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Oxidative damage and exocrine dysfunction of ovine fetal pancreas are induced by maternal nutritional restriction

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ABSTRACT - This study investigated oxidative damage and exocrine dysfunction of fetal pancreas caused by maternal nutritional restriction. Eighteen ewes carrying singleton fetus were randomly divided into control group (CG, *ad libitum*, 0.67 MJ ME/$BW^{0.75}$/d, n = 6), restricted group 1 (RG1, 0.33 MJ ME/$BW^{0.75}$/d, n = 6), and restricted group 2 (RG2, 0.18 MJ ME/$BW^{0.75}$/d, n = 6) at d 90 of pregnancy. Maternal undernutrition was imposed from d 90 to 140 of pregnancy. At 140 d of gestation, fetal blood and pancreas tissue were collected to determine fetal pancreatic extracellular matrix, antioxidant capacity, and indicators of exocrine dysfunction. With the decrease of maternal nutrition, the fetal body weight, pancreatic weight, and DNA content were reduced in RG2 compared with CG, and increased and thickened collagen fibers were observed in RG2. Fetuses in RG2 exhibited increased collagen 3 (COL3) and fibronectin (FN) levels relative to CG, and the COL1:COL3 ratio was lower than that of the CG. For RG1, we found increased COL3 compared with CG. Malondialdehyde, serum amylase, and serum lipase in fetal pancreas in RG2 increased, but the total antioxidant capacity (T-AOC) decreased compared with the CG. The impaired ovine fetal pancreas growth, antioxidant imbalance, and pancreatic exocrine dysfunction are induced by maternal undernutrition during late pregnancy.

Keywords: antioxidant capacity, extracellular matrix, indicators of pancreatic exocrine, maternal undernutrition

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1. Introduction

The pancreas is a compound exocrine and endocrine gland located behind the abdominal cavity, and the synergistic function of exocrine chamber and endocrine chamber is very important in promoting the digestion of nutrients and the subsequent regulation of blood glucose homeostasis (Larsen and Grapin-Botton, 2017). Generally, the endocrine chamber of the pancreas predates the exocrine chamber during embryogenesis (Dall'Aglio et al., 2017). As a leading cause of perinatal death (Yates et al., 2018), intrauterine growth restriction (IUGR) induces 10 times higher incidence rate and mortality rate of newborns (Hay et al., 2016). A large number of epidemiological data indicate that the fetal liver (Gao et al., 2014), cardiopulmonary (Darby et al., 2020) and immune system (Liu et al., 2018) are procedural damaged in an adverse maternal environment. The environmental constraints on fetal growth leads to permanent changes in organogenesis, such as β-cell dysfunction of pancreas and insulin resistance, which are known to be the major pathophysiologic factors driving type 2 diabetes (Berney et al., 1997; Taylor, 2013).

Although maternal undernutrition during pregnancy affects the fetal and neonatal pancreatic function, the pancreas injury and pancreatic dysfunction remains poorly understood. Improving the knowledge about fetal pancreatic extracellular matrix (ECM), associated with fibrogenesis and antioxidant capacity during IUGR fetal pancreas development, might provide essential parameters for impaired fetal pancreatic function. In addition, the pancreatic function in nutrient digestion is important; however, information regarding fetal digestive enzymes in response to maternal nutrition restriction during pregnancy is still limited (Trotta et al., 2020). Since the biological response of the fetus to a compromised maternal nutrition can be assessed directly from the uterus and the process of ovine fetal organogenesis and development are relatively similar to humans, sheep have been extensively used as a model of IUGR for humans (Anderson et al., 2006; Morrison, 2008). Therefore, the objective of this study was to investigate the effects of maternal undernutrition on the ovine fetal pancreatic ECM, antioxidant capacity, and indicators of pancreatic exocrine function during late pregnancy.

2. Material and Methods

2.1. Animals and treatments

The experiment was carried out in Hohhot, Inner Mongolia, P. R. China (48°48'11" S and 111°41'28" W). Animal research was conducted according to the guidelines of the Institutional Committee on Animal Use (case no. 2020-022). Second- or third-parity Mongolia ewes were mated by two Mongolia rams at synchronized estrus, and eighteen ewes carrying singleton, which had similar live weights (52.82±2.67 kg), were selected and allocated into three groups randomly (Table 1): restricted group 1 (RG1, 0.33 MJ ME/$BW^{0.75}$/d, n = 6), restricted group 2 (RG2, 0.18 MJ ME/$BW^{0.75}$/d, n = 6), and control group (CG, *ad libitum*, 0.67 MJ ME/$BW^{0.75}$/d, n = 6). All ewes were supplied chopped hay (mainly *Leymus chinensis*, ME: 8.90 MJ/kg; DM: 88.42%; CP: 10.09%; EE: 4.34%; NDF: 71.98%; ADF: 35.82%; ASH: 4.67%; Ca: 0.57%; P: 0.09%) in individual pens from d 90 to d 140 of gestation. The CG ewes were fed three times daily to keep approximately 10% feed refusals of the total amount offered according to the requirement of local Mongolia sheep (Technical Supervision Council of Inner Mongolia Autonomous Region, 1992). The feed refusals in CG were collected before feeding at 08:30 h and sampled (AOAC, 1984). The ewes from restricted groups were fed twice at 08:30 and 16:00 h each day. The amounts of chopped hay offered for the RG1 and RG2 were invariable during restriction period.

Table 1 - Maternal nutrition plans in different groups during late pregnancy

Nutrition level	CG (*ad libitum*)	RG1	RG2
Mean daily grass intake (g/d)[1]	1689.05	842.49	440.18
Mean daily crude protein intake (g/d)[1]	170.43	85.01	44.41
Daily metabolizable energy intake[2] (MJ ME/$BW^{0.75}$/d)	0.67	0.33	0.18

CG - control group; RG1 - restricted group 1; RG2 - restricted group 2.
[1] Natural basis.
[2] Dry matter basis.

2.2. Slaughtering procedures and histology

At slaughter on the d 140 of gestation, the gravid uterine tissue and fetuses were removed, and then the fetal body weight was recorded. A portion of umbilical cord blood was coagulated, then centrifuged (3500 × *g*, 15 min), and the serum was stored at −80 °C. Samples of fetal pancreas were collected and weighted. Part of the pancreas samples were immediately frozen in liquid nitrogen and stored at −80 °C. While the other portion was fixed in 0.1 mol·L^{-1} paraformaldehyde (pH 7.4), and then the tissues were dehydrated and paraffin-embedded. The 4-μm sections were stained

using corresponding commercially available Masson stain kits for collagen fibers (D026, NJJCBIO, Nanjing, China) and photographed for microscopic examination. The content of collagen fiber was analyzed with Image-Pro 6 software in five random high-power fields (Costa et al., 2021).

2.3. DNA contents and protein:DNA ratios

A 0.5-g sample of pancreas was homogenized in 20 mL of buffer (0.05 M Na_3PO_4, 2.0 M NaCl, 0.002 M EDTA, pH 7.4), and the DNA concentration was analyzed with Hoescht 33258 (Sigma, B2338, 1 µg/mL), and the calf thymus type I DNA was used as the standard (Sambrook and Russell, 2001). Concentrations of protein in pancreas homogenates were determined with Bradford method with BSA as the standard (Swanson et al., 1999).

2.4. Extracellular matrix in fetal pancreas

Collagen1 (COL1), collagen3 (COL3), laminin (LN), and fibronectin (FN) were measured by enzyme-linked immunosorbent assay (ELISA) kits (NJJCBIO, Nanjing, China). According to the manufacturer's recommendations, about 0.5 g of the fetal pancreas was rinsed and homogenized in 0.85% chilled normal saline to obtain a 10% pancreas homogenate, then 50 µL aliquots of the supernatants were added to the microtiter plates coated by sheep antibodies of COL1, COL3, LN, and FN labeled with horse radish peroxidase, and incubated at 37 °C for 30 min to become antibody-antigen -enzyme-antibody complex. After washing with PBS twice, 50 µL staining solution of tetramethyl benzidine was added (Li et al., 2021). The reaction was terminated 15 min later by a sulfuric acid solution, then determined at 450 nm in Microplate Reader (ELX800, BIO-TEKINSTUMENTS, Northern Vermont, USA).

2.5. Total antioxidant capacity (T-AOC), superoxide dismutase (SOD), peroxidase (POD), and malondialdehyde (MDA) in ovine fetal pancreas

The pancreas was rinsed and homogenized in 0.85% chilled normal saline to obtain a 10% homogenate. The content of malondialdehyde (MDA) and activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were determined using commercial spectrophotometrically kits (NJJCBIO, Nanjing, China). Total antioxidant capacity (T-AOC) was examined by a spectrometric commercial kit (A015; NJJCBIO). In the reaction mixture, ferric ion was reduced by antioxidant reducing agents, and blue complex Fe^{2+}-TPTZ (2,4,6-tri (2-pyridyl)-s-triazine) was produced. One unit of T-AOC was defined as the amount that increased OD520 by 0.01 at 37 °C.

2.6. Serum amylase and serum lipase in ovine fetal blood

Commercial kits were purchased to determine the serum amylase and serum lipase (NJJCBIO, Nanjing, China) in fetal blood according to the manufacturer's recommendations. In the kits, purified sheep antibodies of serum amylase and serum lipase labeled with horse radish peroxidase are used to coat microtiter plate wells.

2.7. Statistical analysis

All data were analyzed using the general linear model procedure in the SAS software (Statistical Analysis System, version 9.4). The model was represented by:

$$Y_i = \mu + M_i + e_i,$$

in which µ is the overall mean, M_i is the fixed effect of the nutrition treatments (i = 1 to 3), and e_i is the random residual error. Since the animals were of the same age, parity number, breed, etc, the variables did not go in the model. Significance was considered at $P \leq 0.05$ (SAS Institute, 2002).

3. Results

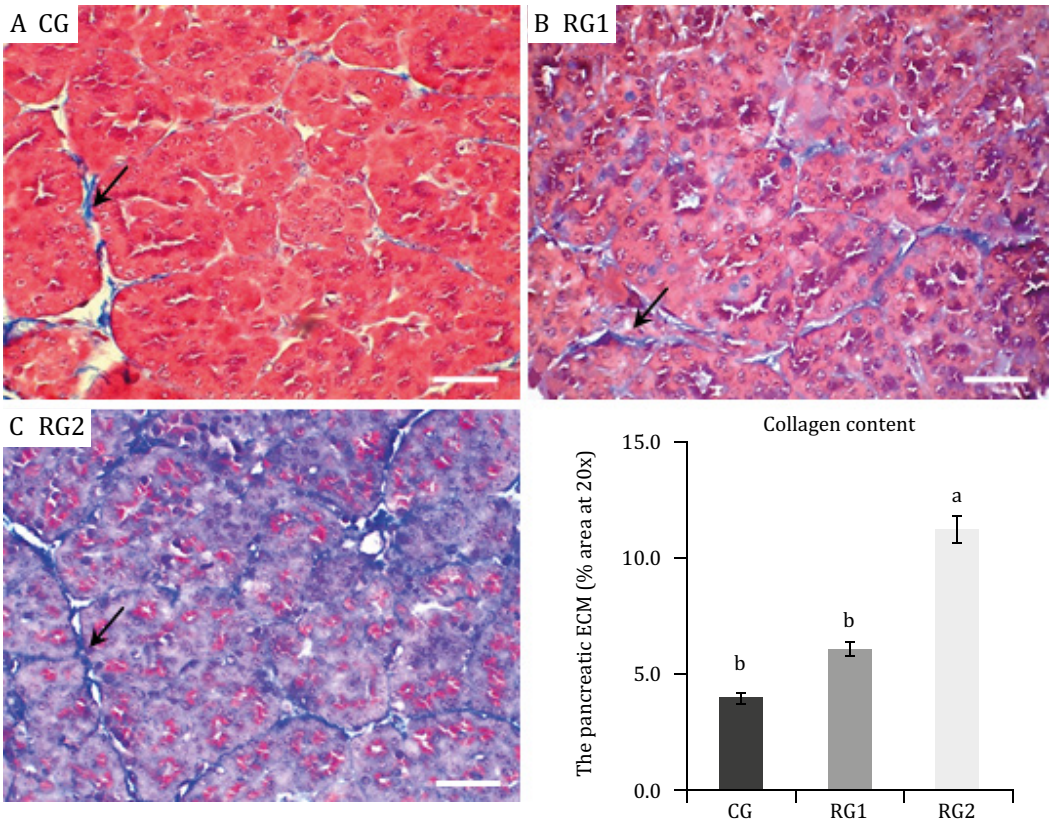
3.1. Ovine fetal pancreatic weight, structure, DNA content, and protein:DNA ratio

Fetal body weight, pancreatic weight, and DNA content were reduced in RG2 (P<0.05) compared with the CG (Table 2). For the RG1, although there were no differences in pancreatic weight, DNA content, and protein:DNA ratio relative to the controls (P>0.05) decreased fetal body weight was found as compared with the controls (P<0.05). According to Masson staining for collagen fibers (Figure 1), increased collagen fibers content was observed in the RG2 (P<0.05).

Table 2 - Effects of maternal undernutrition on ovine fetal pancreas weight, DNA content, and protein:DNA ratio during late pregnancy

Item	CG (n = 6)	RG1 (n = 6)	RG2 (n = 6)	SEM	P-value
Fetal weight (g)	3977.67a	3572.60b	3111.00c	74.83	0.0001
Pancreas weight (g)	3.19a	2.94ab	2.48b	0.15	0.0191
DNA content (μg)	38.17a	23.92ab	20.83b	4.69	0.0417
Protein:DNA ratio	7.32	8.66	7.76	1.96	0.7354

SEM - standard error of the mean.
CG - control group, *ad libitum*, 0.67 MJ ME/$BW^{0.75}$/d; RG1 - restricted group 1, 0.33 MJ ME/$BW^{0.75}$/d; RG2 - restricted group 2, 0.18 MJ ME/$BW^{0.75}$/d.
a-c - Within a row, means with a different letter differ (P<0.05).

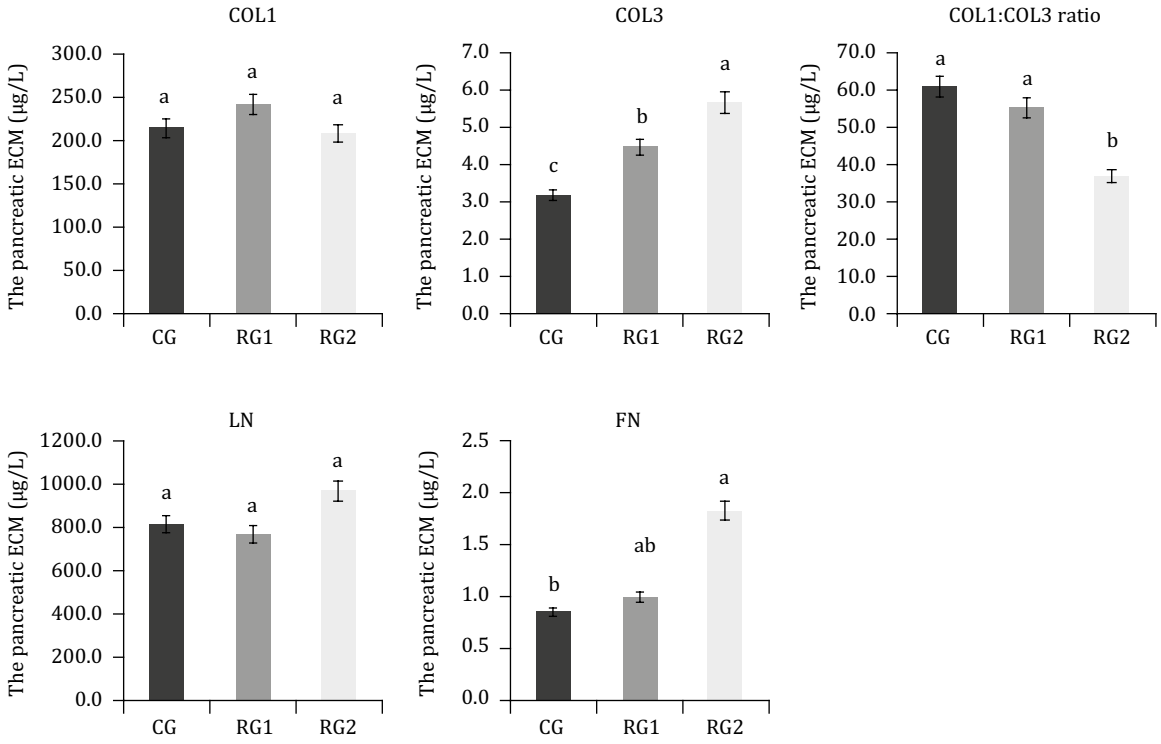


CG - control group, *ad libitum*, 0.67 MJ ME/$BW^{0.75}$/d; RG1 - restricted group 1, 0.33 MJ ME/$BW^{0.75}$/d; RG2 - restricted group 2, 0.18 MJ ME/$BW^{0.75}$/d.
Frames A to C from sections of fetal pancreas tissues show Masson stain for collagen fibers; Magnification: 20×; the arrow indicates a collagen fiber; bars: 100 μm.

Figure 1 - Effect of maternal undernutrition during late pregnancy on collagen fiber content in fetal pancreas.

3.2. Extracellular matrix of ovine fetal pancreas

Fetuses in RG2 exhibited decreased COL1:COL3 ratio (P<0.05) relative to the controls (Figure 2), and their COL3 and FN levels were higher than those of the CG (P<0.05). For RG1, increased COL3 was found, but no difference was observed in COL1, LN, and FN compared with the CG (P>0.05).



CG - control group, *ad libitum*, 0.67 MJ ME/BW$^{0.75}$/d; RG1 - restricted group 1, 0.33 MJ ME/BW$^{0.75}$/d; RG2 - restricted group 2, 0.18 MJ ME/BW$^{0.75}$/d.
COL - collagen; LN - laminin; FN - fibronectin.
a-c - Means with a different letter differ (P<0.05).

Figure 2 - Effects of maternal undernutrition on ovine fetal pancreatic extracellular matrix.

3.3. T-AOC, SOD, POD, and MDA in ovine fetal pancreas

The MDA concentration in fetal pancreas in the RG2 (P<0.05) was higher than that of the CG (Table 3); however, the T-AOC concentration was reduced compared with the CG (P<0.05). For the RG1, there were no differences in T-AOC, MDA, SOD, and POD relative to the controls (P>0.05).

Table 3 - Effects of maternal undernutrition on antioxidant capability in ovine fetal pancreas during late pregnancy

Item	CG (n = 6)	RG1 (n = 6)	RG2 (n = 6)	SEM	P-value
T-AOC (U/mL)	0.19a	0.14bc	0.11c	0.01	0.0051
SOD (U/mL)	22.27	18.61	17.51	1.86	0.2216
POD (U/mg prot)	0.73	0.79	0.83	0.093	0.7798
MDA (nmol/mL)	0.56b	0.63b	1.45a	0.21	0.0237

T-AOC - total antioxidant capacity; SOD - superoxide dismutase; MDA - malondialdehyde; POD - peroxidase; SEM - standard error of the mean.
CG - control group, *ad libitum*, 0.67 MJ ME/BW$^{0.75}$/d; RG1 - restricted group 1, 0.33 MJ ME/BW$^{0.75}$/d; RG2 - restricted group 2, 0.18 MJ ME/BW$^{0.75}$/d.
a-c - Within a row, means with a different letter differ (P<0.05).

3.4. Serum amylase and serum lipase in ovine fetal blood

The serum amylase and serum lipase in RG2 (P<0.05) were higher than those of the CG (Table 4). For the RG1, no difference was found relative to the controls (P>0.05).

Table 4 - Effects of maternal undernutrition on serum amylase and serum lipase in ovine fetal blood during late pregnancy

Item	CG (n = 6)	RG1 (n = 6)	RG2 (n = 6)	SEM	P-value
Serum amylase (IU/mL)	16.45b	21.33ab	24.76a	1.56	0.0392
Serum lipase (pg/mL)	331.99b	290.37b	519.26a	62.43	0.0052

SEM - standard error of the mean.
CG - control group, *ad libitum*, 0.67 MJ ME/$BW^{0.75}$/d; RG1 - restricted group 1, 0.33 MJ ME/$BW^{0.75}$/d; RG2 - restricted group 2, 0.18 MJ ME/$BW^{0.75}$/d.
a-b - Within a row, means with a different letter differ (P<0.05).

4. Discussion

As the center that controls energy consumption and metabolism (Zhou and Melton, 2018), the pancreas is composed of two components with different morphology and function: exocrine pancreas (acinar cells and ductal cells) and endocrine pancreas (islets of Langerhans). The pancreatic exocrine acinar cells produce a series of digestive enzymes to decompose fat, protein, and carbohydrates for absorption, while the endocrine glands α cell and β cells directly secrete insulin and other hormones into the blood to maintain glucose homeostasis (Leung, 2010; Logsdon and Ji, 2013; Zhou and Melton, 2018). In the present study, the decreased fetal pancreatic weight and DNA content in the RG2 indicated that the growth and development of ovine fetal pancreas was retarded. In addition, increased collagen fiber was observed in the RG2 with the decrease of maternal nutrition, which suggested that the fibrosis was induced in fetal pancreas by maternal undernutrition. When tissue is damaged, the body uses enhanced collagen secretion to repair the lost or damaged extracellular matrix and restore tissue integrity (Hinz, 2016).

Extracellular matrix (ECM) is a non-cellular three-dimensional macromolecular network composed of collagens, proteoglycans/glycosaminoglycans, elastin, fibronectin, laminins, and several other glycoproteins (Theocharis et al., 2016). Laminin (Hamill et al., 2010), fibronectin (Pulido et al., 2017), collagen fibers (Gjaltema and Bank, 2017), and other major adhesion proteins are very important for the formation, structure, and function of basement membrane (Janković and Kosanović, 2008; Wysocki, 1992; Amann et al., 2017). In general, excessive ECM production and aberrant ECM turnover characterize progressive organ fibrosis (Herrera et al., 2018), which will lead to changes in tissue structure, sclerotic stroma, and malignant tumors (Piersma et al., 2020). In the present study, the increases in COL3 activity were found in both RG1 and RG2, and the COL1:COL3 ratio was also suppressed by maternal undernutrition in the RG2 fetal pancreas, which indicated that COL3 production might be more sensitive to maternal undernutrition than COL1 in fetal pancreas. Collagen is the principal building block of connective tissue, and its upregulation is a critical event in the development of tissue fibrosis (Perez-Aso et al., 2013). The excessive deposition of collagen within the IUGR fetal pancreas induced by maternal undernutrition, especially type III (COL3), is one of the major contributing factors implicated in the pathogenesis of fibrosis. Oxidative stress occurs as a consequence of the imbalance between natural cellular antioxidative defenses and the prooxidant state (Abd Hamid et al., 2011), which is usually accompanied by increased expression of ROS and MDA and decreased expression of T-AOC and SOD (Yang et al., 2019). Malondialdehyde, the final decomposition product of cell membrane lipid peroxidation, will destroy the structure and function of cell membrane, and eventually lead to cell aging or death (Qin et al., 2020; Zhao et al.,

2021). In this study, the higher concentration of MDA and reduction of T-AOC in the fetal pancreas suggest that oxidative stress was induced by maternal undernutrition. Increased oxidative stress is a common pathological feature of fibrosis (Liu and Gaston Pravia, 2010).

Regarding the fetal pancreatic exocrine function, some results show that nutrient restriction of ewes during mid-to-late gestation had no effect on fetal pancreatic digestive enzymes (Keomanivong et al., 2016); however, more results demonstrated that pancreatic exocrine function is affected by both nutrition and pregnancy (Awda et al., 2017; Keomanivong et al., 2017). In the present study, increased serum amylase and serum lipase in RG2 were observed in IUGR fetal pancreas, which indicated that exocrine function in fetal pancreas was affected by maternal nutritional restriction during late pregnancy. The possible mechanisms of pancreatic exocrine function regulation are complex (Swanson et al., 2000). The retarded growth of IUGR fetal pancreas accompanied with oxidative damage and fibrosis in this study might be a possible mechanism of impaired pancreatic exocrine function, which might prove essential in the identification of parameters for impaired fetal pancreatic function.

5. Conclusions

The impaired ovine fetal pancreas growth with fibrosis, antioxidant imbalance, and pancreatic exocrine dysfunction are induced by maternal undernutrition during late pregnancy. Increased fibrosis, oxidative stress, and higher serum amylase and serum lipase are potential identification parameters for fetal pancreatic dysfunction when exposed to an adverse nutritional environment in late pregnancy.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: Y. Liu and F. Gao. Data curation: C. Ma and L. Zhao. Validation: Y. Yang. Writing – original draft: Y. Zi. Writing – review & editing: Y. Liu and F. Gao.

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