BASIC SCIENCE: OBSTETRICS

Decreased placental X-linked inhibitor of apoptosis protein in an ovine model of intrauterine growth restriction

Juan A. Arroyo, PhD; Russell V. Anthony, PhD; Henry L. Galan, MD

OBJECTIVE: The objective of the study was to assess placental apoptosis at both midgestation and near term in an ovine model of placental insufficiency (PI) and intrauterine growth restriction (IUGR).

STUDY DESIGN: At 40 days’ gestational age (dGA), 2 groups of 4 ewes were exposed to hyperthermic conditions for either 55 days or 80 days to induce IUGR with necropsies at 95 (midgestation) and 130 dGA (term = 140 dGA), respectively. Blood gases were assessed and placental tissues obtained for apoptosis analyses.

RESULTS: PI-IUGR pregnancies showed: (1) a decrease in fetal O2 saturation and pO2 (P < .04), (2) an increase in placental villi apoptosis (P < .05) at midgestation and near term, and (3) a decrease of cotyledon X-linked inhibitor of apoptosis protein (XIAP) at both gestational periods (P < .04) with no differences in caruncle XIAP protein.

CONCLUSION: Placental villous apoptosis is increased at midgestation and near term in our ovine model of IUGR, and this increase is associated with a significant decrease in XIAP protein in the cotyledon of IUGR animals.

Key words: ewe, hyperthermic, intrauterine growth restriction, midgestation, necropsy, placental apoptosis, placental insufficiency, X-linked inhibitor of apoptosis protein


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Normal placenta and placental development are critical for a successful pregnancy and mediate important steps such as implantation, immune protection of the fetus, maternal blood flow to the placenta, and delivery of nutrients to the fetus. Abnormal placenta and maternal adaptation may result in pregnancy wastage and complications later in pregnancy, such as preeclampsia and intrauterine growth restriction (IUGR), that are associated with long-term adverse sequelae for the newborn and adult.1-4 Programmed cell death, or apoptosis, is a component of normal development and differentiation in most tissues.5,6 This is an active process of cellular destruction that serves an essential function in multicellular organisms.7 Apoptosis is important during pregnancy, particularly during implantation and placentaation.8 Placenta of growth-restricted pregnancies have demonstrated a number of pathologic findings such as reduced syncytiotrophoblast surface area, increased thickness of the exchange barrier formed by the trophoblast and fetal capillary endothelium, and an increase in placental apoptosis at term.5,6,9-14

The inhibitors of apoptosis proteins (IAPs) are a family of proteins that regulate cell death.15-17 These proteins include the neuronal apoptosis inhibitor protein, X-linked inhibitor of apoptosis protein (XIAP), c-inhibitor of apoptosis-1 and -2, and survivin.17,18 XIAP is the most potent member of the group IAPs that regulate cell death.19 XIAP protects trophoblast cells from fas-mediated apoptosis, suggesting an important role for XIAP in the regulation of trophoblast apoptosis.20 This protein is also present in trophoblasts throughout placental development. Expression is significantly decreased near delivery when apoptosis is maximal, but little is known about apoptosis across gestation in pathologic pregnancies such as IUGR.21

We chose to study apoptosis in an ovine model of IUGR induced by hyperthermic (HT) exposure. This established model has numerous features characteristic of IUGR in humans, including asymmetric fetal growth and reduced placental mass, reduced uterine and umbilical blood flows, abnormal umbilical arterial and aortic Doppler velocimetry, and many others.22-26 The process of placental apoptosis has not been evaluated in this model, and because placental weight is reduced at both midgestation and near term in our ovine IUGR model, we hypothesize that hyperthermic exposure early in ovine pregnancy disrupts fetal and placental development and increases apoptosis in the placental villi at midgestation, as well as near term in this model.

We further hypothesize that with an increase in villous apoptosis, there will be a concomitant decrease in the antiapoptotic molecule XIAP, the expression of which also remains unknown. To test our hypotheses, apoptosis was examined in sheep whole placentomes by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end la-
bouillet) ewes with time-dated singleton of 16 mixed-breed (Columbia-Ram) Animal Care and Use Committee. A total of 20 pregnant ewes were used in this study and equally divided into 2 groups based on gestational age at necropsy. In group 1 (Gp1), 4 ewes were housed in an environmental chamber for 55 days beginning at 40 dGA (term = 147 days), and 4 ewes were housed at ambient temperature (20 ± 2°C) to serve as controls. Gp1 animals were killed at 95 dGA.

In group 2 (Gp2), 4 ewes were exposed to HT conditions for 80 days and were removed to control conditions at approximately 120 days’ gestation. Ewes were removed from the environmental chamber at 120 days of gestation after 80 days of exposure to minimize fetal deaths. Four ewes were kept at ambient temperature for 130 dGA to use as controls. All animals from Gp2 were killed at 130 dGA (near term).

**Materials and Methods**

**Animal care**

This study was approved by the University of Colorado Health Sciences Center Animal Care and Use Committee. A total of 16 mixed-breed (Columbia-Rambouillet) ewes with time-dated singleton pregnancies were used in this study and equally divided into 2 groups based on gestational age at necropsy. In group 1 (Gp1), 4 ewes were housed in an environmental chamber for 55 days beginning at 40 dGA (term = 147 days), and 4 ewes were housed at ambient temperature (20 ± 2°C) to serve as controls. Gp1 animals were killed at 95 dGA.

In group 2 (Gp2), 4 ewes were exposed to HT conditions for 80 days and were removed to control conditions at approximately 120 days’ gestation. Ewes were removed from the environmental chamber at 120 days of gestation after 80 days of exposure to minimize fetal deaths. Four ewes were kept at ambient temperature for 130 dGA to use as controls. All animals from Gp2 were killed at 130 dGA (near term).

**TUNEL**

TUNEL was performed on paraffin-embedded whole placental sections. The TUNEL protocol was followed as suggested by the manufacturer (Chemicon, Inc, Temecula, CA). Briefly, slides were dewaxed with 100% xylene. Tissue slides were postfixed using a solution of ethanol:acetic acid (2:1) for 5 minutes. The equilibration buffer was added directly to the tissue slide for 10 seconds followed by incubation with the deoxynucleotidyl transferase enzyme for 1 hour at 37°C. Following the enzyme treatment, the antidigoxigenin conjugate was incubated on the slide for 30 minutes. 4’,6-diamidino-2-phenylindole,dihydrochloride was used for nuclear staining in our slides followed by mounting with a glass coverslip. Slides were viewed using fluorescein excitation and emission filters.

Microscopic analysis was performed in 2 cotyledons per animal (3 controls; 3 IUGR). To ensure homogeneity of sampling, the microscopic fields were assessed for apoptosis by counts performed in a vertical fashion of sequential fields from the cotyledon depression to the caruncular surface. This was re-

All ewes were pair fed and offered water ad libitum. The environmental conditions to which the ewes were exposed are similar to that previously described and consisted of the following: (1) temperature maintained at 40°C for 12 hours during the day and decreased to 35°C at night; and (2) humidity was kept between 35% and 40%. Prior to necropsy, umbilical vein blood was sampled for blood gas analysis using the ABL 520 analyzer (Radiometer America, Inc, Westlake, OH).

At the time the animals were killed, fetal and placental weights were recorded. The placentomes were separated using forceps into cotyledon (fetal) and caruncle (maternal) components, which were frozen in liquid nitrogen for Western blot analysis. The midsections of placentomes were obtained across the central depression of the cotyledon to the caruncule side of the placentome, placed in 10% formalin and paraffin-embedded for histology and immunolocalization studies.

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This study was approved by the University of Colorado Health Sciences Center Animal Care and Use Committee. A total of 16 mixed-breed (Columbia-Rambouillet) ewes with time-dated singleton pregnancies were used in this study and equally divided into 2 groups based on gestational age at necropsy. In group 1 (Gp1), 4 ewes were housed in an environmental chamber for 55 days beginning at 40 dGA (term = 147 days), and 4 ewes were housed at ambient temperature (20 ± 2°C) to serve as controls. Gp1 animals were killed at 95 dGA.

In group 2 (Gp2), 4 ewes were exposed to HT conditions for 80 days and were removed to control conditions at approximately 120 days’ gestation. Ewes were removed from the environmental chamber at 120 days of gestation after 80 days of exposure to minimize fetal deaths. Four ewes were kept at ambient temperature for 130 dGA to use as controls. All animals from Gp2 were killed at 130 dGA (near term).

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| TABLE |
|---|---|---|
| **Fetal blood gas data of IUGR and control animals** | **Control** | **IUGR** | **P value** |
| **Blood gas data, midgestation** | | | |
| pH | 7.26 ± 0.02 | 7.17 ± 0.05 | .117 |
| pCO₂ (mm Hg) | 61.93 ± 2.06 | 68.04 ± 7.13 | .302 |
| pO₂ (mm Hg) | 48.63 ± 15.8 | 23.23 ± 2.3 | .018 |
| O₂ saturation (%) | 55.3 ± 0.061 | 38.1 ± 0.047 | .035 |
| **Blood gas data, near term** | | | |
| pH | 7.37 ± 0.01 | 7.4 ± 0.05 | .161 |
| pCO₂ | 45.9 ± 4.61 | 50.98 ± 3.95 | .06 |
| pO₂ (mm Hg) | 18.9 ± 1.47 | 13.9 ± 1.9 | .001 |
| O₂ saturation (%) | 52.2 ± 7.03 | 33.05 ± 10.98 | .007 |

peated in juxtaposed fields until 20-30 fields were counted. There was no attempt to differentiate the degree of apoptosis within the areas of the cotyledon between the central depression and the caruncular layer. For graphical purposes, the percent apoptosis was calculated in the placentomes as the number of TUNEL-positive cells divided by the total number of cells in 20-30 fields.

**Deoxyribonucleic acid (DNA) fragmentation analysis**

The DNA degradation protocol was followed as suggested by the manufacturer (R&D Systems, Minneapolis, MN). Briefly, 0.1 g of ground frozen midgestation cotyledon tissue was resuspended in 200 μL of sample buffer for each sample. To this, 20 μL of 10 × tissue buffer was added and samples were incubated at 50°C for 12-18 hours. Following this, 100 μL of lysis solution 1 was added to 100 μL of the tissue suspension and samples were mixed. A volume of 700 μL of extraction solution 2 was added to the samples followed by the addition of 400 μL of extraction buffer 3. Samples were vortexed and centrifuged at 12,000 g for 5 minutes.

The upper (aqueous) layer was transferred to a new microcentrifuge tube, and 0.1 volume of sodium acetate 4 was added to the aqueous DNA samples. To the total volume in the microcentrifuge tube, an equal volume of 2-propanol was added and mixed. Samples were centrifuged at 12,000 g for 10 minutes and the supernatants were removed and discarded without disturbing the DNA pellet.

Pellets were washed with 1 mL of 70% ethanol and centrifuged at 12,000 × g for 5 minutes once more. Supernatants were removed and the pellets were dried by inverting the tube on a laboratory tissue. DNA pellets were resuspended in 100 μL of DNase-free water and quantified in a spectrophotometer. To 0.1 μg/μL of...
DNA, 2 μL of gel-loading buffer was added and samples were loaded onto a 1.5% TreviGel 500 gel. Gel was stained for 15 minutes in 0.5 g/mL ethidium bromide, and DNA was visualized using an ultraviolet transilluminator.

**Western blot analysis**

Cotyledonary and caruncular tissues were homogenized in protein lysis buffer (10 mM of phenylmethylsulfonyl fluoride, 10 mM of Na3VO4,1/100 Triton TX-100, 150 mM NaCl, 20 mM Tris base, 5 M of 4-(2-aminoethyl)benzene sulfonyl fluoride, 5 M of EDTA, 10 nM of leupeptin, and 10 ng/mL of apro tinin). Protein tissue lysates (50 μg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were incubated with an antibody against mouse XIAP (at a dilution of 1:200; Transduction Laboratories, Lexington, KY). A secondary antimouse immunoglobin (Ig)-horseradish peroxidase antibody (dilution 1:10,000); Upstate Cell Signaling Solutions, Lake Placid, NY) was incubated for 1 hour at room temperature.

The membranes were incubated with chemiluminescent substrate (Pierce, Rockford, IL) for 5 minutes and the emission of light was digitally recorded by using a charge-coupled device camera. To determine loading consistencies, each membrane was stripped of antibodies and reprobed utilizing antibody against mouse beta-actin (dilution 1:4,000; MP Biomedicals, Aurora, OH) to determine the amount of total protein present in each lane. Presence of these proteins was confirmed by densitometry and quantified. Results were compared with the untreated controls.

**Immunohistochemistry**

Immunohistochemistry (IHC) was performed on paraffin-embedded whole placentome sections. Sections were dewaxed with 100% xylene. Sections were blocked for 45 minutes with a biotin-labeled antimouse secondary antibody. Slides were washed in 1× PBS and incubated in streptavidin-biotin-horseradish peroxidase solution and developed with diaminobenzidine (DAB) or NovaRED using the Vectastain ABC, DAB, and NovaRED kit (Vector Laboratories, Inc, Burlingame, CA). NovaRED was used to label the cytokeratin-positive cells, and DAB was used to stain for the XIAP-positive cells in a serial placentome section. Hematoxylin was used for nuclear counterstaining. Slides were mounted using Permount mounting media.

**Statistical analysis**

Data are shown as mean ± SE and a P value of < .05 was considered significant for the statistical comparisons that follow. Comparisons between control and IUGR groups using a rank sum test (Mann-Whitney U test) were made for the following: fetal and placental weights, TUNEL-positive cell ratio to all cells, blood gas values, and XIAP Western blot analysis. For comparison between study groups for the number of microscopic fields showing apoptosis by immunofluorescence, the f-test was used to assess equality of variance. This showed the variance to be equal, thus, the t test assuming equal variance was used to assess for differences in apoptosis between groups. Differences between groups were determined using student’s t test with P < .05 considered significant.
**RESULTS**

HT-exposed sheep showed a significant decrease in placental weight (2.4-fold; 440 ± 50 vs 186 ± 18; P < .004) but not fetal weight at midgestation (Gp1; see Figure 1). In contrast, the HT sheep in the near-term studies (Gp2) showed a significant decrease for both placental (2.0-fold; 348.7 ± 21.02 vs 168.7 ± 43.2; P ≤ .004) and fetal (1.8-fold; 2914 ± 201 g vs 1718 ± 433 g; P ≤ .008) weights. At both gestational time periods (95 and 130 dGA), there was a significant decrease in umbilical vein O₂ saturation (P < .04) and pO₂ (P < .03) associated with IUGR pregnancies (Table). There were no pregnancy losses in our studies.

For the TUNEL studies, approximately 300 microscopic fields were available for analysis (HT group = 147 fields; control group = 156 fields). The TUNEL assay showed a significant increase in apoptosis (4-fold) during hyperthermia at midgestation in the villi of the sheep. A representative picture for TUNEL-positive apoptotic cells is shown in Figure 2, A for the Gp1 midgestation studies. Similar results were observed for apoptosis (2.4-fold increase) for Gp2 (near-term) HT sheep. A representative picture for the TUNEL assay at this point is shown in Figure 2, B. To confirm that apoptosis is occurring as an early event in the cotyledons, DNA degradation and cleavage of cytokeratin 18 (M30) was performed. DNA degradation showed an increase in DNA laddering during hyperthermia in the sheep (Figure 3). Immunohistochemistry for M30 showed the presence of cytokeratin 18 cleavage in treated animals (Figure 4).

XIAP protein was significantly decreased at both midgestation (1.7-fold) and near term (2.4-fold) in the cotyledons of HT-treated animals (Figure 5). In contrast, caruncle XIAP protein content was similar between treatment groups at both midgestation and near term in the sheep (Figure 6).

Figure 7 shows localization of XIAP protein in the placentome of treated and control animals (Figure 7, A and B). XIAP was colocalized to the cytokeratin-positive cells in the villi of the ovine placentome (Figure 7, C and D). In these immunohistochemistry studies, NovoRED identifies cytokeratin positive cells, indicating the trophoblast origin, and in a serial section using DAB, XIAP protein is colocalized to the cytokeratin-positive cells. XIAP staining was found primarily in the trophoblast cells.

Correlations between cotyledon XIAP concentration and oxygen saturation between treatment groups at both 95 and 130 dGA were observed (Figure 5).

**FIGURE 5**

HT and control cotyledon XIAP protein at midgestation and near term

<table>
<thead>
<tr>
<th>95 dGA</th>
<th>130 dGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>IUGR</td>
</tr>
<tr>
<td>XIAP (54 kDa)</td>
<td></td>
</tr>
<tr>
<td>ACTIN</td>
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A 1.7-fold and 2.4-fold decrease was observed for XIAP protein in the cotyledon of HT IUGR animals during midgestation and near term, respectively.

**FIGURE 6**

HT and control caruncle XIAP protein at midgestation and near term

<table>
<thead>
<tr>
<th>95 dGA</th>
<th>130 dGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>IUGR</td>
</tr>
<tr>
<td>XIAP (64 kDa)</td>
<td></td>
</tr>
<tr>
<td>Actin</td>
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</table>

XIAP protein did not change during midgestation or near term in the caruncle of treated animals vs controls.
130 dGA is shown in Figure 8. At 95 dGA, a strong relationship between oxygen saturation and XIAP protein concentration was found for the control group, but the opposite was seen in the HT animals. At 130 dGA, the HT group also showed a strong inverse relationship between oxygen saturation and XIAP protein levels.

**COMMENT**

Compared with control pregnancies, we observed that placentome apoptosis was increased in the villous layer of hyperthermic exposed pregnancies at both midgestation and near term. The near-term apoptotic result in our study is consistent with other studies showing an increase in placental apoptosis shown by TUNEL assay at term during human IUGR. Interestingly, increased villous apoptosis was also observed at midgestation during IUGR in this model.

To understand the apoptotic mechanism associated with this increase in apoptosis, XIAP protein levels were determined in the cotyledon and caruncle of treated and control animals. We found that XIAP protein expression in the cotyledon was significantly decreased in HT treated animals as compared with controls for both gestational time periods. In contrast, there were no differences observed between treatment groups for caruncle XIAP protein at these time points. In addition, IHC experiments showed that XIAP was localized to the villous trophoblast of the placentome, suggesting that the protective effect of this protein is preferentially expressed in the very metabolically active trophoblast cells. Umbilical vein cord gases demonstrated that the placental circulation is hypoxic at both mid- and late gestation.

We chose to discuss and assess blood tensions in the umbilical vein because this reflects blood coming directly from the placenta. These data suggest that the growth-restricted placentae in this model of IUGR already transfer oxygen poorly. The apoptotic process, which is active at midgestation, may contribute to the poor trophoblast transfer function. However, the converse of this may also be true in that hypoxia is known to induce apoptosis in these cells.

When correlating oxygenation data with XIAP concentrations at 95 dGA, a strong positive correlation was noted in control pregnancies. We speculate that under normal conditions, higher oxygen levels allow for normal XIAP concentrations. In contrast, we observed XIAP concentrations to be inversely correlated with oxygen saturation levels in HT IUGR pregnancies at both 95 and 130 dGA. This association is opposite to that seen in controls and suggests that the hyperthermic process and associated hypoxia do not allow for the normal inhibitory activity on apoptosis by XIAP, resulting in an early increase in apoptosis. Although the number of animals for this correlation is small, the findings and implications are interesting, and in vitro studies are in progress to confirm these effects of hypoxia on XIAP.

Insufficient or excessive apoptosis can contribute to pathological conditions such as cancer, autoimmune deficiency syndrome, and autoimmune disease. Cell death or apoptosis had been shown to be present in the placenta during gestation, suggesting a role for apoptosis during normal pregnancy. Trophoblasts are specialized epithelial cells that are critical for a successful pregnancy. These cells have specialized functions that facilitate the exchange of nutrients and wastes between maternal and fetal compartments. Aberrant trophoblast function and apoptosis are associated with clinical obstetric pathology such as that observed in pregnancies with isolated IUGR and in pregnancies associated with both IUGR and preeclampsia. Increased trophoblast apoptosis is associated with IUGR in humans at term.

Results from the current study, including the TUNEL assay, DNA degradation, and cleavage of cytokeratin 18 suggest that apoptosis in the placenta occurs as a much earlier event than has
been previously described in IUGR. We speculate that the increase in apoptosis could be a factor in the decreased placental weight observed in our model. Interestingly, at midgestation there were no differences in fetal weights, whereas a significant reduction in fetal weight was observed near term. In contrast, placental weight showed to be reduced at both midgestation and near term.

This finding is also seen in several animal models of IUGR. For example, in the rat dietary restriction model by Ozaki et al.34 there was no significant decrease in fetal weight by day 20 of gestation, but growth restriction was present at birth. Similar results have been described in the guinea pig IUGR model with uterine artery ligation.35 The authors specified that placental weight was reduced prior to the fetal weight decrease observed at near term. In an insulin-like growth factor-II–inactive IUGR model, placental weight was constantly decreased through mid and late gestation, whereas fetal growth restriction was seen only toward the end of gestation.36

Collectively, these results suggest that decreased placental weight at midgestation precedes decreased fetal weight seen later in pregnancy. We found that placental apoptosis preceded the decreased fetal weight observed in this model of IUGR, and this may partly be responsible for the decrease in placental weight at midgestation in this model and others described above. We speculate that the increase in midgestation cotyledon apoptosis may result in placental functional changes that fail to meet the fetal demands required for normal growth, particularly because the fetus just begins to enter the slope of maximal growth at this gestational age. The insufficient placental nutrient transfer, previously described in this model,22 subsequently leads to reduced fetal weight in late gestation.

In summary, the current study shows that apoptosis is increased in the cotyledon, which is seen in the villous layer of the placenta with no changes observed in the caruncle tissues. This suggests that hyperthermia has a preferential impact on the fetal side of the placenta and, more specifically, the villous trophoblast. In addition, XIAP protein expression is decreased in the cotyledon at both midgestation and near term in this model of IUGR, and it is localized to the villous trophoblast in this tissue. Thus, we speculate that a possible mechanism for the increased apoptosis observed in the placenta of treated animals is secondary to a decrease in XIAP expression in the cotyledon of treated animals as compared with controls. To our knowledge this is the first report to show a decrease in XIAP protein associated with an increase in placental apoptosis during IUGR in animal or human studies. Further mechanistic studies are needed to determine the role of XIAP in the activation of caspases 3 and 9 in this model of IUGR in the sheep.

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