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Maternal Undernourishment in Guinea Pigs Leads to Fetal Growth Restriction with Increased Hypoxic Cells and Oxidative Stress in the Brain

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Maternal undernourishment · Fetal growth restriction · Fetal hypoxia · Brain development · Hypoxyprobe-1

Abstract
Background: We determined whether maternal nutrient restriction (MNR) in guinea pigs leading to fetal growth restriction (FGR) impacts markers for brain hypoxia and oxidative stress. ***Methods:*** Guinea pigs were fed ad libitum (control) or 70% of the control diet before pregnancy, switching to 90% at mid-pregnancy (MNR). Near term, hypoxyprobe-1 (HP-1) was injected into pregnant sows. Fetuses were then necropsied and brain tissues were processed for HP-1 (hypoxia marker) and 4HNE, 8-OHdG, and 3-nitrotyrosine (oxidative stress markers) immunoreactivity (IR). ***Results:*** FGR-MNR fetal and brain weights were decreased 38 and 12%, respectively, with brain/fetal weights thereby increased 45% as a measure of brain sparing, and more so in males than females. FGR-MNR HP-1 IR was increased in most of the brain regions studied, and more so in males than females, while 4HNE and 8-OHdG IR were increased in select brain regions, but with no sex differences. ***Conclusions:*** Chronic hypoxia is likely to be an important signaling mechanism in the FGR brain, but with males showing more hypoxia than females. This may involve sex differences in adaptive decreases in growth and normalizing of oxygen, with implications for sex-specific alterations in brain development and risk for later neuropsychiatric disorder.

Introduction

Fetal growth restriction (FGR) with failure to achieve growth potential in utero is a known risk factor for later adverse health outcomes including cardiovascular disease, metabolic syndrome, and neurodevelopmental adversity [1–4]. This has led to the notion that restriction of fetal growth as an adaptation to impaired nutrient delivery can negatively affect the structure and functional development of organ tissues thereby programming risk for later health adversity [1, 2, 5]. For the brain, these include deficits in motor skills, cognition, memory and academic ability, and neuropsychological dysfunction with poor attention and hyperactivity [3, 4]. Since these adversities often relate to memory and learning, hippocampal brain regions may be particularly vulnerable [3, 4].

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Fetal growth is dependent on placental development and maternal nutrition which impact nutrient availability, with placental insufficiency and maternal undernutrition thereby major causes of FGR [6, 7]. Clinical studies of placental insufficiency-related FGR report aberrant placental development with reduced trophoblast exchange area, altered vascularization, and decreases in umbilical blood flow [8, 9], leading to chronic fetal hypoxemia as a primary signaling mechanism for growth restriction in these pregnancies [10, 11]. Likewise, clinical studies of maternal undernutrition-related FGR report placentas with reduced surface area for nutrient exchange and altered vascularization [12, 13], and lower cord oxygen values at birth [11], implicating chronic fetal hypoxemia in these pregnancies.

Guinea pigs deliver precocial young after a relatively long pregnancy with many developmental events occurring during fetal life similar to that in humans including that in the brain [14, 15]. Accordingly, moderate maternal nutrient restriction (MNR) in guinea pigs at 70% of an ad libitum diet preconception until mid-pregnancy increasing to 90% thereafter has been utilized for inducing FGR and studying fetal growth and developmental outcomes. We [16] and others [17, 18] have shown that this experimental paradigm leads to moderate/severe FGR with fetal weights decreased by 30–40% near term, associated with aberrant placental development, asymmetrical growth, and polycythemia and hypoglycemia. Recently, we [19] have shown evidence for chronic hypoxia in the liver and kidneys of these FGR-MNR fetuses using immunoreactivity (IR) for hypoxyprobe-1 (HP-1) as a widely used marker of tissue hypoxia [20–22] and implicating systemic hypoxemia in these animals. This may be a consequence of the altered vascularization and structural changes in the placentas of these animals [13, 17] and decreased placental blood flow as seen in rodent pregnancies with dietary restriction [23].

While chronic hypoxemia in the systemic circulation appears to be a primary signaling mechanism for the growth restriction seen in humans and animal models with both placental insufficiency- and maternal undernutrition-related FGR, this has not been clearly shown for the brain. Here the increase in blood flow in response to chronic hypoxemia will serve to lessen the fall in tissue oxygenation although this is unlikely to be normalized, leading to brain sparing and asymmetrical growth restriction, but with brains still smaller [3, 4, 16, 18, 24–27]. Furthermore, sex differences in tissue oxygenation have not been studied in the FGR brain despite the likelihood that brain development will be impacted by sex [28], and the finding that males and females have differing risk for neuropsychiatric disorders, some of which are likely to be developmental in origin [3, 29]. We have therefore used moderate MNR in guinea pigs leading to moderate/severe FGR to test the hypothesis that IR for HP-1 will be increased in the brain as a marker of tissue hypoxia and with sex differences. 4HNE, 8-OHdG, and 3-nitrotyrosine IR have additionally been examined as markers for lipid peroxidation, oxidatively modified DNA, and protein oxidation, respectively [30], given the potential for low-grade oxidative stress as a mechanistic pathway for altered brain development with chronic fetal hypoxia [31–33]. Brain cell counts have also been assessed as a measure of cellular density and basis for reporting the immunopositive cell counts for HP-1 and the oxidative stress biomarkers.

Material and Methods

Animal Cohorts and Tissue Collection

An established model of moderate MNR in guinea pigs [16–19] was used with experimental procedures approved by Western University Animal Use Subcommittee (2012-060). Animal feeding, maternal weights and food consumption, and pregnancy outcomes have previously been reported [16]. Briefly, sows were assigned to a control group fed ad libitum or an MNR group fed 70% of the average food intake of the control animals on a per kilogram of body weight basis, from 4 weeks pre-conception until mid-pregnancy increasing to 90% thereafter. This dietary regime resulted in actual food consumption by the MNR animals of ~65–70% of that consumed by the control animals throughout pregnancy [16]. On day 60–61 of pregnancy (term = ~68 days), the hypoxia marker, pimonidazole hydrochloride (HP-1, 60 mg/kg, Chemicon, Temecula, CA, USA) was injected intraperitoneally into control and MNR sows and allowed to circulate for 120 min. Animals were then sedated followed by laparotomy and delivery of each of the fetuses. Body and placental weights and crown rump length were recorded for all live-born fetuses. Fetuses were considered to be appropriate for gestational age (AGA) if ≥80 g and FGR if <80 g, which is in accord with the weight criteria we [27] and others [34] have used for categorizing AGA and FGR fetal weights in guinea pigs near term. Subsequently, only AGA fetuses from control litters and FGR fetuses from MNR litters were subjected to full necropsy which included weighing of the brain and serial coronal sections beginning at the mammillary bodies (corresponding to the coronal level of the mid-hippocampus) prepared for histological analysis. This establishment of an AGA-control cohort group, all of whom were ≥80 g, and an FGR-MNR cohort group, all of whom were <80 g, has the advantage of avoiding confounding effects of tissue/metabolite study in AGA fetuses from MNR pregnancies and FGR fetuses from control pregnancies, and better reflects the human situation where AGA and FGR birth weight distributions are separate [3, 4, 7].

Histological Sample Preparation

Tissue blocks for histochemical analyses were immersion fixed in 4% paraformaldehyde for 72 h, washed in phosphate buffered saline for 3 days, and then placed in 70% ethanol for 14–21 days. They were then processed and embedded in paraffin, and subsequently cut at 5 μm thickness on a rotary microtome and mounted on Superfrost Plus slides (VWR Scientific, Westchester, PA, USA).

Prior to staining, all slides were deparaffinized with three 5-min washes in xylene and then rehydrated in a series of ethanol baths (100, 100, 90, 90, and 70%) lasting 2 min each. Tissue sections were then rinsed in deionized water for 5 min. For all histological staining, all slides for each parameter studied were stained on the same day using the same solutions to minimize variation in intensity of stain.

Hypoxyprobe-1 Immunohistochemistry

HP-1, a pimonidazole hydrochloride, is reduced by nitroreductases in relatively hypoxic cells (pO_2 <10 mm Hg) to form covalent protein adducts that can then be detected immunohistochemically using the Hypoxyprobe-1 kit [19, 20–22]. HP-1 IR was assessed using the ImmPRESS HRP anti-rabbit IgG (Peroxidase) Polymer Detection Kit (Vector Laboratories, Burlingame, CA, USA). Negative controls were performed by omitting the primary antibody and replacing it with purified pre-immune rabbit IgG to rule out non-specific binding and confirm absence of staining. Tissue sections were processed as described, subjected to antigen retrieval in 10 mM sodium citrate at pH 6.0 for 20 min in a 90 °C vegetable steamer, then incubated in 3.0% hydrogen peroxide for 10 min to block endogenous peroxidase activity, followed by incubation with 2.5% normal horse blocking serum for 30 min. Tissue sections were incubated overnight at 4 °C with the primary antibody, anti-pimonidazole rabbit antisera (1:100; PAb2627AP, Hypoxyprobe Inc., Burlington, MA, USA), and the following day, rinsed and incubated for 40 min at room temperature with the secondary antibody (ImmPRESS HRP anti-rabbit IgG, MP-7401). The bound antibody was visualized with SIGMAFAST 3,3′-diaminobenzidine tablets (Sigma-Aldrich, St. Louis, MO, USA) with 6 min exposure. Slides were then dehydrated through graded ethanol baths, cleared in xylene, mounted with Permount (Fisher Scientific, Toronto, ON, Canada), and dried.

4HNE, 8-OHdG, and 3-Nitrotyrosine Immunohistochemistry

4HNE and 3-nitrotyrosine IR were assessed using anti-4HNE (1:50; HNEJ-2, ab48506, Abcam, Cambridge, MA, USA) and anti-nitrotyrosine (1:200; 39B6, sc-32757, Santa Cruz Biotechnology), respectively, as the primary antibody with overnight incubation and ImmPRESS HRP anti-mouse IgG polymer (MP-7402, Vector Laboratories) as the secondary antibody with 40 min incubation, followed by 3,3′-diaminobenzidine visualization. 8-OHdG IR was assessed using anti-8-OHdG (1:1,000; N45-1, ab48508, Abcam) as the primary antibody with 60 min incubation and ImmPRESS AP anti-mouse IgG polymer (MP-5402, Vector Laboratories) as the secondary antibody with 30 min incubation, followed by visualization with ImmPACT Vector Red Alkaline Phosphatase substrate (SK-5105, Vector Laboratories) for 20 min. Processing of tissue sections was otherwise as previously described, and slides were additionally counterstained for 30 s in Harris Hematoxylin (Fisher Scientific).

Hematoxylin and Eosin Immunohistochemistry

Serial adjacent sections were stained with hematoxylin and eosin (H&E) for assessing total brain cell counts. Tissue sections were processed as described, then immersed in Harris Modified Hematoxylin (Fisher Scientific) for 30 s. The stain was differentiated in 1% acid ethanol for ~1 s and then flushed with running water for 1 min. Tissue sections were then stained with eosin (Fisher Scientific) for ~1 s, followed by dehydration in a series of ethanol baths (70, 70, 100, and 100%), three 5-min washes in xylene, and mounting with Permount (Fisher Scientific).

Imaging and Quantification

Immunopositive cell counts for HP-1, 4HNE, 8-OHdG, and 3-nitrotyrosine and total brain cell counts, were quantified in 4–6 randomly selected high-power fields (20× magnification) from comparable areas in the gray matter layers 2–5, periventricular white matter, pyramidal cell layer in the hippocampus CA1 and CA3 and granule cell layer of the dentate gyrus, and the thalamus. Imaging was performed using a Zeiss Axiolmager Z1 microscope (Carl Zeiss Canada, Toronto, ON, Canada). Identical illumination settings were used for all brain regions and analysis was performed using Image Pro Premier 9.2 software (Media Cybernetics Inc., Rockville, MD, USA), with the analyst blinded as to animal group. To ensure consistency and impartial evaluation, an automated macro incorporating a binary colour intensity cutoff threshold was set up for each of the stains used based on advanced testing of a random sample of images including screening against negative controls to select and count only cell bodies deemed to be positively stained. For total brain cell counts, the number of nuclei were counted manually using H&E staining. All cell types including neurons, glial cells, and endothelial cells were assessed in the analysis and only cells with a defined nuclear membrane were counted.

Data Acquisition and Statistical Analysis

Fetal population characteristics and immunohistochemistry findings are shown as group means ± SEM and were compared using two-way analysis of variance to determine the effects of MNR and sex. HP-1 group variances were unequal with log transformation of data prior to analysis. HP-1 group findings showed interactions between MNR and sex with post hoc testing carried out using non-paired Student *t* test with log transformation of data to determine the effect of MNR in males and females separately (Graphpad Software, San Diego, CA, USA). For all analysis, statistical significance was assumed for $p < 0.05$.

Results

Fetal Population Characteristics

While 12 sows were bred under control conditions and 18 under MNR conditions, 3 animals from each of these groupings failed to become pregnant and 3 MNR animals delivered preterm. The remaining 9 control and 12 MNR animals had pregnancies continuing to necropsy at 60/61 days of gestation with 31 and 42 fetuses, respectively, whose body and placental weights have been reported [16]. Litter size did not differ between the 2 study groups averaging 3–4. The 80-g threshold for categorizing AGA and FGR fetal weights resulted in 20 AGA-control fetuses

Table 1. Fetal population characteristics

	Male		Female		*p* (ANOVA)		
	AGA-control (*n* = 9)	FGR-MNR (*n* = 9)	AGA-control (*n* = 9)	FGR-MNR (*n* = 9)	G	S	G × S
Fetal wt, g	102±3	62±4	101±3	64±4	***	ns	ns
Brain wt, g	2.76±0.07	2.45±0.09	2.72±0.08	2.36±0.07	**	ns	ns
Liver wt, g	4.7±0.2	2.6±0.3	4.7±0.4	2.7±0.2	***	ns	ns
Brain/fetal wt, %	2.70±0.06	4.10±0.25	2.72±0.09	3.76±0.16	***	ns	ns
Brain/liver wt	0.59±0.02	1.01±0.08	0.60±0.05	0.90±0.05	***	ns	ns

Data presented as means ± SEM. ** $p < 0.01$, *** $p < 0.001$. AGA, appropriate for gestational age; FGR, fetal growth restricted; MNR, maternal nutrient restricted; wt, weight; G, group; S, sex; ns, not significant.

Table 2. Total brain cell counts per mm^2

	Male		Female		*p* (ANOVA)		
	AGA-control (*n* = 9)	FGR-MNR (*n* = 9)	AGA-control (*n* = 9)	FGR-MNR (*n* = 9)	G	S	G × S
GM 2–5	2,271±119	2,697±200	2,422±161	2,301±127	ns	ns	ns
PVWM	3,689±166	3,600±234	3,396±215	3,357±259	ns	ns	ns
CA1	6,370±248	6,353±346	6,199±235	5,900±257	ns	ns	ns
CA3	3,159±157	3,050±256	3,603±220	3,001±224	ns	ns	ns
DG	10,945±466	11,015±586	10,659±496	10,456±302	ns	ns	ns
Thalamus	1,649±48	1,628±125	1,720±90	1,590±75	ns	ns	ns

Data presented as means ± SEM. AGA, appropriate for gestational age; FGR, fetal growth restricted; MNR, maternal nutrient restricted; G, group; S, sex; ns, not significant; GM, gray matter, PVWM, periventricular white matter; DG, dentate gyrus.

(10 males and 10 females) and 25 FGR-MNR fetuses (11 males and 14 females) with the body, organ, and placental weights from these animals also previously reported [16]. Eighteen AGA-control fetuses and 18 FGR-MNR fetuses (each 9 male and 9 female) were presently studied and were selected on the basis of no more than 1 male and 1 female from each litter to limit litter effects as a covariate. These animals were representative of the mean fetal weights for their respective cohort groups with their population characteristics shown in Table 1. Changes in these population characteristics in the FGR-MNR fetuses from that of the AGA-control fetuses were similar for both males and females with no sex differences noted. Briefly, FGR-MNR fetal weights were decreased ~38% while brain weights were decreased ~12%. Accordingly, the brain/fetal weight as a measure of brain sparing was increased ~45% in the FGR-MNR fetuses. It is of note that brain/fetal weights were increased more in the FGR-MNR males than females at 52 vs. 38% when compared to respective AGA-controls, although this difference was not significant. FGR-MNR liver weights were decreased ~44% with the brain/liver weights as a measure of asymmetrical growth thereby increased ~60% in the FGR-MNR fetuses compared to that of the AGA-controls.

Total Brain Cell Counts

Total cell counts per mm^2 for the brain regions of the AGA-control and FGR-MNR fetuses are shown in Table 2. While there was considerable change in total cell counts across the brain regions studied from a low of ~1,650 cells/mm^2 in the thalamus to a high of ~10,760 cells/mm^2 in the dentate gyrus, there were no significant group or sex effects for any of the brain regions studied.

Hypoxyprobe-1 IR

Representative photomicrographs for HP-1 IR are shown in Figure 1 while HP-1-positive cell counts per mm^2 for the brain regions of the AGA-Control and FGR-

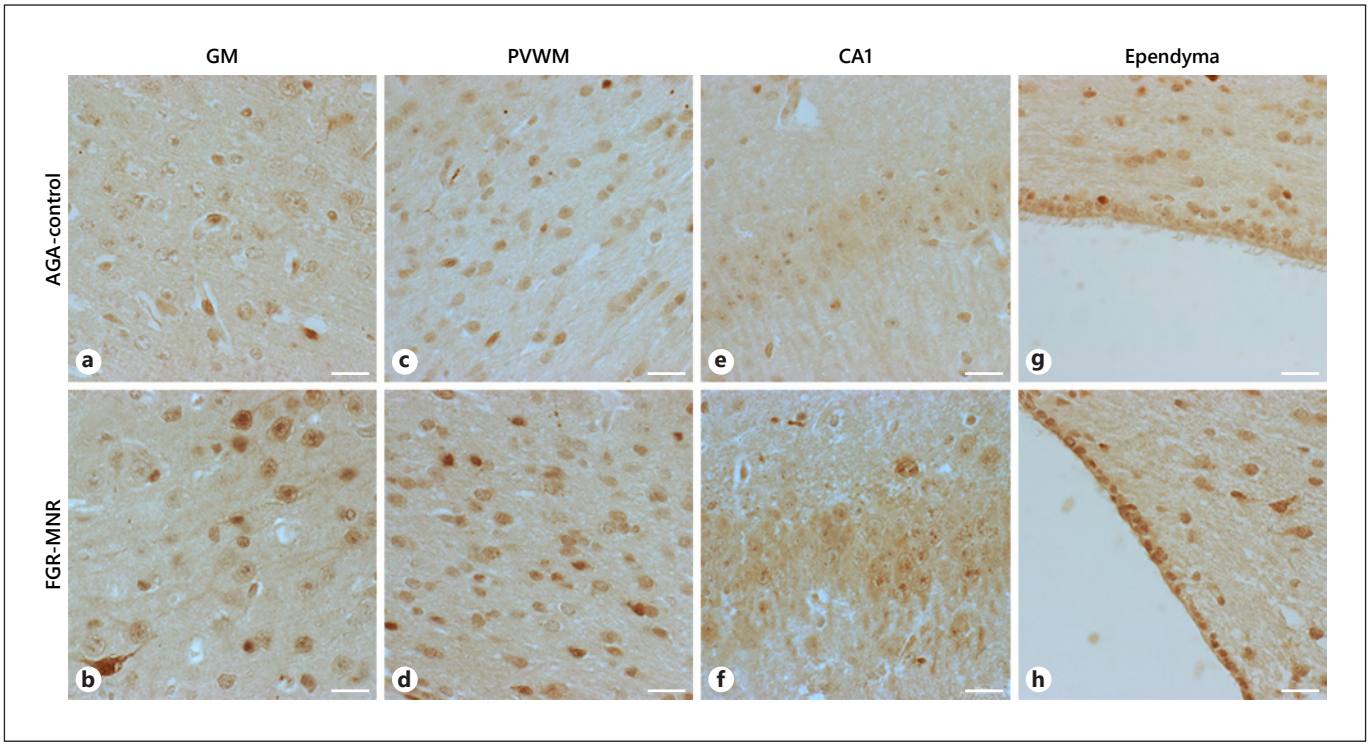

Fig. 1. Representative photomicrographs illustrating positively stained cells for hypoxyprobe-1 (brown) in AGA-control and FGR-MNR fetuses in the gray matter (**a**, **b**), periventricular white matter (**c**, **d**), CA1 (**e**, **f**), and ependyma (**g**, **h**), showing the staining to be increased in FGR-MNR fetuses in all of these brain areas and especially prominent in the ependymal cells. Bar, 20 μm. AGA, appropriate for gestational age; FGR, fetal growth restriction; MNR, maternal nutrient restriction; GM, gray matter; PVWM, periventricular white matter.

Table 3. Hypoxyprobe-1-positive cell counts per mm^2

	Male		Female		*p* (ANOVA)		
	AGA-control (*n* = 7)	FGR-MNR (*n* = 8)	AGA-control (*n* = 7)	FGR-MNR (*n* = 8)	G	S	G × S
GM 2–5	165±82	1,194±233**	335±67	754±193	*	ns	*
PVWM	235±126	2,101±492**	336±96	724±308	*	ns	*
CA1	180±106	1,859±444*	282±81	607±314	*	ns	*
CA3	104±61	871±210*	156±22	167±81	ns	ns	0.05
DG	167±84	2,278±860**	413±109	848±388	*	ns	**
Thalamus	200±101	652±192	211±61	223±71	ns	ns	ns

Data presented as means ± SEM. * $p < 0.05$, ** $p < 0.01$. AGA, appropriate for gestational age; FGR, fetal growth restricted; MNR, maternal nutrient restricted; G, group; S, sex; ns, not significant; GM, gray matter, PVWM, periventricular white matter; DG, dentate gyrus.

MNR fetuses are shown in Table 3. In the AGA-controls, HP-1-positive cell counts/mm^2 and as a percent of the total brain cell counts averaged ~260 or 10% in the gray matter, 290 or 7% in the white matter, 240 or 4% in the CA1, 130 or 4% in the CA3, 290 or 3% in the dentate gyrus, and 210 or 12% in the thalamus, with no sex-related differences. HP-1 IR was affected by MNR with an interactive effect with fetal sex such that HP-1-positive cell counts/mm^2 were increased in FGR-MNR males compared to AGA-control males in the gray matter ~6 fold

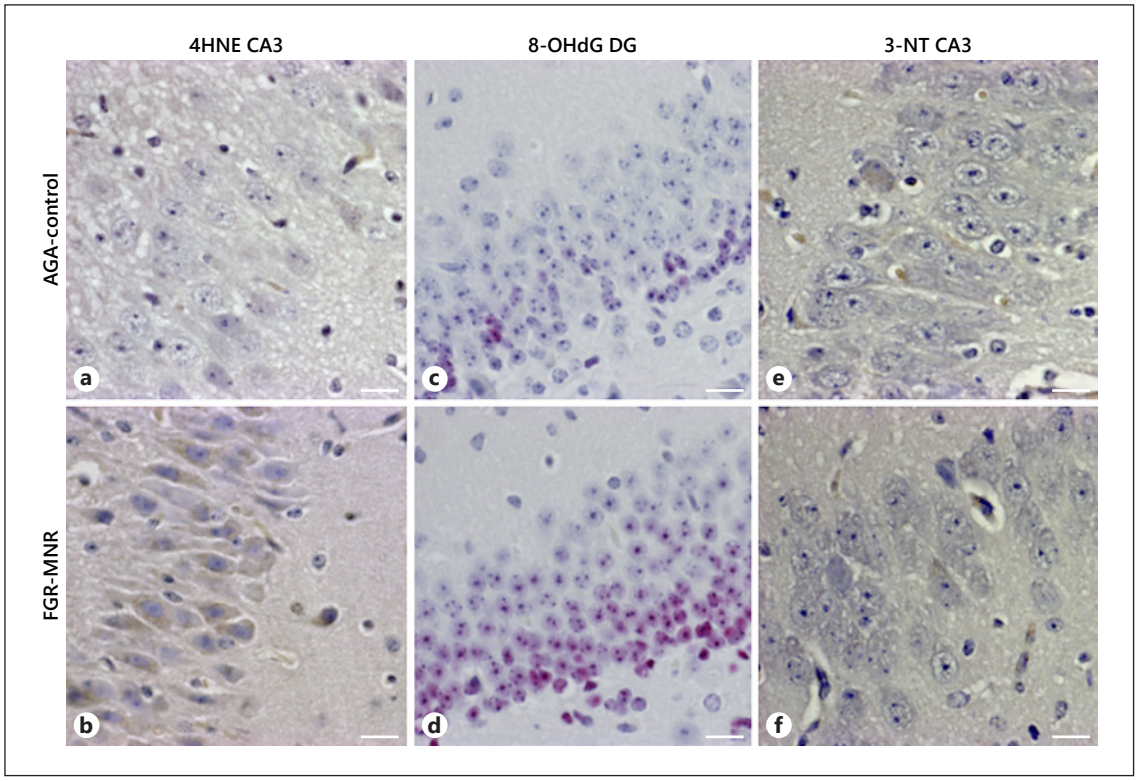

Fig. 2. Representative photomicrographs of 4HNE immunostaining in the CA3 of an AGA-control (**a**) and FGR-MNR (**b**) fetus showing localization within the cytosol and increased in the FGR-MNR fetus, of 8-OHdG immunostaining in the dentate gyrus of an AGA-control (**c**) and FGR-MNR (**d**) fetus showing localization more so within the nucleus and increased in the FGR-MNR fetus, and of 3-nitrotyrosine immunostaining in the CA3 of an AGA-control (**e**) and FGR-MNR (**f**) fetus showing localization within the cytosol, but little change between the animal groups. Sections were also counterstained with hematoxylin. Bar, 25 μm. AGA, appropriate for gestational age; FGR, fetal growth restriction; MNR, maternal nutrient restriction; DG, dentate gyrus; 3-NT, 3-nitrotyrosine.

($p < 0.01$), white matter ~8 fold ($p < 0.01$), CA1 ~9 fold ($p < 0.05$), CA3 ~7 fold ($p < 0.05$), and dentate gyrus ~13 fold ($p < 0.01$), and overall with similar increases in HP-1-positive cell counts as a percent of the total brain cell counts. HP-1-positive cell counts were variably increased in the brain regions of the FGR-MNR females compared to that of the AGA-control females; however, none of these differences were significant. While cell specificity was not determined, HP-1 IR was present in cells in the gray matter, hippocampus, and thalamus that were neuronal in morphology, and in cells in the periventricular white matter, which, based on their size and distribution were likely to be oligodendroglia (Fig. 1). Of note, HP-1 IR visually appeared to be highest in the ependymal cells lining the ventricles where most of these cells appeared to be positive in both the AGA-control and FGR-MNR animals (Fig. 1), although positive cell counts were not possible because of inconsistent delineation of adjacent ependymal cells.

4HNE, 8-OHdG, and 3-Nitrotyrosine IR

4HNE and 3-nitrotyrosine immunostaining was localized primarily within the cytosol, while 8-OHdG immunostaining was localized more so within the nucleus as shown in the representative photomicrographs in Figure 2. The mean number of 4HNE-, 8-OHdG-, and 3-nitrotyrosine-positive cells per mm^2 are shown in Figures 3–5, respectively, with the male and female findings for the AGA-control and FGR-MNR groups combined since there were no sex differences for any of the brain regions examined. While 4HNE-positive cell counts were increased in all of the brain regions of the FGR-MNRs when compared to that of the AGA-controls, this was only significant for the periventricular white matter and CA3 (both $p < 0.05$). Likewise, 8-OHdG-positive cell counts were increased in all of the brain regions of the FGR-MNRs, but this was only significant for the dentate gyrus and thalamus (both $p < 0.05$). Conversely, 3-nitrotyro-

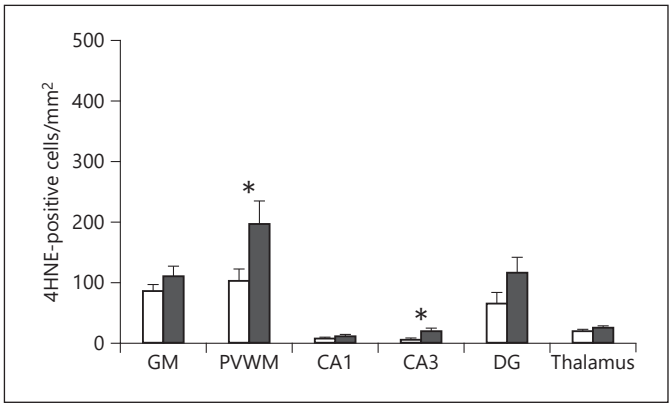

Fig. 3. 4HNE-positive cells per mm^2 in the brain regions of the AGA-control fetuses (open bars, n = 18) and FGR-MNR fetuses (gray bars, n = 18). Data presented as mean ± SEM. *$p < 0.05$. AGA, appropriate for gestational age; FGR, fetal growth restriction; MNR, maternal nutrient restriction; GM, gray matter; PVWM, periventricular white matter; DG, dentate gyrus.

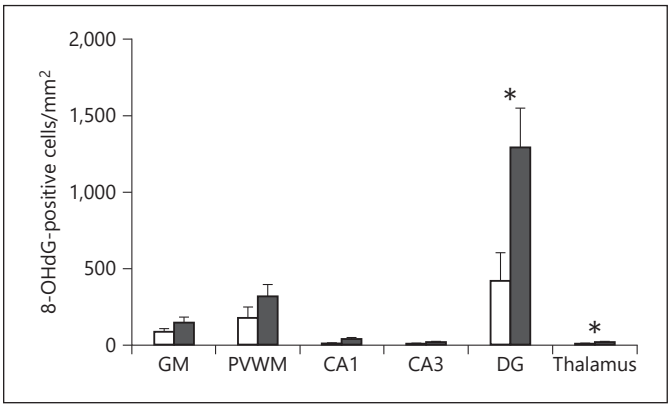

Fig. 4. 8-OHdG-positive cells per mm^2 in the brain regions of the AGA-control fetuses (open bars, n = 18) and FGR-MNR fetuses (gray bars, n = 18). Data presented as mean ± SEM. *$p < 0.05$. AGA, appropriate for gestational age; FGR, fetal growth restriction; MNR, maternal nutrient restriction; GM, gray matter; PVWM, periventricular white; DG, dentate gyrus.

sine-positive cell counts showed no significant change between the respective brain regions. 4HNE, 8-OHdG, and 3-nitrotyrosine IR were again present in cells in the gray matter, hippocampus, and thalamus that were neuronal in morphology, and in cells in the periventricular white matter that were likely to be oligodendroglia (Fig. 2).

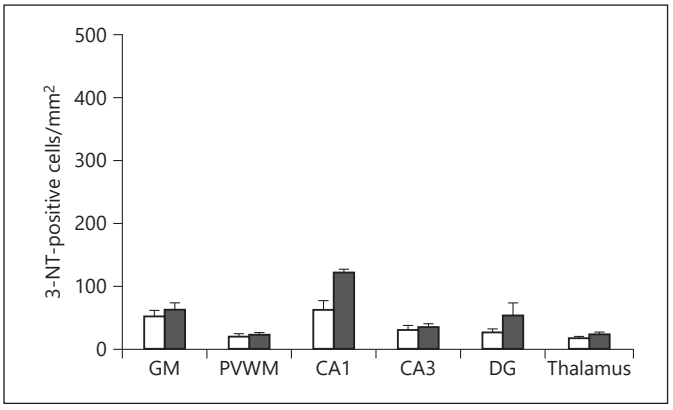

Fig. 5. 3-Nitrotyrosine-positive cells per mm^2 in the brain regions of the AGA-control fetuses (open bars, n = 18) and FGR-MNR fetuses (gray bars, n = 18). Data presented as mean ± SEM. AGA, appropriate for gestational age; FGR, fetal growth restriction; MNR, maternal nutrient restriction; GM, gray matter; PVWM, periventricular white matter; DG, dentate gyrus; 3-NT, 3-nitrotyrosine.

Discussion

FGR-MNR fetal weights were decreased ~38%, brain weights were decreased ~12%, and liver weights were decreased ~44% compared to the AGA-controls, indicating brain sparing and asymmetrical growth restriction. These findings are well described in human FGR with the degree of asymmetrical growth better aligned with the degree of growth restriction than causative factors per se [3, 4, 7, 24]. This sparing of brain growth is likely due in part to blood flow redistribution favoring the vital organs including the brain in response to chronic hypoxemia, which is seen in sheep with placental embolization leading to FGR [25], and clinically in humans with more advanced FGR [3, 4]. However, while the increase in brain blood flow will lessen the fall in tissue oxygenation compared to that in non-vital organs, some degree of hypoxia remains likely as a signaling mechanism for altered growth and development [26]. As such, it is not surprising that FGR offspring in human and animal studies, while showing brain sparing, still have smaller brains with increased risk for abnormal neurodevelopment, whether due to placental insufficiency or maternal undernutrition [3, 4, 16, 18, 24, 27].

Total brain cell counts/mm^2 which included neurons, glial cells, and endothelial cells, were unchanged between the AGA-control and FGR-MNR fetuses indicating that cellular density as measured was also unchanged. While smaller head size, and reduced brain volumes and total neuronal cell counts have been widely reported in human and animal studies in relation to FGR severity [3, 4], cel-

lular density has been less studied. Late pregnancy maternal hypoxia-induced FGR in guinea pigs results in selective decreases in neuronal density [32, 35] whereas carunclectomy-induced FGR in sheep with chronic hypoxia earlier in pregnancy results in selective increases in neuronal density [36]. Accordingly, while FGR brains are generally smaller, changes in cellular density appear to depend upon the stage of brain development in relation to the nature and timing of the causative insult, and brain regions and cell types assessed with most study involving neuronal density rather than total cell density as in the present study.

HP-1 staining was observed in the AGA-control animals in each of the brain regions studied indicating some degree of local tissue hypoxia even under normal physiologic conditions, which was also seen in the liver and kidneys of these animals [19]. This can be explained as oxygen consumption in cells close to blood vessels creating oxygen gradients for more distal cells, which is then amplified by the lower partial pressure for oxygen normally seen during fetal development. HP-1-positive cell counts as a percent of the total brain cell counts in the AGA-controls was lowest in the hippocampal brain regions at 3–4% which was half to a third that seen in the gray matter, white matter, and thalamus at 7–12%. This regional differential in HP-1-positive cells/total cells suggests higher tissue oxygen levels in the hippocampus than these other brain regions and thereby higher oxygen delivery relative to oxygen consumption as the primary determinants of tissue oxygen levels [37]. Of interest, HP-1 staining appeared to be highest in the ependymal cells lining the lateral ventricles with virtually all of these cells positively stained and suggesting lower tissue oxygen levels. This finding is consistent with a high rate of oxidative metabolism relative to oxygen delivery as might be expected in these cells with their specialized cilia, microvilli, and neuroproliferative activities, along with watershed blood flow support [38].

HP-1-positive cell counts/mm^2 in the FGR-MNR males were significantly increased for all of the brain regions studied except for the thalamus, indicating lower levels of oxygenation in these tissues compared to those of the respective AGA-control males. While HP-1-positive cell counts were variably increased in the FGR-MNR females, none of these increases were significant and all were substantially less than that seen for the FGR-MNR males, indicating that any lowering of oxygen levels in these brain regions compared to those of the respective AGA-control females was also less. Growth and related developmental processes are known to account for a consequential fraction of nutrient/oxygen consumption in the fetus near term, including that of the brain [26]. As such, growth restriction in response to decreases in systemic nutrient/oxygen levels in these FGR-MNR animals [16, 19] can be protective by lowering tissue oxidative needs thereby minimizing the lowering of tissue oxygenation and sustaining oxidative metabolism for more essential metabolic needs [26]. In the present study, brain/fetal weights were increased more in the FGR-MNR males than females at 52 vs. 38% when compared to respective AGA-controls, although this difference was not significant with the small sample size relative to population variance. Nonetheless, this finding suggests that females decrease brain growth to a greater extent than males with a given degree of tissue hypoxia with more normalizing of oxygen levels and thereby HP-1-positive cells. Of note, larger heads for body weight in males have also been reported with FGR in humans [24], further supporting the conjecture of sex differences in brain sparing with growth restriction. Additionally, small for gestational age neonates as a metric for FGR show higher cerebral oxygen saturation than AGA neonates and more so in males than females [39], which could be attributed to increased cerebral vascularization in response to lower tissue oxygenation during fetal development with the same sex differences.

Biomarkers of oxidative stress are increased in cord blood and placental tissues of small for gestational age/FGR infants at birth, implicating a role in adverse development of organ tissues with chronic fetal hypoxia and increasing risk for later health adversities [5, 40]. Accordingly, these biomarkers have been studied in animal models with mid-late pregnancy hypoxia-induced FGR [5, 32, 33, 40, 41] and shown to be increased in several organ tissues. However, study in the brain has been limited with 4HNE increased in the cortex and white matter tracts in fetal sheep after uterine artery ligation [33] and nitric oxide synthases increased in the cortex, hippocampus, and thalamus in fetal guinea pigs after late pregnancy maternal hypoxia [32], and with no study with MNR-induced FGR. The oxidative stress biomarkers in the FGR-MNR animals herein studied were variably increased for most of the brain regions, although this was only significant for 4HNE in the periventricular white matter and CA3, and for 8-OHdG in the dentate gyrus and thalamus. These findings are qualitatively similar to that seen in other organs as well as the brain with mid-late pregnancy hypoxia-induced FGR [5, 32, 33, 40, 41], but the changes in oxidative stress biomarkers appear less pronounced. This may reflect the insidious nature and earlier onset of MNR-induced FGR compared to the abrupt nature of mid-late pregnancy hypoxia-induced FGR, as well as the lesser degree of tissue hypoxia anticipated for the fetal brain with blood flow redistribution. There addition-

ally were no sex-related differences in the oxidative stress biomarkers in the FGR-MNR animals despite the HP-1 findings, likely contributed to by the low level change in these markers and population variance against which this was measured. Nonetheless, the increases in oxidative stress biomarkers herein seen support our hypothesis that chronic hypoxia is a primary signaling mechanism in the FGR brain as it is in other body tissues and implicates these stressors in their adverse development [5, 32, 33, 40, 41], whether resulting from placental insufficiency- or maternal undernutrition-related FGR.

In the present study, total brain cell counts were assessed as a measure of cellular density and basis for reporting the immunopositive cell counts for HP-1 and oxidative stress biomarkers, and found to be unchanged between the animal groups. As such, a change in cellular density cannot be seen to impact these measurements or their interpretation. However, cellular composition may have changed as seen with uterine artery ligation-induced FGR in rats [42], and HP-1- and oxidative stress-positive cell counts were not assessed in relation to cell type, although neurons and glial cells appeared to be involved as noted. Accordingly, changes in cellular composition may have contributed to the HP-1 and oxidative stress findings to the extent that cell types in the developing brain have differing intracellular oxygenation and thereby susceptibility to HP-1 reduction by nitroreductases, and vulnerability to oxidative stress. Notably, even if cellular composition has changed in the FGR-MNR brains, the increases in HP-1-positive cell counts, and 4HNE- and 8-OHdG-positive cells are still indicative of an increase in brain tissue hypoxia and oxidative stress, respectively.

HP-1-positive cell counts were increased in most of the brain regions studied in the FGR-MNR animals and more so in males than females as the major finding herein. This further supports our hypothesis that chronic hypoxia is a primary signaling mechanism in the FGR brain for growth alterations as it is systemically for overall growth restriction whether resulting from placental insufficiency- or maternal undernutrition-related FGR, but with sex differences whereby FGR brain hypoxia is greater in males than females. This sex difference may involve the decrease in growth processes and normalizing of oxygen levels with a given degree of brain hypoxia, being less in males than females as discussed [24, 26, 39]. As reviewed by McCarthy et al. [28], the majority of sex differences in the developing brain are likely caused by gonadal hormones and especially testicular hormones, leading to differences in morphology and physiology and interactions with the environment. While mechanisms underlying the present sex differences in HP-1 findings are unknown, differences in gonadal hormones and their impact on growth processes in the developing brain and responses to hypoxia are thereby likely to be a contributing factor. Adaptive responses to chronic hypoxia in the fetal brain thus appear to differ depending on sex, with males showing less reduction in overall growth leading to more tissue hypoxia, and females showing more reduction in overall growth leading to less tissue hypoxia. As such, these adaptive responses may differ in the extent to which growth and related developmental processes are protected or compromised, contributing to sex-specific alterations in brain development as we have seen with increased apoptosis in these FGR males [43], and sex-specific risk for later neuropsychiatric disorder [3, 29].

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Statement of Ethics

The experimental procedures were approved by Western University Animal Use Subcommittee (2012-060).

Disclosure Statement

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Author Contributions

Y.M. participated in animal care, animal study, immunohistochemistry study and analysis, statistical analysis, manuscript preparation. K.N. and R.R.H. participated in immunohistochemistry study and analysis, manuscript preparation. T.R.H.R. participated in study design, animal study, statistical analysis, manuscript preparation. B.S.R. participated in study design, animal study, immunohistochemistry study and analysis, statistical analysis, manuscript preparation.

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