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Increased placental XIAP and caspase 3 is associated with increased placental apoptosis in a baboon model of maternal nutrient reduction (MNR)

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Abstract

Objective—Our objective was to determine signaling molecules and apoptosis rate in the term placenta of a baboon model of maternal nutrient reduction (MNR).

Study Design—Female baboons were fed *ad libitum* for controls (CTR; n=7) or 70% of CTR diet (MNR; n=6) from 30 to 165 days of gestation (dG) with necropsy at 165 dG. Placental tissues were collected, fixed for immunohistochemistry or snap frozen to measure ERK, AKT, JNK, XIAP and caspase 3. Placental villous apoptosis was determined by TUNEL and cytokeratin 18 cleavage.

Results—Compared to CTR, MNR placentas demonstrated: reduced placental weight (p<0.02), decreased p-ERK (p<0.04), increase placental villous apoptosis (p<0.001), increased villous cytokeratin 18 cleavage, increased XIAP protein (p<0.007) and increased active caspase 3 (p<0.02).

Conclusion—We conclude that placental apoptosis is increased in this baboon model of MNR at term and that the increase in XIAP may be a protective mechanism against this apoptosis.

Keywords

Apoptosis; XIAP; Caspase 3; placenta; MNR

Introduction

Placental development is important for fetal growth and development. Abnormal placentation is associated with the development of complicated pregnancies in humans. Poor maternal nutrition is a common problem in the developing world, which can have many adverse effects on the fetus including intrauterine growth restriction (IUGR).¹ A higher degree of syncytiotrophoblast apoptosis is found in placentas from pregnancies complicated by fetal growth restriction and pre-eclampsia.^{2–5} Apoptosis, an active process of cellular destruction,

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serves an essential remodeling function in multi-cellular organisms and is a component of normal development and differentiation in most tissues including the placenta. ^{6, 7} Abnormal regulation of apoptosis in different tissues has been implicated in the onset and progression of a broad range of diseases. ²

The X-linked inhibitor of apoptosis protein (XIAP) is the most potent member of a group of inhibitor of apoptosis proteins (IAPs) that regulate cell death. ⁴ IAP possesses a defining structural motif, called Baculovirus Inhibitor of Apoptosis Repeat (BIR), which is responsible for the inhibition of caspases 3, 7 and 9. ⁸ Caspases are a family of cysteine proteases that play a central role in initiating and executing the apoptosis cascade. ^{6, 9} In trophoblast cells, caspases are known to cleave the cytokeratin 18 protein which produces a neo-peptide that is used as a marker of apoptosis. ¹⁰ Controlling the activity of caspases is essential for the appropriate execution of cell death and the regulation of cell survival. XIAP is present in trophoblast throughout placental development, but expression is significantly decreased near delivery when apoptosis is maximal. ¹¹ A decrease in XIAP protein is associated with the presence of increased apoptosis during IUGR in the pregnant sheep. ¹² This suggests a role for XIAP in the regulation of trophoblast apoptosis in normal and abnormal processes in pregnancy.

Signal transduction cascades serve to elicit a cellular response initiated at the cell surface. These cascades contain multiple components involving series of phosphorylation steps. The extracellular signal-regulated kinase 1/2 (ERK1/2) and the protein kinase B (AKT) pathways are found in many cell types and play various roles. These proteins mediate biological responses like cellular growth, proliferation and cell survival. ¹³ The c-Jun Amino Terminal Kinase (JNK) is another signaling molecule that is activated to diverse stimuli including DNA damage, UV radiation, heat shock and others. ¹⁴ There is a reduction of these signaling proteins in other models of IUGR. ¹⁵

While there are many studies on the effects of controlled reductions in maternal nutrient intake on apoptosis in rodents and sheep, data are lacking in non-human primates. ^{16–19} In the present study, we evaluated placental apoptosis and changes in key signaling molecules in the placentas of control pregnant baboons fed *ad libitum* and mothers exposed to nutrient reduction (MNR) fed 70 % of the feed consumed by the controls. Previous studies on the placentas showed that MNR decreased placental size near-term when villous volume and surface area, capillary surface area, and the villous isomorphous coefficient were all decreased and intervillous space hydraulic diameter was increased. ^{1, 20}

The pregnant baboon presents several advantages for the study of effects of maternal nutrition on placental and fetal growth compared with other non-human primates including fetal size, and similarities in placentation and placental structure with those of human pregnancy. ^{1, 16} We hypothesized that maternal nutrient reduction would increase placental villous apoptosis and decrease abundance of XIAP at the end of gestation. Since this model is associated with a decrease in placental size, we further hypothesized an increase in cytokeratin 18 cleavage and active caspase 3 in placentas from MNR mothers. We also sought to determine changes in cell signaling proteins associated with cell proliferation (ERK), cell survival (AKT) and cell stress (JNK) in the placenta of controls and MNR animals.

Materials and Methods

Animal care

All procedures were approved by the Southwest Foundation for Biomedical Research (SFBR) Institutional Animal Care and Use Committee and conducted in AAALAC-approved facilities. Animal care was done as described by Schlabritz-Loutsevitch et al. (2007). ^{1, 16} Briefly, the subjects of this study were 13 female baboons aged 8–15 years maintained in group housing

and bred as previously described in detail. 1· 16 Baboons were observed twice a day for wellbeing and three times a week for turgescence (sex skin swelling) and signs of vaginal bleeding to enable timing of pregnancy. ¹ Pregnancy was dated initially by observing the changes in the swelling of the sex skin and confirmed at 30 days of gestation by ultrasonography. Animals (*Papio* sp.) were fed Purina Monkey Diet 5038 (Purina, St. Louis, MO). Diet was closely monitored in seven animals fed *ad libitum* (control group) and their food intake calculated weekly on a per kilogram basis. From confirmation of pregnancy, the six pregnant baboons in the MNR group received 70% of the average daily amount of feed eaten (on a weight-adjusted basis) by control (CTR) animals at the same gestational age. Cesarean sections were performed at 165.9±1.1 days of gestation (dG) in CTR and 162.1±4.6 dG in MNR animals. Placental tissues were obtained from a central area of a cotyledon and fixed in formalin (10% buffered formalin), and embedded in paraffin for immunohistochemistry or snap frozen in liquid nitrogen for protein studies.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)

Placental sections were used for these experiments. TUNEL protocol was followed as suggested by the manufacturer (Chemicon, Inc.). Briefly, slides were dewaxed and post-fixed using a solution of ethanol:acetic Acid (2:1) for 5 min. The equilibration buffer was added to the slide for 10 sec followed by incubation with the TdT enzyme for 1hr at 37°C. Following this, the anti-digoxigenin conjugate was incubated on the slide for 30 min and 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) was used for nuclear staining. Slides were viewed using fluorescein excitation and emission filters. Analysis was performed in 2 placental slides per animal (3 controls; 3 MNR, selected randomly) and 20–30 fields were counted per slide with a mean count generated per slide for analysis purposes. The percent apoptosis was calculated in the placental slides (12 slides total) as the number of TUNEL positive cells divided by the total number of cells in 20 to 30 fields × 100.

Western blot analysis

Western blot was performed as previously described by Arroyo et al., (2008). ¹² Briefly, frozen placental tissues were homogenized and protein tissue lysates (50µg) were separated on 4–12% Bis-Tris gel SDS-PAGE 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) or antibodies against rabbit active (cleaved) caspase 3, caspase 3, phospho-AKT, total AKT, phospho-ERK or total ERK, and phospho-JNK or total JNK (all at 1:500; Cell Signaling Technology, Dancers, MA). A secondary anti-mouse or anti-rabbit Ig-horseradish peroxidase (HRP) antibody (dilution 1:10,000) (Upstate Cell Signaling solutions, Lake Placid, NY) was incubated for 1 hr at room temperature. The membranes were incubated with ECL substrate (Amersham, Princeton, NJ) for 5 min and the emission of light was detected using x-ray film. To determine loading consistencies, each membrane was stripped of antibodies and reprobbed utilizing an antibody against mouse beta-actin at a dilution of 1:4,000 (MP Biomedicals, Aurora Ohio). Presence of these proteins was confirmed and quantified.

M30 Cytodeath Immunohistochemistry (IHC)

IHC was performed on paraffin-embedded placental sections. Slides were de-waxed with 100% xylene. Slides were washed in PBS and sections were blocked for 1 hr using 10% normal goat serum / PBS. Slides were then incubated for 1 hr with a mouse monoclonal primary antibody against M30 Cytodeath (the neo-peptide produced from cytokeratin 18 cleavage; dilution 1:200) (Roche, Indianapolis, IN) or a mouse IgG1 (dilution of 1:500) for negative control. Sections were washed in 1X PBS. Sections were then incubated for 45 min with a biotin-labeled anti-Mouse secondary antibody. Slides were washed in 1X PBS, incubated in streptavidin-biotin-horseradish peroxidase solution, and developed with diaminobenzidine (DAB)

Vectastain ABC DAB kit (Vector Laboratories, Inc., Burlingame, CA). DAB (brown) was used to stain for the M30 positive cells in serial placental sections. Hematoxylin was used for nuclear counterstaining. Slides were mounted using Permount mounting media.

Statistical analysis

Comparisons were made between control and MNR groups using a rank sum test (Mann-Whitney U test) for the following: fetal and placental weights, and XIAP, caspase 3, ERK and AKT western blot analysis. For TUNEL assay, comparison of mean number of apoptotic cell counts per slide between both groups (n=5) slides per group were checked for normality followed by student's t-test. Data are shown as mean \pm SE and a P value of <0.05 was considered significant for the statistical comparisons.

Results

MNR baboons showed a significant decrease in placental weight ($177\text{g}\pm 8$ vs. $145\text{g}\pm 7$; $p<0.02$) and mean fetal weight decreased by 7% ($744\text{g}\pm 32$ vs. $669\text{g}\pm 33$; $p=0.1$) at 165 dG (Figure 1). In our study, there were no pregnancy miscarriages or fetal demises, fetal growth restriction or fetal abnormalities.

Placental apoptosis

For the TUNEL studies, approximately 265 microscopic fields were available for analysis (MNR group = 120 fields; control group = 145 fields). Figure 2 demonstrates the difference in apoptosis achieved by TUNEL staining in A) placenta of control *ad libitum* fed mother and B) placenta of mother in the nutrient reduction group. It can be seen (Fig. 2C) that the percentage of apoptosis increased by approximately 100% in the presence of maternal nutrient reduction. Immunofluorescence was performed to identify trophoblast cells depicted in red in Figure 3 A. Figure 3 shows the presence of M30 in negative control (Panel B), treated (Panel D) and control animals (Panel C). Panel A depicts immunofluorescence staining for pan-cytokeratin to identify the trophoblast cells. Immunohistochemistry for M30 showed an increased cytokeratin 18 cleavage in the trophoblast of placentas from mothers in the MNR group (3D) compared to controls (Figure 3C).

Placental XIAP and caspase 3 proteins

XIAP protein was significantly increased at 165 dG (1.5-fold) in the placenta of mothers in the nutrient reduction group (Figure 4). Similarly, active caspase 3 protein (5.8-fold) was also increased in the placenta of mothers in the nutrient reduction group (Figure 5).

ERK AKT and JNK

pERK protein increased 10.0-fold ($p<0.04$; Figure 6 A) while neither pAKT (Figure 6 B) nor pJNK (Figure 6 C) show differences in the placentas of the two groups.

Comments

The baboon model of MNR is characterized by a decrease in villous number and volume and a decrease in overall placental size near-term. ^{1, 16} We found that the smaller placentas of the MNR baboon group were characterized by an increase in apoptosis (TUNEL studies), which was further validated, by a qualitative increase in M30 from the IHC studies. To further elucidate the underlying mechanisms of apoptosis, XIAP and caspase 3 protein levels were determined in the placenta of MNR and control animals. XIAP protein was significantly increased in MNR treated animals as compared to controls. Similarly, active caspase 3 was

also increased in the placenta of MNR animals. Suggesting that the increase in XIAP protein could be a result of the increased apoptosis found at this point.

Cell death or apoptosis is normally present during cell growth and in the development of tissues, including the placenta.^{21, 22} Increased trophoblast apoptosis is present in clinical obstetrics pathology such as preeclampsia and intrauterine growth restriction.^{2, 7, 12, 23, 24} Trophoblast are epithelial cells that facilitate the exchange of nutrients and wastes between maternal and fetal compartments. Proper functioning of these cells is critical for placental development leading to a successful pregnancy. Results from the current study, including the TUNEL assay, and cleavage of cytokeratin 18, indicate that apoptosis is present in the placenta in this baboon model of MNR and that is greater than in the matched controls. This may contribute to the decreased placental weight observed in our model. Interestingly, the decrease in weight seen in the placenta was not seen in the fetus between groups near-term. Although unexpected, this finding has been described in other animal models of MNR. Ozaki et al showed that there was no significant decrease in fetal weight by day 20 of gestation, but growth restriction was present at birth in the rat dietary restriction model.²⁵ Collectively, these results suggest that decreased placental weight and apoptosis does not affect fetal size near-term in this baboon model of MNR although it remains to be seen if fetal organ systems are impacted by these placental changes.²⁵

In addition, experiments were performed to determine signaling molecules associated with the above findings during MNR. More specifically, the protein activation (phosphorylation) of ERK, AKT and JNK was studied. There was a significant decrease in ERK activation in the MNR placenta as compared to controls. In contrast, no significant differences were observed in the activation of placental AKT or JNK proteins during MNR in the baboon. The association of decreased ERK with the decreased in placental size and increased apoptosis is not entirely surprising as ERK is commonly associated with cell proliferation. While the above findings seem logical and consistent with published literature in apoptosis, some findings were not as easily interpreted. We did not observe any changes in activation of AKT or JNK proteins. This was unexpected because these proteins are usually involved in apoptosis and cell stress. At the same time we observed an increase in the anti apoptotic protein XIAP and an increase in caspase 3 activation. Given that XIAP normally inhibits caspase activation and subsequent apoptosis, this increase may represent a compensatory mechanism for the trophoblast for survival in the placenta of these MNR animals. We speculate that the XIAP increase could be involved in protecting the trophoblast from stress resulting in the lack of activation of AKT or JNK leading to improved trophoblast survival and function during the maternal food restriction in this baboon model. The fact that there is an increase in activation of caspase 3 and M30 staining, suggests that other mechanism independent of XIAP could be a factor inducing this activation and leading to the apoptosis that we see in the MNR placenta. While there is no direct clinical application of the findings in this study, it remains relevant to normal and abnormal human placental nutrient transport for several reasons. The mechanisms that underlie the effect of diminished maternal nutrition on fetal-placental development as well as its role in fetal origins of adult disease has received considerable over the last few decades and has received worldwide interest.²⁶⁻²⁷ The Barker Hypothesis²⁸ is based on end organ changes in function and structure during fetal life that subsequently leads to disease as adults. Several studies have shown that moderate degrees of nutrient restriction can impact organ structure and function including the kidney, heart, liver and placenta.²⁹⁻³⁰ The finding of increased placental apoptosis in this baboon model of MNR at term is a finding that has been described the human IUGR placenta.³¹ Why the finding of an increase in XIAP, which is known to be protective against apoptosis, did not prevent apoptosis compared to controls is not known. It may be that without XIAP, apoptosis is not controlled, a question that may only be answered with knock-out models.

Finally we would note that both groups of baboons were of mixed age and parity. The extent to which these factors affect the changes we report remains to be determined. We have recently shown that placental efficiency calculated as fetal divided by placental weight is higher in mature adult multiparous than primiparous baboons of the same weight (unpublished observations). The degree to which that may be due to a lesser degree of apoptosis in multiparity remains to be evaluated.

In summary, apoptosis is increased in the baboon placenta leading to a reduction in placental weight in this MNR baboon model. In addition, XIAP protein expression is increased in the placenta near-term in this model of MNR. We speculate that a possible mechanism of protection for the increased apoptosis observed in the placenta of treated animals is by increasing XIAP expression in the placenta of MNR animals as compared to controls. Also, we speculate that although XIAP was increased, this increase is not sufficient to prevent the decrease placental weight in this model of MNR. To our knowledge this is the first report to show an increase in XIAP protein associated with an increase in placental apoptosis during MNR in animal or human studies. Further studies are needed to determine the role of XIAP and trophoblast apoptosis earlier in gestation in this baboon model of MNR.

Acknowledgments

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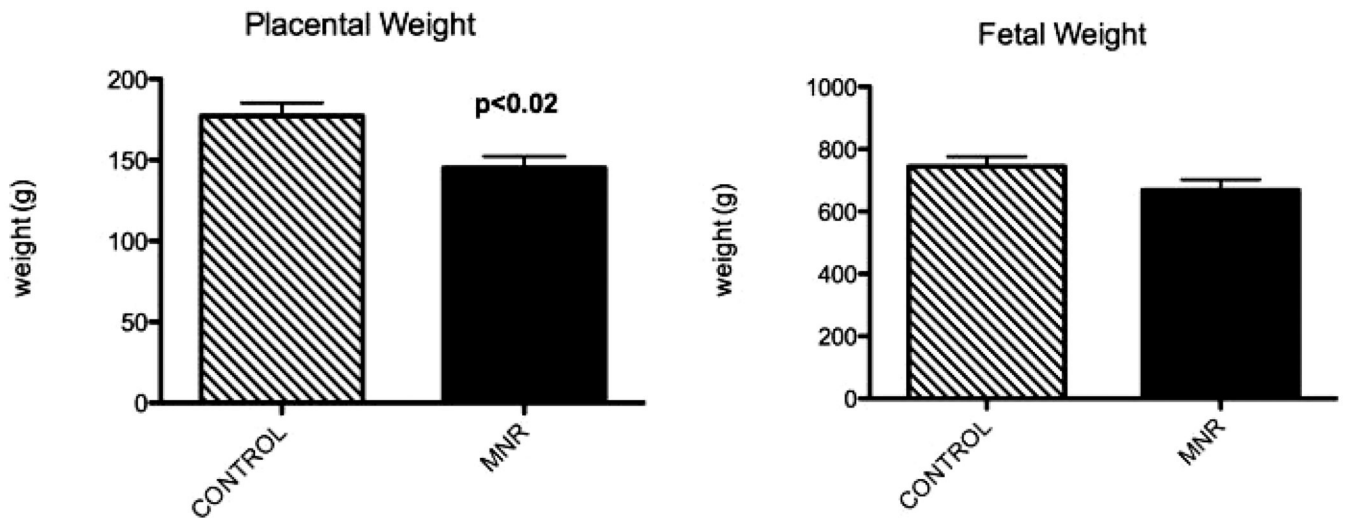


Figure 1. Fetal and placental weights in fetuses of control and maternal nutrient reduction (MNR) groups at 165 days of gestation

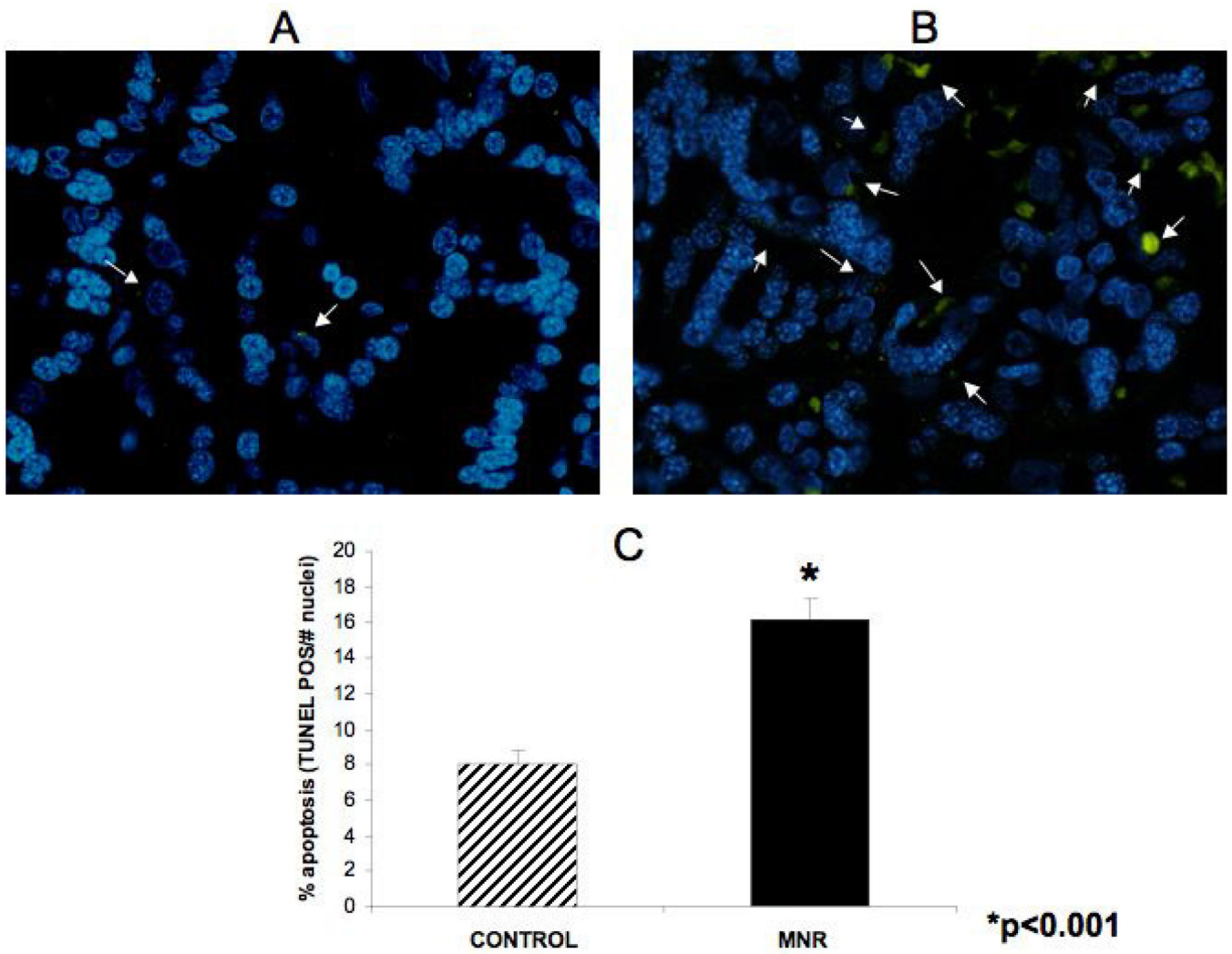


Figure 2. Apoptosis demonstrated by TUNEL staining in A) placenta of a control *ad libitum* fed mother and B) placenta of a nutrient restricted mother. Quantification of percent apoptotic cells between groups (C).

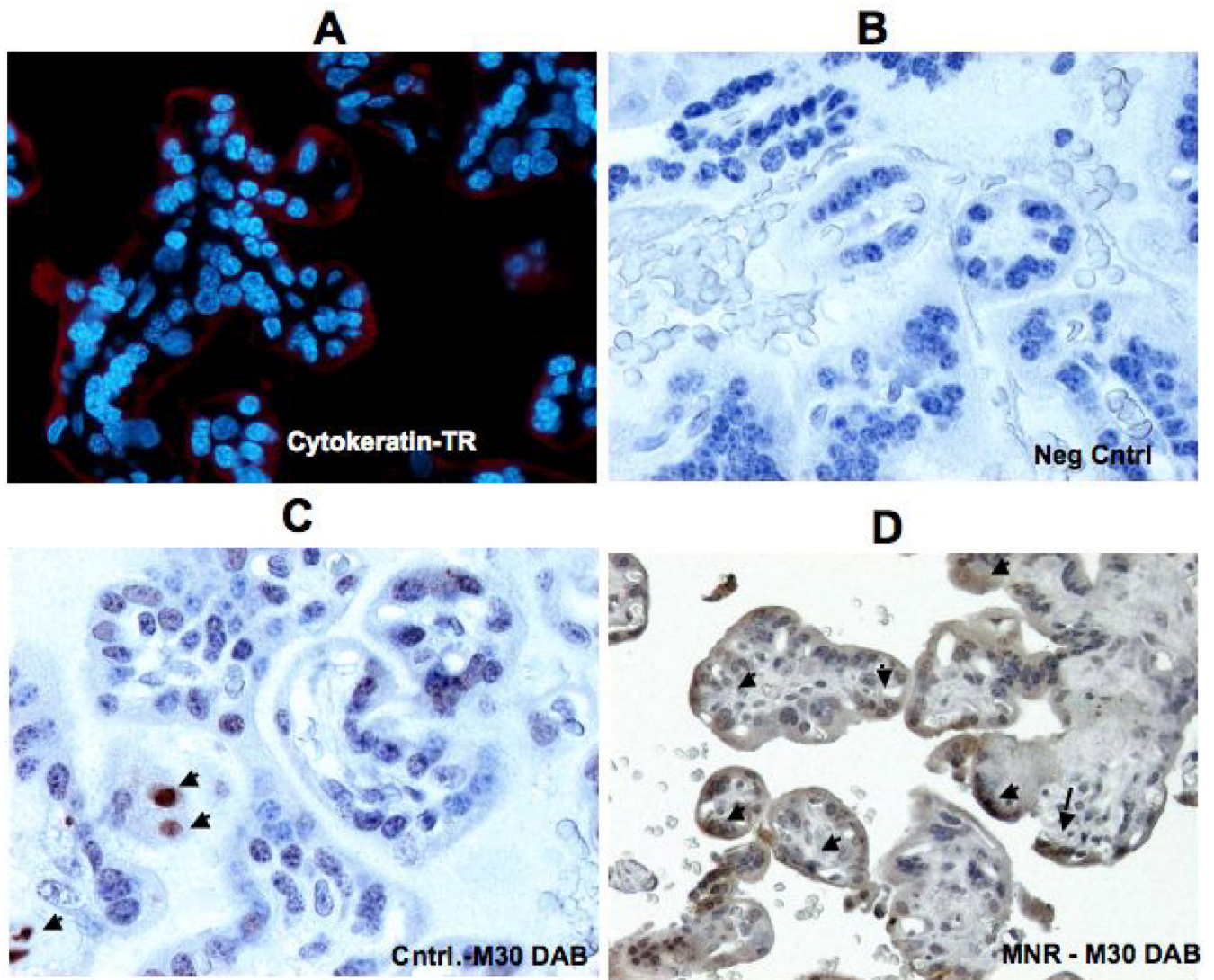


Figure 3. Cytokeratin 18 cleavage (M30) in placenta of control and MNR animals. Cytokeratin 7 immunofluorescence (red) in the baboon placenta (A). Placental sections for neg control (B), control *ad libitum* fed mother (C), and maternal nutrient reduction group (D).

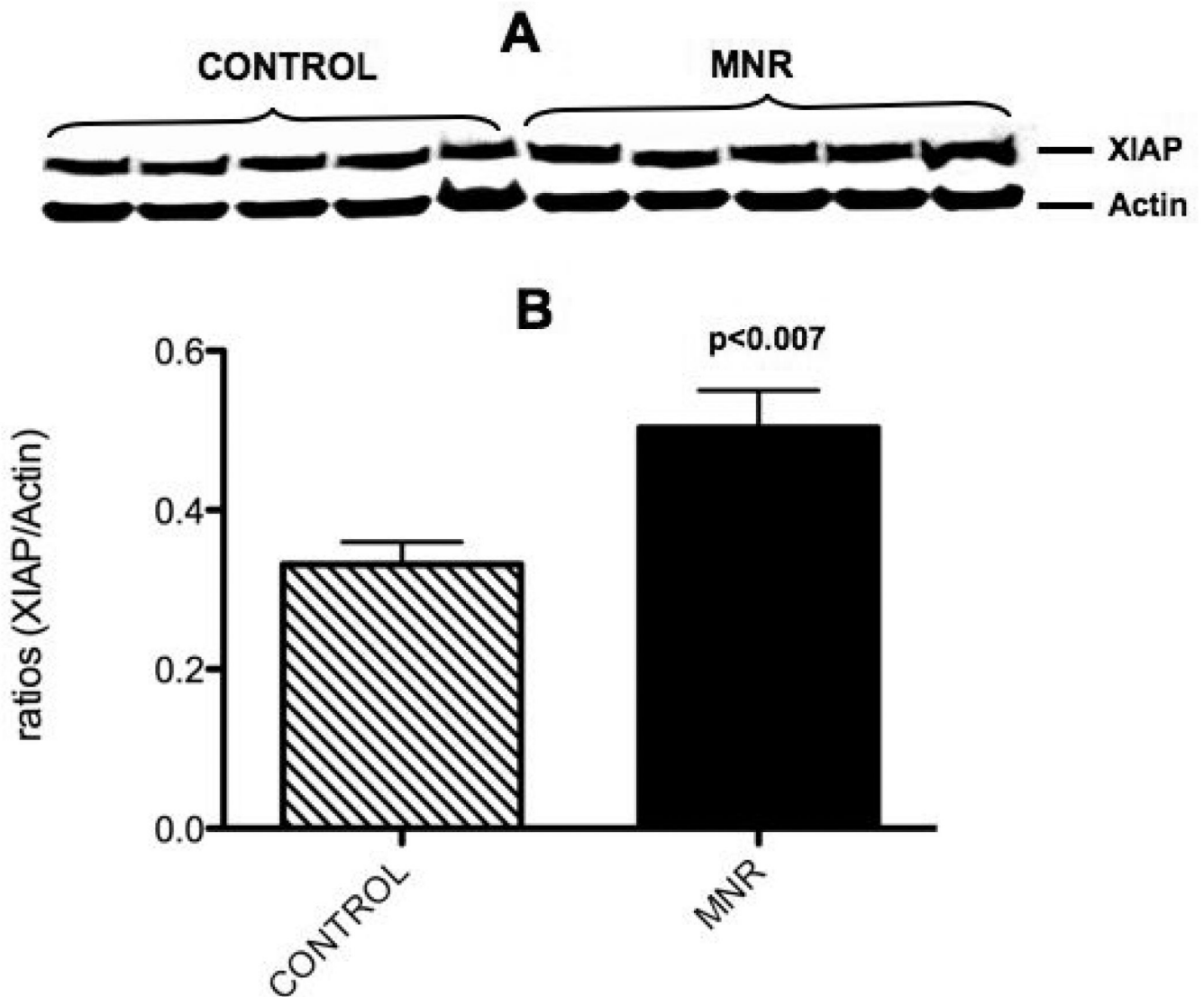


Figure 4. Placental villi XIAP protein from the control *ad libitum* fed and maternal nutrient reduction groups. Characteristic western blot of XIAP (A) and the corresponding densitometry quantification (B)

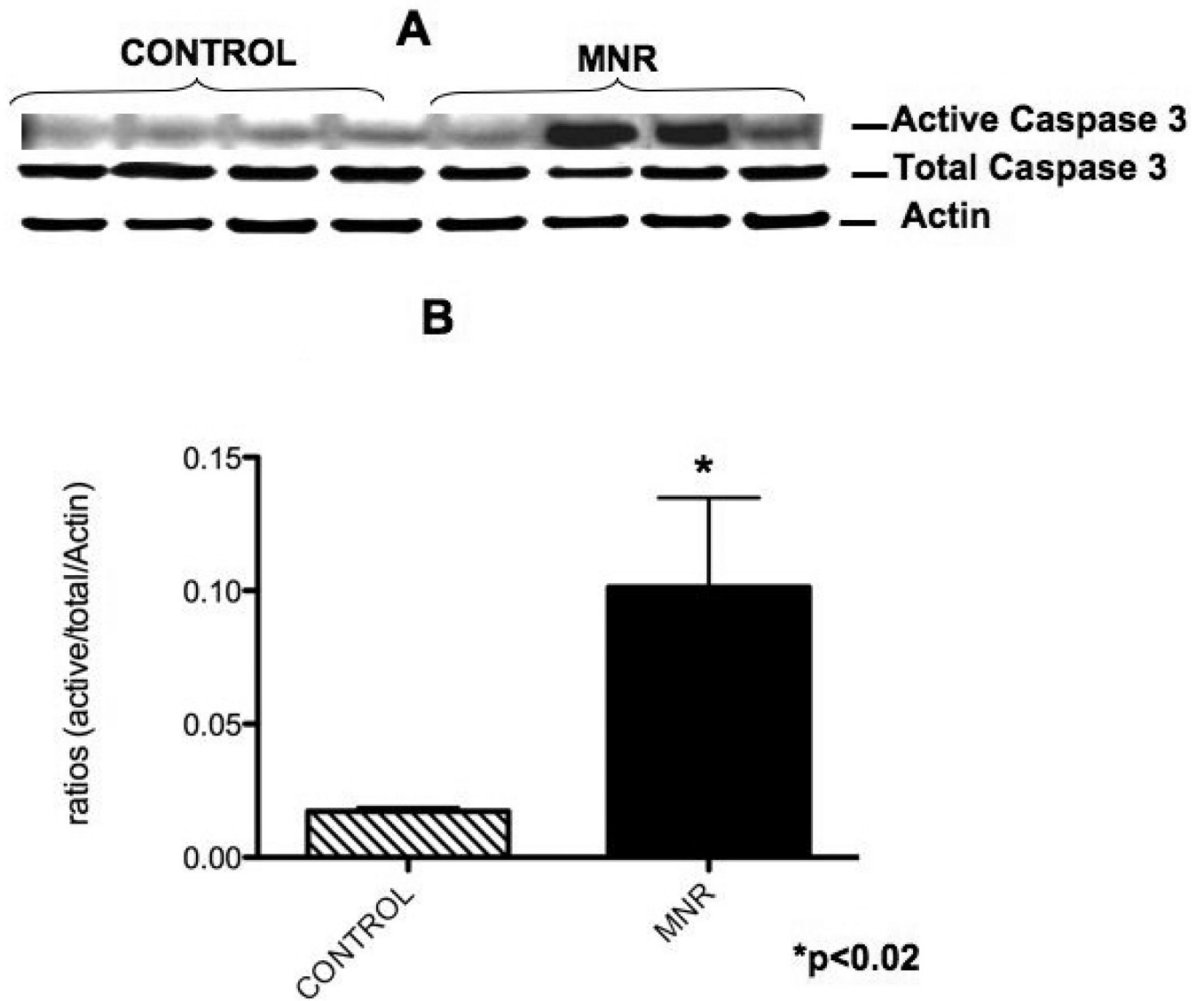


Figure 5. Placental villi active caspase 3 protein from control *ad libitum* fed and maternal nutrient reduction groups. Panel A shows a characteristic western blot, and panel B reflects the corresponding densitometry quantification.

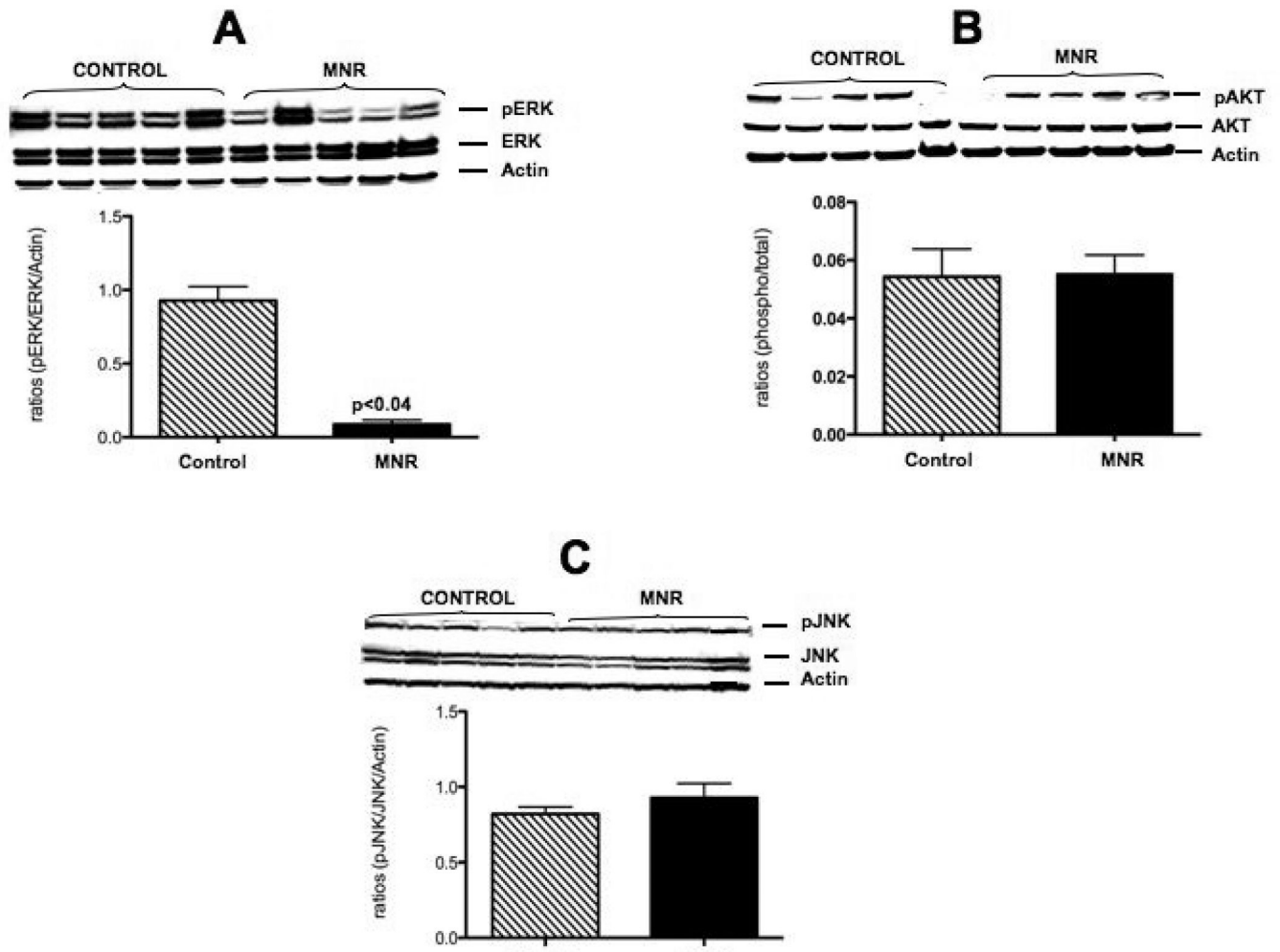


Figure 6. Characteristic western blots and protein quantification for ERK (A), AKT (B) and JNK (C) in placental villi from control *ad libitum* fed and maternal nutrient reduction groups.