Maternal Glucose Supplementation in a Murine Model of Chorioamnionitis Alleviates Dysregulation of Autophagy in Fetal Brain

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Abstract

Fetal brain injury induced by intrauterine inflammation is a major risk factor for adverse neurological outcomes, including cerebral palsy, cognitive dysfunction, and behavioral disabilities. There are no adequate therapies for neuronal protection to reduce fetal brain injury, especially new strategies that may apply promptly and conveniently. In this study, we explored the effect of maternal glucose administration in a mouse model of intrauterine inflammation at term. Our results demonstrated that maternal glucose supplementation significantly increased survival birth rate and improved the neurobehavioral performance of pups exposed to intrauterine inflammation. Furthermore, we demonstrated that maternal glucose administration improved myelination and oligodendrocyte development in offspring exposed to intrauterine inflammation. Though the maternal blood glucose concentration was temporally prevented from decrease induced by intrauterine inflammation, the glucose concentration in fetal brain was not recovered by maternal glucose supplementation. The adenosine triphosphate (ATP) level and autophagy in fetal brain were regulated by maternal glucose supplementation, which may prevent dysregulation of cellular metabolism. Our study is the first to provide evidence for the role of maternal glucose supplementation in the cell survival of fetal brain during intrauterine inflammation and further support the possible medication with maternal glucose treatment.

Keywords

maternal glucose supplementation, fetal brain injury, autophagy, intrauterine inflammation

Introduction

Chorioamnionitis remains a significant cause of death and long-term neurological disability worldwide, with high costs to the individual and the family, plus a heavy economic burden for society.¹⁻³ The adverse developmental sequelae include cognitive dysfunction and cerebral palsy.^{4,5} Intrauterine inflammation is a major causative factor of fetal brain injury.⁶⁻¹⁰ In fact, chorioamnionitis, clinically associated with intrauterine inflammation, contributes 11% or higher and confers a 4-fold overall increased risk of cerebral palsy in term infants, much higher than preterm infants,¹¹ indicating worse sequelae for term infants. Currently, there are no adequate therapies for fetal neuroprotection, resulting in the rise of total number of children with lifelong health problem, although the fetal survival rate continues to improve.¹²

In the cascade of events of fetal brain injury, maternal pro-inflammatory cytokines and subsequent fetal inflammatory responses are the key links between the maternal inflammatory exposure and fetal neurodevelopmental impairment.^{13,14} These processes develop within hours after the inflammation.¹⁵⁻¹⁷

Therefore, it is imperative to develop new strategies that can aid in reducing the fetal brain injury promptly and conveniently, especially at immediate insult period.

During the development of fetal brain, glucose not only plays a critical role, as the primary substrate for energy production,¹⁸ but also helps to proceed normal biosynthetic processes.^{19,20} Studies have shown that lipopolysaccharide (LPS),

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a major membrane component of gram-negative microbes, induces hypoglycemia in mice.^{21,22} Low glucose concentration in amniotic fluid is a sensitive biomarker of chorioamnionitis.^{23,24} Furthermore, glucose supplementation during hypoxia-ischemia is protective in the immature brain.²⁵ As such, maternal glucose treatment may alleviate fetal brain injury induced by intrauterine inflammation.

Associated with glucose metabolism, autophagy is a normal physiological process that promotes homeostasis through protein degradation and turnover of cell organelles.^{26,27} Although there is solid evidence that autophagy plays a role in the mechanisms of cancer, neurodegeneration diseases, and inflammation,²⁸⁻³⁰ little is known whether autophagy is involved in the fetal brain injury caused by intrauterine inflammation.³¹ Studies have shown that autophagy leads to decreased apoptosis,^{32,33} which is one of major mechanisms of fetal brain injury.³⁴⁻³⁶ A better understanding of the mechanism of fetal brain injury would provide possible targets to our ongoing efforts on prevention.

In this study, we hypothesized that maternal glucose administration may be effective at reducing fetal brain injury induced by intrauterine inflammation. Furthermore, we hypothesized that maternal glucose administration ameliorates fetal brain injury following exposure to intrauterine inflammation by preventing autophagy dysregulation. Therefore, we aimed to determine (1) whether maternal glucose supplementation can provide neuroprotection against fetal brain injury at term caused by intrauterine inflammation and (2) the potential mechanisms of the glucose protective action on autophagy. The overall goal of these studies was to contribute to the development of possible therapeutic strategies for fetus/neonate with fetal brain injury in the context of chorioamnionitis.

Materials and Methods

Animals

All animal care and treatment procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. Animals were handled according to the National Institutes of Health guidelines. Timed pregnant CD-1 outbred mouse strain was obtained from Charles River Laboratories (Wilmington, Massachusetts).

An established model of intrauterine inflammation was utilized for the study.^{15,37} Briefly, at embryonic day (E18) of gestation (term), mice were placed under isoflurane/oxygen anesthesia continuously, and a minilaparotomy was performed in the lower abdomen. The LPS (*Escherichia coli*, 055: B5; Sigma-Aldrich, St Louis, Missouri) at a dose of 25 µg in 100 µL of phosphate-buffered saline (PBS) was infused between the first 2 gestational sacs in the lower right uterine horn. Routine closure was applied after the intrauterine injection. Surgical control dams received the same volume of intrauterine injection of PBS. After surgery, intraperitoneal (IP) injection of 200 µL of sterile 10% dextrose (Hospira, Inc, Lake Forest, Illinois) or PBS (vehicle) was given at 1, 2, 3, 4, and 5 hours.

Dams with surgery were divided into 4 groups: PBS plus PBS posttreatment (PBS + PBS), PBS plus glucose posttreatment (PBS + Glu), LPS plus PBS posttreatment (LPS + PBS), and LPS plus glucose posttreatment (LPS + Glu). One additional insulin group (INS) without surgery was added, and IP injection of insulin (1 U/kg; Thermo Fisher, Halethorpe, Maryland) was given.

Survival Rate

Dams were recovered in individual cages after surgery. After 2 days, live pups were examined in each cage and survival birth rate was calculated for each group. Any live pup from each dam was added to the survival number as 1. The survival rate came from the ratio of the number of dams who have live pup divided by the total number of dams.

Neurodevelopmental Behavior Assessment

The behavioral tests were performed as previously described.¹⁷ A developmental milestone scoring system³⁸ was used with modifications to evaluate pups. The negative geotaxis test measured the ability to turn 180° when placed head down on a 45° inclined flat surface. The cliff aversion test measured the ability to turn and crawl away from an edge. Tests were performed on the pups at postnatal days (PND) 5, 9, and 13.

Glucose, Adenosine Triphosphate, Free Fatty Acid, Lactate, and Glycogen Measurements

Maternal glucose levels were measured at 0, 2, 4, and 6 hours after surgery using portable blood glucometer (FreeStyle, Los Angeles, California) from tail vein of dams. Placentas and fetal brains were harvested after 6 hours of surgery and were immediately fresh frozen on dry ice, followed by storage at -80°C until utilized. Glucose, adenosine triphosphate (ATP), free fatty acid, lactate, and glycogen in fetal brain were measured using assay kits purchased commercially (Abcam, Cambridge, Massachusetts). Following the manufacture's protocol, the plates were read using CLARIOstar system (BMG Labtech, Cary, North Carolina) and final data were normalized by protein concentration.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Western Blotting

Fresh frozen fetal brains were applied to Western blotting WB. To prepare tissue lysate, samples were homogenized on ice in RIPA lysis buffer (Amresco, Solon, Ohio) along with phosphatase inhibitor cocktail 2 (Sigma-Aldrich) and proteinase inhibitor (Sigma-Aldrich). The homogenized mixture was then placed on ice for 20 minutes and centrifuged at 14,000 rpm for 15 minutes at 4°C. The resulting supernatants were retained for further experiments. Protein concentrations were determined in duplicates using the Bicinchoninic Acid kit (Thermo, Rockford, Illinois). Protein (50 µg) was separated by sodium

dodecyl sulfate–polyacrylamide gel electrophoresis using 4% to 15% gels (Bio-Rad Laboratories, Hercules, California) and then electrotransferred onto nitrocellulose membranes (Bio-Rad Laboratories). The quality was determined by Ponceau S staining of the nitrocellulose membranes. Membranes were blocked with 10% bovine serum albumin (BSA) (Sigma-Aldrich) in Tris-buffered saline + 0.1% Tween-20 (TBS-T, pH 7.5), incubated with primary antibodies in TBS-T containing 10% BSA for 1 hour at room temperature, and washed in TBS-T. The following were used as primary antibodies: rabbit against LC3 (Novus, Littleton, Colorado) and rabbit anti- β -actin (Abcam). Goat anti-rabbit IR Dye-800CW (Li-Cor, Lincoln, Nebraska) was used as the secondary antibody. Imaging was performed using the Li-Cor Odyssey Near Infra-Red System and analyzed using Image J (v1.48, <http://imagej.nih.gov/ij/>; National Institute of Health, Bethesda, Maryland).

Immunohistochemistry and Histology Staining

Mice at 6 hours after surgery at E18 and PND 9 were euthanized with carbon dioxide, and neonatal brains were fixed overnight at 4°C in 4% paraformaldehyde. Using Leica CM1950 cryostat, 20 μ m thickness samples were cut and mounted on positive charge slides (Fischer Scientific, Hampton, New Hampshire), followed by immunohistochemical (IHC) staining or histochemical staining. Slides were washed with PBS, which was followed by incubation in PBS solution containing 0.05% Triton X-100 and 5% normal goat serum (Invitrogen, Carlsbad, California) for 30 minutes. Brain tissues were incubated with following primary antibodies overnight at 4°C: rabbit anti-myelin basic protein (anti-MBP; Abcam) or rabbit anti-oligo 2 (Millipore, Billerica, Massachusetts). The next day, sections were rinsed with PBS and then incubated with donkey anti-rabbit Alexa Fluor 568 or 488 (Life Technologies, Grand Island, New York) fluorescent secondary antibody diluted in 1:500 for 3 hours at room temperature. The sections were further stained with 4', 6-diamidino-2-phenylindole (DAPI; Roche, Indianapolis) for 2 minutes at room temperature followed by mounting with Fluoromount-G (eBioscience, San Diego, California).

For enhanced IHC staining, fetal brain sections were incubated in 1% hydrogen peroxide for 30 minutes to inactivate the endogenous peroxidase, followed by rabbit anti-LC3 incubation overnight at 4°C and then applied to tyramide signal amplification (TSA) systems (PerkinElmer, Akron, Ohio) according to the protocol of manufacture.

Using myelination histological staining kit (Hitobiotech, Kingsport, Tennessee), myelin was visualized in brains according to the manufacture's instruction. Images were attained using Axioplan 2 Imaging system (Carl Zeiss, Thornwood, New York).

Transmission electron microscope (TEM)

Fetal brains (1 mm³) were fixed in 4% formaldehyde and 1% glutaraldehyde in 0.1 M PB (pH 7.4) for at least 2 hours to

overnight, followed by postfix 1% osmium tetroxide in 0.1 M PB 1 hour. After embedding in beam capsules, tissue blocks of 0.5 μ m thickness were cut. The precise location of cortex was confirmed using microscope, and the ultrathin sections of 60 nm thickness were cut, followed by staining with uranyl acetate for 15 minutes and lead citrate for 5 minutes. Images were obtained from Hitachi 7600 TEM (Hitachi High Tech America Inc., Clarksburg, MD).

The RT-qPCR

Fresh fetal brains after 6 hours of surgery were dissected and frozen at –80°C until utilized. RNA was prepared from the homogenates with RNEasy Mini kit (Qiagen, Valencia, California) and complementary DNA (cDNA) was prepared using cDNA Synthesis kit (Bio-Rad) following the manufacturer's protocol. Quantitative Polymerase Chain Reaction (qPCR) was performed in triplicate in 20 μ L of reactions for 40 cycles, using manufacturer's suggested protocols for temperature cycling (Bio-Rad). The reactions were run on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad), using SensiFAST Probe No-ROX (Bioline, Taunton, Massachusetts). Primers used were obtained from Integrated DNA Technologies (Coralville, Iowa) for GLUT1, Mm.PT. 58.42079875, GLUT3, Mm.PT. 58.32642005, β -actin, Mm.PT.58.33540333, and 18 S, ribosomal RNA, X03205.1. Data analysis was performed with CFX Manager software (Version 3.1) (Bio-Rad).

Cell Counting

Oligo 2-positive IHC staining cell counting in fetal and PND 9 brains was used to evaluate the oligodendrocytes development and relationship with myelination. All photographs used for quantification were taken with Zeiss AxioPlan 2 Microscope System (Carl Zeiss, Thornwood, NY) through a 40 $\times$ objective lens. Cells were counted (field of view) based on oligo 2 staining by using Image J on randomly chosen 25 fields in corpus callosum. The experiments were performed in 5 repeats for all groups.

Statistical Analysis

Statistical analyses were performed using Prism 5 (GraphPad Software, Inc, La Jolla, California). For survival birth rate, each litter was considered as number of 1 and was analyzed using chi-square test. One-way analysis of variance (ANOVA) with Bonferroni post hoc test was used for multiple comparisons of normally distributed data and Kruskal-Wallis with Dunn's multiple comparisons for nonparametric data where it is appropriate.

Results

Maternal Glucose Treatment Prevents Maternal Hypoglycemia

Maternal glucose level exposed to intrauterine LPS (n = 18) demonstrated a decrease trend within the observation period, reaching the statistical significance after 4 ($P < .001$, 2-way

Figure 1. Maternal glucose concentration exposed to intrauterine inflammation. Pregnant dams were injected intraperitoneally with 200 μ L of sterilized 10% dextrose or PBS at 1, 2, 3, 4, and 5 hours after intrauterine lipopolysaccharide (LPS) infusion. Maternal glucose concentrations were measured at 0, 2, 4, and 6 hours after surgery using portable blood glucometer from tail vein. Two-way ANOVA, ^aLPS + glucose (blue), $P < .05$ compared to LPS + PBS (red); ^bPBS + PBS (green), $P < .001$ compared to LPS + PBS, ^cPBS + PBS (green), $P < .01$ compared to LPS + glucose (blue); ^dPBS + PBS (green), $P < .01$ compared to LPS + PBS (red); ^ePBS + PBS (green), $P < .05$ compared to LPS + glucose (blue). ANOVA indicates analysis of variance; PBS, phosphate-buffered saline.

ANOVA; Figure 1) and 6 hours ($P < .01$, 2-way ANOVA; Figure 1) of surgery, compared to surgical control (PBS + PBS, $n = 12$). Maternal glucose supplementation ($n = 17$) significantly increased maternal glucose level ($P < .05$, 2-way ANOVA; Figure 1) compared to LPS intrauterine injection 2 hours after surgery. However, maternal glucose levels in LPS plus PBS and LPS plus glucose groups were decreased significantly compared to surgical control at 4 and 6 hours after surgery ($P < .05$, 2-way ANOVA; Figure 1).

Maternal Glucose Treatment Increases Survival Rate

In comparison with surgical control ($n = 7$), intrauterine LPS-exposed mice ($n = 12$) demonstrated 50% drop in survival rate ($P < .05$, chi-square test; Table 1). Maternal glucose administration, following LPS ($n = 20$), significantly increased survival rate to 85% ($P < .05$, chi-square test; Table 1). Negative (PBS + glucose, $n = 10$) and maternal insulin controls (insulin, $n = 10$) were not affected on survival rate (Table 1).

Maternal Glucose Treatment Improves Neurobehavioral Performance

Cliff aversion and negative geotaxis tests were applied to access the behavioral effects of maternal glucose supplementation in intrauterine LPS-exposed pups. Similar to our prior

Table 1. Survival Rate.

Treatment	Survival Rate (%)
PBS + PBS ($n = 7$)	7/7 (100)
LPS + PBS ($n = 12$)	6/12 (50 ^a)
LPS + glucose ($n = 20$)	17/20 (85 ^b)
PBS + glucose ($n = 10$)	10/10 (100)
Insulin ($n = 10$)	10/10 (100)

Abbreviations: PBS, phosphate-buffered saline; LPS, lipopolysaccharide.

^aCompared to PBS + PBS, $P < .05$.

^bCompared to LPS + PBS, $P < .05$.

Figure 2. Neuromotor and developmental behavioral changes in response to intrauterine exposure to lipopolysaccharide (LPS). Surviving offspring were assessed neurological development on postnatal days (PND) 5, 9, and 13 using cliff aversion (A) and negative geotaxis tests (B). Prenatal LPS exposure induced significant changes in cliff aversion (PNDs 5, 9, and 13) and negative geotaxis (PNDs 5 and 9) performance in LPS groups compared to surgical control ($P < .05$, 1-way ANOVA). Maternal treatment with glucose significantly improved both behavioral tests, compared to LPS exposure group ($P < .05$, 1-way ANOVA). * $P < .05$ compared to PBS + PBS; n (pups) = 39 PBS + PBS (4 litters), 50 LPS + PBS (6 litters); 40 LPS + glucose (6 litters); 51 PBS + glucose (5 litters); 63 insulin (5 litters). ANOVA indicates analysis of variance; PBS, phosphate-buffered saline.

data,¹⁷ intrauterine LPS-exposed pups ($n = 50$, 6 litters) displayed a deficit in cliff aversion and negative geotaxis with a significant longer performing duration at PNDs 5, 9, and 13, compared to surgical control ($n = 39$, 4 litters, 1-way ANOVA; Figure 2). These motor behavioral impairments improved significantly in maternal glucose-treated LPS-exposed offspring ($n = 40$, 6 litters, 1-way ANOVA; Figure 2).

Maternal Glucose Treatment Improves Myelination and Oligodendrocyte Development

Histochemical staining of myelin (Figure 3A) at E18, IHC staining (Figure 3B) of MBP and TEM (Figure 3C) at PND 9 were applied to access the development of myelination.

Figure 3. Maternal glucose administration increases myelination in brain. Brains of pups were collected at embryonic (E) day 18 or postnatal day (PND) 9. Morphology of myelination in the corpus callosum was examined using histochemical, immunohistochemical (IHC), and transmission electron microscopy (TEM) technology. A, Representative histochemical staining of myelination at E18 six hours after surgery. B, Representative IHC images of myelin basic protein at PND 9. C, Representative TEM images of myelin at PND 9. The bottom panel was the magnification of squares in the top panel ($n = 5$ for each group).

Histology demonstrated a lighter myelin staining in corpus callosum of intrauterine LPS-exposed fetal brains, compared to surgical control and glucose-treated groups (Figure 3A). Furthermore, at PND 9, MBP immunostaining (Figure 3B) and TEM (Figure 3C) showed the similar changes in LPS-exposed pup brain, with less myelination in the corpus callosum compared to surgical control. Maternal glucose treatment increased myelin expression.

To determine whether the decreased myelination in neonatal brain exposed to intrauterine inflammation was due to the improper development of myelin-producing oligodendrocyte, we did oligo 2 IHC staining. At E18, 6 hours after surgery, in the corpus callosum area, the number of oligodendrocyte decreased significantly ($n = 5$) in LPS-exposed group, compared to the surgical control ($n = 5$; $P < .001$, 1-way ANOVA; Figure 4A and B). Maternal glucose supplementation increased

Figure 4. Immunohistochemical (IHC) analysis of oligodendrocytes. IHC of oligo 2, an oligodendrocyte marker was performed in the corpus callosum of brain at embryonic (E) day 18. A, Representative images of oligodendrocytes 6 hours after surgery. B, Cell counting demonstrated a significant decrease in oligodendrocytes in LPS + PBS group, compared to surgical control ($P < .001$, 1-way ANOVA, $n = 5$ for each group). Maternal glucose treatment increased the number of oligodendrocytes ($P < .001$, 1-way ANOVA, $n = 5$ for each group). Scale bar = 50 μm . ANOVA indicates analysis of variance; LPS, lipopolysaccharide; PBS, phosphate-buffered saline.

the oligodendrocyte number significantly ($n = 5$, $P < .001$, 1-way ANOVA; Figure 4A and B).

Maternal Glucose Treatment Downregulates ATP Level in Fetal Brain

To evaluate the metabolic changes in fetal brain exposed to intrauterine LPS injection and the role of maternal glucose supplementation, energy resources, including ATP, glucose, lactate, glycogen, and free fatty acid, were measured. In fetal brain, 6 hours after surgery, intrauterine LPS significantly increased the ATP level ($n = 5$, $P < .05$, 1-way ANOVA; Figure 5A), compared to surgical control ($n = 5$), while maternal glucose treatment significantly decreased ATP level ($n = 5$, $P < .05$, 1-way ANOVA; Figure 5A). Different from the changes in ATP level, there was a significant decrease in the glucose level in intrauterine LPS-exposed fetal brain with ($n = 5$) or without maternal glucose supplementation ($n = 5$), compared to surgical control ($n = 6$, $P < .05$, 1-way ANOVA; Figure 5B). There were no significant changes in lactate (Figure 5C), glycogen (Figure 5D), and free fatty acid (Figure 5E) levels in the fetal brain ($P > .05$, 1-way ANOVA).

Since glucose transporter 1 (GLUT1) and glucose transporter 3 (GLUT3) are important membrane proteins to facilitate the transport of glucose in brain, qPCR was performed on fetal brain at E18. There was a significant decrease in insulin group ($n = 5$) for messenger RNA of GLUT1, compared to surgical control ($n = 6$, $P < .05$, 1-way ANOVA; Supplementary Figure S1A). There was no significant difference between other groups ($P > .05$, 1-way ANOVA; Supplementary Figure S1A). The qPCR of GLUT3 demonstrated that there was no significant change between groups ($P > .05$, 1-way ANOVA; Supplementary Figure S1B).

Maternal Glucose Treatment Alleviates Intrauterine LPS-Induced Inhibition of Autophagy

To explore whether autophagy is involved in the mechanism of fetal brain injury induced by intrauterine LPS exposure and thereby the role of maternal glucose supplementation on autophagy, we studied autophagy markers, microtubule-associated protein light chain 3 (LC3). During the process of autophagy, LC3 I is required to form into LC3 II.^{39,40} At E18, LC3 was expressed in the cortex of fetal brain (Figure 6A). Western blotting was applied to differentiate LC3 I and LC3 II (Figure 6B). There was no significant change in LC3 I between groups (Figure 6C). However, LC3 II was significantly increased in maternal glucose-treated groups ($n = 5$), compared to intrauterine LPS-exposed group ($n = 5$, $P < .01$, 1-way ANOVA; Figure 6D). Furthermore, the total LC3 was also significantly increased in maternal glucose supplementation ($n = 5$, $P < .001$, 1-way ANOVA; Figure 6E). We observed the ultrastructure of cells in fetal brain by TEM. Intrauterine LPS-exposed fetal brain demonstrated swelled mitochondria with damaged inner structure, disorganized rough endoplasmic reticulum

(RER), and detached ribosome was dispersed in cytoplasm (Figure 6F, middle). Glucose treatment improved the morphology of RER and mitochondria (Figure 6F, right). In intrauterine LPS-exposed fetal brain ($n = 3$), autophagosome was absent in LPS-exposed group (Figure 6G, middle). The autophagosome at early stage was observed in both surgical control ($n = 3$; Figure 6G, left) and maternal glucose-treated groups ($n = 3$; Figure 6G, right).

Discussion

In this study, we demonstrated that maternal glucose treatment improved the survival rate and the neurobehavioral outcomes of offspring in our mouse model of chorioamnionitis at term. These findings were associated with the decrease in ATP levels and the alleviation of dysregulated autophagy, improved oligodendrocyte maturation, and myelination development in fetal brain.

Myelination is the process of myelin sheath formation around axon, to allow nerve impulses move more quickly.⁴¹ If the myelin sheath is damaged or lost, transmission of nerve impulses is slower or blocked. These changes may lead to a variety of symptoms, such as sensory impairment, difficulties in controlling movement, and problems with bodily functions. In children, dysregulation of myelination is related to a number of disabilities, including autism, Down syndrome, and schizophrenia.⁴²⁻⁴⁴ Oligodendrocyte is the myelin-producing cell in nervous system.⁴⁵ In rodents, oligodendrocytes mature prenatally and then differentiate during the first 2 postnatal weeks, whereas in humans, they mostly develop between 23 and 37 weeks of gestation.⁴⁶ In our study, intrauterine inflammation decreased fetal myelination at term, likely through the interruption of oligodendrocyte maturation, which is similar to previous studies.⁴⁷⁻⁴⁹ Maternal glucose treatment increased the number of oligodendrocytes significantly, thereby promoting myelination of fetal brain and alleviating neural dysfunction (ie, neurobehavioral deficits).

Our data have shown that intrauterine LPS exposure increased the ATP levels in fetal brain, while maternal glucose supplementation decreased the ATP levels. Though intracellular ATP is recognized as a direct energy storage format for living processes, a solid body of evidence has demonstrated that extracellular ATP is required to trigger several types of immune responses including those by microglia and macrophage, leading to cellular disintegration, mitochondrial damage, and apoptosis.^{50,51} During intrauterine inflammation, ATP may be released by activated platelets, leukocytes, damaged cells, and parenchymal cells such as epithelial or endothelial cells⁵² and may result in high levels of extracellular ATP, in combination with downregulation of extracellular ATP degradation.⁵³ Maternal glucose supplementation prevented the increased ATP levels in fetal brain exposed to intrauterine inflammation, suggesting that the alleviation of maternal glucose dysregulation is of benefit to fetal ATP metabolism.

The maternal glucose supplementation did not recover the glucose levels in fetal brain induced by intrauterine

Figure 5. Energy substrates in fetal brain exposed to intrauterine inflammation. At embryonic (E) day 18, fetal brains were collected 6 hours after surgery. The contents were measured using assay kits, including ATP (A), glucose (B), lactate (C), glycogen (D), and free fatty acid (E). (* $P < .05$; ** $P < .01$, I-way ANOVA, $n = 5$ for each group). ANOVA indicates analysis of variance; ATP, adenosine triphosphate.

inflammation but improved the neurological performance of pups. This may indicate that the alleviation of fetal brain injury by maternal glucose supplementation was not majorly associated with energy substrates. In fact, physiologically the blood glucose is at the lowest in the first few hours or days of newborns due to the transition from the placental supply to milk feeding.⁵⁴ We speculate that acute low glucose level to

some extent in neonatal brain is not the main cause of fetal brain injury during chorioamnionitis at term. Previous studies have shown that chorioamnionitis is associated with hypoxia–ischemia in neonatal brain^{55,56} and maternal glucose supplementation improved development of immature brain during hypoxia–ischemia by assisting other metabolic processes, such as cytosolic and mitochondrial oxidation–reduction.²⁵

Figure 6. Autophagy in fetal brain exposed to intrauterine inflammation. Fetal brains were harvested 6 hours after surgery at embryonic (E) day 18. A, Immunohistochemical staining of microtubule-associated protein light chain 3 (LC3) in cortex (arrows). B, Western blot (WB) was performed to differentiate the expression of LC3 I and LC3 II. C, Quantitative analysis of LC3 I in WB. D, Quantitative analysis of LC3 II in WB. E, Quantitative analysis of total LC3 in WB. F, Representative transmission electron microscope (TEM) images of cells in cortex of fetal brain (white arrows: mitochondria; black arrows: rough endoplasmic reticulum). G, Representative TEM images of autophagosome in cytoplasmic of cells (black arrows: autophagosome).

The fetal brain development, including neural induction, proliferation, migration, maturation, and differentiation, continuously requires protein synthesis and degradation.⁵⁷ The process of proper quality and quantity control is highly manipulated by autophagy, to facilitate the removal of defective or

superfluous cytosolic proteins, organelles, and other cellular constituents, therefore, to ensure the reservation of energy consumption and the continuous recycling required by fetal brain development.⁵⁸ In normal condition, autophagy is a physiological process occurring at a baseline level. In addition,

autophagy has a critical role in cytoprotection by preventing the accumulation of toxic proteins and through its action in various aspects of immunity, including the elimination of invasive microbes and its participation in antigen presentation.⁵⁹ In our study, though there was no significant difference between surgical control and LPS groups at protein level, maternal glucose supplementation significantly increased the LC3 II protein expression (activation of autophagy) in fetal brain. The TEM demonstrated that intrauterine LPS exposure resulted in the absence of autophagosome in fetal brain, whereas autophagy increased in the maternal glucose supplementation group. We speculate that the elevated autophagy may help to recycle damaged cellular components and eliminate injured cells in fetal brain exposed to maternal intrauterine inflammation.

In conclusion, our study demonstrated that maternal glucose may be a potential and easily accessible worldwide therapeutic intervention to alleviate fetal brain injury due to with chorioamnionitis and exposure to intrauterine inflammation at term. Further translational and clinical studies should investigate the utility of this single therapeutic intervention for the health of mother and child.

Declaration of Conflicting Interests

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Supplemental Material

Supplementary material for this article is available online.

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