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Myra Batool , Charles L. Cai , [Jacob V. Aranda](#) , [Kay D. Beharry](#) *

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Article

Early Versus Late Caffeine and/or Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) for Prevention of Intermittent Hypoxia Induced Neuroinflammation in the Neonatal Rat

Myra Batool ¹, Charles L. Cai ¹, Jacob V. Aranda ^{1,2} and Kay D. Beharry ^{1,2,*}

¹ Department of Pediatrics, Division of Neonatal-Perinatal Medicine, State University of New York, Downstate Health Sciences University, Brooklyn, NY, 11203 USA (myra.batool@gmail.com; Charles.cai@downstate.edu; jacob.aranda@downstate.edu; kay.beharry@downstate.edu)

² Department of Ophthalmology, State University of New York, Downstate Health Sciences University, Brooklyn, NY, 11203 USA (jacob.aranda@downstate.edu; kay.beharry@downstate.edu)

* Correspondence: kay.beharry@downstate.edu

Abstract: Preterm infants often experience frequent intermittent hypoxia (IH) episodes which is associated with neuroinflammation. We tested the hypotheses that early caffeine and/or non-steroidal inflammatory drugs (NSAIDs) confer superior therapeutic benefits for protection against IH-induced neuroinflammation than late treatment. Newborn rats were exposed to IH or hyperoxia (50% O₂) from birth (P0) to P14. For early treatment, the pups were administered: 1) daily caffeine citrate (Cafcit, 20 mg/kg IP loading on P0, followed by 5 mg/kg from P1-P14); 2) ketorolac topical ocular solution in both eyes from P0 to P14; 3) ibuprofen (Neoprofen, 10 mg/kg loading dose on P0 followed by 5 mg/kg/day on P1 and P2); 4) caffeine+ketorolac co-treatment; 5) caffeine+ibuprofen co-treatment; or 6) equivalent volume saline. On P14, animals were placed in room air (RA) with no further treatment. For late treatment, pups received similar treatments from P15-P21 (caffeine and/or ketorolac), or P15-P17 (ibuprofen). RA control were similarly treated. At P21, whole brains were assessed for histopathology, apoptosis, myelination, and biomarkers of inflammation. IH caused significant inflammation, reduced myelination, and apoptosis in preterm rat brains, an effect that was improved predominantly with combined caffeine/NSAID early treatment. Early caffeine/NSAID co-treatment confers synergistic neuroprotection against IH-induced damage.

Keywords: brain; caffeine; cytokines; ibuprofen; intermittent hypoxia; myelination; neuroinflammation; non-steroidal anti-inflammatory drugs

1. Introduction

Every year in the United States, about 63,000 infants are born with a very low birth weight (VLBW; ≤ 1500 g) [1]. This group represents 1-5% of all live births and has increased gradually over the past decade due to increased survival (50-70%) [2,3]. Disability in this subset exceeds 50% in most studies [1,3], with neurocognitive delays in the later preterm population unaccounted for. In terms of neurologic impairments, preterm births account for cognitive, behavioral, attention or socialization deficits in 25–50% and major motor deficits (e.g., cerebral palsy) in 5–10 % [3,4]. The epiCURE study described structural brain disease in 17% of the extremely low birth weight (ELBW) babies, with parenchymal cysts and/or hydrocephalus [5]. These impairments lead to reduced quality of life and productivity, increased healthcare services utilization, higher dependency and need for assisted living [6,7]. Insults to the central nervous system (CNS) account for over 40% of the disability adjusted life years (DALYs) in neonates, mainly because of prematurity and hypoxic ischemic encephalopathy (HIE) [5–7].

Several animal studies have investigated mechanisms of injury that lead to increased neurologic morbidity [8–10]. The effect from hypoxia/hypoxemia appears to be an important and dominant mechanism of injury [11]. Current continuous recordings of oxygen saturation reveal a much higher frequency of intermittent hypoxia (IH) events that were previously un-documented in medical charts and provide insight to high-risk patterns associated with both short-term and long-term morbidity [12,13]. Although interpretation of absolute values of cerebral tissue oxygenation have yet to be determined, recent data have shown a greater adverse impact from IH than bradycardic events [14]. Regarding hypoxia exposure, chronic IH (CIH) has been observed to cause greater extent and degree of damage to the premature brain than chronic continuous hypoxia (CCH) [15,16] due to increased inflammation as CCH is unlikely to stimulate adaptive responses in neurons [17,18]. Various regions in the CNS, such as the sub cortex and especially the hippocampus have been shown to be more vulnerable than others to hypoxic exposure [18]. CIH can result in considerable damage to the hippocampal formation and IH exposure can lead to apoptosis in the hippocampal CA1 region [18–20]. The atlas of brain maps has proven useful in several publications for illustrating and comparing the distribution patterns of neuroanatomical data on templates and providing detailed mapping results [21]. As the pathology of brain injury becomes clearer, there is increased interest in developing methods to ameliorate it. The excessive production and release of proinflammatory cytokines are hallmarks of neuroinflammation and these cytokines serve as neurotoxic factors involved in the pathogenesis of white matter injury after IH in the immature brain [22,23]. Therapeutic approaches to protect the premature brain from inflammatory damage is of utmost importance.

Caffeine (Caff) and non-steroidal anti-inflammatory drugs (NSAIDs), such as Ibuprofen (Ibu) are often used concomitantly in ELBW babies for apnea of prematurity (AOP) and patent ductus arteriosus (PDA) closure, respectively [24]. Caff is a respiratory stimulant that reduces the incidence of AOP, and ameliorates clinical symptoms associated with AOP [25,26] and has become the drug of choice in neonatal care for improvement of respiratory outcomes [25]. Its use for neuroprotection in neonates and prevention of hypoxemic episodes has been investigated and proven effective [27,28]. Ibuprofen use has been shown to reduce inflammation in the brain of neonatal rats [29] and growth restricted piglets [23,30] suggesting a potential therapeutic intervention to prevent white matter damage. However, its chronic use appears to worsen the outcomes [31]. Ketorolac (Keto), another NSAID, is used to reduce the need for more traditional pain medication such as opioid analgesics [32–34]. A systematic review involving preterm and term infants showed that Keto in appropriate doses is a viable analgesic and does not increase the risk of bleeding or adverse renal effects [35]. Keto has been used in preterm infants to prevent retinopathy of prematurity (ROP), but the results were not consistent [36,37]. Both Ibu and Keto act by decreasing prostaglandin (PG) synthesis through non-selective inhibition of cyclooxygenase (COX)-1 and -2. However, there are no studies investigating whether Keto confers neuroprotection by reduction of inflammation, or the synergistic effects of Caff and NSAIDs for reduction of IH-induced neuroinflammation.

Using our neonatal IH model, which consistently produces oxidative stress in neonatal rats [38,39] we tested the hypothesis that early caffeine and/or NSAIDs confer superior therapeutic benefits for protection against IH-induced neuroinflammation than late treatment. Our hypothesis was tested with the following objectives: 1) To examine the effects of neonatal IH on inflammation in the neonatal rat brain; 2) To compare the effects of caffeine, ketorolac or ibuprofen given individually during neonatal IH on brain inflammation; 3) To establish whether Caff and/or NSAID co-treatment has synergistic effects for reducing brain inflammation and apoptosis; 4) To examine whether topical ocular Keto has effects in the brain; and 5) To investigate whether early treatment during IH exposure is more beneficial than late treatment during recovery/reoxygenation. The primary outcome was brain histopathology and morphometry, and the secondary outcomes were biomarkers of oxidative stress, inflammation, myelination, and apoptosis.

2. Materials and Methods

2.1. Animals

All experiments were approved by the State University of New York, Downstate Medical Center Institutional Animal Care and Use Committee, Brooklyn, NY (Protocol #11-10255). Certified infection-free, timed-pregnant Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) at 18 days gestation. The animals were housed in an animal facility with a 12-hour-day/12-hour-night cycle and provided standard laboratory diet and water ad libitum until delivery of their pups.

2.2. Experimental Design

Within 2-4 hours of birth, newborn rat pups delivering on the same day were pooled and randomly assigned to expanded litters of 18 pups/litter (9 males and 9 females). Gender was identified by the anogenital distance. The expanded litter size was used to simulate poor nutrition and relative postnatal malnutrition of ELBW infants who are at increased risk for severe oxidative stress and need for caff and/or NSAIDs. Two phases of treatment were conducted: 1) **Early Treatment:** Animals were exposed to IH from P0 to P14 and treated with either intraperitoneal (IP) and topical ocular saline (Sal/Sal), IP Caff, topical ocular Keto, IP Ibu, combined IP Caff and topical ocular Keto (Caff/Keto), or combined IP Caff and Ibu (Caff/Ibu). Treatment with Caff consisted of 20 mg/kg (diluted in sterile normal Sal) loading dose on P0 followed by 5 mg/kg/day from P1 to P14 (20 μ L volume). Treatment of Keto consisted of a single drop of Acuvail (4% ocular solution), into both eyes from P0 to P14. Treatment with Ibu consisted of 10 mg/kg loading dose on P0 followed by 5 mg/kg/day on P1 and P2. Treatment with Caff/Keto consisted of equivalent doses and volumes of IP Caff and topical ocular Acuvail, and treatment with Caff/Ibu consisted of equivalent doses and volumes of Caff and Ibu. Treatment with Sal/Sal consisted of equivalent IP and topical ocular volumes from P0 to P14. Rats were allowed to recover in room air (RA) until P21 with no further treatment; and 2) **Late Treatment:** Animals were exposed to IH from P0 to P14 after which they were removed from IH conditions and placed in RA until P21. The pups were administered doses as described above, of Sal, Caff, or Keto from P14 to P21, Ibu from P15 to P17, Caff/Keto from P15-P21; and Caff (P15-P17)/Ibu (P15-P17). A flow chart representing the early and late phases is presented in Figure 1.

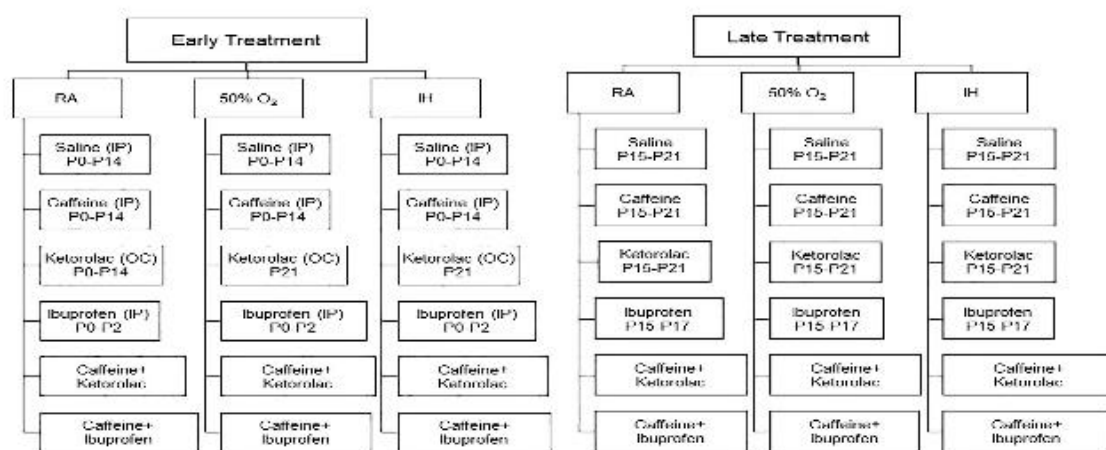


Figure 1. Experimental Design of Early and Late Treatment. RA (room air); 50% O₂ (50% oxygen); IH (intermittent hypoxia); IP (intraperitoneal), OC (topical ocular); P0-P2 (postnatal day 0 to postnatal day 2); P0-P14 (postnatal day 0 to postnatal day 14); P15-P17 (postnatal day 15 to postnatal day 17); P15-P21 (postnatal day 15 to postnatal day 21).

2.3. Neonatal Intermittent Hypoxia (IH) Profiles

Animals randomized to IH were placed with the dams in specialized oxygen chambers attached to an oxycycler (BioSpherix, NY, USA). The IH profile consisted of an initial exposure of hyperoxia (50% O₂) for 30 minutes followed by three brief, 1-minute, clustered hypoxic events (12% O₂), with a 10-minute re-oxygenation in 50% O₂ between each hypoxic event. Recovery from IH occurred in 50% O₂ following each clustered IH event for 2.5 hours for a total of 8 clustering IH episodes per day for 14 days, as previously described [38,39]. Oxygen saturation was confirmed on a sentinel un-anesthetized rat pup from each group using the MouseOx Pulse Oximeter and WinDaq Waveform Browser software (STARR Life Sciences Corp., Oakmont PA) before and after IH exposure.

2.4. Sample Collection & Processing

At euthanasia (P21), whole brains were removed, weighed and placed in 10% neutral buffered formalin (NBF). Fixed coronal sections were placed in cassettes and sent to the Histowiz, Inc. (Long Island City, NY USA) for processing, paraffin embedding, sectioning and hematoxylin and eosin (H&E) staining using standard laboratory techniques. Unstained sections were used for TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling), Luxol fast blue, and immunohistochemistry (IHC). Fresh brain samples excised from the cerebral cortex (n=6 samples/group) were placed in sterile Lysing Matrix D 2.0 mL tubes containing 1.4 mm ceramic spheres (MP Biomedicals, Santa Ana, CA, USA) and 1.0 mL PBS then snap-frozen in liquid nitrogen. Samples were stored at -80°C until analysis of cytokines using enzyme-linked immunosorbent assay (ELISA).

2.5. Brain histopathology and Morphometry

H&E stained images were captured at 20X magnification (scale bar=20 µm) using an Olympus BX53 microscope, DP72 digital camera, and CellSens imaging software (Olympus, Center Valley, PA), attached to a Dell Precision T3500 computer (Dell, Round Rock, TX). Morphometric analyses included total brain width (frontal-occipital and inter-parietal); and width of layers I-VI of the cerebral cortex [40]. Twelve sections were analyzed for each group. Measurements were made using the count and measure tool of the Olympus CellSens imaging software

2.6. Apoptosis (TUNEL Stain)

Apoptosis was determined in the unstained brain sections using the TUNEL assay kit purchased from Abcam (Waltham, MA, USA), according to the manufacturer's protocol. Sections were counterstained with Methyl Green.

2.7. Myelin Stain

Myelination was determined using the Luxol fast blue (LFB) stain (VitroVivo Biotech, Rockville, MD, USA). Briefly, unstained cross-sections were de-paraffinized with xylenes and alcohols prior to overnight incubation with Luxol fast blue solution at 56°C. Slides were washed and differentiated in lithium carbonate solution and 70% ethanol, then counter stained with Cresyl Violet solution, washed and mounted with Permount. Images were captured at 40X magnification using an Olympus BX53 microscope, DP72 digital camera, and CellSens imaging software (Olympus, Center Valley, PA, USA), attached to a Dell Precision T3500 computer (Dell, Round Rock, TX, USA). Stain intensity was determined using the count and measure on region of interest tool of the CellSens imaging software (12 measurements/group).

2.8. IHC Assays

Unstained sections were used for IHC of phosphorylated (p) nuclear factor kappa B (NFκB), IκB, and pro-inflammatory cytokines, including interleukin-1(IL-1)β, IL-6, tumor necrosis factor (TNF)α, and IL-10. Unstained sections (2 sections per slide) were de-paraffinized with xylenes and alcohols

prior to unmasking of antigens. Sections were washed several times in PBS containing triton X-100 (PBS-T) and incubated in blocking solution (Cell Signaling Technologies, Danvers, MA) for 1 hour. The blocking solution was removed and primary antibodies for cytokines (Santa Cruz Biotechnology, Inc., Dallas, TX) were added and sections were incubated overnight. The sections were washed and incubated with Signal Boost antibody serum secondary antibodies (Cell Signaling) for 1 hour. After washing, sections were counterstained with hematoxylin. Images were captured at 20X magnification (scale bar=20 μ m) using an Olympus BX53 microscope, DP72 digital camera, and CellSens imaging software (Olympus, Center Valley, PA), attached to a Dell Precision T3500 computer (Dell, Round Rock, TX). Twelve sections were analyzed for each group. Measurements were made using the count and measure tool of the Olympus CellSens imaging software.

2.9. ELISA Assays

All samples were analyzed on the same day. On the day of analyses, the tubes were allowed to defrost on ice and placed in a high-speed FastPrep-24 instrument (MP Biomedicals, Santa Ana, CA, USA), which utilizes a unique, optimized motion to efficiently homogenize biological samples within 40 seconds via multidirectional simultaneous beating of the Lysing Matrix ceramic beads on the tissue. The homogenates were then centrifuged at 4°C at 10,000 rpm for 20 minutes. The supernatant was filtered, and the filtrate was used for the assay of total cellular proteins, IL-1 β , TNF α , IL-6, and IL-10 using commercially available kits purchased from Millipore Sigma (St. Louis, MO, USA) and MyBioSource (San Diego, CA). All samples were processed and assayed according to the manufacturer's protocol. A total of 6 samples per experimental group were analyzed. All data were standardized using total cellular protein levels.

2.10. Total Cellular Protein Levels

On the day of assays, an aliquot (10 μ L) of the cerebral cortex homogenates was used for total cellular protein levels using the Bradford method (Bio-Rad, Hercules, CA USA) with bovine serum albumin as a standard.

2.11. Statistical Analysis

To determine differences among the treatment groups, a test for normality was first conducted using Bartlett's test. Normally distributed data were analyzed using two-way analysis of variance (ANOVA) with Dunnett's post-hoc tests. Non-normally distributed data were analyzed using Kruskal Wallis test with Dunn's multiple comparison test. Data are presented as mean \pm standard error of the mean (SEM) and a *p*-value of <0.05 was considered as statistically significant, using SPSS version 26.0 (SPSS Inc., Chicago, IL, USA). Graphs were prepared using GraphPad Prism version 7.03 (GraphPad, San Diego, CA, USA).

3. Results

3.1. Brain Weight

Whole brain weight and brain to body weight ratios are presented in Figure 2. Panels A and C represent body weight and brain/body weight ratios for the early treatment groups, respectively; and panels B and D represent the corresponding late treatment groups. The solid white bar represents the groups exposed to room air (RA), the checked bar represents the groups exposed to 50% O₂ (hyperoxia); and the solid black bar represents the groups exposed to neonatal IH. In the early treatment groups, animals exposed to neonatal IH and treated with Sal/Sal or Sal/Ibu had significantly higher brain/body weight ratios compared to their RA or hyperoxia littermates (Figure 2C). In the late treatment groups, animals exposed to hyperoxia and neonatal IH and treated with ketorolac, ibuprofen with and without caffeine had persistently higher brain to body weight ratios (Figure 2D).

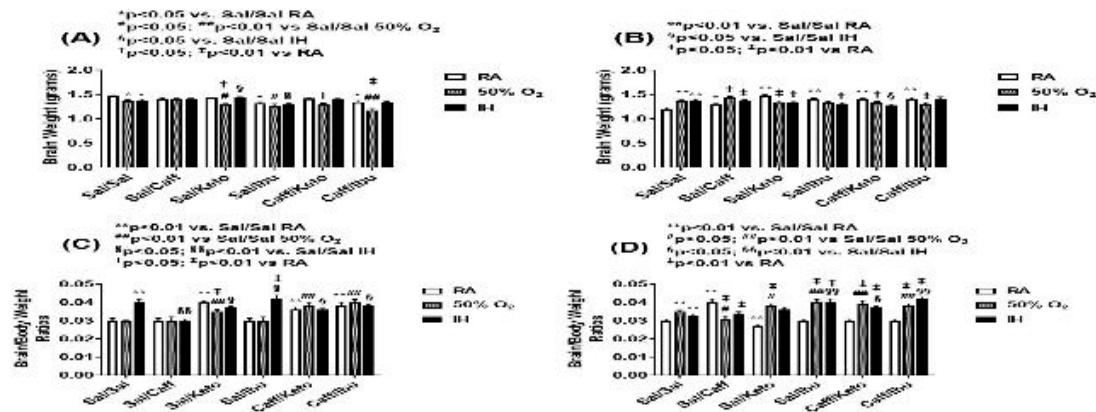


Figure 2. Effect of neonatal intermittent hypoxia (IH) and early or late caffeine/NSAIDs treatment on brain weight and brain/body weight ratios. Panels A and C represent the early treatment, respectively; and panels B and D represent the late treatment, respectively. The solid white bar represents the groups exposed to room air (RA), the checked bar represents the groups exposed to 50% O₂; and the solid black bar represents the groups exposed to neonatal IH. Groups are labeled as follows: Sal/Sal (ocular saline/IP saline); Sal/Caff (ocular saline/IP caffeine); Sal/Keto (IP saline/ocular ketorolac); Sal/Ibu (ocular saline/IP ibuprofen); Caff/Keto (IP caffeine/ocular ketorolac); and Caff/Ibu (IP caffeine/IP ibuprofen). Data are mean \pm SEM (n=18 samples per group).

3.2. Histopathology

Representations of the H&E-stained cerebral cortex sections for the early treatment groups are presented in Figure 3. The upper panels represent the groups exposed to RA; the middle panels represent the groups exposed to hyperoxia; and the lower panels represent the groups exposed to neonatal IH. Treatment groups are labeled in the top panels. Microscopically, the following horizontal organizations of cellular layers (Laminae) were seen as previously described [40] in the cortex: Molecular (Plexiform) layer I consists of apical dendritic tufts of the pyramidal neurons, horizontally organized axons, Cajal-Retzius cells and glial cells. External granular layer II consists of mainly granular cells and small pyramidal cells. External pyramidal layer III consists of small and medium-sized pyramidal cells. Internal granular layer IV has small round granular cells (spiny stellate cells, spiny interneurons) and small pyramidal cells. Internal pyramidal layer V has the largest pyramidal cells. Axons of the pyramidal cells leave the cortex and reach subcortical areas. Multiform (Polymorphous) layer VI: Excitatory cells like spiny stellate cells, pyramidal cells, inverted pyramidal neurons, bipolar/fusiform cells, and odd-shaped cells are present in layer VI. It also holds inhibitory interneurons.

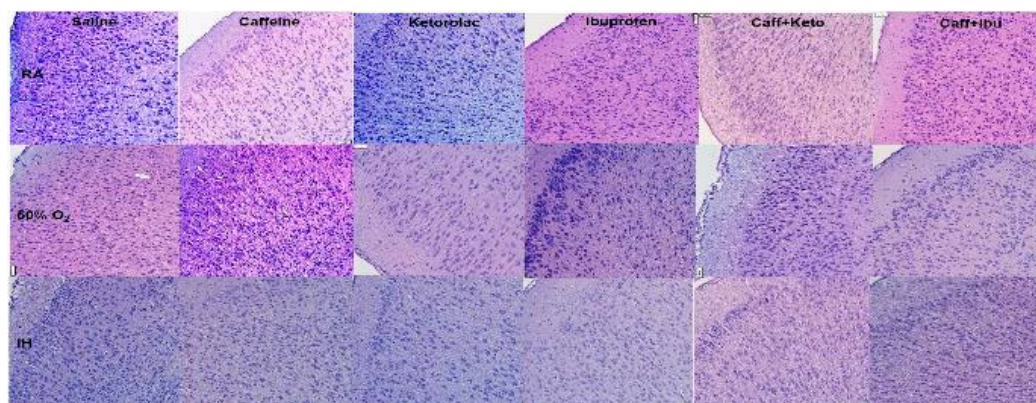


Figure 3. Representations of the H&E stained cerebral cortex sections for the early treatment groups. The upper panels represent the groups exposed to RA; the middle panels represent the groups exposed to hyperoxia (50% O₂); and the lower panels represent the groups exposed to neonatal IH. Images are 20X magnification, scale bar is 50 μ m.

In the RA-exposed group, cellular layers were wider and showed increased cellularity in groups treated with Ketorolac, both alone and in conjunction with Caff. Caff, Ibu and the Caff/Ibu groups all displayed increased brain dimensions on gross specimen analysis. However, cellular layers V and VI with Caff/Ibu co-treatment were reduced in thickness to a significant degree. In the hyperoxia early treatment group, saline treatment resulted in showed significantly reduced frontal-occipital diameter compared to RA controls, but on cellular morphometric analysis, increased cortical thickness in all 6 layers was found. Increased brain dimensions were grossly appreciated in NSAID treated groups; however cellular layer analysis showed layer III was significantly increased in thickness in all treatment subgroups. In the neonatal IH treatment groups, Caff and Keto individual treatment showed marked increase in size of the specimen as well as increased width of layers III, IV and VI, and this effect was maintained in the groups subjected to co-treatment of the 2 agents also. Ibuprofen treatment also conferred increased brain size as well as increase in cell layer width of layers III and V, both alone and in conjunction with Caff. Overall, layers IV and VI were narrower in all treatment groups compared to the control IH group. All histological findings were corroborated with morphometric analyses presented in Table 1.

Representations of the H&E-stained cerebral cortex sections for the late treatment groups are presented in Figure 4. The upper panels represent the groups exposed to RA; the middle panels represent the groups exposed to hyperoxia; and the lower panels represent the groups exposed to neonatal IH. Treatment groups are labeled in the top panels. In groups exposed to RA, Caff treatment conferred significant increased biparietal length and increased width of layers I and II as compared to controls. Keto treatment increased width of cell layer II, holding pyramidal cells, to a greater extent as compared to layer III. Ibu treatment showed increased frontal-occipital (FO) diameter, however only cell layer II showed increase in width. Caff/Keto co-treatment showed an increase in biparietal diameter of the gross brain specimen, while Caff/Ibu increased FO diameter as well increased width of cell layers II, V and VI. In the hyperoxia groups, control animals, as well as all treatment groups were found to have increased brain volumes as shown by increased cross sectional diameters compared to RA counterparts, perhaps due to increased cellular proliferation attributable to higher oxygenation. Caff alone showed increased layer I, and Ibu showed increased layer III cells, together their co-treated groups showed increased width in layers I, II and V. In the neonatal IH groups, all treatment groups were found to have increased brain volumes as shown by increased cross sectional diameters compared to RA counterparts. Cellular layer morphometric analysis showed increased cell I layer width in treatment with Caff or Ibu separately but not in the co-treatment group, which instead had wide layers III and V. Caff/Keto co-treatment had significantly wider layers V and VI compared to the control IH group. All histological findings were corroborated with morphometric analyses presented in Table 2.

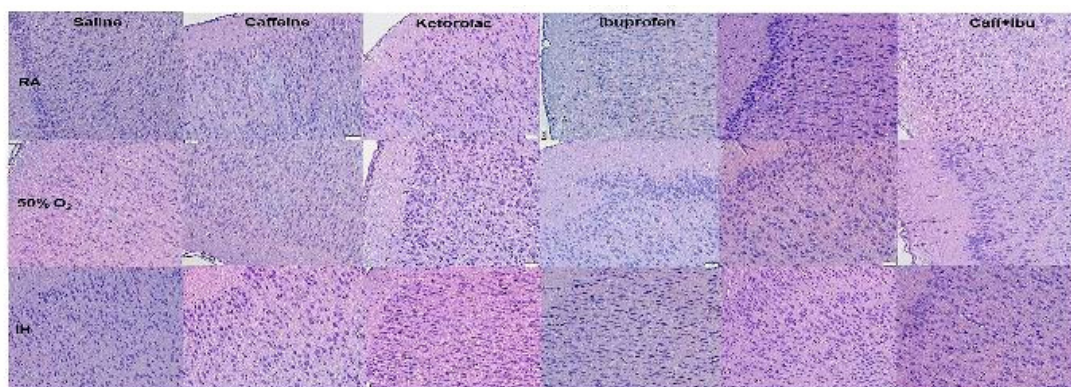


Figure 4. Representations of the H&E stained cerebral cortex sections for the late treatment groups. The upper panels represent the groups exposed to RA; the middle panels represent the groups exposed to hyperoxia (50% O₂); and the lower panels represent the groups exposed to neonatal IH. Images are 20X magnification, scale bar is 50 μ m.

Table 1. Morphometric Analyses (Early).

G	Total Brain Width (frontal-occipital)	Total Brain Width (inter-parietal)	Layer I	Layer II	Layer III	Layer IV	Layer V	Layer VI
RA:								
Sal	7836.6±88.0	12337.9±87.7	109.1±8.0	80.5±7.8	101.7±7.8	146.2±21.4	236.7±12.6	185.4±17.3
Caff	5591.1±7.1**	10406.4±17.8**	134.9±7.4	63.4±6.9	109.3±9.0	130.3±9.2	120.1±7.9**	137.7±12.2
Keto	8543.5±22.3**	14631.0±289.0**	182.6±15.4**	96.6±7.1**	294.1±22.8**	239.9±18.5**	133.2±8.4**	155.4±15.7
Ibu	6794.7±45.4**	10041.3±14.2**	136.9±10.4	101.0±7.1	142.4±26.3	120.8±20.1	223.3±20.4	143.6±10.8
Caff+Keto	8439.3±35.0**	14048.7±397.4**	150.8±10.1*	76.9±5.2**	228.8±23.3**	242.4±13.1**	125.9±10.5**	155.1±18.0
Caff+Ibu	6310.7±57.3**	10917.3±36.9**	123.5±3.4	82.6±9.8	133.0±20.3	142.6±13.9	150.3±17.0**	113.3±12.9**
50% O₂:								
Sal	5890.2±33.0**	9471.5±159.4	109.0±11.3	71.5±1.8	174.0±18.7	241.5±17.0	198.0±28.9	137.3±11.1
Caff	5851.9±20.5†	9312.3±23.7	1174.8±27.0##†	2726.4±144.2##†	2000.5±214.6##†	1973.7±46.9##†	1749.2±302.3##†	1327.3±118.4**†
Keto	8387.7±256.4##	13652.5±915.8##	214.9±13.9##	99.3±13.0	235.5±15.4	260.2±34.3	276.9±90.6	122.0±16.7
Ibu	7667.6±55.7##†	8820.2±1820.7†	122.0±3.0	79.0±5.4	154.6±13.2†	244.5±25.3†	258.9±62.4	149.2±20.9
Caff+Keto	6894.5±92.7##†	12334.0±145.3†	153.4±8.4	82.5±7.5	238.8±0.87†	323.8±9.6	122.5±8.0	153.4±8.4
Caff+Ibu	7313.6±33.0##†	11509.6±70.7†	121.5±5.40	88.9±4.7	170.4±13.0†	244.0±25.5†	165.3±15.0	144.6±10.6
Neonatal IH:								
Sal	8270.1±25.2	11218.5±21.6	312.1±115.9	299.3±135.6*	567.9±265.9	575.4±185.0	290.8±89.2	416.1±107.4*
Caff	8063.0±116.2†	12813.3±17.7†	153.2±34.0	130.4±17.7	158.2±26.4†	310.5±44.7§§†	228.3±55.8	147.6±36.3§§
Keto	9106.0±136.5§§†	13711.7±56.2	156.3±12.2	140.5±12.0	303.1±46.3	294.2±47.8§§	210.5±41.5	150.0±36.0§§
Ibu	8840.0±116.4§§†	12924.0±79.5†	144.4±8.6	122.3±13.3	186.3±9.9†	273.4±47.8§§†	415.5±179.5	179.5±17.3§§
Caff+Keto	6935.5±76.4§§†	12410.0±133.3†	156.0±7.5	88.4±8.9	173.0±23.4§§†	182.9±17.6§§	201.4±37.9	182.6±27.9§§
Caff+Ibu	8711.1±116.6§†	12730.0±42.4†	158.9±14.6§†	87.2±24.9	160.3±26.8§	221.5±36.8§§	314.3±53.5†	188.7±15.1§†

RA (room air); 50% O₂ (hyperoxia); IH (intermittent hypoxia); Caff (Caffeine); Keto (Ketorolac); Ibu (Ibuprofen). Animals were treated with caffeine citrate from birth (P0) to P14; ketorolac eye drops from P0 to P14; or ibuprofen from P0 to P3. Data were analyzed using two-way ANOVA (n=6/group). *p<0.05, **p<0.01 vs. Saline RA; #p<0.05##p<0.01 vs. Saline 50% O₂; §p<0.05, §§p<0.01 vs Saline IH; †p<0.05, ‡p<0.01 vs RA.

Table 2. Morphometric Analyses (Late).

G	Total Brain Width (frontal-occipital)	Total Brain Width (inter-parietal)	Layer I	Layer II	Layer III	Layer IV	Layer V	Layer VI
RA:								
Sal	7705.2±67.0	13309.8±276.3	154.5±6.1	56.1±3.6	147.8±33.9	202.9±19.1	241.0±9.2	127.0±16.2
Caff	7651.1±109.7	11572.0±113.5**	104.0±5.1**	85.9±5.6**	241.5±47.9	259.8±34.0	292.5±30.5	140.4±19.5
Keto	7595.6±219.0	12598.4±250.0	149.6±10.4	101.4±7.6**	280.1±52.3*	211.2±5.1	247.0±16.4	165.7±13.8
Ibu	8404.0±81.8**	13263.0±530.0	166.6±12.9	87.2±5.1**	180.9±15.6	258.3±25.0	234.4±25.9	145.3±12.2
Caff+Keto	7902.7±30.3	11506.7±88.2**	133.7±7.5	70.7±5.1	205.0±26.8	181.5±30.2	186.5±25.0	158.4±7.3
Caff+Ibu	8281.1±207.2*	13327.4±280.4	136.6±8.3	86.5±5.6**	191.1±23.9	270.6±18.9	405.0±29.7**	198.9±9.1**
50% O₂:								
Sal	8947.5±21.2**	13534.8±255.2	179.8±15.2	102.4±10.4	243.0±23.4	237.8±33.6	192.5±18.5	151.3±7.0
Caff	9335.5±122.5##‡	14038.0±174.4‡	130.8±6.5§	83.5±8.4	211.0±20.2	298.0±31.0	305.2±49.2	155.5±13.8
Keto	8773.5±195.7‡	11511.0±165.7##‡	141.8±2.4†	100.0±6.8	222.0±12.2	277.3±16.9†	190.8±39.0	164.0±17.5
Ibu	9093.5±76.8‡	13285.5±288.6	182.6±8.0	111.6±8.4	240.3±16.4†	238.5±18.1	313.6±23.1	162.3±12.7
Caff+Keto	7656.5±7.1##†	13158.5±297.0‡	161.1±6.6	126.9±8.0‡	240.3±23.4	253.4±18.5	218.5±24.9	132.6±10.0
Caff+Ibu	9272.0±36.4‡	13344.7±194.4	171.0±8.7‡	107.4±4.3‡	201.5±15.0	235.8±22.4	299.8±37.6†	154.0±16.4
Neonatal IH:								
Sal	8270.1±29.1**	11218.5±217.6**	187.7±57.9	163.8±65.6	367.6±118.3*	369.8±98.4	284.9±41.8	260.9±65.0*
Caff	8817.0±18.3	13775.3±280.9	178.6±7.7‡	114.5±19.6	324.5±42.2	243.4±56.9	242.3±32.1	178.9±8.5
	§§‡	§§‡						
Keto	7501.7±39.8§§	11466.0±160.1‡	141.4±10.8	90.3±5.8	275.5±47.9	254.3±24.0	240.6±27.1	171.8±25.9
Ibu	9481.3±68.4	15107.3±280.5	226.6±9.6‡	116.5±24.2	227.8±17.7	285.6±30.1	229.7±42.6	178.0±9.4
	§§‡	§§‡						
Caff+Keto	7461.7±93.7	698.0±91.1§§‡	143.0±3.9	97.1±15.9	250.3±29.8	252.6±17.5	127.3±6.1§§	125.0±11.9§
	§§‡							
Caff+Ibu	9465.5±206.6§§‡	14610.0±242.4	124.4±2.2	93.4±3.5	325.0±32.1‡	256.0±17.1	169.7±20.8	180.4±23.1
		§§‡					§‡	

RA (room air); 50% O₂ (hyperoxia); IH (intermittent hypoxia); Caff (Caffeine); Keto (Ketorolac); Ibu (Ibuprofen). Animals were treated with caffeine citrate from birth (P0) to P14; ketorolac eye drops from P0 to P14; or ibuprofen from P0 to P3. Data were analyzed using two-way ANOVA (n=6/group). *p<0.05, **p<0.01 vs. Saline RA; #p<0.05##p<0.01 vs. Saline 50% O₂; §p<0.05, §§p<0.01 vs Saline IH; †p<0.05, ‡p<0.01 vs RA.

3.3. Apoptosis

Apoptosis, as evidenced by the TUNEL stain for early and late treatment groups are presented in Figures 5 and 6, respectively. Apoptosis is indicated by the brown stain. Sections were counter stained with methyl green which indicates negativity. In the early treated groups, apoptosis was less evident in the Sal, Keto, and Caff/Keto groups exposed to RA. Ibu treatment caused the highest TUNEL positivity. In hyperoxia, TUNEL positivity was highest in the Sal and Ibu groups. Although there was some positive staining with Keto and Caff/Keto, there was also a high presence of methyl green. Caff appeared to have the most effect for reducing apoptosis in hyperoxia. In neonatal IH, all groups showed TUNEL positivity, but treatment with Keto and Caff/Keto were most effective for reducing apoptosis (Figure 5). The late treatment groups apoptosis was highest with Caff/Keto and lowest with Sal and Keto in RA. In hyperoxia, Keto and Caff/Keto reduced apoptosis compared to all other groups. In neonatal IH, treatment with Caff, Keto, and Caff/Keto reduced apoptosis compared to Sal, Ibu and Caff/Ibu (Figure 6). Quantitative analysis of the stain intensity is presented in Tables 3 and 4 for early and late treatment, respectively.

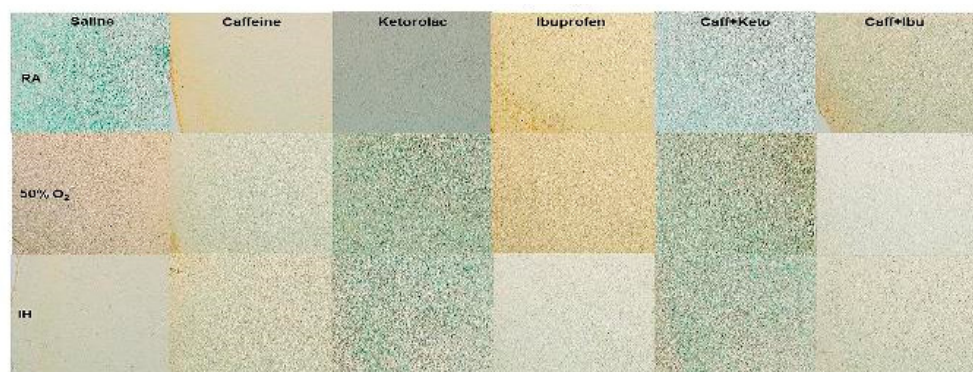


Figure 5. Representations of the TUNEL stained cerebral cortex sections for the early treatment groups. The upper panels represent the groups exposed to RA (room air); the middle panels represent the groups exposed to hyperoxia (50% O₂); and the lower panels represent the groups exposed to neonatal IH (intermittent hypoxia). Caff (caffeine); Keto (ketorolac); Ibu (ibuprofen). Apoptosis is indicated by the brown stain. Sections were counter stained with methyl green which indicates negativity. Images are 20X magnification, scale bar is 50 μ m.

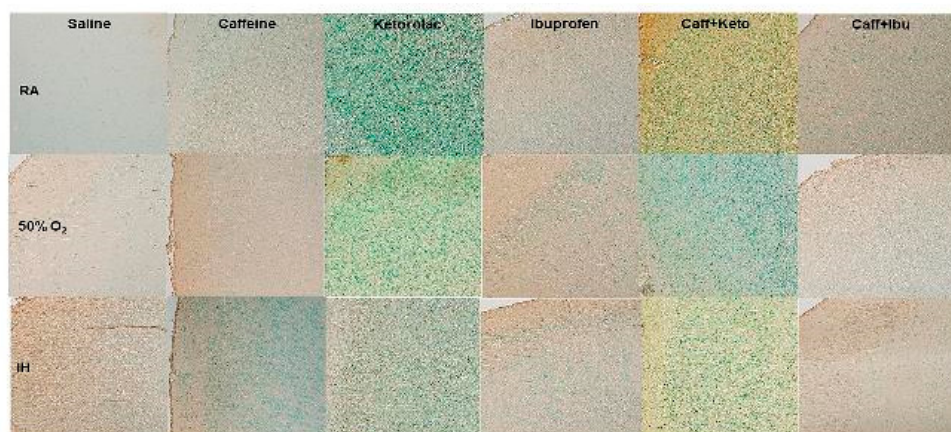


Figure 6. Representations of the TUNEL stained cerebral cortex sections for the late treatment groups. The upper panels represent the groups exposed to RA (room air); the middle panels represent the groups exposed to hyperoxia (50% O₂); and the lower panels represent the groups exposed to neonatal IH (intermittent hypoxia). Apoptosis is indicated by the brown stain. Sections were counter stained with methyl green which indicates negativity. Images are 20X magnification, scale bar is 50 μ m.

Table 3. IHC Quantification (Early).

Group	Apoptosis (TUNEL)	Myelin (LFB)	pNFkB	IkB	IL-1 β	IL-6	TNF α
RA:							
Sal	489.5 \pm 178.2	1369.5 \pm 265.7	0.8 \pm 0.36	181.3 \pm 19.8	391.6 \pm 37.3	381.7 \pm 29.1	3134.4 \pm 193.9
Caff	4387.7 \pm 724.1**	1038.7 \pm 106.5	2.3 \pm 1.3	51.1 \pm 5.2	61.6 \pm 15.5**	1.5 \pm 0.3**	88.2 \pm 21.6**
Keto	225.3 \pm 17.5	2797.6 \pm 124.0**	18.5 \pm 12.3	3277.7 \pm 470.2**	29.6 \pm 7.5**	20.3 \pm 5.6**	1460.8 \pm 551.0**
Ibu	6809.3 \pm 283.7**	3374.5 \pm 203.0**	3444.3 \pm 475.3**	70.5 \pm 4.6	46.1 \pm 5.8**	214.6 \pm 42.5	298.1 \pm 39.0**
Caff+Keto	4462.7 \pm 594.3**	27.8 \pm 2.0**	196.5 \pm 61.3	25477.3 \pm 1440.6**	56.8 \pm 20.4**	63.3 \pm 10.3*	795.7 \pm 131.0**
Caff+Ibu	8517.8 \pm 182.8**	2653.0 \pm 441.5**	29.4 \pm 7.2	331.3 \pm 14.3	8.4 \pm 3.0**	453.4 \pm 165.8	737.3 \pm 133.7**
50% O₂:							
Sal	2917.6 \pm 390.8**	18.4 \pm 2.9**	2049.9 \pm 952.0*	195.2 \pm 19.8	72.4 \pm 11.4**	19.0 \pm 6.1**	131.7 \pm 43.8**
Caff	6915.8 \pm 292.0##†	4.9 \pm 1.5‡	144.8 \pm 31.0	51.7 \pm 8.2	29.7 \pm 3.8	34.5 \pm 7.2‡	2258.9 \pm 368.0##†
Keto	1297.2 \pm 75.7##‡	26.0 \pm 14.4‡	711.9 \pm 77.9‡	467.0 \pm 92.1##‡	23.2 \pm 3.7	0.31 \pm 0.19	184.6 \pm 67.9†
Ibu	3940.6 \pm 593.7‡	0 \pm 0##‡	2275.3 \pm 274.7†	51.7 \pm 4.6	77.8 \pm 6.5	8.2 \pm 1.8‡	198.9 \pm 62.4
Caff+Keto	365.0 \pm 41.7##‡	0 \pm 0‡	3068.9 \pm 320.3‡	1701.1 \pm 137.0‡	59.9 \pm 6.4	14.7 \pm 3.8‡	338.1 \pm 97.1
Caff+Ibu	5658.0 \pm 310.9##†	0 \pm 0##‡	3225.3 \pm 699.0‡	60.9 \pm 7.2†	330.8 \pm 40.7##‡	163.3 \pm 42.6##	2556.8 \pm 300.0##‡
Neonatal IH:							
Sal	306.4 \pm 63.1**	0 \pm 0**	1302.6 \pm 162.2	182.3 \pm 11.2	66.0 \pm 8.9**	50.6 \pm 11.3**	1688.9 \pm 186.6**
Caff	2400.0 \pm 329.1‡	0 \pm 0‡	1026.7 \pm 111.0‡	85.8 \pm 13.8†	77.7 \pm 8.9	5.5 \pm 1.6	10.6 \pm 3.4§§
Keto	777.3 \pm 73.7§‡	0 \pm 0‡	1379.3 \pm 202.6‡	885.8 \pm 89.4§§‡	61.4 \pm 22.5	209.4 \pm 49.2§§‡	805.1 \pm 289.4§§
Ibu	3670.5 \pm 582.7‡	0 \pm 0‡	149.2 \pm 20.6§§‡	204.8 \pm 29.6‡	189.7 \pm 40.3§§‡	41.4 \pm 25.3‡	3.2 \pm 0.57§§‡
Caff+Keto	1851.3 \pm 396.9‡	0 \pm 0‡	971.7 \pm 179.1	93.9 \pm 6.9‡	53.3 \pm 10.8	67.6 \pm 11.7	919.5 \pm 6.9§§
Caff+Ibu	8004.7 \pm 1326.2§§	0 \pm 0‡	369.0 \pm 53.1§§	905.1 \pm 134.4§§‡	22.5 \pm 3.6	56.7 \pm 8.8†	382.8 \pm 66.8§§

RA (room air); 50% O₂ (hyperoxia); IH (intermittent hypoxia); Caff (Caffeine); Keto (Ketorolac); Ibu (Ibuprofen); pNFkB (phosphorylated nuclear factor kappa B); IL (interleukin); TNF (tumor necrosis factor). . Animals were treated with caffeine citrate from birth (P0) to P14; ketorolac eye drops from P0 to P14; or ibuprofen from P0 to P3. Data were analyzed using two-way ANOVA. *p<0.05, **p<0.01 vs. Saline RA; #p<0.05##p<0.01 vs. Saline 50% O₂; §p<0.05, §§p<0.01 vs Saline IH; †p<0.05, ‡p<0.01 vs RA.

Table 4. IHC Quantification (Late).

G	Apoptosis (TUNEL)	Myelin (LFB)	pNFkB	IkB	IL-1 β	IL-6	TNF α
RA:							
Sal	65.4 \pm 17.5	916.2 \pm 165.5	0.69 \pm 0.41	38.3 \pm 3.6	1699.9 \pm 230.6	43.5 \pm 9.8	4268.9 \pm 681.2

Caff	16.0±2.5	5027.7±723.4**	253.8±66.4	93.9±5.6	2155.9±598.8**	1691.8±502.1**	1992.7±379.7**
Keto	2315.2±462**	1600.0±306.4	4425.4±990**	1765.7±223.7**	9113.9±419.7**	13.2±1.8	256.2±116.8**
Ibu	262.0±24.1	5576.5±470.0**	24.3±4.4	174.5±15.0	52.0±12.6**	423.4±203.1	524.2±235.1**
Caff+Keto	1526.1±251.9**	563.3±47.8	4751.7±607.3**	653.9±79.9**	4.9±0.87**	50.7±7.0	281.5±43.7**
Caff+Ibu	5.1±1.5	5495.0±693.9**	29.4±4.3	34.8±6.3	205.8±32.2**	41.4±5.8	2479.9±368.4**
50% O₂:							
Sal	876.2±108.4**	13042.9±438.2**	3239.5±800.3**	30.0±2.8	1151.5±175.2*	4.1±0.9	2742.2±746.7
Caff	33.5±8.7##	4083.8±764.4##	12668.9±2030	89.2±5.2	5365.3±531.0##‡	86.5±27.8#‡	5411.4±735.4##‡
Keto	106.8±23.7##‡	2348.2±579.3##	2924.9±1037.3	869.2±202.6##	1875.3±690.3‡	61.2±27.2	2522.4±202.6‡
Ibu	4.7±1.4##‡	1710.2±346.9##‡	2275.3±274.7‡	186.9±17.0	135.7±56.0	29.7±14.2†	169.0±48.2##
Caff+Keto	1116.6±54.7†	1172.7±118.9##†	22.1±3.6##‡	645.6±77.9##	213.6±86.9	61.4±7.7#	630.3±120.1##
Caff+Ibu	44.2±15.6##†	1692.1±370.8##‡	14.5±2.6##	31.1±5.9	3.7±1.5‡	99.4±16.9##	559.1±186.3##‡
Neonatal IH:							
Sal	270.0±39.3	41.3±4.5	852.3±140.0	81.9±7.5**	32.4±5.5**	545.4±71.3*	168.0±36.9*
Caff	21.0±11.7	1279.1±229.4§§‡	1454.9±395.0	59.3±4.0‡	3731.0±481.4§§	312.6±51.9§§‡	643.4±234.3
Keto	543.3±135.5‡	1364.7±100.8§§	735.1±109.3‡	2139.4±398.3§§	123.3±15.4‡	4.1±1.1§§	17.3±6.9
Ibu	194.3±46.2	1744.7±153.3§§‡	343.1±54.4	71.0±6.1‡	18.1±4.7	5.5±2.2§§†	1031.7±184.7§§
Caff+Keto	441.3±110.9‡	998.8±60.0§§	3363.9±234.0§§‡	3227.0±320.6§§‡	40.9±9.6	56.9±18.8§§	720.3±310.0
Caff+Ibu	75.4±5.8‡	993.0±87.2§§‡	579.9±53.5‡	210.6±12.2‡	50.6±38.0‡	319.4±50.1§§‡	254.3±83.3‡

RA (room air); 50% O₂ (hyperoxia); IH (intermittent hypoxia); Caff (Caffeine); Keto (Ketorolac); Ibu (Ibuprofen); pNFkB (phosphorylated nuclear factor kappa B); IL (interleukin); TNF (tumor necrosis factor). Animals were treated with caffeine citrate from birth (P0) to P14; ketorolac eye drops from P0 to P14; or ibuprofen from P0 to P3. Data were analyzed using two-way ANOVA (n=6/group). *p<0.05, **p<0.01 vs. Saline RA; #p<0.05##p<0.01 vs. Saline 50% O₂; §p<0.05, §§p<0.01 vs Saline IH; †p<0.05, ‡p<0.01 vs RA.

3.4. Myelination

Representative cerebral cortex sections stained for myelination as indicated by the LFB stain for early and late treatment groups are presented in Figures 7 and 8, respectively. In the early RA groups, there was increased myelination with Ibu and Caff/Ibu treatment. In early hyperoxia, myelination was significantly increased in the Sal group compared to the Sal RA group, and declined with the other treatments in hyperoxia, although treatment with Caff and Caff/Ibu produced the highest myelination compared to the Keto, Ibu, and Caff/Keto groups. Myelination was reduced in all groups exposed to early IH (Figure 7). In the late treatment groups, myelination was highest with Caff, Keto, Ibu, and Caff/Ibu treatment in RA. However, exposure to hyperoxia and neonatal IH decreased myelination in all groups (Figure 8). Quantitative analysis of the stain intensity is presented in Tables 3 and 4 for early and late treatment, respectively.

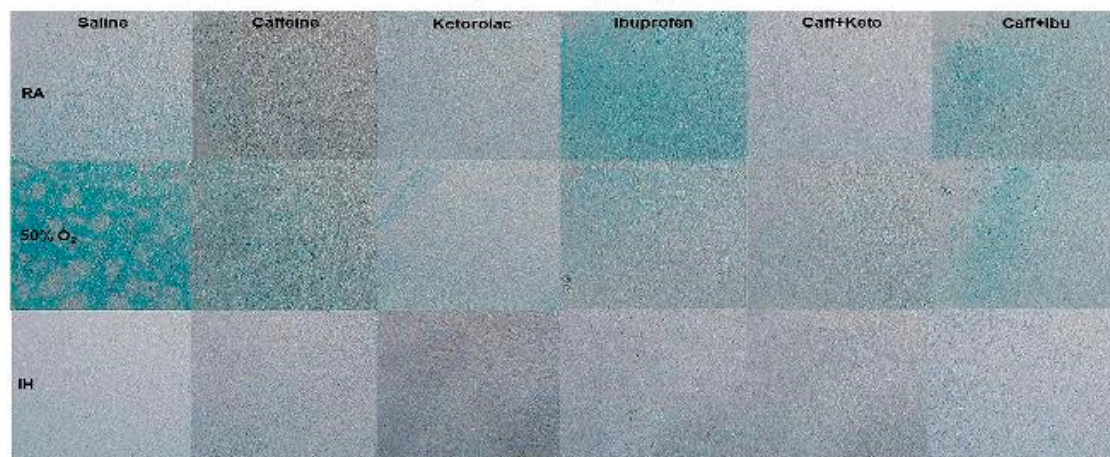


Figure 7. Representations of the Luxol fast blue (LFB) stained cerebral cortex sections indicating myelination, for the early treatment groups. The upper panels represent the groups exposed to RA (room air); the middle panels represent the groups exposed to hyperoxia (50% O₂); and the lower panels represent the groups exposed to neonatal IH (intermittent hypoxia). Caff (caffeine); Keto (ketorolac); Ibu (ibuprofen). Myelination is indicated by the blue stain. Images are 20X magnification, scale bar is 50 μ m.

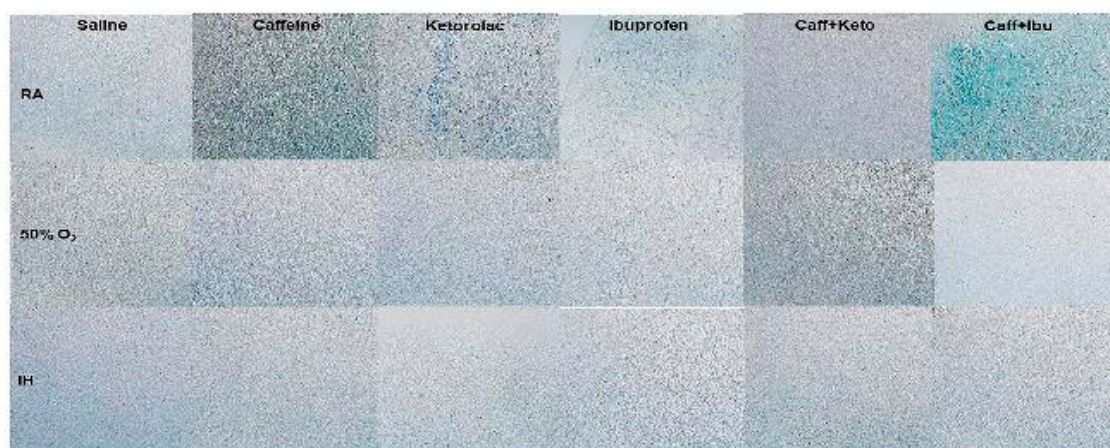


Figure 8. Representations of the Luxol fast blue (LFB) stained cerebral cortex sections indicating myelination, for the late treatment groups. The upper panels represent the groups exposed to RA (room air); the middle panels represent the groups exposed to hyperoxia (50% O₂); and the lower panels represent the groups exposed to neonatal IH (intermittent hypoxia). Caff (caffeine); Keto (ketorolac); Ibu (ibuprofen). Myelination is indicated by the blue stain. Images are 20X magnification, scale bar is 50 μ m.

3.5. Cytokines Levels

Cytokine levels in the brain homogenates for the early and late treatment groups are presented in Figures 9 and 10, respectively. All cytokines were elevated in the Sal-treated groups exposed to neonatal IH. This response was reversed with Caff, Keto, and Caff/Keto. However, IL-1 β and IL-10 were also reduced with Ibu and Caff/Ibu, but not TNF α and IL-6. For the late treatment groups, all cytokines were lower with all treatments and exposures compared to saline controls in RA. Overall, cytokine levels were lower in the hyperoxia and neonatal IH groups. Of all groups Caff/Keto most effectively reduced cytokines in the neonatal IH exposed groups.

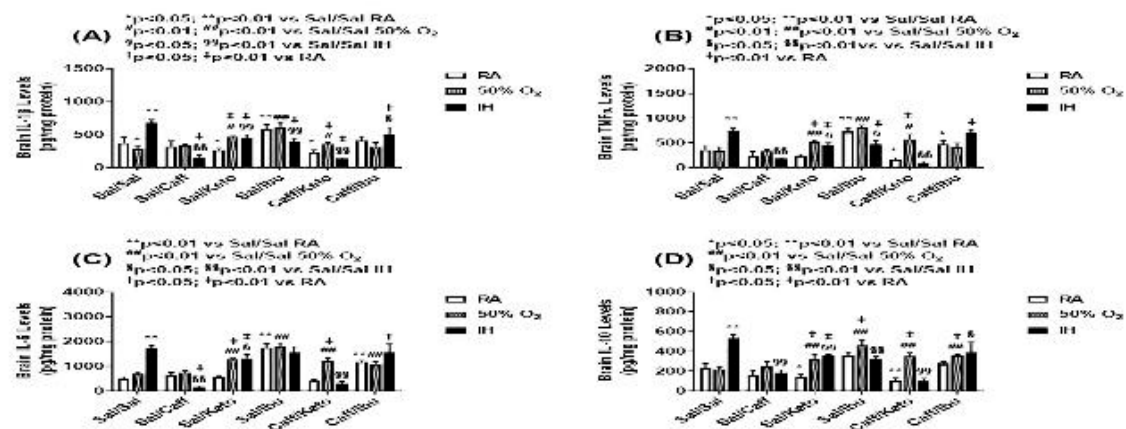


Figure 9. Effect of neonatal intermittent hypoxia (IH) and early caffeine/NSAIDs treatment on cytokines in the cerebral cortex homogenates. The groups are as described in Figure 2. Data are mean \pm SEM (n=6 samples per group).

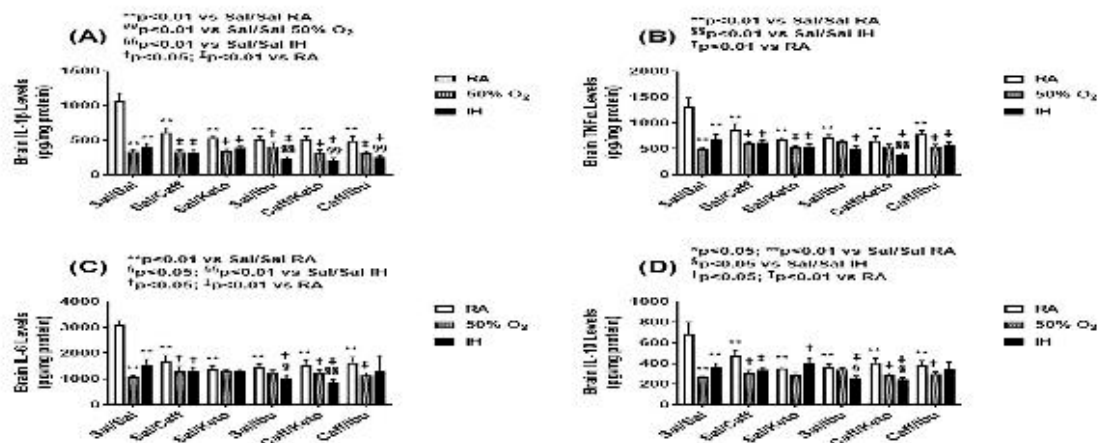


Figure 10. Effect of neonatal intermittent hypoxia (IH) and late caffeine/NSAIDs treatment on cytokines in the cerebral cortex homogenates. The groups are as described in Figure 2. Data are mean \pm SEM (n=6 samples per group).

3.6. IHC

Immunoreactivity of I κ B, IL-1 β , and TNF α (brown stain) are presented in Figures 11–16. No differences were noted among the groups for NF κ B, IL-6 and IL-10 (data not shown). IHC of I κ B in the early and late treatment groups is presented in Figures 11 and 12, respectively. In early RA, I κ B was induced with Keto and to a lesser degree Caff/Keto. Hyperoxia decreased I κ B in all groups, but treatment with Keto and Caff/Keto showed mild immunostaining. This effect was also evident in neonatal IH, but to a greater degree. In the late treatment groups, I κ B was also induced with Keto

and Caff/Keto in all oxygen environments. Overall, Keto and Caff/Keto treatment appears to have a positive influence on I κ B induction regardless of early or late treatment. Quantitative analysis of the stain intensity is presented in Tables 3 and 4 for early and late treatment, respectively. IHC of IL-1 β in the early and late treatment groups is presented in Figures 13 and 14, respectively. In the RA groups, only Keto treatment increased IL-1 β , and to a lesser degree, Caff/Ibu. There was no staining in any of the groups exposed to hyperoxia and exposure to neonatal IH show minor staining in the cerebral cortex layer I with Caff, and punctate staining with Ibu. Late treatment also showed no appreciative IHC staining for IL-1 β , except for minor staining in the cerebral cortex layer I with Caff as shown with early treatment. TNF α IHC in the early and late treatment groups are presented in Figures 15 and 16, respectively. In the early RA groups, treatment with Sal, Ibu and Caff/Ibu showed highest staining in RA, while in hyperoxia, the highest staining was noted with Sal, Caff, Caff/Keto and Caff/Ibu. In IH, minor staining was noted with Caff and Caff/Keto late treatment. Quantitative analyses of the cytokine staining intensities are presented in Tables 3 and 4 for early and late treatment, respectively.

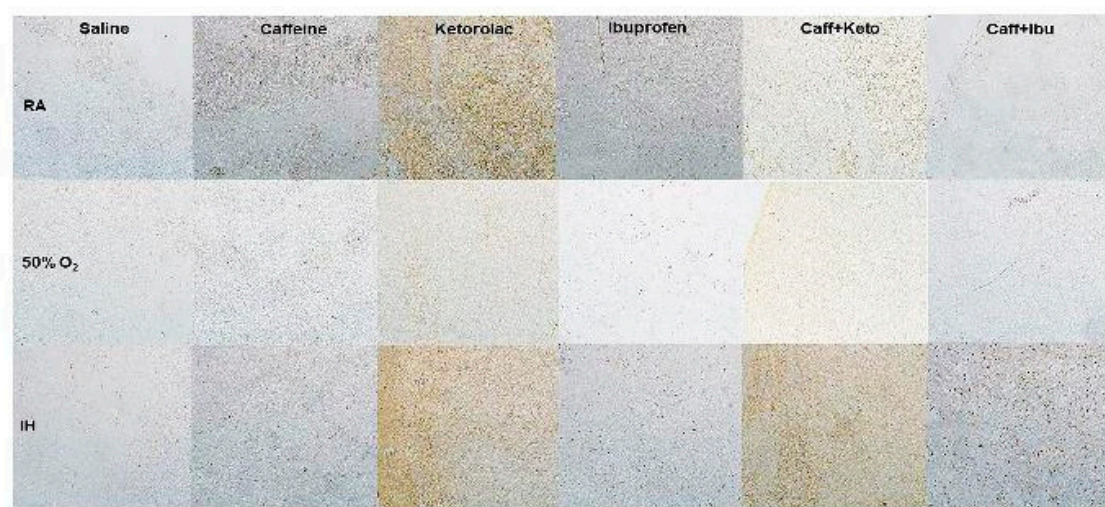


Figure 11. Representative image of immunoreactivity of I κ B in the early treatment groups. I κ B is indicated by the brown stain. The sections were counterstain with hematoxylin (blue). Images are 20X magnification, scale bar is 50 μ m.

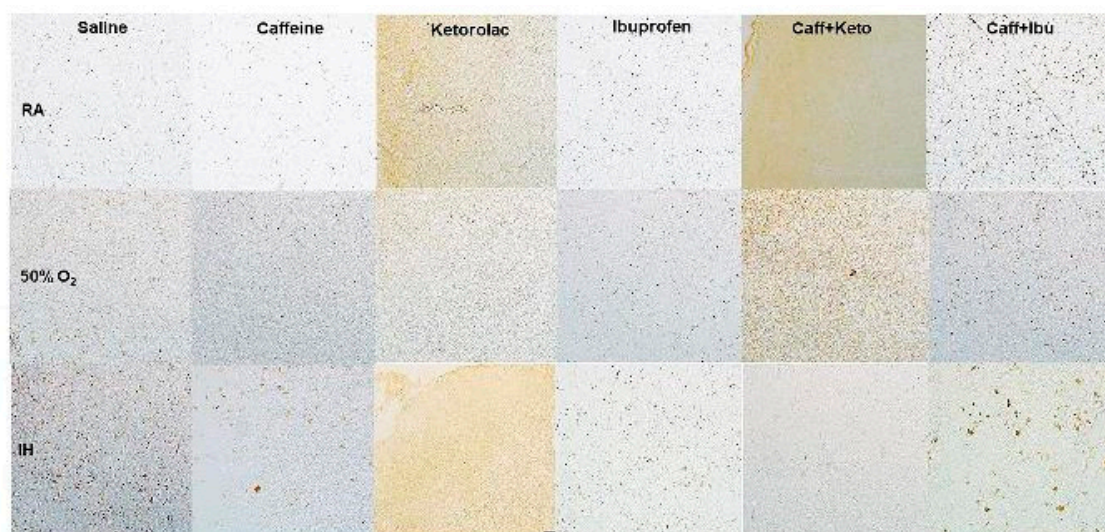


Figure 12. Representative image of immunoreactivity of I κ B in the late treatment groups. I κ B is indicated by the brown stain. The sections were counterstain with hematoxylin (blue). Images are 20X magnification, scale bar is 50 μ m.

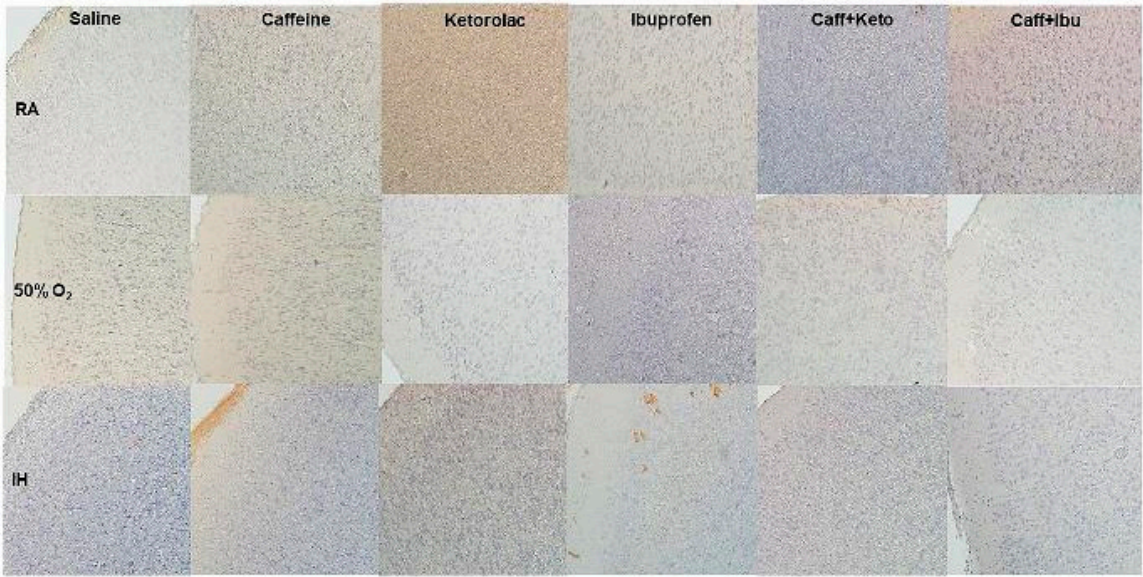


Figure 13. Representative image of immunoreactivity of IL-1 β in the early treatment groups. IL-1 β is indicated by the brown stain. The sections were counterstain with hematoxylin (blue). Images are 20X magnification, scale bar is 50 μ m.

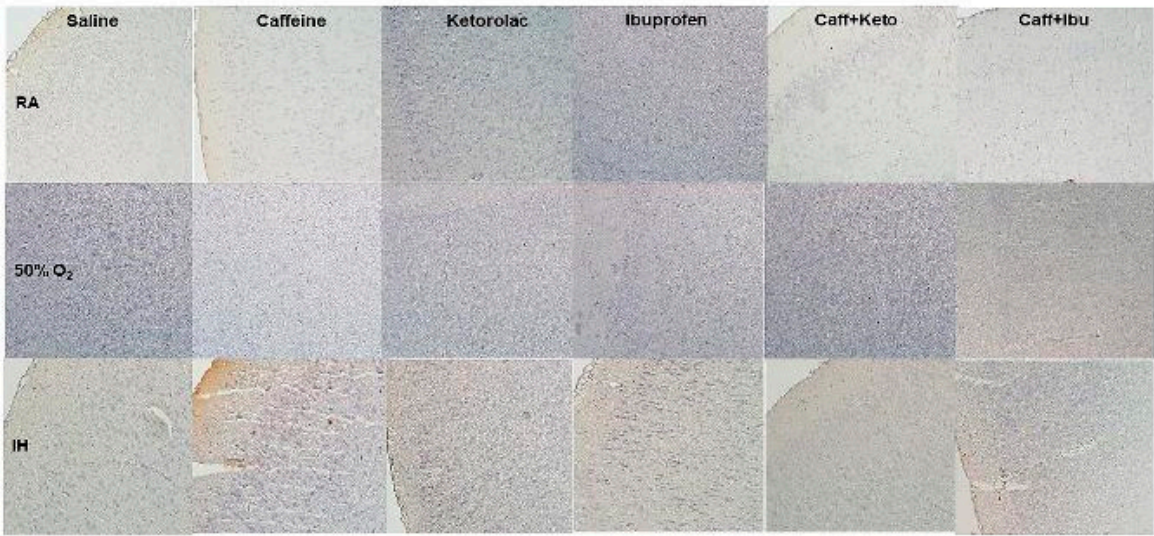


Figure 14. Representative image of immunoreactivity of IL-1 β in the late treatment groups. IL-1 β is indicated by the brown stain. The sections were counterstain with hematoxylin (blue). Images are 20X magnification, scale bar is 50 μ m.

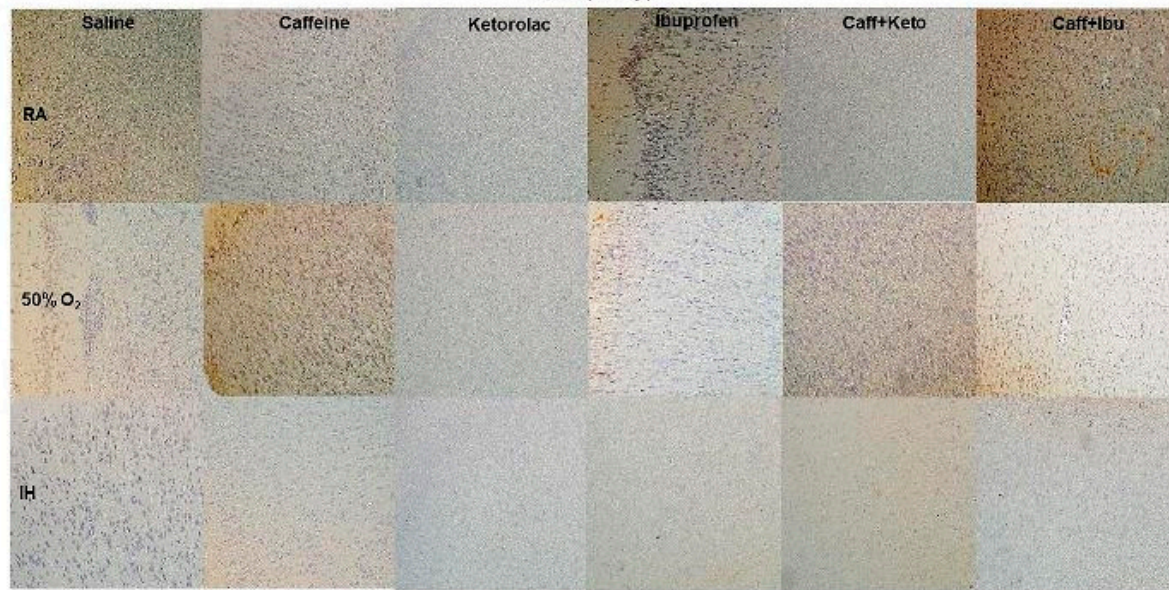


Figure 15. Representative image of immunoreactivity of TNF α in the early treatment groups. TNF α is indicated by the brown stain. The sections were counterstain with hematoxylin (blue). Images are 20X magnification, scale bar is 50 μ m.

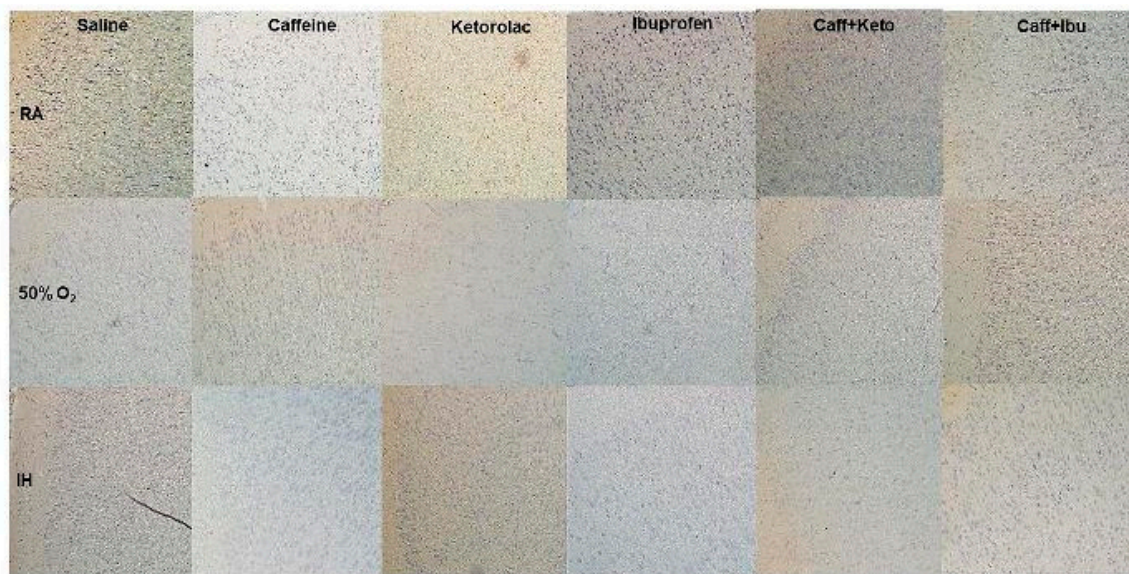


Figure 16. Representative image of immunoreactivity of TNF α in the late treatment groups. TNF α is indicated by the brown stain. The sections were counterstain with hematoxylin (blue). Images are 20X magnification, scale bar is 50 μ m.

4. Discussion

The present study was conducted to address specific questions regarding: 1) the effects of early exposure of hyperoxia and neonatal IH on inflammation in the neonatal rat brain; 2) the comparative efficacy of Caff, Keto, or Ibu on inflammation in the neonatal rat brain; 3) synergistic effects of Caff and/or NSAID co-treatment for reducing hyperoxia- and IH-induced brain inflammation; 4) topical ocular Keto effects in the brain; and 5) whether early treatment during hyperoxia or IH exposure is more beneficial than late treatment during recovery/reoxygenation. We utilized a model of neonatal IH developed in our laboratory that consistently produces oxidative stress, as previously reported [38,39]. Using rodent models of brain injury, studies have shown that rodents at postnatal day 7-10 is developmentally similar to a term human infant [41-44]. For our experiments, we used newborn

rats on the first day of life which corresponds to a very preterm infant brain. Early treatment correlated with the preterm infant brain and late treatment with the term infant brain. The major findings of these experiments were: 1) early exposure of the neonatal brain to either hyperoxia or neonatal IH impairs brain weight and development, and causes induced inflammation; 2) Given individually, Caff, and Keto, but not Ibu were most beneficial for reducing apoptosis and inflammation; 3) Caff/Keto demonstrated better synergistic effects for inducing I κ B and reducing apoptosis and inflammation than Caff/Ibu; 4) topical ocular Keto enters the brain and confers beneficial effects with or without Caff; and 5) early treatment during exposure is more beneficial for inducing myelination, reducing apoptosis and overall improvement in neuro inflammation and cortical organization than late treatment during recovery/reoxygenation. These findings support our hypothesis and demonstrate that not only the choice of treatment also the timing of its administration has significant effects in the premature brain.

Exposure to hyperoxia and IH showed significant elevations in brain/body weight ratios, in all early groups except Caff and in late Ibu, Caff/Keto and Caff/Ibu groups. This may be due to the lower body weights in response to abnormal oxygenation [45]. Organ weight is one of the most sensitive indicators of drug toxicity and organ weight relative to body weight accounts for changes in overall body weight [46], and may suggest an overall physiological response to the drug. Regarding cerebral cortex organization, the RA groups showed an overall increase in cell differentiation and cortical thickness that was relatively preserved in the earlier cohort, however in the late treatment groups, the effects of increased thickness of layers I-V became less pronounced and only layer II in NSAID treatment groups remained prominent. Literature shows that layer 3 consists of pyramidal cells mainly and serves as association and commissural corticocortical fibers [42]. With an ever-increasing body of evidence in favor of dysmaturation of white matter as the underlying mechanism of neurodysregulation of the ELGAN's brain [47–49]. Our findings shed further light on the effects of hyperoxia and neonatal IH on cortical cell organization and deserving further research. In the hyperoxia groups, all treatment groups were found to have increased brain volumes as shown by increased cross sectional diameters compared to RA counterparts, perhaps due to increased cellular proliferation attributable to higher oxygenation that persisted, albeit to a lesser extent, in the late treatment littermates. In cellular layer analysis, however, effects on layer 3 were largely positive in the early treatment cohort but less so in the later one. It remains to be seen whether this increase in cell mass was due to hypertrophy or hyperplasia, as this study did not aim to find the cytopathologic pathologic nature of hyperoxia-induced cell changes, but studies by Kato et. al [9,10] seem to suggest this.

In the IH exposed groups, gross size of the specimen was enlarged in both early and late groups even in controls, which seems to suggest that this effect was conferred by IH and not by the treatment. Further examination of the cell layers revealed that layer III remained mostly unchanged, however layers IV and VI became narrower in the early treatment groups but cell layers in late treatment cohort did not show appreciable changes from controls. This may suggest that early treatment, at a time when the brain is still developing, is detrimental to the internal granular layer. This layer, also known as the main input cortical station, receives sensory input from periphery, and layer VI or the fusiform/multiform layer comprised of axons of the fusiform cells which distribute commissural fibers and corticothalamic projections ending in the thalamus [40]. As the early treatment coincides with cell migration stage in the preterm population, perhaps reduced cell migration and axonal projection lead to suppression of abnormal circuitry that contribute to sensory and emotional disturbances that are a hallmark of neonatal brain injury. Furthermore, late treatment did not produce similar cell layer findings suggesting that after migration, cells are less susceptible to the adverse effects of hyperoxia and/or IH.

The $t_{1/2}$ of Caff is about 4 days, while Keto and Ibu are eliminated much faster, having a half-life of 4-6 hours. This would suggest that treatment with Ibu, as happens with PDA closure, would confer short term anti-inflammatory effects while Caff would provide more sustained and pervasive effects in the tissue. The findings from our study suggest however that the effect of the NSAID's on brain differentiation is related more to the dosing being timed with stages of neural tissue development

than their duration of action. The effects of topical ocular Keto alone and in conjunction with Caff indicate that the drug crosses the blood-ocular barrier and enters into the brain, and provides further evidence of a connection between the eye and the brain [50].

Both early and late treatment with Keto and Caff/Keto reduced apoptosis or programmed cell death in all exposures, while Caff, Ibu or their combination was not effective. This seems to suggest that Caff may have diminished neuroprotective in chronic hyperoxia and IH even with Ibu co-treatment. This must also be seen in the context of apoptosis that occurs in later stages of preterm brain maturation as part of normal neuronal development once cells have reached their “programmed” destination in the brain [49]. Many studies have shown that Caff alone [51] and with morphine induces apoptosis [52]. The mechanism of Caff induction of apoptosis even in RA conditions, remains to be determined.

Myelination is considered a proxy for white matter (WM) development and has been positively correlated with brain development [53,54]. In our study, early Ibu and Caff/Ibu in RA as well as hyperoxia seemed to favor, while late treatment caused reduced myelination. This confirms that late treatment may be more injurious. Overall, IH seems to negatively affect the process of myelination regardless of early or late treatment. This finding confirms previous reports which shows irreversible dysmyelination with chronic hypoxia and intermittent hypoxia [55]. Although several of the treatment groups showed higher myelination, it is debatable whether function is correlated with these quantities. WM dysmaturation studies have shown that increased activity of pre-OL's is responsible for abnormal corticofugal tracks, helping the authors form the theory that perhaps better markers of “meaningful myelination” need to be found as predictors of brain development [49].

Studies have shown that separate I κ B molecules preferentially inhibit distinct Rel/NF- κ B protein dimers [56]. This interaction enables the I κ B proteins to mask the nuclear localization signal and prevent nuclear translocation of Rel/NF κ B protein dimers. Many aspects of the inflammatory responses are also controlled by NF κ B including the production of acute-phase proteins such as serum amyloid A, angiotensinogen and factors of the complement cascade and the induction of pro-inflammatory cytokines such as IL-1 (both α and β) and TNF α [57]. I κ B is the most studied inhibitor of NF κ B. During activation of NF κ B, numerous stimuli, including IL-1 and TNF α , activate a complex of I κ B kinases to degrade I κ B [58]. While there were no appreciable differences in NF κ B, we showed that Ibu alone and in combination with Caff induces I κ B, although early treatment was more effective than late treatment. Since Ibu is a potent anti-inflammatory drug, it is likely that those effects may be in part due to induction of I κ B. To our knowledge, this is the first report of Ibu effect on I κ B in the brain, in the setting of hyperoxia or IH. However, studies have shown a suppressive effect of Ibu on NF κ B in respiratory cells [59].

The other cytokines namely IL-1 β , IL-6, and TNF α , seem to vary in their relationship as predictors of response to drug treatment in different oxygen environments. In the CNS, cytokines exert their function through both traditional engagement of their receptors, which are expressed by both glial and neuronal cells and through less traditional means such as modulation of neurotransmitter receptor function. For example, IL-1 modulates γ -aminobutyric acid-responsive neurotransmitter receptors to enhance inhibitory responses, and both IL-1 and IL-6 modulate synaptic plasticity through inhibiting formation of long-term potentiation [60]. Excitotoxicity is a major pathway of neuronal cell death that is associated with ischemia, trauma, and neurodegenerative diseases and results from an uncontrolled elevation in intracellular calcium that enters the cell through chronically activated N-methyl-D-aspartic acid receptors. Agents such as antioxidants, growth factors, and certain cytokines protect against excitotoxicity [61]. In RA, IL-1 β , IL-6, and TNF- α seem to show similar trends, with a reduction in both early and late NSAID treatment groups. In the presence of hyperoxia, IL-1 β , and TNF α behave similarly and are reduced in response to early NSAID treatment, but IL-6 seems to rise, suggesting a mechanism of action different than the other 2. IL-6 has often been proposed as the “exercise factor” as it rises in response to strenuous exercise [62]. Perhaps hyperoxia seems to affect IL-6 differently by virtue of its response to increased oxygen as seen in exercise and our hyperoxia model. Response to Caff is interesting in that all cytokines show an

increase in early group, suggesting perhaps that Caff alone can work through cyclic AMP and adrenaline to increase their levels. In later treatment, this effect is seen in the Caff/Ibu group, suggesting decreased effect of Ibu and confirming beneficial synergism with Caff. In IH, all cytokines seem to decrease with treatment, suggesting a role for use these neuroprotective agents in IH.

Although the present study has clinical significance, there are limitations. We did not examine the responses at P14. This would have provided further information regarding immediate effects of the exposures with or without treatment. We also did not differentiate between the outcomes of male and female as gender may be confounding factor in response to neuroprotective therapies. Nevertheless, our study shows that the use of Caff and/or NSAIDs during early postnatal life appears to confer better neurologic outcomes in terms of cortical development and neuroinflammation, when given in the setting of hyperoxia or neonatal IH. In contrast, later treatment has reduced efficacy for promoting myelination and normal cortical development. These findings suggest that achieving better oxygenation to ensure desired effects of neonatal management with Caff and/or NSAIDs should remain a priority. We also showed that the developing brain responds to hypoxia by upregulating programmed cell death, confirming previous studies [63,64].

5. Conclusions

In conclusion, the effects of medication in the neonate are a challenge for pharmacists and pharmacologists due to their unique physiologic attributes. Ascertaining safety takes precedence over efficacy, yet pursuit of both is desirable for the achievement of the ultimate goal, a healthy infant. While neonatal medicine has made significant improvements in care and ventilation of preterm infants, the incidence of apnea of prematurity and frequent intermittent hypoxia remain high. Further studies are needed to determine whether our findings can be extrapolated to the human situation.

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