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Protective Effects of Caffeine via Microglia in a Rodent Model of Preterm Hypoxic Ischemic Brain Injury

Serena Beri
serena.beri@uconn.edu

University of Connecticut

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Serena Beri

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Thesis Supervisor: Roslyn Holly Fitch

GA Supervisor: Ruth McLeod

Honors Advisor: Joseph LoTurco

Abstract

This study investigated the neuroprotective effects of caffeine, a common treatment for infant respiratory distress (1, 2). Caffeine is often given to preterm infants to enhance respiratory drive. Many of these same infants are at risk for hypoxia-ischemia (HI) -- a brain injury that occurs due to a lack of blood and oxygen supply to the brain and involves tissue loss (4). This injury can lead to major consequences such as learning and motor disorders such as cerebral palsy and other neurodevelopmental disorders. Although caffeine has been widely used as treatment for respiratory drive in preterm infants, some emergent research suggests it may have secondary benefits in protecting against brain damage (1). However, the mechanism for how caffeine acts on the brain as a neuroprotectant is not well understood. The current research addresses this gap by investigating the protective mechanism of caffeine on microglia activation. Infant rats were separated into four conditions that included an injury simulating HI or a sham injury, and treatment with saline or caffeine solution. Groups thus included: Sham saline, Sham caffeine, HI saline, and HI caffeine. Subjects were sacrificed 48 hours after birth and measured for chromatin condensation (using a DAPI stain) and microglial soma area (using an IBA stain). Given putative beneficial effects of caffeine on HI, we posited that caffeine may act by reducing microglial over activation. If true, the HI saline groups should have the largest microglial soma area and smallest DAPI density (indicating chromatin condensation), while HI caffeine subjects should look more like shams. Preliminary research supports this hypothesis in males only, but not females -- even though protective effects of caffeine were observed in both sexes. Applications of the current data will provide valuable insights in modifying the caffeine HI therapy technique that can be used to prevent neural tissue loss and poor outcomes in preterms.

Protective Mechanism of Caffeine on Microglia in Preterm Hypoxic Ischemic Injury

Hypoxia Ischemia (HI) is identified as the third leading cause of death in the United States, as it affects over half a million new victims annually (3). This injury is especially damaging in severely preterm infants, who experience an HI incidence rate of over 60% (10). HI has been categorized as a brain dysfunction that occurs when low oxygen and/or blood levels prevent the brain and other vital organs from generating enough ATP. This results in neuronal death and tissue loss, as the neuronal ion gradients and energy demands of the cell are not met (4,11). Once these neurons die, they cannot regrow, resulting in later learning disabilities and harmful cognitive effects (3). Premature infants experience an increased susceptibility as minimal blood oxygen deviations can cause major brain tissue loss, resulting in impairments affecting learning, memory, language, and motor abilities (5). These impairments include neurodevelopmental disorders like cerebral palsy, epilepsy, and ADHD (7). For these reasons we sought to investigate methods that can protect and treat the preterm brain from HI related damage.

HI can occur in term infants; however, incidence rates are higher in preterm infants (5). In term infants HI results from complications occurring during pregnancy, labor, or delivery. These complications involve problems with blood flow to the placenta, umbilical cord problems, trauma to the brain or skull, abnormal fetal position of the infant, and respiratory failure or cardiac arrest (7). The risk for hypoxia-ischemia in preterm infants can stem from these factors, and in addition can also stem from breathing issues associated with prematurity. These issues include sepsis, infections, apnea, bradycardia, and anemia (7). These additional risk factors increase the likelihood that a preterm baby will experience hypoxic-ischemic injuries compared to a term baby.

Treatments addressing HI typically involve therapeutics like brain cooling. Brain cooling is a method of inducing hypothermia to the infant brain through the use of a cooling cap. It works by causing the infant's metabolic rate to slow, allowing nerve cell recovery (8). Evidence suggests that moderate cerebral hypothermia delivered within a few hours after severe HI can reduce neuronal loss, as well as improve behavioral recovery in term infants and adults (9). However, not much research has been done to assess the use of hypothermia in vulnerable preterm infants, as it can pose potential detrimental risks such as intracranial hemorrhages, infections, and potential respiratory damage (9). Therefore, current approaches to treat HI in preterm infants involve novel strategies like pharmaceutical neuroprotection.

Hypothermia is one form of neuroprotection, which means an intervention that protects neurons against damage. In general, neuroprotection cannot reverse existing damage, but it can be used to prevent further nerve damage and slow degeneration (8). Since hypothermia cannot be used in preterm infants, other novel interventions are needed. Recent clinical studies suggest that high dose erythropoietin (EPo) treatment reduces disability from HI in infants (11). EPo is a kidney hormone that produces red blood cells, which can then increase the available oxygen transport to the brain (11). However, even though EPo is associated with increased neuronal function following HI injury, a unique concern to the preterm population is whether EPo might increase certain risks like damage to the eyes (e.g., retinopathies, 11). Other neuroprotection studies investigate the use of magnesium sulfate, a maternal pretreatment. A study concluded that a multifaceted knowledge translation strategy reduced the likelihood of cerebral palsy in the child, with no important neonatal risks (6). The use of magnesium sulfate is still controversial; another study has shown that even though it prevents preterm motor disabilities, it can also cause possible harmful effects on the cerebral cortex and hippocampus of the mother (12). Because of

the harmful effects that these neuroprotectants can induce, caffeine has been identified as another possible treatment against hypoxia ischemia in preterm infants. We discuss these results further below.

Caffeine is a respiratory stimulant that was previously thought to aid in increasing diaphragm contraction, coordination of breathing, and oxygen consumption in preterm infants (25). Caffeine is also believed to have long term benefits as well, which led researchers to consider caffeine as a therapy for HI in preterms. Even among infants with no recorded history of HI, results show that administering caffeine within the first two days leads to better cognitive outcomes later in life (26). Previous research in our lab also suggests that caffeine treatment significantly improved some behavioral outcomes and partially rescued neural networks in rodents with HI injury (22). These findings support that caffeine improves behavioral outcomes by acting as a neuroprotectant, specifically in reducing inflammation through its role on adenosine. Adenosine acts as a neuromodulator in the brain meaning that it regulates the release of various neurotransmitters (13). It has a dominant role in the brain as it is released from nearly every cell, and its receptors are dispersed throughout the brain. These receptors include the four types A1, A2A, A2B, and A3. Since caffeine is a non-selective adenosine antagonist, it has the ability to act on each of these receptors (14).

The A1 receptor has both presynaptic and postsynaptic effects, presynaptically causing inhibition of neurotransmitter release, and postsynaptically causing hyperpolarization and inhibitory postsynaptic potential (14). Furthermore, antagonism of this receptor by caffeine would create a stimulating effect in the brain. However, the A1 receptor has a twofold effect that can increase or reduce inflammation (15). In fact, A1 activation can release proinflammatory factors such as Interleukin IL-6 and IL-1b, and conversely is also involved in inflammation

resolution and tissue repair through a reduced microglial effect (15). Therefore, it is believed that this receptor is not the major target of caffeine's anti-inflammatory response. The A3 and A2B receptors are typically proinflammatory in the brain, however they both can have dual effects as well (16). The A3 receptor is associated with increased inflammation in the lungs and release of the same interleukins in A1 activation, yet it can also have an anti-inflammatory response as A3 agonism is used in treatment options against disorders like rheumatoid arthritis and psoriasis (15, 17). Because of these dual effects, the A3 receptor is also not thought to be the main target of caffeine. The A2B receptor, like the two previously mentioned receptors, has a dual purpose for inflammation as well. In most of the body, A2B activation is anti-inflammatory, conversely in the lungs A2B activity is pro-inflammatory (18). Since preterm infants commonly experience inflammation in their lungs and gut, it is proposed that the A2B receptor does play a small role in caffeine's anti-inflammatory response in preterm infants (18). Finally, A2A receptors have a pro-inflammatory action in the body. Activation results in an increase of cellular calcium, which leads to increased neurotransmitter release (14). A2A receptors are expressed highly in immune cells in the body and brain. For this reason, researchers believe that caffeine mainly acts on A2A receptors by inhibiting them leading to anti-inflammation (14).

Microglia are also affected by A2A receptors, as activation increases microglial activation, and increases the release of neurotoxic factors like, TNF- alpha and IL-1 (19). These neurotoxic factors all increase inflammation and result in cell death to stop injury from spreading through the brain. Since caffeine is an A2A antagonist, it may act to block these receptors and thus decrease microglial activation (20). Caffeine could concurrently result in immunosuppression and protection of cells from inflammatory damage (19). Although this might

seem like an adverse effect as caffeine decreases the immune response in the brain, it is actually beneficial as it decreases microglial overactivation and reduces cell death.

Although caffeine has previously been thought to be beneficial for its anti-inflammatory properties, the mechanism of action has not been understood. No research to our knowledge has identified its action on microglia in animal studies. The current study helps to address this gap by investigating the effects of caffeine treatment on microglial activation in a rodent model of preterm HI injury. In addition, we investigate whether comparable effects are seen for males and females, which could be important given sex differences in prematurity and outcomes (21, 22). Findings will have important implication for treatment of at-risk preterm infants.

Methods

Subjects

Subjects were male (n=35) and female (n=21) rats. Pups were housed at an animal facility in UConn Health. After being born at P1, litters were culled and fostered into litters of 8 (4 males and 4 females) until P6 when treatments began.

HI induction

When pups reached P6, they were assigned to one of 4 treatment conditions: HI with saline treatment; HI with caffeine treatment; Sham with saline treatment; and Sham with caffeine treatment. Sham with caffeine and sham with saline were grouped together for males (n=35, (HI caffeine=12, HI saline=11, sham=12)) and females (n=21, (HI caffeine=8, HI saline=7, sham=6)). HI was induced using the Rice-Vannucci protocol (23). Pups were first anesthetized using isoflurane. The right carotid artery was located by making a small incision to the right of the midline in the neck on the ventral side of the rodent. The artery was cauterized to induce ischemia. Following cauterization, the incision was sutured, and pups were given bupivacaine for

analgesia. Pups were then given a foot tattoo for identification and recovered from anesthesia in a warming tank. After the surgery they were returned to their dams. Sham animals were placed under anesthesia and received the same incision, without cauterization of the carotid artery, therefore no ischemia was induced. Sham pups were also given bupivacaine, sutured, tattooed, and recovered in the same manner as other groups.

To induce hypoxia, HI rodents were placed in closed hypoxia chamber with 8% oxygen (balance nitrogen). Sham subjects were placed in open chambers with room air (21% oxygen) as to not experience hypoxia. Rodents remained in respective tanks for 90 minutes.

Caffeine Treatment

After removal from tanks, rodents were given respective injections. Injections occurred intraperitoneal, meaning through the peritoneum of the abdomen. Pups were injected with either caffeine (25mg/kg) or an equivalent amount of sterile physiological saline as a vehicle. The caffeine dose was based on equivalent dosage in human infants adjusted to weight and measured from a sterile vial of 20ml/kg of caffeine citrate (exact dosage was calculated using the pups' weight). After injection, all pups were returned to their dams.

IBA-1 and DAPI Staining

48 hours post-surgery rats were perfused using formalin, and their brains were dissected and embedded in paraffin. Coronal slices were obtained using a cryostat. Slices were stained for chromatin condensation using 4',6-diamidino-2-phenylindole (DAPI), and for microglia using ionized calcium binding adaptor molecule 1 antibody (IBA-1). Images were then taken using a Nikon Confocal florescent microscope, with a blue fluorescent filter for DAPI, and a green florescent filter for IBA-1.

Image and Data Analysis

All image analysis occurred using Fiji Film *ImageJ* on 80x images of the left and right cortex and hippocampus, taken from a serial set of mounted sections for each subject. To measure cell death, chromatin condensation was measured by the optical density of the DAPI stain. A lower DAPI optical density indicates higher chromatin condensation, and thus more cell death. Microglia activation was analyzed by measuring the microglia soma area, where a greater area indicated increased microglia activation (24). Data analysis was run using IBM SPSS Statistics, using an ANOVA test to determine if there was a significant relationship between each condition and cell death or microglia activation. Analysis was run on each sex separately, with Sex run as a covariate, and with Sex split data. Sham-caffeine and sham-saline animals were consolidated into one larger Sham group as there were no significant differences on any measures. DAPI optical density results were also combined for males and females as there were no significant differences in measures. Results were limited to the right cortex where ischemia injury occurred.

Results

Male histology: Data is presented for a set of 35 male rats with P6 HI (n=35, (sham=12, HI saline=12, HI caffeine=12)). Regarding IBA measures, there was a marginally significant main effect of treatment in the right cortex soma area ($F=2.384$, $P= 0.108$) with HI saline animals having a larger soma area than caffeine treated animals, and sham animals showing the lowest soma area as expected. A specific comparison between HI saline and sham showed that sham animals had a significantly lower soma area than HI saline treated animals ($P= 0.106$).

Figure 1

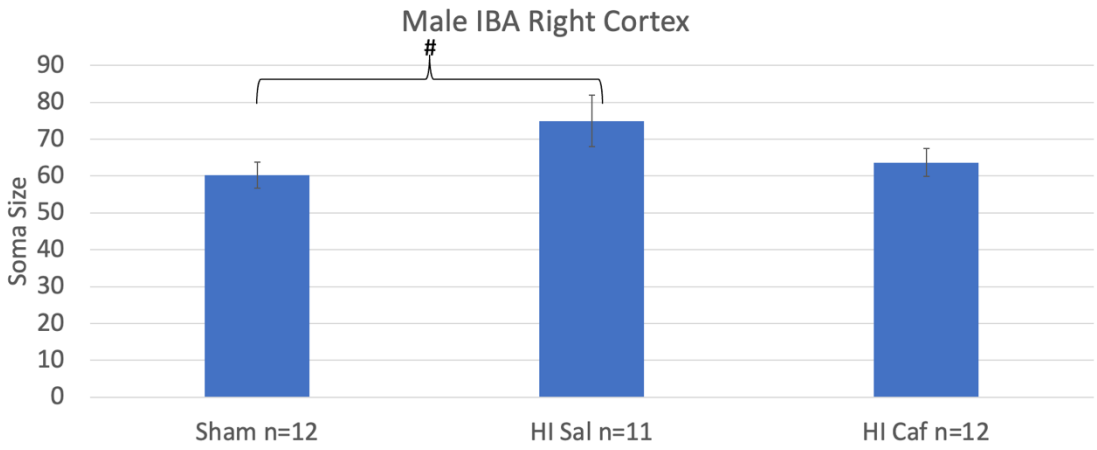

Figure 1: For soma area measures of the right cortex, HI-saline males showed a larger average soma area than sham animals (P=0.106). HI-caffeine treated males had a lower area than HI-saline treated animals (P=0.108), but not a full reduction to sham levels. # = marginally significant (P is slightly greater than 0.05)

Female Histology: Data is presented for a set of 21 female rats with P6 HI (n=21, (sham=6, HI saline=7, HI caffeine=8)). Regarding IBA measures, there was a marginally significant main effect of treatment in the right cortex soma area (F=3.157, P= 0.067) with HI caffeine females having a larger soma area than HI saline treated animals and sham animals. Variance in HI saline data showing low microglia activation similar to sham animals is likely due to high error bars.

Figure 2

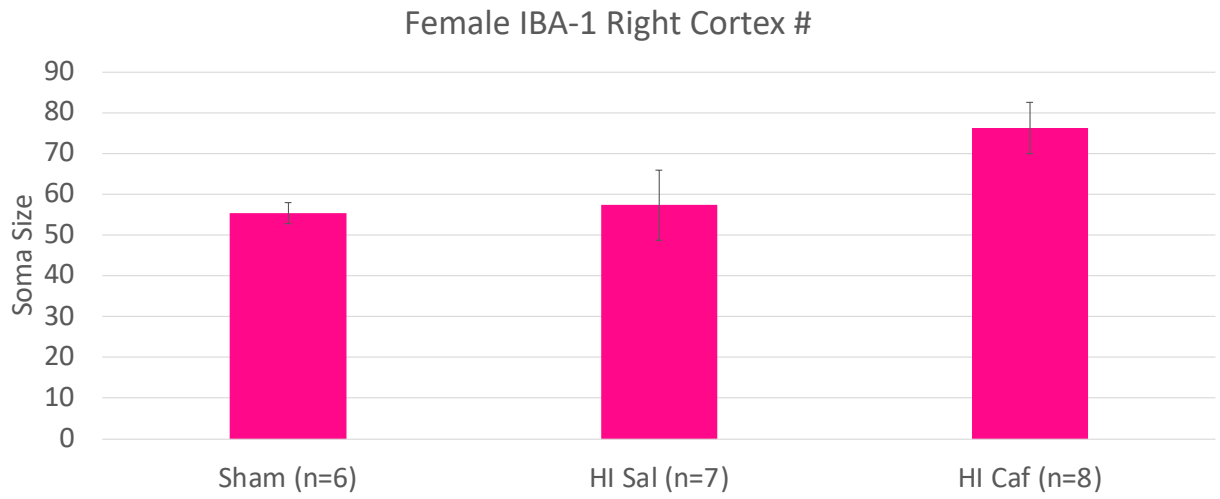

Figure 2: For soma area measures of the right cortex, HI caffeine females showed a larger average soma area than HI saline and sham animals (P=0.067).

Combined Male and Female Histology: Data for DAPI optical density is presented for both male and female rats with P6 HI (n=56, (sham=18, HI saline=18, HI caffeine=20)). Regarding DAPI measures, a significant main effect showed HI-saline animals experienced the most cell death, while HI-caffeine treated animals had less cell death than HI-saline treated animals, but not a full recovery to sham levels (P=0.011). A specific comparison between HI saline and sham showed that saline animals experienced significantly higher cell death than sham animals (P= 0.008).

Figure 3

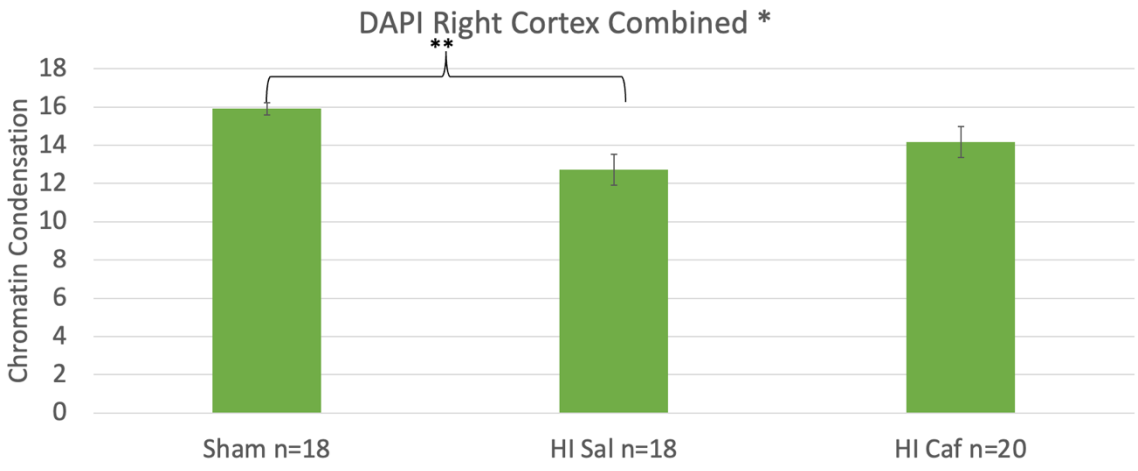

Figure 3: For DAPI optical density, HI-saline males showed the most cell death, while sham animals experienced the least cell death with HI caffeine animals in the middle (P=0.011). HI saline animals also showed significantly more cell death than sham animals (P=0.008). Lower values indicate increased cell death. ** = P< 0.05

Discussion

One of the main findings of this study was that caffeine reduced cell death in the right cortex of male and female rodents with an HI injury. This data was significant, showing that HI saline animals of both sexes experienced greater cell death relative to sham animals, with HI caffeine rodents in the middle (P=0.011). We expected to see these results, based on prior findings with caffeine, and because saline would not provide any protection against HI. Also, notably, the HI caffeine treated rodents experienced some protection and tissue preservation from the caffeine but did not show full recovery to sham animals with no injury.

In addition to our cell death results, caffeine was shown to reduce microglia activation in male rodents with HI as well. HI-caffeine treated males had a lower soma area than HI-saline treated animals (P=0.108), while HI-saline males showed a larger average soma area than sham animals (P=0.106). Since HI caffeine treated male rodents experienced lower soma area than HI

saline males, it is supported that caffeine reduced microglia activation. Although it was hypothesized that caffeine reduced microglia activation, no prior study has demonstrated this mechanism in animals.

However, this finding was not supported in female rodents. Regarding the right cortex, HI-saline females and sham females had marginally lower soma areas than HI caffeine treated rodents who experienced the largest average soma area (P=0.067). Since HI saline and sham female rodents had lower microglia activation than HI caffeine treated females, it can be theorized that in females caffeine is not acting through microglia. This suggests sex differences in male and female rodents in which caffeine reduces microglial activation in males but not females. Further research must be done to determine the mechanism for caffeine in females and why this occurs.

Overall, the results of this study show that caffeine does have an effect on microglia by reducing microglial activation in males with HI. This is beneficial as it leads to decreased inflammation and increased tissue preservation. Caffeine protection was also observed in both sexes, as male and female rodents with HI and caffeine treatment experienced reduced cell death. Results also show that there may be some sex differences in how caffeine works to provide neuroprotection.

Conclusions and Future Directions

This study helps to address a gap in previous research by investigating the effects of caffeine treatment on microglial activation and demonstrating this effect in rodent models. By understanding this mechanism, researchers can modify how caffeine is administered to preterm infants, either by regulating dosage or providing treatment earlier. This will help to promote the overall survival of preterms against hypoxia ischemia. In future, validation of the results from

this study will require testing with a larger sample size to account for any variables in HI surgery induction or human error in image analysis. Future work will also focus on validating the effects of caffeine in females and how these effects differ from males. This will require an in-depth analysis of human preterm neurodevelopmental data. Future studies should focus on how sex differences may affect HI treatment efficiency to hopefully lead to improved treatment for preterm infants suffering from hypoxia ischemia.

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