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Effects of intrauterine growth restriction during late pregnancy on the cell apoptosis and related gene expression in ovine fetal liver

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ABSTRACT

This study investigated the effect of intrauterine growth restriction (IUGR) during late pregnancy on the cell apoptosis and related gene expression in ovine fetal liver. Eighteen time-mated *Mongolian* ewes with singleton fetuses were allocated to three groups at d 90 of pregnancy: Restricted Group 1 (RG1, 0.18 MJ ME kg $BW^{-0.75}$ d^{-1}, n = 6), Restricted Group 2 (RG2, 0.33 MJ ME kg $BW^{-0.75}$ d^{-1}, n = 6) and a Control Group (CG, *ad libitum*, 0.67 MJ ME kg BW $^{-0.75}$ d $^{-1}$, n = 6). Fetuses were recovered at slaughter on d 140. Fetal liver weight, DNA content and protein/DNA ratio, proliferation index, cytochrome c, activities of Caspase-3, 8, and 9 were examined, along with relative expression of genes related to apoptosis. Fetuses in both restricted groups exhibited decreased BW, hepatic weight, DNA content, and protein/DNA ratio when compared to CG ($P < 0.05$), as well as reduced proliferation index ($P < 0.05$). However, the increased numbers of apoptotic cells in fetal liver were observed in both restricted groups ($P < 0.05$). Fetuses with severe IUGR (RG1) exhibited increased ($P < 0.05$) activities of Caspase-3, 8, 9, as higher levels of mitochondrial cytochrome c in fetal liver; intermediate changes were found in RG2 fetuses, but the difference were not significant ($P > 0.05$). Hepatic expression of gene related to apoptosis showed reduced *protein 21* (*P21*), *B-cell lymphoma 2* (*Bcl-2*) *and apoptosis antigen 1 ligand* (*FasL*) expression in RG1 and RG2 ($P < 0.05$). In contrast, the increased hepatic expression of *protein 53 (P53), Bcl-2 associated X protein* (*Bax*) and *apoptosis antigen 1* (*Fas*) in both IUGR fetuses were found ($P < 0.05$). These results indicate that the fetal hepatocyte proliferation were arrested in G1 cell cycle, and the fetal hepatocyte apoptosis was sensitive to the IUGR resulted from maternal undernutrition. The cell apoptosis in IUGR fetal liver were the potential mechanisms for its retarded proliferation and impaired development.

1. Introduction

Intrauterine growth restriction (IUGR) resulting from maternal undernutrition during pregnancy has been associated with altered development of the major organ systems [1–3], such as heart, pancreas, and lung, which are strongly predisposed to the development of disease in later life [4–9]. As one of the most important body's organs, the successful development and functional maturation of the fetal liver is vital to fetal survival as well as postnatal health, but the developing fetal liver is sensitive to damage from both internal and external sources including teratogens, infection and nutritional deficiencies [10]. Impaired growth of IUGR fetal liver caused by maternal undernutrition had been observed [3,11–13], and altered gene expression of hepatic PRL-GH-IGF axis were found in prenatal and postnatal animals [11,12,14,15] In addition, IUGR affects the activities of key metabolic enzymes in the fetal liver, and the abnormal metabolism of nutrients and ammonia, reduced antioxidative capacity, and impaired cell growth in fetal pigs [16]. The precise mechanisms responsible for programming fetal liver size remain unknown, although they may be mediated by nutritional alterations in both hepatic mitogenic and apoptotic factors [10]. The objective of this study, therefore, was to investigate the effect of intrauterine growth restriction during late pregnancy on the cell apoptosis and related gene expression in ovine fetal liver.

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2. Materials and methods

2.1. Animals and treatments

All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in China [17]. According to the previous results, when the maternal nutrition density during late pregnancy is lower than the ensured "threshold" level (0.33 MJ ME kg $BW^{-0.75}$ d^{-1}), it may lead to *Mongolian* ovine fetal pathological changes and the ability of compensatory growth of postnatal offspring is suppressed or even lost [13,18,19]. For this study, it is a companion study, and the details of animals, experimental design and detailed procedures have been presented previously [20]. Briefly, three groups comprising eighteen Mongolian ewes carrying singletons were allocated at d 90 of pregnancy: a severely restricted group (Restricted Group 1: RG1, 0.175 MJ ME kg $BW^{-0.75}$ d^{-1}, n = 6), a restricted group at the "threshold" level (Restricted Group 2: RG2, 0.33 MJ ME kg $BW^{-0.75}$ d^{-1}, n = 6) and a Control Group (CG, *ad libitum,* 0.67 MJ ME kg $BW^{-0.75}$ d^{-1}, n = 6). Second or third parity ewes were mated at a synchronized estrus, after treatment for 12 d with intravaginal progestagen pessaries (each contained 0.3 g progesterone in inert silicone elastomer) and an injection of pregnant mare serum gonadotropin (PMSG), and had similar live weights (mean 52.82 ± 2.67 kg) during the 90 d of pregnancy. Pregnancies were confirmed by ultrasound scanning at approximately d 50 of gestation (Medison-SA-600, Shanghai, China). All animals were housed in individual pens, and chopped hay (mainly *Leymus chinensis*) was supplied *ad libitum* until d 90 of gestation. Based on the fact that the fetus is considered to achieve 80%–85% of its final birth weight during the last two months of gestation [1,21], maternal undernutrition was imposed from d 90 to d 140 of pregnancy. At the beginning of restriction, the ME and chemical composition in the hay were measured (Table 1), and then the daily intake of the hay offered in RG1 and RG2 was calculated by the ewe body weight, nutrition value of hay, and the designed energy plane in the restricted groups. Restricted ewes were fed at 08:30 and 16:00 h each day, and the amount of feed offered was constant throughout the restriction period (Table 2). The ewes in the Control Group were offered feed at 08:30, 11:00 and 16:00 h daily (the feed refusals were approximately 10% of the total amount offered). The animals had free access to water and mineral mixture blocks (containing per kilogram: Ca, 15 g; P, 11.5 g; Mg as $MgSO_4 \cdot H_2O$, 1 g; Fe as $FeSO_4 \cdot 7H_2O$, 500 mg; Cu as $CuSO_4 \cdot 5H_2O$, 250 mg; Zn as $ZnSO_4$, 175 mg; Mn as $MnSO_4$, 100 mg; Co as $CoCl_2 \cdot 6H_2O$, 20 mg; I as KI, 40 mg; Se as $Na_2SeO_3 \cdot 5H_2O$, 1.5 mg; Yuantongweiye Co., Ltd., Inner Mongolian, China). All feed refusals were collected daily before feeding at 08:30, weighed and sub-sampled for chemical analysis.

Table 1
Composition of grass hay and refusals during the restriction period.

	Grass hay	Refusals
ME[a], MJ/Kg	8.90	–
DM, %	88.42	91.99
CP, %	10.09	9.27
EE, %	4.34	2.72
NDF, %	71.98	71.19
ADF, %	35.82	36.60
ASH, %	4.67	4.39
Ca, %	0.57	0.68
P, %	0.09	0.08

[a] ME, metabolizable energy; DM, dry matter; CP, crude protein; NDF, neutraldetergent fiber; ADF, acid detergent fiber, EE = Ether extract; Ca = Calcium; P = Phosphorus.

2.2. Slaughtering procedures

The detailed procedures of slaughtering were described previously [13,20]. Briefly, all fetuses were removed at 140 d of gestation, and fetal BW and liver weights were recorded. About 0.5 g fresh samples of liver tissue was collected for analysis of proliferation index, and some was snap-frozen in liquid nitrogen and held at −80 °C. Portions of the livers were fixed with paraformaldehyde (0.1 mol L^{-1}, pH 7.4) and paraffin-embedded.

2.3. Contents of DNA and protein in fetal liver

About 0.5 g of the frozen fetal liver was homogenized in 20 mL buffer (0.05 M Na_3PO_4, 2.0 M NaCl, 0.002 M EDTA, pH 7.4), then supernatants were used for analyzing concentrations of DNA and protein, each measured in duplicate. The DNA concentration was measured with Hoescht 33,258 (Sigma, B2338, 1 μg mL^{-1}) as described by Sambrook and Russell [22], and standards were DNA Type I from calf liver. Protein concentrations were determined by the method of Bradford with bovine serum albumin (BSA) as the standard [23].

2.4. Proliferation index in fetal liver

Portions of the freshly collected fetal liver were washed with phosphate-buffered saline (PBS, pH 7.4) and minced, then passed through a stainless steel mesh (300 mesh). The Proliferation index in fetal liver was measured with PI staining solution (containing 0.5% propidium iodide, 0.25% TritonX-100 and 10 mg ml^{-1} Rnase, 4ABIO, Beijing, China). Data were analyzed with CellQuest software (Becton Dickinson), and the proliferation index was calculated as the percentages of S, G2 and M phase cells occupying the different phases of the cell cycle.

2.5. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining of fetal liver in histologic section

Paraffin-embedded sections of fetal liver of 4 mm thickness were deparaffinized, and treated with 50 mg/ml of proteinase K (KeyGEN Biotech. CO., LTD., Nanjing, China) at 37 °C for 40 min. After rinsing thrice with PBS, fragmented DNA in apoptotic cells were examined by TUNEL technique according to the intructions of a commercial assay kits (KGA7032, KeyGEN Biotech. CO., LTD., Nanjing, China). Berifly, the sections were incubated with working strength terminal deoxynucleotidyl transferase (TdT) solution

Table 2
Maternal feed consumption in late pregnancy.

Treatments	CG (*ad* libitum)[1]	RG2	RG1	SEM	*P*-value
Mean daily grass intake, g d^{-1}	1693[a]	853[b]	444[c]	8	<0.0001
Mean daily crude protein intake, g d^{-1}	171[a]	86[b]	45[c]	1	<0.0001
Daily metabolizable energy intake, MJ ME kg $BW^{-0.75}$ d^{-1}	0.67	0.33	0.18		

[a-c]Within a row, means without a common superscript differ ($P < 0.05$).
[1]CG = control group; RG2 = restricted group2; RG1 = restricted group1.