Protective Effect of *N*-acetylcysteine on Liver Damage During Chronic Intrauterine Hypoxia in Fetal Guinea Pig

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Kazumasa Hashimoto, MD¹, Gerard Pinkas, BS¹,
LaShauna Evans, BS², Hongshan Liu, MD¹,
Yazan Al-Hasan, PhD¹, and Loren P. Thompson, PhD¹

Abstract

Chronic exposure to hypoxia during pregnancy generates a stressed intrauterine environment that may lead to fetal organ damage. The objectives of the study are (1) to quantify the effect of chronic hypoxia in the generation of oxidative stress in fetal guinea pig liver and (2) to test the protective effect of antioxidant treatment in hypoxic fetal liver injury. Pregnant guinea pigs were exposed to either normoxia (NMX) or 10.5% O₂ (HPX, 14 days) prior to term (65 days) and orally administered *N*-acetylcysteine ([NAC] 10 days). Near-term anesthetized fetuses were excised and livers examined by histology and assayed for malondialdehyde (MDA) and DNA fragmentation. Chronic HPX increased erythroid precursors, MDA (NMX vs HPX; 1.26 ± 0.07 vs 1.78 ± 0.07 nmol/mg protein; $P < .001$, mean $\pm$ standard error of the mean [SEM]) and DNA fragmentation levels in fetal livers (0.069 ± 0.01 vs 0.11 ± 0.005 OD/mg protein; $P < .01$). *N*-acetylcysteine inhibited erythroid aggregation and reduced ($P < .05$) both MDA and DNA fragmentation of fetal HPX livers. Thus, chronic intrauterine hypoxia generates cell and nuclear damage in the fetal guinea pig liver. Maternal NAC inhibited the adverse effects of fetal liver damage suggestive of oxidative stress. The suppressive effect of maternal NAC may implicate the protective role of antioxidants in the prevention of liver injury in the hypoxic fetus.

Keywords

fetal hypoxia, oxidative stress, guinea pig, *N*-acetylcysteine, fetal liver

Introduction

Chronic hypoxia in utero is a major cause of fetal growth restriction and fetal and neonatal morbidity and mortality.¹⁻³ Yet, the impact of chronic exposure to hypoxia on fetal organ function has had limited study. The hypoxic fetus redistributes its cardiac output to favor both the heart and the brain at the expense of the fetal liver.⁴⁻⁶ Thus, the fetal liver is vulnerable to damage under conditions of chronic fetal hypoxia.

The fetal liver plays a primary role in hemopoiesis during the fetal period and transitions to a more metabolic role near term or after birth depending on the animal species. The fetal liver consists of hemopoietic cells, hepatocytes, as well as, sinusoidal and bile ductal cells during its development.^{7,8} During the fetal period, erythropoietin is produced in hepatocytes and fibroblast-like Ito cells as evidenced by its gene expression in the liver.^{9,10} With the gestational proliferation of hepatocytes near term, hepatocytes begin to play an important role in carbohydrate¹¹ and lipid¹² metabolism, as well as, synthesis and secretion of liver proteins¹³ associated with angiogenesis and growth factors, such as insulin-like growth factor binding protein 1 (IGFBP-1).¹⁴⁻¹⁶ Thus, as gestation nears term, the fetal liver is progressing from a hemopoietic to a metabolic function in preparation for postnatal life.

Fetal hypoxia can lead to local tissue formation of reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, lipid peroxides, and hydroxyl radicals.^{2,17} Excess generation, which may lead to oxidative stress, can result from a variety of sources that include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, mitochondrial dysfunction, and reduced antioxidant capacity.¹⁸ Oxidative stress has been reported to contribute to cellular injury in a variety of organs such as brain,¹⁹ heart,²⁰ and liver.²¹ An excess of ROS generation that surpasses its antioxidant capacity has damaging effects that disrupt plasma membrane integrity via lipid peroxidation^{20,22} and nuclear oxidative damage via DNA fragmentation.^{20,21} Chronic hypoxia impairs carbohydrate metabolism¹¹ and insulin signaling²³ in the fetal

¹Department of Obstetrics, Gynecology and Reproductive Sciences, University of Maryland, Baltimore, MD, USA

²Department of Physiology, University of Maryland, Baltimore, MD, USA

Corresponding Author:

Loren P. Thompson, Department of Obstetrics, Gynecology and Reproductive Sciences, University of Maryland School of Medicine, Bressler Research Building, Room 11-040, 655W Baltimore Street, Baltimore MD, USA
Email: lthompson1@umm.edu

sheep liver, having a programming effect on insulin resistance in the offspring.²⁴ We propose that fetal hypoxia is likely to have a significant impact on normal liver function in utero via oxidative stress, contributing to altered fetal growth and metabolism.

The objectives of the study were to (1) quantitate the effect of chronic hypoxia on the fetal liver in generation of oxidative stress as an important stimulus for inducing fetal liver damage and (2) to test the protective effect of maternal administration of an antioxidant, *N*-acetylcysteine (NAC), against the fetal liver damage in the hypoxic fetus. This was assessed by measuring the tissue levels of malondialdehyde (MDA) as an index of lipid peroxidation and DNA fragmentation as an index of nuclear damage and apoptotic cell death.

Materials and Methods

Animal Treatment and Sample Preparation

Pregnant guinea pigs (Dunkin-Hartley, term = 65 days) were placed in either a hypoxic (HPX) chamber (10.5% O₂ for 14 days prior to fetal excision, N = 6) or room air (normoxic or NMX, N = 5-7) as previously described.^{25,26} The selected gestational period is a rapid growth phase in fetal guinea pig development.²⁷ The hypoxic level (10.5% O₂) was the minimal oxygen level that could be imposed without causing pregnancy termination. These conditions generate fetal hypoxia as evidenced by increased RBC content and reticulocyte number in fetal guinea pig plasma,²⁸ increased hypoxia inducible factor 1 α (HIF-1 α) protein levels in hypoxic fetal cardiac ventricles,²⁹ and increased staining of hypoxyprobe 1 in hypoxic fetal livers.³⁰ In addition, a separate group of HPX and NMX animals were treated with NAC (500-600 mg/kg per d) by placing it in their drinking water for 10 days during the hypoxic period or during the age-matched gestational period in NMX animals, respectively. The dose was based on studies shown to inhibit an inflammatory cytokine response to LPS in rats³¹ and effects of ROS in guinea pigs.³² Also, we have reported that NAC inhibits MDA levels in fetal guinea pig heart ventricles.³³ Both food (g/d; Tekblad Guinea Pig Diet, Harlan Laboratories, Frederick, MD) and water volume (mL/d) intake rates were measured throughout the duration of treatment.

At near term, 63 days of gestation (term = 65 days), pregnant sows were anesthetized (ketamine, 1 mg/kg; xylazine, 80 mg/kg) and fetuses were delivered by hysterotomy. Fetal body, liver, placenta, brain, and heart weights were measured. Right liver lobes were excised and either fixed for staining or snap frozen in liquid nitrogen and stored at -80°C. The study protocol was approved by University of Maryland Animal Care and Use Committee and conforms to the Guide for the Care and Use of Laboratory Animals published by the US NIH Publication No. 85-23, 1996.

Histopathology

Right lobes of NMX and HPX fetal guinea pig livers were excised and fixed in 10% buffered formalin (Sigma, St Louis,

Missouri) for hematoxylin and eosin (H&E) staining. Livers were dehydrated through ascending ethanol concentrations (70%, 95%, and 100%), paraffin embedded, and sectioned at 5 μ m. Fetal liver sections (5 μ m) were deparaffinized and rehydrated through decreasing ethanol concentrations and sections were stained in hematoxylin solution (10-15 seconds) and counterstained with eosin (5-10 seconds) according to the standard histological protocol. The sections were dehydrated, cleared, coverslipped and imaged using bright field microscopy.

Thiobarbituric Acid Reactive Substances Assay

Frozen livers of NMX and HPX animals with and without NAC were homogenized and extracted for measurement of MDA. Malondialdehyde was quantified using a thiobarbituric acid reactive substances assay (TBARS assay; Caymen Chemical, Ann Arbor, Michigan), measured in triplicate, and normalized to milligram protein of each sample. All samples were normalized to protein content using an RC-DC protein assay (Bio-Rad, Hercules, California).

Quantification of Nuclear DNA Injury

Frozen livers of NMX and HPX fetuses treated with and without NAC were quantified for nuclear DNA injury. Histone-complexed DNA fragments (oligonucleosomes) were detected by enzyme-linked immunosorbent assay (ELISA) system (Cell Death Detection Kit, Roche, Indianapolis, IN) as an index of nuclear injury and apoptosis. Samples were run in triplicate and optical density (OD) was measured at 405 nm by VERSAmax 3.13 spectrophotometer (Molecular Devices, Sunnyvale, California). Protein levels were determined by an RC-DC protein assay (Bio-Rad) for each sample and values were expressed as OD^{405nm/mg protein}.

Data Analyses

Values were expressed as mean $\pm$ standard error of the mean (SEM). Single fetuses (N values) were obtained from separate litters from pregnant sows. Assay values were normalized to milligram fetal liver protein. Differences among groups were tested by a 2-way analysis of variance (ANOVA) with hypoxia and treatment as independent factors. If differences were detected, a post hoc Student Newman-Keuls test was performed to compare the differences between groups. A *P* value <.05 was used for statistical significance.

Results

Fetal Guinea Pig Body and Organ Weights

The effect of chronic hypoxia and NAC treatment on fetal body weight and liver weight is shown in Figure 1. The average fetal body weight of NMX fetal guinea pigs was 88.8 $\pm$ 4.5 g. Hypoxia significantly decreased (*P* < .01) fetal body weight (59.5 $\pm$ 2.4 g) by 33% compared to NMX controls. Maternal

Figure 1. Fetal guinea pig body and liver weights. Fetal guinea pig body and liver weights of normoxic (NMX) and hypoxic (HPX; 10.5% O₂ for 14 days) animals treated without (control) and with *N*-acetylcysteine (NAC). Liver weight (g) was normalized to its respective fetal body weight (BW; g) and expressed as a ratio (g/g). Values are mean $\pm$ standard error of the mean ([SEM] N values: NMX: control = 7, NAC = 6; HPX: control = 6; NAC = 6).

administration of NAC had no effect on fetal body weight under conditions of either normoxia (88.8 ± 4.5 g vs 76.6 ± 7.8 g; NMX vs NMX-NAC) or hypoxia (59.5 ± 2.4 g vs 59.8 ± 2.4 g; HPX vs HPX-NAC). Mean liver weight of NMX fetuses was 4.59 ± 0.41 g. Hypoxia reduced liver weight (2.88 ± 0.18 g) compared to its NMX controls by 37.2% ($P < .05$). Mean fetal liver weights of NMX-NAC and HPX-NAC were 3.96 ± 0.42 g and 3.21 ± 0.18 g, respectively. When normalized to its fetal body weight, relative liver weights were similar among all groups (0.048 ± 0.002 , 0.049 ± 0.003 , 0.052 ± 0.001 , and 0.054 ± 0.002 for NMX, HPX, NMX-NAC, and HPX-NAC group, respectively). Chronic hypoxia increased ($P < .05$) the relative weights of placenta, brain, and heart (Figure 2), demonstrating a brain and heart sparing effect of hypoxia. Chronic hypoxia did not reduce the absolute weight of the placenta (4.69 ± 0.24 vs 4.59 ± 0.33 g) although significantly ($P < .05$) reduced brain and heart weights compared to NMX controls (2.62 ± 0.06 vs 2.35 ± 0.06 g; 0.46 ± 0.03 vs 0.38 ± 0.01 g, HPX vs NMX, respectively).

Food and water intake was affected differently by chronic hypoxia and NAC treatment. Measured over the same period of HPX treatment, the average food intake of NMX and HPX

animals was 34.6 ± 3.7 vs 37.9 ± 2.2 g/day, respectively. *N*-acetylcysteine treatment did not affect food intake in either NMX (34.6 ± 3.7 vs 35.6 ± 1.9 g/d, respectively) or HPX (37.9 ± 2.2 vs 35.8 ± 2.9 , untreated vs treated, respectively) animals. However, water intake (mL/d) was increased ($P < .05$) in HPX (76.6 ± 17.4 vs 136.9 ± 17.8 mL/d, NMX vs HPX, respectively) and decreased by NAC to similar levels in both NMX (53.5 ± 3.6) and HPX (64.7 ± 5.8) animals.

Histopathology

Representative images of fetal livers stained with H&E are illustrated in Figure 3. Hepatocytes (open black arrows) of NMX fetal livers exhibit clearly identifiable plasma membranes, granular cytoplasm, and intact nuclei (both mono- and binucleated). Granularity is associated with mitochondrial organelles and endoplasmic reticulum. Erythroid precursor cells (or erythroid progenitors, black triangles) are identified by dark nuclei and clustered in small groups.³⁴ Chronic hypoxia increased aggregation of the clustered erythroid cells and also appeared to disrupt the plasma membrane structure of hepatocytes compared to NMX controls. Maternal NAC

Figure 2. Fetal guinea pig placenta, brain, and heart weights. Fetal guinea pig placental, brain, and heart weights of normoxic (NMX) and hypoxic (HPX; 10.5% O₂ for 14 days) animals treated without (control) and with *N*-acetylcysteine (NAC). Organ weights (g) were normalized to their respective fetal body weight (BW; g) and expressed as a ratio (g/g). Values are mean $\pm$ standard error of the mean ([SEM] N values: NMX: control = 7, NAC = 6; HPX: control = 6; NAC = 6).

administration had no effect on fetal liver histology under conditions of NMX. *N*-acetylcysteine in the presence of HPX decreased the number of erythroid cell aggregates compared to HPX alone.

Lipid Peroxidation

The effect of chronic hypoxia and NAC treatment on lipid peroxidation is shown in Figure 4. Chronic hypoxia significantly increased ($P < .001$) MDA levels by 41% (NMX vs HPX; 1.26 ± 0.07 vs 1.78 ± 0.07 nmol/mg protein). Maternal administration of NAC reduced ($P < .05$) MDA levels in HPX fetal livers (1.51 ± 0.08 ; HPX-NAC) and had no effect on NMX tissues (1.49 ± 0.09 ; NMX-NAC).

DNA Fragmentation

The effect of chronic hypoxia and NAC treatment on DNA fragmentation is shown in Figure 5. Chronic hypoxia significantly increased ($P < .01$) DNA fragmentation by 59% (0.069 ± 0.01 vs 0.11 ± 0.005 nmol/mg protein; NMX vs HPX). Maternal administration of NAC decreased DNA

fragmentation in HPX fetal livers (0.11 ± 0.005 vs 0.064 ± 0.004 ; HPX vs HPX-NAC) but had no effect on NMX fetal livers (0.069 ± 0.01 vs 0.075 ± 0.01 ; NMX vs NMX-NAC) compared to the respective controls.

Comment

This study demonstrates that chronic exposure to hypoxia induces oxidative stress in the fetal liver, resulting in cellular and nuclear damage. Histological examination of fetal livers exposed to chronic hypoxia displays an alteration in cellular membrane integrity and aggregation of erythroid cells. Further, chronic hypoxia increases MDA levels as an indication of lipid peroxidation. Consistent with oxidative stress, exposure to fetal hypoxia results in a significant increase in liver DNA fragmentation. Fetal liver damage in response to chronic intrauterine hypoxia was reversed by NAC as evidenced by a return to normal histology, MDA levels, and DNA fragmentation levels compared to NMX controls. These findings identify the impact of chronic hypoxia in generating oxidative stress in the fetal liver.

Figure 3. Histological examination of fetal guinea pig liver. Fetal livers obtained from normoxic (NMX), hypoxic (HPX), NMX + N-acetylcysteine (NAC), and HPX + NAC-treated guinea pigs. Sections (5 μ m) were stained with hematoxylin and eosin (H&E) and hepatocytes (open arrows) and erythroid progenitor cells (closed triangles) identified in figures at magnification $\times 100$ (Inset = $\times 400$).

Effect of Chronic Intrauterine Hypoxia on Fetal Liver Growth

Chronic hypoxia induces a decrease in fetal body weight associated with an increase in the relative placenta weight as previously described.²⁸ There was no change in the relative fetal liver weight despite hypoxia-induced changes in lipid peroxidation and DNA fragmentation. Further, NAC did not reverse fetal growth restriction despite the ability of NAC to reverse lipid peroxidation and DNA damage in the fetal liver.

Chronic hypoxia reduces liver weight in proportion to fetal body weight. The changes in liver weight of hypoxic fetuses is likely the result of either poor oxygenation due to perfusion heterogeneity or oxygen-sensitive mechanisms^{13,35} that are unaffected by NAC directly since NAC had no effect on absolute liver weights or liver/body weight ratios. Perfusion of the liver has a direct relationship to its growth and function.³⁶ In human fetuses, fetal liver perfusion is met by 80% of blood

flow from umbilical vein and 20% of splanchnic origin.³⁷ Of the umbilical blood flow, 20% to 50% bypasses the fetal liver and is directed toward the inferior vena cava by the ductus venosus.^{6,38} Fetal hypoxia diverts blood away from the fetal liver as an adaptive response for redistributing blood flow to the heart and brain.^{39–41} This makes the fetal liver vulnerable to hypoxic stress as a result of redistribution of oxygen away from hepatic tissue. In contrast to human fetuses, the fetal guinea pig lacks a functional ductus venosus.^{42,43} In growth-restricted guinea pig livers, total hepatic blood flow is reduced and flow is distributed away from the right lobe preferentially.⁴⁴ Thus, the hypoxic guinea pig liver (ie, right lobe) experiences a similar flow limitation as the hypoxic human liver with a ductus venosus. In the long term, the proportional decrease in liver growth is likely to have important implications in normal growth and metabolism of the fetus as well as contribute to neonatal morbidity via programming mechanisms.

Figure 4. Fetal hypoxia induces lipid peroxidation of fetal livers. Malondialdehyde (MDA) levels (nmol MDA/mg protein) of fetal guinea pig livers of normoxic (NMX) and hypoxic (HPX; 10.5% O₂ for 14 days) animals treated without (control) and with *N*-acetylcysteine (NAC). Values are mean $\pm$ standard error of the mean ([SEM] N values: NMX: control = 5, NAC = 5; HPX: control = 5; NAC = 6).

Figure 5. Fetal hypoxia induces DNA damage of fetal livers. DNA fragmentation levels (OD value_{405 nm}/mg protein) of fetal guinea pig liver of normoxic (NMX) and hypoxic (HPX; 10.5% O₂ for 14 days) animals treated without (control) and with *N*-acetylcysteine (NAC). Values are mean $\pm$ standard error of the mean ([SEM] N values: NMX: control = 7, NAC = 6; HPX: control = 6; NAC = 6).

In contrast to fetal liver, the relative placenta weight was increased by hypoxia and reversed by NAC. Despite the lack of changes in relative brain and heart weights by NAC, the effect of NAC on placenta differs from the fetal liver, brain, and heart. This may reflect the difference of NAC on factors modifying placental growth and/or the effect of oxidative stress in the hypoxic placenta.

Effect of Hypoxia on Cellular Integrity of Fetal Liver

The fetal liver plays an important role in hemopoiesis as the primary site during fetal development.^{7,8} Initially, the yolk sac blood islands are responsible for hemopoiesis during embryogenesis. During fetal development, progenitor cells seed the fetal liver, which then becomes the predominant site.⁴⁵ Hepatocytes make up the majority of the parenchymal tissue, which

are derived from terminal differentiation of progenitor cells.^{7,8} In response to hypoxia, stress erythropoiesis, a response that generates an increase in erythrocytes under conditions of reduced oxygen supply,⁴⁶ is induced in the fetal liver. We have previously measured an increase in plasma RBC content and reticulocyte count in hypoxic versus normoxic fetal guinea pigs under similar conditions.²⁸ In the current study, chronic hypoxia increases aggregation of erythroid progenitor cells in fetal guinea pig liver, suggesting a compensatory response to reduced oxygenation. The decrease in aggregate hemopoietic cell clusters following NAC administration suggests a response to oxidative stress and not to reduced oxygenation since hypoxic conditions still remain with NAC as evidenced by sustained heart and brain sparing effects. Since NAC did not completely eliminate this effect, it is possible that a combination of both oxidant molecules and other

hypoxia-related mechanisms (ie, HIF-1 α expression)⁴⁷ may contribute.

Oxidative stress induces cellular damage to molecules such as lipids, proteins, and DNA. As a result, phospholipids of plasma membranes are vulnerable to peroxidation by superoxide anions.⁴⁸ Superoxide anions can induce marked fragmentation of nuclear DNA.^{48,49} Defense mechanisms against oxidative stress is dependent on the relative activities of Cu/Zn superoxide dismutase (SOD) and Mn-SOD. Fetal liver expresses lower levels of antioxidant enzymes compared to adult liver,^{50–53} suggesting a reduced antioxidant capacity.⁴⁷ The fetal liver consists mainly of hemopoietic (or erythroid progenitor) cells during the early fetal period and increases in cell population to hepatocytes, sinusoidal cells, and bile duct cells later in gestation.⁵⁴ Hemopoietic cells but not hepatocytes have been shown to exhibit marked DNA fragmentation as gestation progresses to term.⁵⁵ As term approaches, there is a progressive change in fetal liver cell composition due to rapid hepatocyte proliferation and regression of hemopoietic cells via apoptosis.⁵⁵ In late gestation, it is possible that oxidative stress may affect liver function in a cell-specific manner with a greater proportion of lipid peroxidation and DNA fragmentation occurring in hepatocytes. Thus, intrauterine oxidative stress may alter the progression pattern of vulnerable cell populations and contribute to fetal liver dysfunction. The discrepancy between reversal of liver cell damage by NAC but not liver weight may indicate that only a limited fraction of the total liver is affected by hypoxia.

Our data do not indicate that NAC has direct adverse effects on the hepatic hemopoietic cells as demonstrated by the lack of histopathology and DNA damage in NMX livers. Further, the overall hemopoietic function may not be damaged by fetal hypoxia since red blood cells (RBCs) and their progenitor, reticulocytes, are increased in fetal plasma in response to hypoxic stress.²⁸ The role of the bone marrow as an additional hemopoietic system under these conditions is unclear. It is possible that chronic hypoxic stress could also trigger erythropoiesis of the bone marrow as a compensatory response.

Effect of NAC on Hypoxia Responses

Reactive oxygen species such as superoxide anions, hydrogen peroxide, and lipid peroxides can play both a physiological and pathological role in the fetal liver. When generated in excess, these oxidant molecules can contribute to fetal toxicity by their interactions with cell organelles within specific fetal organs. *N*-acetylcysteine is an antioxidant that crosses the placenta and has previously been shown to inhibit oxidative stress during pregnancy.^{56–60} In the current study, the hypoxic fetal guinea pig liver exhibits histopathological damage, increased lipid peroxidation, and DNA fragmentation.

Malondialdehyde is a by-product of lipid peroxidation generated in the presence of oxidant molecules such as superoxide anions and used to assess hypoxic stress and the effectiveness of antioxidants such as NAC. Oxidative stress generated by increased superoxide anions can directly cause DNA breaks,

which may contribute to the increased fragmentation levels. Additionally, there may be indirect effects of oxidative stress that alter the ability of DNA to repair DNA breaks. The protective effect of maternal NAC treatment on the hypoxic fetal liver is supportive of oxidant damage. In cultured fetal rat liver cells, exogenously added NAC, reduced glutathione (GSH), or L-cysteine inhibited the DNA fragmentation that occurs in the perinatal period in hemopoietic cells but not hepatocytes.⁵⁵ Yet, addition of superoxide dismutase or catalase enzymes that decrease superoxide anion and hydrogen peroxide accumulation, respectively, did not reverse the DNA damage in these same cells. Thus, antioxidant thiols have a protective role against hemopoietic cell injury, which may identify a cell-specific, and perhaps a ROS-specific, response to oxidative stress.

It is possible that NAC affects hypoxia signaling rather than reversing oxidative stress. This is supported by evidence in cultured cancer cells showing that NAC inhibits HIF-1 α expression.^{61,62} However, there is no direct evidence that NAC affects either hypoxia signaling or oxygen sensing (ie, chemoreceptor cells) in vivo in the fetus.⁶³ Lastly, while the direct effect of NAC on uteroplacental blood flow was not measured in this study, it is likely to be insignificant due to the persistence of fetal hypoxia in the presence of NAC administration.

In conclusion, this study identifies the fetal liver as sensitive and vulnerable to reduced oxygenation and that oxidative stress plays a causal role in fetuses exposed to chronic intrauterine hypoxia. This is mediated by impairment of cell membrane integrity by lipid peroxidation and DNA fragmentation. The impact of oxidative stress on fetal liver damage may have several consequences including contributing to fetal growth restriction and altering both the hemopoietic response to hypoxia⁶⁴ and the cellular integrity of the fetal liver. The protective effect of NAC confirms the damaging effects of oxidative stress in the hypoxic fetal liver and indicates that antioxidants may be a potential therapeutic approach for the prevention of subsequent fetal and neonatal morbidity associated with chronic intrauterine hypoxia.

Declaration of Conflicting Interests

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