latency to reach the platform, scores from Trials 1 and 2 were averaged for each animal for each day. Repeated measures ANOVA, followed by LSD post-hoc analysis, were conducted on latency (Figure 2) and path length (Figure 3) respectively. LSD post-hoc analysis revealed a significant difference between sham and vehicle ($p \leq 0.015$), but not between vehicle and treatment groups. There was also a significant effect of days on path length. Similar to latency, LSD post-hoc analysis revealed a significant difference between sham and vehicle ($p \leq 0.014$) but not between vehicle and treatment groups.

Memory Paradigm. For probe measures, duration of swimming within the probe quadrant during the first 30 s of the trial was measured. A one-way ANOVA, followed by LSD post-hoc analysis, was conducted on duration of swimming within the probe quadrant (Figure 4). There was a significant difference between groups ($p \leq 0.017$). The most significant difference in performance was between sham (33.10 ± 2.38 s) and vehicle groups (18.24 ± 1.81 s, $p \leq 0.003$). Furthermore, there was a significant difference in performance between vehicle and P8 (30.48 ± 3.88, $p \leq 0.010$) and between vehicle and P32 (29.58 ± 2.69, $p \leq 0.017$), but not between vehicle and P16 (23.16 ± 3.92, $p \leq 0.282$). Vehicle only rats recovered approximately 55% compared to sham animals, while P8 rats recovered 92%, P16 rats 70%, and P32 rats 89% compared to sham animals.

Histology

Interestingly, in spite of significant changes in blood flow during occlusion and reperfusion (Figure 6), as defined by laser doppler inclusion criteria, cresyl violet staining did not reveal decreased intensity of staining in the infarct region of many animals within each treatment group. For the purpose of infarct measurement and analysis, three animals with maximum infarct volume (as initially defined in the methods) from each group were selected and infarct size quantified using Image J software. A One-way ANOVA, followed by LSD post-hoc analysis, revealed a significant difference in infarct volume (expressed as % of intact contralateral tissue) between vehicle and P8 (11.58 ± 3.76 compared with 1.93 ± 0.39, $p \leq 0.05$) and between vehicle and P16 (11.58 ± 3.76 compared with 1.61 ± 0.63, $p \leq 0.05$), but not between vehicle and P32 (11.58 ± 3.76 compared with 4.19 ± 1.4). The average P8 rat infarct was 16 %, P16 was 14 % and P32 36 % the average size of vehicle infarct. Upon further histological analysis, rats that did not appear to have decreased tissue volume measured as percent intensity of staining relative to the contralateral hemisphere exhibited severe behavioral impairments. Subsequent microscopic evaluation of the brain sections revealed significant gliosis and pyknotic tissue, indicative of neuronal loss and inflammation in the region of infarct (Figure 7) compared with the contralateral hemisphere (Figure 8).

Discussion

The primary objective of this study was to determine the optimal dose of PROG in the treatment of transient ischemic stroke, a devastating neurological event that PROG has already shown promise in treating (Ishrat *et al.*, 2010; Sayeed *et al.*, 2006). The results indicate that the

optimal dose of PROG in the treatment of transient ischemic stroke is 8 mg/kg (Table 1). P8 animals were able to reach baseline performance levels on the accelerating rotarod task by day 21 post-occlusion, compared with vehicle, which only achieved 67% of baseline performance (Figure 1). This suggests that PROG is able to preserve motor function, a deficit frequently experienced by those that suffer a MCA stroke (Slater *et al.*, 2011). Furthermore, P8 animals demonstrated improved memory retention during the MWM probe trial (Figure 4) and exhibited a significantly decreased infarct volume when compared with vehicle treated rats (Figure 5).

Similar to P8 rats, P32 animals also exhibited significantly improved rotarod (Figure 1) and MWM probe (Figure 4) performance, however, they did not exhibit significantly reduced infarct volume (Figure 5) when compared to vehicle treated rats.

In contrast, P16 rats demonstrated significantly improved outcome on the accelerating rotarod (Figure 1) and significantly decreased infarct volume (Figure 5) when compared with vehicle treated rats; they did, however, exhibit cognitive deficits similar to vehicle treated rats, on the MWM probe trial (Figure 4).

In this study, histological results distinguish P8 from P32. It is important to emphasize the need for more sophisticated imaging techniques to identify region of infarct at 22 days post-ischemia. Previous studies have demonstrated that during stroke recovery, reactive glia and neural stem cells begin migrating to the region of infarct (Jin *et al.*, 2003). The intervening days between occlusion and perfusion allowed for progressive degeneration to proceed in conjunction with natural adaptations that would allow the animals to compensate for deficits resulting from the stroke. Differing degrees of visible infarction, gliosis and pyknotic tissue were probably the result of differential stroke progression and adaptive responses between individual rats, but may also have been affected by treatment; as mentioned previously, PROG has been implicated in

reducing pro-inflammatory and pro-apoptotic cascades, which should hypothetically reduce the volume of pyknotic tissue in the region of infarct (Stein & Hurn, 2009). In order to quantify volume of tissue lost due to the ischemic event in the current cresyl violet stained sections, cell counts of healthy and gliotic cells may be counted in order to quantify volume of tissue lost. However, as gliotic and pyknotic tissue are indistinguishable in these slides, I propose that future studies use immunohistochemistry to differentiate between mature neurons, neuronal stem cells, and reactive glia by staining for specific markers for each (NeuN (Wiltrout, Lang, Yan, Dempsey & Vemuganti, 2007), MAP-2 (Wiltrout *et al.*, 2007), GFAP (Eng & Ghimikar, 1994) and nestin (Lin, Matesic, Marvin, McKay & Brustle, 1995) for mature neurons, neuronal stem cells, dormant glia and reactive glia respectively). Western blot analysis maybe used to measure levels of apoptotic markers, including caspase-9, -3, -8, and -12 or Fas (Das, Ghosh, Manna & Sil *et al.*, 2011).

Considering the limitations of the histological analysis of this study, it is important to further support the selection of 8 mg/kg of PROG as the best dose for the treatment of transient ischemic stroke. First, previous studies on PROG as a treatment for both transient and permanent MCAO models of stroke used 8 mg/kg PROG (Ishrat *et al.*, 2010; Sayeed *et al.*, 2006). These studies demonstrated significant improvements in behavioral outcomes and preservation of neuronal tissue in PROG compared with vehicle treated rats, findings that were replicated in the current study. Furthermore, studies conducted in other neural insult models, such as traumatic brain and spinal cord injuries, have also evaluated 8 mg/kg of PROG and found that it effectively attenuated behavioral and cognitive deficits compared with vehicle treated animals and positively altered the histochemical profile of the injured neural tissue (Ishrat *et al.*, 2010; Stein & Hurn, 2009). One study, conducted by Goss *et al.* (2003), strongly

parallels the current study. Goss *et al.* (2003) generated a dose-response curve for PROG in the treatment of TBI. They identified 8 mg/kg and 16 mg/kg PROG as optimal doses in facilitating MWM performance and attenuating forepaw somatosensory neglect in a medial bilateral prefrontal cortex contusion model of TBI; 32 mg/kg PROG did not worsen outcome compared to vehicle, and was therefore not considered toxic. It is possible that the MWM is more sensitive to spatial deficits in subjects with bilateral frontal cortex injuries, which are more typical of TBI than of MCAO stroke, if the hippocampus is also impaired. It appears that MCAO deficits are more dramatic in motor tasks, such as rotarod, grip-strength and gait. The deficits observed in the MWM after MCAO may be more of a combination of the motor impairments along with cognitive and impairments caused by hippocampal damage, not just memory impairment.

One significant difference between this study and those previously discussed is the animal model. While TBI is predominantly an issue in younger populations, aging is actually a major risk factor for stroke ("Heart disease", 2010). Many individuals without any other obvious stroke-risk factors end up suffering a stroke. Rats used in this study were healthy and meant to model transient ischemic stroke in these individuals without any pre-existing and potentially confounding factors. Future studies will examine the effect of PROG on stroke recovery in populations at high risk for stroke, such as among diabetic populations. They will also examine the effects of PROG on stroke recovery in females. Numerous sex-specific differences exist between males and females, including differential systemically circulating levels of PROG, significant differences in brain morphology, connectivity and gross and cellular chemistry (Berry *et al.*, 2009; Pletzer *et al.*, 2010; Stein, 2007; Stein & Hurn, 2009; Waldron, McCourtly & Lecanu, 2010). Any such variances might contribute to the differential progression of and

recovery from stroke, as well as interact differentially with any drug treatment administered. In fact, current guidelines now ask that sex differences be examined and the interaction of sex hormones be evaluated for safety and efficacy for any new medications ("Inclusion of", 1987; Stein, 2004).

In summary, few treatments exist to treat stroke – a common and devastating neurological insult that begins with a primary injury that initiates a cascade of secondary events responsible for a significant amount of tissue damage and ultimately experienced deficits. PROG has been demonstrated to be effective at attenuating many of these secondary cascades in other models of CNS injury as well as two models of stroke. Data from the other CNS injury models in addition to the behavioral and histological data collected in this study indicate 8 mg/kg as an optimal dose of PROG for the treatment of transient ischemic stroke in middle aged rats. Future studies should confirm these findings in additional models of stroke, including permanent ischemic and hemorrhagic models of stroke, and in female and at risk populations, in order to expand the potential for PROG to progress to clinical trials for the treatment of general stroke. This has to potential to significantly impact society by providing an effective treatment for all the millions of Americans who suffer a stroke and perhaps minimizing the total cost to society.

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Tables and Figures

Table 1.

Summary of the statistically significant findings of the histological and behavioral data for each treatment group. (+) represents a significant ($p \leq 0.05$) difference between treatment group and vehicle, while (-) represents the lack of a significant difference between treatment group and vehicle.

Treatment	P8	P16	P32
Rotarod	+	+	+
MWM: Latency	-	-	-
MWM: Distance Traveled	-	-	-
MWM: Probe	+	-	+
Infarct Volume	+	+	-

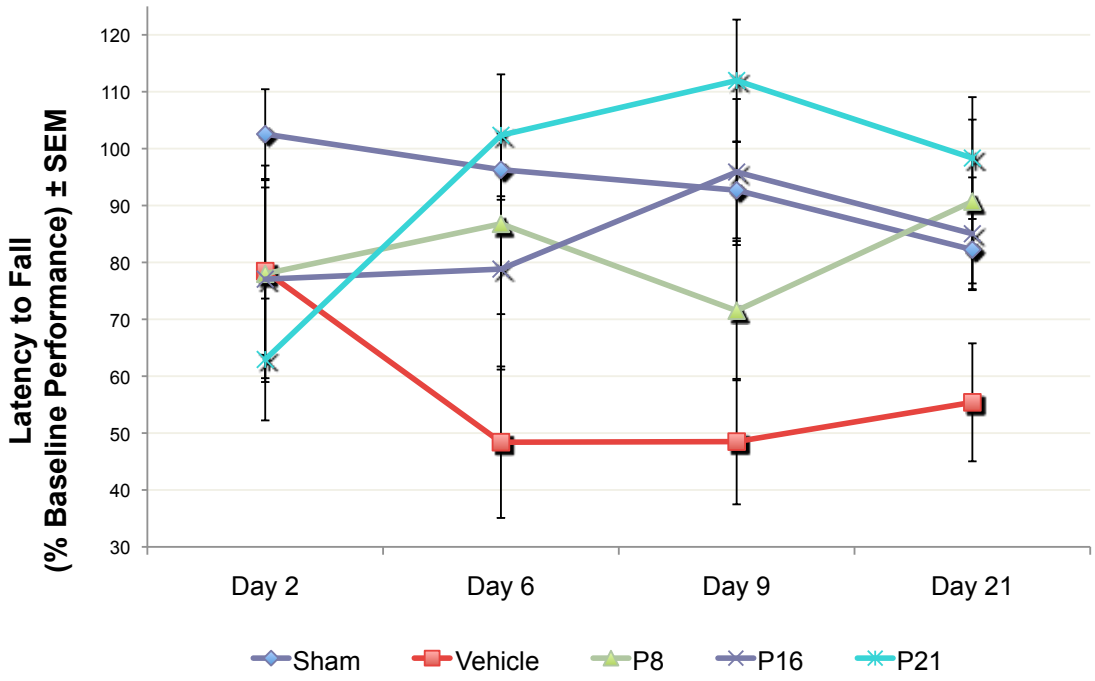

Figure 1. Latency to fall from the accelerating rotarod expressed as % baseline performance ± the standard error of the mean. Sham, P8, P16 and P32 rats all performed significantly better ($p \leq 0.05$) than vehicle on days 6-21.

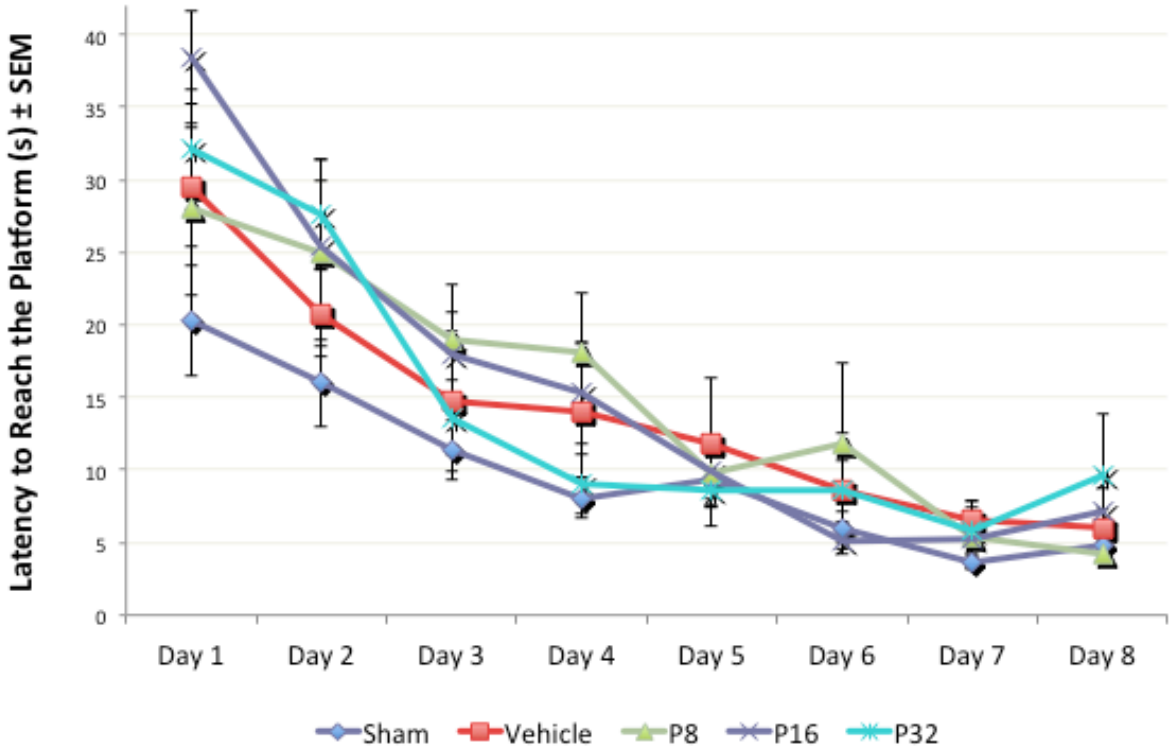

Figure 2. Latencies (s) of rats to reach the hidden platform of the Morris water maze ± the standard error of the mean on days 1 – 8 of training. Values are the average of the latency to reach the platform in trials 1 and 2. Overall, there was a significant difference between sham and vehicle only ($p \leq 0.015$) treated groups, but not between vehicle and P8, P16 or P32 treatment groups.

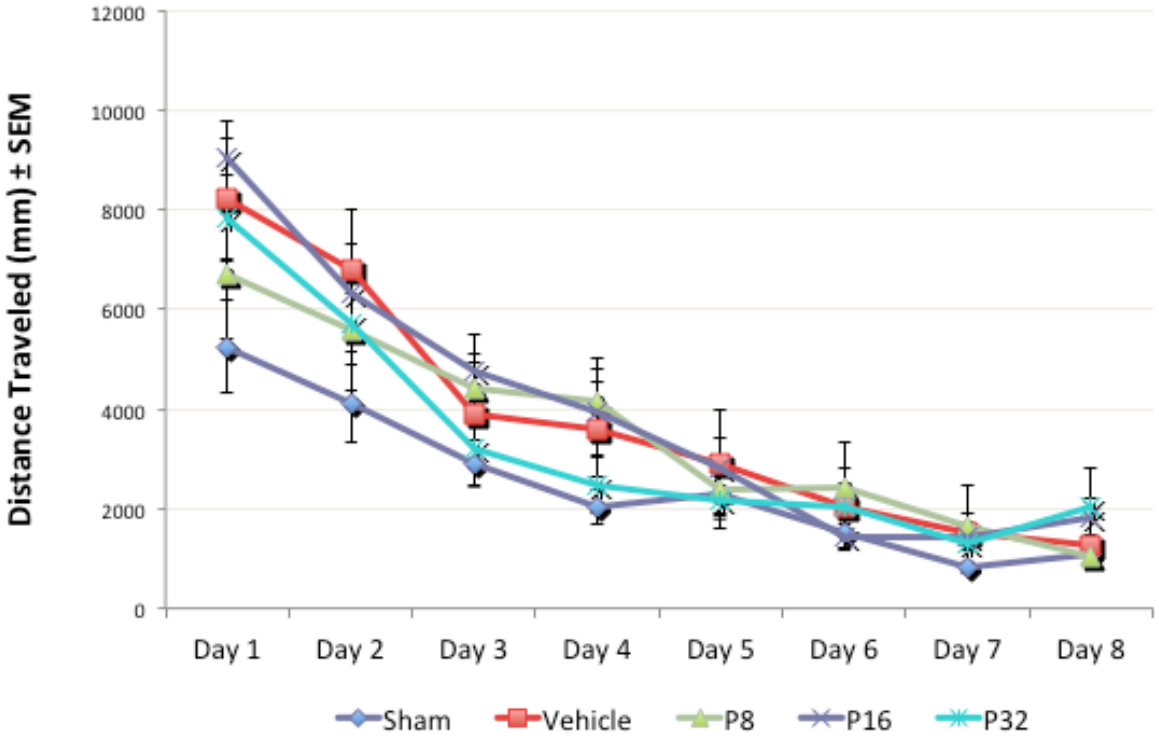

Figure 3. Distance (mm) traveled to reach the hidden platform of the Morris water maze on days 1 – 8 of training. Values are the average of distance traveled in trials 1 and 2. Overall, there was a significant difference between sham and vehicle only ($p \leq 0.014$) treated groups, but not between vehicle and P8, P16 or P32 treatment groups.

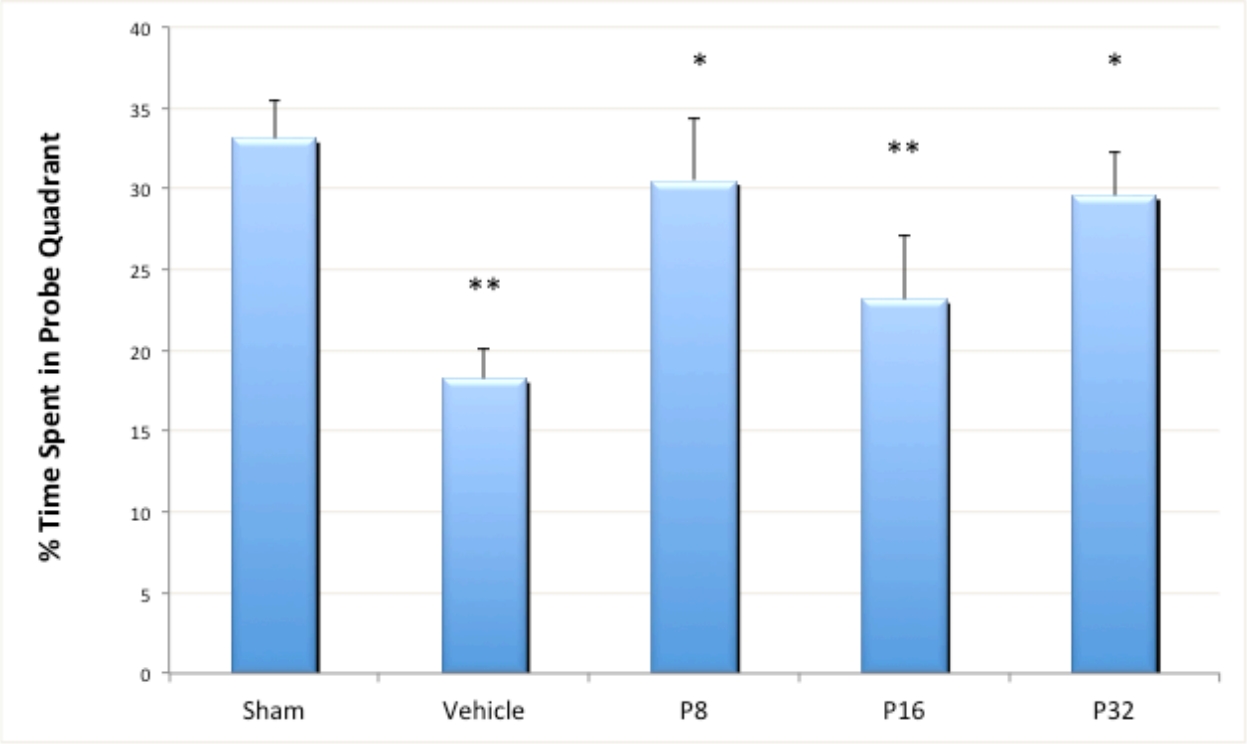

Figure 4. Morris water maze probe trial examining the effect of progesterone treatment on memory formation, measured as the % time spent in the platform quadrant during the first 30 s of the probe trail. One asterisk indicates a significant ($p < 0.05$) difference relative to sham values while two asterisks indicates a significant ($p < 0.05$) difference relative to vehicle values.

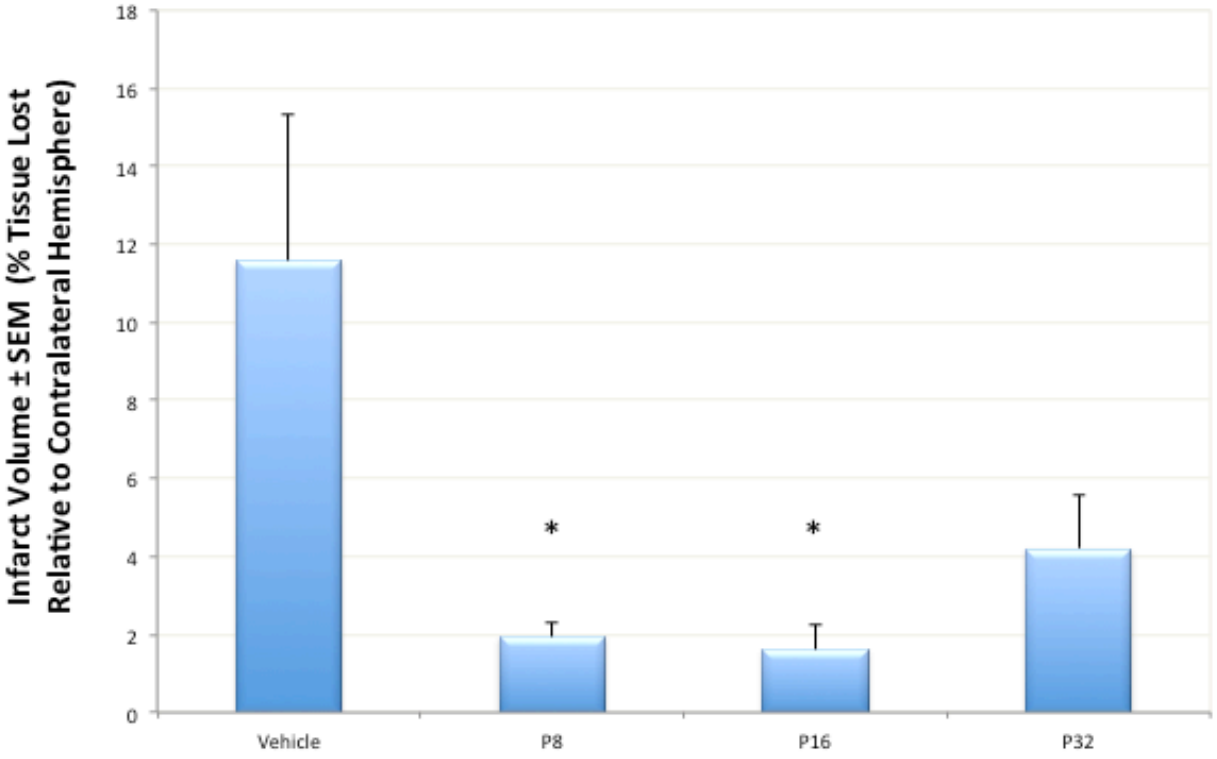

Figure 5. Infarction Volume. Cresyl violet stain was used to determine infarction volume. Infarction volume is reported as % tissue lost relative to the contralateral hemisphere of the animal. There was a significant difference ($p \leq 0.05$) between P8 and vehicle treated rats and between P16 and vehicle treated rats.

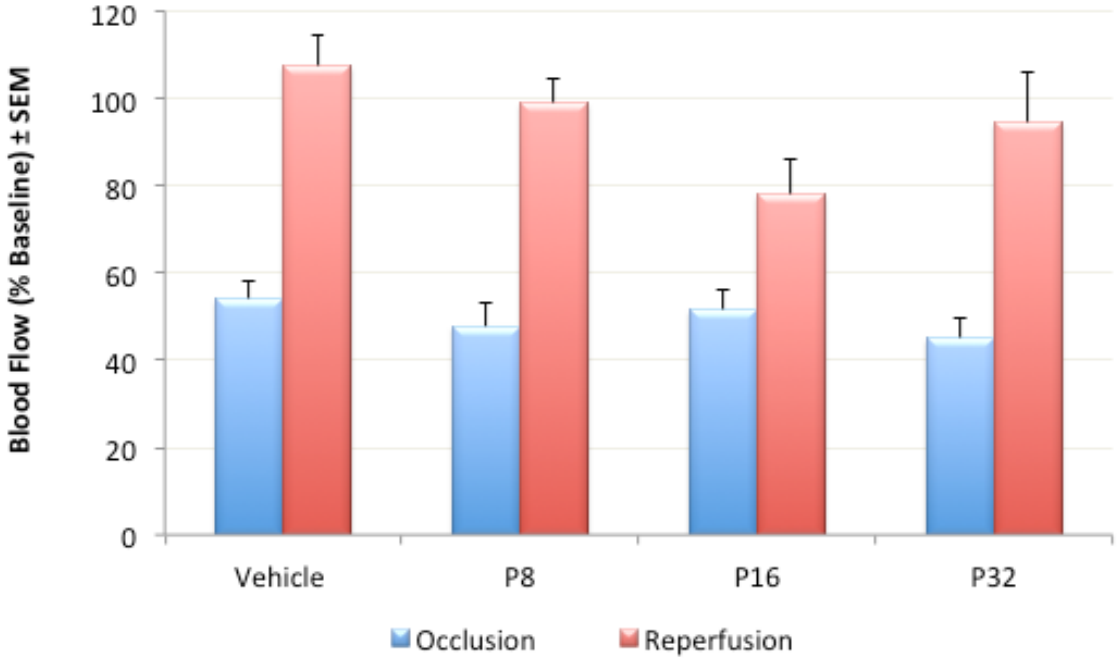

Figure 6. Measure of laser doppler blood flow during ischemic surgery expressed as percent baseline blood flow. All groups exhibited decreased blood flow during occlusion and increased blood flow comparable to baseline during reperfusion.

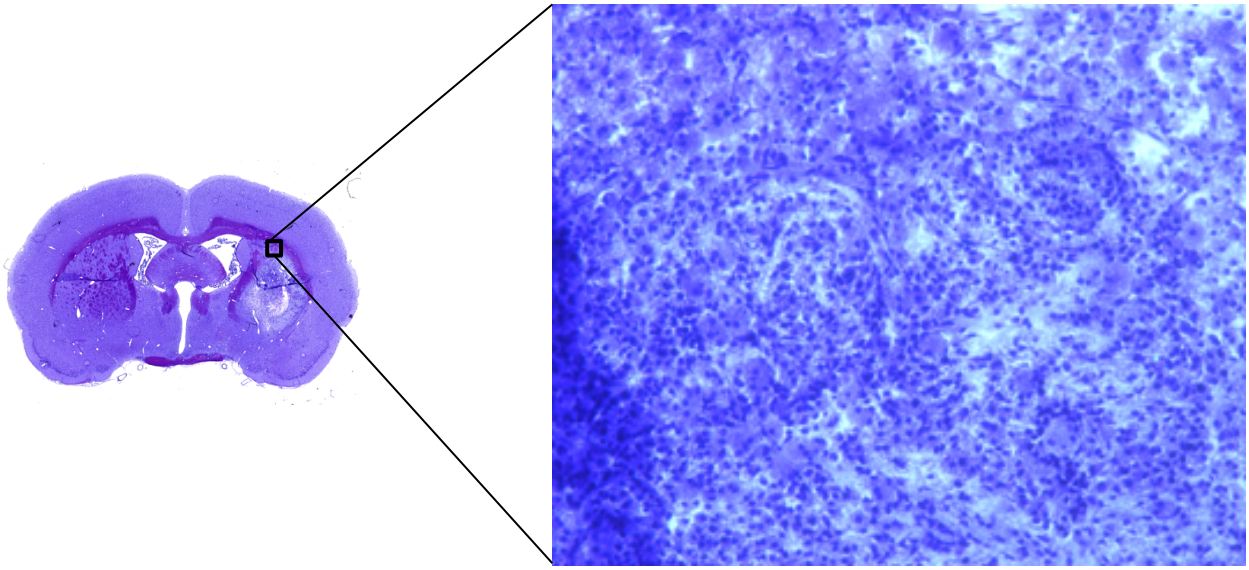

Figure 7. Microscopic analysis of cresyl violet stained infarction revealed significant levels of gliotic and pyknotic tissue.

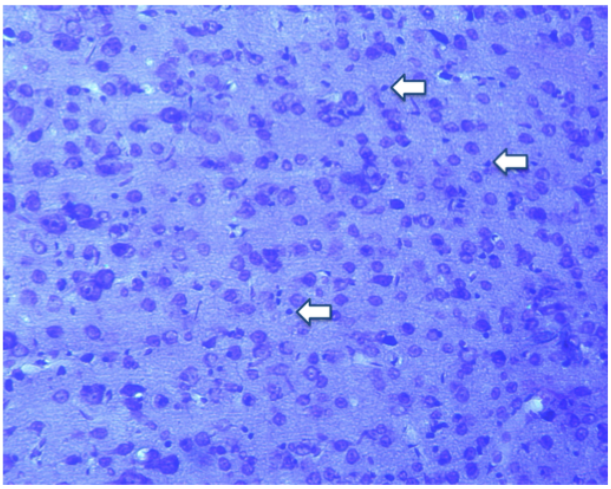

Figure 8. Contralateral hemisphere stained with cresyl violet exhibits significantly less gliotic and pyknotic tissue compared with the region of infarction (see Figure 7 for comparison).