Differential Effect of Intrauterine Hypoxia on Caspase 3 and DNA Fragmentation in Fetal Guinea Pig Hearts and Brains

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Abstract

The aim of this study is to quantify the effect of intrauterine hypoxia (HPX) and the role of nitric oxide (NO) on the apoptotic enzyme, caspase 3, and DNA fragmentation in fetal heart and brain. Hypoxia and NO are important regulators of apoptosis, although this has been little studied in the fetal organs. We investigated the effect of intrauterine HPX on apoptosis and the role of NO in both fetal hearts and brains. Pregnant guinea pigs were exposed to room temperature (N = 14) or 10.5% O₂ (N = 12) for 14 days prior to term (term = 65 days) and administered water or L-N6-(1-iminoethyl)-lysine (LNIL), an inducible nitric oxide synthase (iNOS) inhibitor, for 10 days. Fetal hearts and brains were excised from anesthetized near-term fetuses for study. Chronic HPX decreased pro- and active caspase 3, caspase 3 activity, and DNA fragmentation levels in fetal hearts compared with normoxic controls. L-N6-(1-iminoethyl)-lysine prevented the HPX-induced decrease in caspase 3 activity but did not alter DNA fragmentation levels. In contrast, chronic HPX increased both apoptotic indices in fetal brains, which were inhibited by LNIL. Thus, the effect of HPX on apoptosis differs between fetal organs, and NO may play an important role in modulating these effects.

Keywords

fetal, hypoxia, apoptosis, nitric oxide, heart, brain

Introduction

Chronic exposure to intrauterine hypoxia (HPX) can occur if the mother lives at high altitude, smokes, or develops a maternal illness such as preeclampsia, uterine ischemia, or placental dysfunction.^{1,2} Hypoxia can compromise cardiac function in the fetus by altering cardiac gene expression, increasing myocyte apoptosis, and inducing myocyte hypertrophy.³⁻⁵ Hypoxia has also been shown to be a potent inducer of programmed cell death, or apoptosis, in the heart.⁶⁻⁸ The impact of HPX stress on cardiac function is dependent on the duration and severity of HPX and the gestational age of the fetus.

Although nitric oxide (NO) has been shown to regulate cardiac apoptosis in the adult heart,⁹ the role of NO in the fetal heart has had limited study. We have previously shown that chronic intrauterine HPX increases inducible nitric oxide synthase (iNOS), messenger RNA (mRNA), and protein levels, as well as NO product (nitrate/nitrite levels) in fetal guinea pig heart ventricles.¹⁰ The HPX-induced increase in NO is attributed to transcriptional regulation of iNOS since the expression levels of endothelial nitric oxide synthase (eNOS) are decreased and neuronal nitric oxide synthase (nNOS) unchanged in fetal cardiac ventricles.¹¹ The functional role of iNOS-derived NO in the fetal heart remains unclear although it likely contributes to the contractile function and metabolic processes in cardiomyocytes. Nitric oxide has also been shown to both induce and inhibit myocardial apoptosis,

depending on the conditions of study.¹²⁻¹⁶ Some of the proapoptotic mechanisms induced by NO involve disruption of the mitochondrial membrane and upregulation of proapoptotic proteins.¹⁵ However, NO can also be antiapoptotic by inhibiting caspase activity, scavenging reactive oxygen species, and upregulating cytoprotective stress proteins.¹⁵

We hypothesize that chronic intrauterine hypoxia induces apoptosis in the fetal heart because of the HPX-induced increase in iNOS-derived NO levels in fetal guinea pig cardiac ventricles.¹⁰ To test this, we will quantify the effect of intrauterine HPX on cardiac apoptosis, by measuring protein and activity levels of the apoptotic enzyme, caspase 3, and DNA fragmentation in the fetal heart. This will be compared with the same apoptotic indices measured in fetal forebrains of the same animals. The role of HPX-induced NO on apoptosis will be studied by administering L-N6-(1-iminoethyl)-lysine (LNIL), a selective iNOS inhibitor, to the pregnant sows.

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Methods

Animal Model

Female Dunkin-Hartley guinea pigs (term = ~65 days) were purchased from a commercial breeder (Elm Hills, Chelmsford, Massachusetts) and time-mated within our facility. Pregnant sows were placed in room temperature (normoxia [NMX], N = 14) or in a HPX chamber (n = 12; 10.5% O₂ for 14 days) as previously described.¹⁰⁻¹¹ To inhibit iNOS-induced NO generation, LNIL was administered to NMX (N = 6) and HPX (N = 11) animals in their drinking water at a dose of 1 to 2 mg/kg per d for 10 days, 4 days after the placement of sows in the HPX chamber. L-N⁶-(1-iminoethyl)-lysine has been reported to inhibit the activity of iNOS at this dose, without affecting the activity of either eNOS or nNOS.^{17,18} In addition, this dose has previously been reported to reduce NO levels in fetal guinea pig cardiac ventricles under identical conditions as the present study.¹⁰ Food and water were supplied ad libitum and intake rates measured during the course of the experiment. At 60 days of gestation, pregnant sows were anesthetized (ketamine, 1 mg/kg; xylazine, 80 mg/kg), fetuses were removed via hysterotomy, and fetal body and organ weights of hearts, brains, and placentas measured. Left cardiac ventricles (apex of hearts) and forebrains (sectioned at temporal lobes) were excised and immediately frozen in liquid nitrogen and stored at -80°C. These methods were approved by the University of Maryland Animal Care Committee and conform to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health [NIH] Publication No. 85-23).

Western Blot Analysis of Caspase 3

Caspase 3 (32, 22, and 15 kDa) protein levels were quantified from NMX and HPX animals. Left ventricles of fetal hearts were frozen in liquid nitrogen, homogenized in ice-cold lysis buffer (Upstate, Billerica, Massachusetts) with protease and phosphatase inhibitors (Roche Molecular Biochemicals, Mannheim, Germany), placed on ice for 30 minutes, and spun at 13 000 rpm at 4°C for 10 minutes. Protein concentrations of the supernatant were analyzed by the Bradford Protein Assay (Bio-Rad Laboratories, Hercules, California). Equal amounts of protein (60 µg) of NMX and HPX fetal cardiac ventricles were loaded onto 7.5% Tris/glycine polyacrylamide gels and separated by gel electrophoresis. Dual-color standard markers (Bio-Rad Laboratories) were used to verify molecular mass in each gel. Proteins were transferred to Immun-Blot poly(vinylidene fluoride) (PVDF) membranes (Bio-Rad Laboratories), blocked in 5% nonfat dry milk for 2 hours, and probed overnight at 4°C. Membranes were incubated with a polyclonal antibody specific for caspase 3 (1:2000, Abcam, Cambridge, Massachusetts) and then with the second antibody (1:10 000, horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin [IgG]) after extensive washing. Protein bands were detected by ECL Western blotting Analysis System (Amersham, Piscataway, New Jersey). Each band was quantified by densitometry (GS-700 Imaging system, Bio-Rad Laboratories) and normalized to α -actin as a loading control.

Immunohistochemistry of Caspase 3

Fetal guinea pig hearts were excised and fixed in 10% formalin (Sigma, St Louis, Missouri) for immunohistochemistry of caspase 3. Hearts were dehydrated through ascending ethanol concentrations, paraffin embedded, and sectioned at 5 µm. Heart sections were deparaffinized, rehydrated through decreasing ethanol concentrations, and, in parallel, sections of each group were immunostained. Each of the sections was boiled in sodium citrate buffer (pH 6), treated with 0.3% H₂O₂, blocked with buffer containing normal goat serum (4%), and incubated separately with anti-caspase 3 (1:50; Cell Signaling, Danvers, Massachusetts) overnight at 4°C. Sections were incubated with biotinylated goat anti-rabbit antibody (1:10 000; Vector Laboratories, Burlingame, California). Avidin-biotin complex reagent (Vector Laboratories) was used in combination with the peroxides substrate solution composed of 10 mL Tris buffer, 2 mg 3,3'-diaminobenzidine (DAB)-tetrahydrochloride dihydrate, and 3% H₂O₂. Negative controls were generated in the absence of primary antibody.

Caspase 3 Activity Assay

Caspase 3 activity was measured in both fetal cardiac ventricles and forebrains using a Fluorometric assay (Sigma). Frozen left cardiac ventricles and forebrains (~20 mg) were homogenized in 100 µL of 1× lysis buffer (provided with assay) and centrifuged at 14 000 g for 15 minutes at 4°C. Using the manufacturer's instructions, 5 µL of sample plus 200 µL of reaction mixture were loaded onto a 96-well plate and read at an excitation wavelength of 360 nm and emission wavelength of 460 nm using a microplate reader (Synergy HT; Bio-Tek, Winooski, Vermont). 7-Amino-4-methylcoumarin (AMC), a caspase 3 substrate, fluoresces when active caspase 3 cleaves acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC). Using a standard curve, caspase 3 activity of samples was determined as picomoles AMC/min per milligram protein.

DNA Fragmentation Assay

DNA fragments are measured as an index of apoptosis using a Cell Death enzyme-linked immunosorbent assay ([ELISA]; Roche Molecular Biochemicals), which measures cytosolic mononucleosomes and oligonucleosomes (180 bp or multiples).¹⁹ Frozen tissue sections of fetal left cardiac ventricles and forebrains were homogenized in lysis buffer supplied in the Cell Death ELISA. DNA fragmentation was quantified in a 96-well plate and was loaded according to the manufacturer's instructions and read at 405 nm wavelength using a microplate reader (VersaMax; Molecular Devices, Sunnyvale, California). Results were calculated as optical density (OD)_{405 nm}/mg protein.

Statistics

Results are expressed as mean $\pm$ standard error of the mean (SEM). Comparisons between means of protein levels for

Figure 1. Western analysis of caspase 3 in normoxic ([NMX] N = 4) and hypoxic ([HPX] N = 4) left ventricles. Density values of each band were analyzed relative to its loading control (α -actin). Values are mean $\pm$ standard error. *Indicates $P < .05$ versus NMX. N indicates number of fetuses.

Western blot analysis were made using Student *t* test. Two-way analysis of variance (ANOVA) was applied for the analysis of differences between means of the 4 groups with HPX and drug treatment as independent variables. Student-Newman-Keuls post hoc test was performed to analyze the differences between treatments. $P < .05$ was used to determine statistical significance, and *n* values indicated the number of fetuses in each group.

Results

Fetal Weights and Food/Water Intake

Hypoxia significantly ($P < .05$) decreased fetal body weight (88.6 ± 6.1 vs 65.5 ± 5.4 g, NMX vs HPX) and increased ($P < .05$) relative brain weight (0.0296 ± 0.0011 vs 0.0381 ± 0.0020 , NMX vs HPX) and relative heart weight (0.0051 ± 0.0002 vs 0.0068 ± 0.0003 , NMX vs HPX). L-N6-(1-iminoethyl)-lysine decreased ($P < .05$) fetal body weight (88.6 ± 6.1 vs 70.4 ± 2.7 , NMX vs NMX LNIL) but had no significant effect on relative (organ weight/body weight ratio) placenta, heart, or brain weight in either NMX or HPX groups. Food and water intake rates over the course of the treatment were similar between NMX and HPX animals. L-N6-(1-iminoethyl)-lysine had no significant effect in either NMX or HPX animals.

Intrauterine HPX Decreases Caspase 3 Protein in the Fetal Heart

Caspase 3 is an effector caspase at which both the extrinsic and intrinsic apoptotic pathways converge. Caspase 3 protein consists of pro- (32 kDa) and active caspase 3 (22 and 15 kDa).

Figure 2. Immunohistochemistry of caspase 3 in normoxic (NMX) and hypoxic (HPX) fetal heart ventricles untreated (control) and in the presence of L-N6-(1-iminoethyl)-lysine (LNIL). Representative figures (magnification $\times 200$) illustrate caspase 3 expression (brown stain) and nuclei of cardiomyocytes (blue stain).

Hypoxia significantly ($P < .05$) decreased protein levels of both pro- and active caspase 3 (15 kDa) compared with NMX controls (Figure 1). There were no significant differences in protein levels of active caspase 3 (22 kDa) between NMX and HPX. Figure 2 illustrates the effect of HPX using immunohistochemistry of caspase 3. Caspase 3 expression was decreased in HPX compared with NMX fetal hearts. L-N6-(1-iminoethyl)-lysine reversed the HPX-induced effect but had no effect on NMX hearts compared with NMX alone.

Caspase 3 Activity of Fetal Hearts and Forebrains

Since there was a decrease in the caspase 3 protein, we tested whether HPX affected the catalytic activity of caspase 3 in a parallel manner. Caspase 3 activity was measured by a fluorometric enzyme activity assay in which degradation of the substrate, Ac-DEVD-AMC, is directly proportional to fluorescence. Figure 3 illustrates caspase 3 activity (picomoles AMC/minute per milligram protein) of NMX and HPX fetal left cardiac ventricles and fetal forebrains in guinea pigs with and without LNIL treatment.

Hypoxia significantly ($P < .05$) decreased caspase 3 activity in left cardiac ventricles (238.8 ± 17.9 pmol AMC/min per mg protein) by 37% compared with NMX controls (382.0 ± 55.1 pmol AMC/min per mg protein). In the presence of LNIL, caspase 3 activity was significantly greater ($P < .05$) than untreated HPX controls by 1.7-fold (410.1 ± 49.2 pmol AMC/min per mg protein). There were no significant differences between NMX alone and NMX $\pm$ LNIL left ventricles.

In fetal forebrains, HPX significantly ($P < .05$) increased caspase 3 activity (172.2 ± 2.8 pmol AMC/min per mg protein) by 30% compared with NMX controls (132.3 ± 9.9 pmol AMC/min per mg protein).

Figure 3. Effect of chronic hypoxia (HPX) and L-N6-(1-iminoethyl)-lysine (LNIL) on caspase 3 activity of fetal guinea pig cardiac ventricles (left) and forebrains (right). Activity levels were measured as picomoles AMC/minute per milligram protein in normoxic ([NMX] N = 4), hypoxic ([HPX] N = 5), and in the presence/absence of LNIL (NMX $\pm$ LNIL, N = 4; HPX $\pm$ LNIL, N = 4). Values are mean $\pm$ standard error. *Indicates $P < .05$ versus NMX. **Indicates $P < .05$ versus HPX. Fetal hearts and forebrains were obtained from the same animals. N indicates number of fetuses; AMC, 7-amino-4-methylcoumarin.

AMC/min per mg protein). In HPX animals with LNIL treatment, the caspase 3 activity was significantly ($P < .05$) decreased (129.3 ± 9.9 pmol AMC/min per mg protein) by 25% compared with untreated HPX controls. There were no significant differences between untreated and LNIL-treated NMX forebrains.

DNA Fragmentation of Fetal Hearts and Forebrains

DNA fragmentation assay measures the amount of mononucleosomes and oligonucleosomes as a result of cleaved DNA. Figure 4 illustrates DNA fragmentation ($OD_{405 \text{ nm}}/\text{mg protein}$) of NMX and HPX fetal left cardiac ventricles and forebrains of animals with and without LNIL treatment.

In fetal heart ventricles, HPX significantly ($P < .05$) decreased DNA fragmentation (0.077 ± 0.005 $OD_{405 \text{ nm}}/\text{mg protein}$) by 24% compared with NMX controls (0.101 ± 0.006 $OD_{405 \text{ nm}}/\text{mg protein}$). There was no difference in cardiac DNA fragmentation levels between NMX alone and NMX $\pm$ LNIL. L-N6-(1-iminoethyl)-lysine had no effect in altering the DNA fragmentation levels of HPX cardiac ventricles compared with HPX alone.

Contrary to fetal cardiac ventricles, HPX significantly ($P < .05$) increased DNA fragmentation (0.077 ± 0.007 $OD_{405 \text{ nm}}/\text{mg protein}$) of fetal forebrains compared with NMX controls (0.043 ± 0.007 $OD_{405 \text{ nm}}/\text{mg protein}$). L-N6-(1-iminoethyl)-lysine significantly ($P < .05$) decreased DNA fragmentation (0.021 ± 0.002 $OD_{405 \text{ nm}}/\text{mg protein}$) by 75% compared with HPX-untreated controls but had no significant effect under conditions of NMX.

Discussion

This study shows that intrauterine HPX activates the apoptotic pathway in an organ-specific manner. Chronic HPX decreased caspase 3 protein and activity levels as well as DNA fragmentation in the fetal cardiac ventricle but increased caspase 3 activity and DNA fragmentation in the fetal forebrain. L-N6-(1-iminoethyl)-lysine reversed the HPX-induced effect on caspase 3 activity in both fetal hearts and forebrains. L-N6-(1-iminoethyl)-lysine inhibited DNA fragmentation in the brain but had no effect in the heart. This suggests a difference between these organs on the role of NO as a regulating factor of apoptosis.

Hypoxic Regulation of Caspase 3 and DNA Fragmentation

The mechanism of HPX-activated apoptosis is associated with upregulation of proapoptotic proteins causing disruption of the mitochondrial membrane potential and stimulating cytochrome c release.¹⁹ The interaction of these proapoptotic factors leads to the cleavage and activation of caspase 3, resulting in downstream DNA fragmentation.¹⁹ The effect of HPX on apoptosis has been well characterized in the fetal brain²⁰⁻²² and associated with the activation of the effector²⁰ caspase, caspase 3. In the present study, HPX increased both caspase 3 activity and DNA fragmentation in the fetal forebrain, secondary responses to the activation of apoptotic mechanisms. The immature or fetal brain has been reported to have a higher apoptotic

Figure 4. Effect of chronic hypoxia (HPX) and L-N6-(1-iminoethyl)-lysine (LNIL) on DNA fragmentation of fetal guinea pig cardiac ventricles (left) and forebrains (right). DNA fragmentation was quantified as OD_{405 nm}/mg protein in normoxic ([NMX] N = 8), hypoxic ([HPX] N = 6), and in the presence/absence of LNIL (NMX ± LNIL, N = 5; HPX ± LNIL, N = 8). Values are mean ± standard error. *Indicates $P < .05$ versus NMX. **Indicates $P < .05$ versus HPX. Fetal hearts and forebrains were obtained from the same animals. N indicates number of fetuses. OD, optical density.

capacity compared to the adult,²³ rendering it more susceptible to HPX stress.²⁰⁻²² This is attributed to higher expression levels of factors in the apoptotic pathway such as caspase 3, proapoptotic factors (eg, Bax), and apoptotic-inducing factor (AIF).²³ Furthermore, NO synthesis, whether generated by nNOS or iNOS, has also been shown to contribute to brain injury by NO's inhibitory action on mitochondrial function leading to the activation of the intrinsic pathway (ie, cytochrome c release). Both NOS isoforms have been shown to be constitutively expressed at levels severalfold higher in fetal relative to postnatal brains,²⁴ rendering fetal brains susceptible to the multiple actions of increased NO levels. In the present study, the inhibitory effect of LNIL suggests that iNOS-derived NO contributed to the HPX-induced increase in both apoptotic indices, caspase 3 activity, and DNA fragmentation. Previous studies have also reported that administration of aminoguanidine,²⁵ an iNOS inhibitor, and 2-iminobiotin,^{24,26} a combined iNOS/nNOS inhibitor, decreased caspase 3 activation under conditions of HPX/ischemia and protected against brain injury. Furthermore, the inhibitory effect of LNIL on DNA fragmentation in the fetal forebrain is consistent with several studies in which a nonselective NOS inhibitor blocked HPX-induced apoptosis in the neonatal brain.²⁷⁻²⁹ In an identical animal model, LNIL has been shown to reduce HPX-induced apoptosis and neuronal loss in the fetal brain by downregulating iNOS mRNA.³⁰

In contrast, chronic HPX decreased rather than increased both the caspase 3 activity and DNA fragmentation in the fetal heart, suggesting an important role of iNOS-derived NO in apoptosis of HPX fetal hearts. L-N6-(1-iminoethyl)-lysine reversed the inhibitory effect of HPX on caspase 3 activity but

had no effect on DNA fragmentation. This suggests a complex regulation of HPX-induced apoptosis in the fetal heart that differs from fetal brain.

Previous studies have reported that HPX increases both caspase 3 protein/activity and DNA fragmentation in adult cardiac myocytes^{6,7} and in fetal rat hearts.⁸ In the latter study, fetuses from pregnant rats exposed to HPX conditions (10.5% O₂ for 6 days) exhibited increased levels of proapoptotic factors (Bax, Fas), caspase 3 expression, and DNA fragmentation,⁸ demonstrating HPX-stimulated apoptosis in fetal hearts. The differences between this and the current study are not clear. The paradoxical decrease in HPX-induced cardiac apoptosis measured in fetal guinea pig hearts may have several explanations that require further study. First, the present study examined a longer time course of 14 versus 6 days in the rats. As a result, there may be a temporal relationship with apoptosis that increases proapoptotic factors during the early time period of HPX but diminishes with prolonged exposure. This may be a protective mechanism against prolonged HPX that helps preserve the existing cardiomyocyte number. Second, the effect of HPX on inducing apoptosis in cardiomyocytes in fetal hearts may be related to differences in the age of maturation of the heart³¹ since fetal guinea pigs are precocial compared with immature fetal rats at near term. In addition, cardiomyocytes are reported to be resistant to apoptosis depending on the levels of pH, glucose, adenosine triphosphate (ATP), and NO which may vary between species, as well as maturational status of the heart.³¹ Lastly, studies using human fetal cardiac myocytes found that the human fetal heart was resistant to HPX-induced apoptosis.^{32,33} They reported a decrease in apoptotic

nuclei during HPX compared with rat cardiomyocytes³² or no change in gene expression of apoptotic proteins, Bcl-2 (anti) and Bax (pro).³³ The current study suggests that the fetal guinea pig response to HPX stress may be more similar to the human fetus than the adult human and fetal rat.

Nitric Oxide Regulation of Apoptosis

We have previously reported that chronic HPX increases both NO product (nitrate/nitrite) and cyclic guanosine monophosphate (cGMP) levels in fetal guinea pig heart ventricles and inhibited by maternal administration of LNIL, a selective iNOS inhibitor.¹⁰ The effect of LNIL treatment in the present study suggests that HPX-induced NO may play an important role in regulating apoptosis in fetal cardiac ventricles. Nitric oxide can regulate caspase 3 activity in both positive and negative manner.¹²⁻¹⁶ Since LNIL reversed the HPX-induced downregulation of caspase 3, this suggests an inhibitory effect of NO on caspase 3 activity in the fetal heart. Enzyme activity of caspase 3 has been reported to be negatively regulated by both nitrosylation and phosphorylation,³⁴⁻³⁹ both of which may occur under the conditions of our study.¹⁰ Thus, NO may inhibit caspase 3 activity by directly nitrosylating the catalytic site and/or by phosphorylating caspase 3 via cGMP/protein kinase G activation.

It is unclear why LNIL had no effect on DNA fragmentation in HPX fetal hearts while it prevented the decrease in caspase 3 activity. This suggests a dissociation between these actions^{40,41} and a difference in the role of NO in regulating these processes. Since the inhibitory effect of NO on caspase 3 activity is reversible with LNIL treatment, it is possible that changes in DNA fragmentation in the fetal heart are not temporally related to changes in caspase 3 activity. Alternatively, the cell types within the heart ventricle that express changes in caspase 3 activity may differ from those that exhibit DNA fragmentation.

Functional Consequence of Apoptosis in the Fetal Heart

The fetus responds to HPX stress by redistribution of cardiac output favoring the heart and brain over other peripheral organs. These are adaptive responses that contribute to organ protection in the presence of reduced oxygen levels. Yet, intrauterine HPX can cause irreversible damage to the fetal heart by a variety of mechanisms that may have long-term consequences postnatally. For example, offspring chronically exposed to HPX in utero have an increased risk of developing cardiovascular disease as an adult.⁵ Furthermore, hearts from offspring exposed to intrauterine HPX are more susceptible to cardiac injury in response to an ischemia/reperfusion challenge.^{42,43} Thus, in the present study, while it remains unknown whether the HPX-induced decrease in apoptosis in the fetal heart is cardioprotective or maladaptive, any changes in cardiomyocyte number, as a result of an increased or decreased apoptotic rate, could have sustained functional consequences in the offspring.^{44,45}

In summary, chronic intrauterine HPX both increases and decreases apoptosis in fetal guinea pig organs. Nitric oxide

plays an important role in regulating apoptosis in both HPX fetal hearts and brains. In the fetal guinea pig heart, the HPX-induced decrease in caspase 3 activity and DNA fragmentation may suggest conditions that prevent cardiomyocyte loss and be cardioprotective. On the other hand, any change in cell number (increase or decrease) may alter the normal heart growth pattern and contribute as a maladaptation, postnatally. In the fetal guinea pig brain, HPX increases apoptosis, which is mediated by NO synthesis. Further study is needed to identify the mechanisms by which NO acts as a positive or negative modulator of apoptosis in HPX fetal organs.

Authors' Note

The content is solely the responsibility of the authors and does not necessarily represent the official view of the National Heart, Lung and Blood Institute or the National Institutes of Health.

Declaration of Conflicting Interests

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