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Effects of L-arginine on endometrial microvessel density in nutrient-restricted Hu sheep

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ABSTRACT

Nutrient deficiency in ruminants can lead to estrus cycle disorders, a decreased pregnancy rate, and reduce birth weight. L-arginine (L-Arg), an important amino acid, can improve uterine homeostasis in pregnant sheep and prevent intrauterine growth restriction (IUGR). However, most studies of L-Arg have been conducted on pregnant sheep and few have reported the effects of L-Arg on microvessel density (MVD) in the non-pregnant ovine endometrium. The processes of normal uterine cyclical development and implantation are dependent on a balanced of endometrial MVD. In this study, female Hu sheep were randomly assigned to either a control group (n = 6), a nutrient-restricted group (n = 6), or an L-Arg supplemented nutrient-restricted group (n = 6). The effects of L-Arg on MVD in ovine endometrium were then studied. Our results showed that ovine endometrial MVD was significantly increased by nutrient restriction, but L-Arg counteracted the effect of nutrient restriction on MVD ($P < 0.05$). Levels of angiogenic growth factors (including VEGFA, VEGFR2, and FGF2) had significant increases ($P < 0.05$) in endometrium of nutrient restriction on sheep, but that L-Arg supplementation substantially decreased ($P < 0.05$) their expressions in nutrient restriction sheep. Furthermore, oxidative stress caused by nutrient restriction was also alleviated by L-Arg supplementation in the ovine endometrium. Overall, the results suggested that L-Arg has crucial roles in maintaining the balance of endometrial MVD and angiogenic growth factors, and increasing anti-oxidation capability in the endometrium of nutrient-restricted sheep. This study will provide a theoretical basis and technical means for the normal development of endometrial microvessels in low nutrition level.

1. Introduction

Nutritional level is one of the key factors in mediating mammalian reproductive performance [1,2]. Malnutrition can affect reproductive performance by disturbing the balance of the reproductive endocrine system of animals [3–5]. During reproduction, insufficient availability of nutrients to the conceptus often results in uterine dysfunction [6]. In addition, nutrient deficiency in ruminants can lead to estrus cycle disorders and decreases in pregnancy rate and birth weight [7,8]. Recent research shows that the development of ovine follicles is inhibited by temporary nutrient restriction [9]. This may be due to the negative energy balance caused by inadequate food intake.

L-Arg, an important amino acid, has multiple physiological functions in animals [10–12]. It has been reported that L-Arg can regulate metabolism in the uterus and improve the reproductive performance of pregnant sheep. Similarly, the survival rate of fetal lambs was improved by parenteral administration of L-Arg [13,14]. Furthermore, dietary supplementation of L-Arg in nutrient-restricted Hu sheep improved the uterine and placental anti-oxidation capabilities, which promote fetal and placental development during gestation [15].

Most studies of L-Arg have been conducted on pregnant sheep [16,17], and few have reported the effects of dietary supplementation of L-Arg on the development of the non-pregnant uterus, especially endometrial microvessel density (MVD). Endometrial MVD is closely related to the cyclical development of the uterus and plays an important role in the process of fertilization and embryo implantation [18,19]. Endometrial MVD in sheep depends on

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angiogenesis. Physiological angiogenesis plays a critical role in endometrial development and differentiation by increasing blood flow and nutrient supply. Furthermore, angiogenesis is a critical step in the formation of the corpus luteum, endometrial growth, embryo implantation, and placentation [20]. Previous researches has shown that angiogenesis in the uterus is directly regulated by various factors, including VEGF and FGF2 [21–23].

As a precursor of polyamines and nitric oxide, arginine enhances placental angiogenes and fetal-placental blood flow. Additionally, arginine is an antioxidant that regulates the balance of oxidation-antioxidation and modulates angiogenesis [15,24]. We hypothesized that L-Arg influences endometrial angiogenesis by regulating VEGF and FGF2. Therefore, the objectives of this study were to determine how L-Arg supplementation in nutrient-restricted Hu sheep affects the (1) morphology of the non-pregnant uterus, especially endometrial MVD; (2) expression of endometrial angiogenic growth factors; and (3) endometrial anti-oxidation capability.

2. Materials and methods

This study was conducted according to the Guide for the Care and Use of Laboratory Animals prepared by the Ethics Committee of Nanjing Agricultural University (SYXK 2011-0036).

2.1. Experimental design

A total of 18 healthy multiparous Hu sheep of similar age (average age: 2.0 ± 0.2 years) were housed under similar conditions in Jiangsu Province, China. Female Hu sheep in this experiment were synchronized with pessaries for 12 days [25]. We assessed estrous behavior with vasectomized rams on the second day after pessary removal, and the end of estrous behavior was considered to be Day 0 of the estrous cycle. All ewes were randomly divided into three groups: Control group (n = 6), Nutritional restriction group (n = 6), and Arg group (n = 6). We assessed the estrous behavior from Day 15 of the estrous cycle and sacrificed ewes on the second day after reinstatement of estrous behavior.

All ewes were fed a total mixed ration diet based on published feeding standards (NY/T816-2004; China). The Control group received a maintenance diet (1040 g/ewe per day), whereas the Nutritional restriction group, and Arg group were fed a nutrient-restricted diet (520 g/ewe per day) diet. The Arg group was administered Arg (155 mmol Arg/kg body weight; Amresco, Solon, OH, USA) three times daily, according to previously published studies [13,25]. Control and Nutritional restriction groups were administered a similar volume of sterile saline in place of L-Arg.

2.2. Preparation of samples

Blood samples (15 mL) were drawn and centrifuged at 1500 × *g* for 10 min at 4 °C. The obtained serum was stored at −20 °C for enzyme-linked immunosorbent assays (ELISA). All ewes were stunned with a captive-bolt gun (Supercash Mark 2, Accles and Shelvoke Ltd., Birmingham, England) prior to slaughter on the second day after estrous behavior initiation, as monitored using vasectomized rams, to ensure insensibility. The mid-part of the uterine horn was surgically removed and divided into two samples (each about 5 g). The endometrium was collected from one sample, immediately frozen in liquid nitrogen and stored at −80 °C until RNA and protein extraction, The second sample was fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 24 h and embedded in paraffin for immunohistochemistry.

2.3. ELISA

The activity of catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase 2 (SOD2) and the levels of glutathione/oxidized glutathione (GSH/GSSG), malondialdehyde (MDA) in the uterus were measured using commercial ELISA kits (Kmaels Co., Ltd., Shanghai, China) according to the manufacturer's instructions and as previously described [25]. Briefly, we added 50 μL of standard or sample to each well and incubated the assays for 2 h at 37 °C. The liquid was removed and each well was washed three times with washing buffer (200 μL), 50 μL of horseradish peroxidase-conjugated avidin was added to each well and incubated for 1 h at 37 °C. The plates were washed five times as described above. Then, 100 μL of TMB substrate was added to each well and incubated for 20 min at 37 °C. Finally, 50 μL stop solution was added to each well. The absorbance at 450 nm was measured using a microplate reader (ELx808IU, Biote Tek, Winooski, VT, USA).

2.4. RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the process provided by the manufacturer [26]. The concentration and quality of the RNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Samples identified to exhibit a 260/280 OD value between 1.8 and 2.0 were selected for further analysis [25]. Then qualified RNA (1 μg/reaction) was reverse transcribed into cDNA using Reverse Transcription Reagent kit (Takara, Dalian, China) and stored at −20 °C until qRT-PCR analysis.

Primers were designed online using Primer 5 software, and evaluated by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) at NCBI (Table 1). PCR amplification efficiency of each pair of primers was assessed before quantification, and was found to be in an acceptable range (between 0.9 and 1.1). SYBR green (Roche, Germany) was used for qRT-PCR, according to a previously described protocol [25]. Briefly, qRT-PCR was performed using the Step One Plus Real-Time PCR System in a reaction volume of 20 μL. The PCR amplifications were carried out at 95 °C for 10 min; followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s; and 72 °C for 10 min. Expression levels of genes were quantified using the $2^{-\Delta\Delta CT}$ method, and data were normalized to a house-keeping gene (*β-actin*).

Table 1
Details of primer sequences and expected product sizes of genes used for qRT-PCR.

Gene	Primer sequence (5′-3′)	Product size, (bp)
VEGFA	F:GCCTTGCCTTGCTGCTCTAC R:GGTTTCTGCCCTCCTTCTGC	76
VEGFR2	F:GAGTGAGGAAGGAGGATGAAG R:GTAGAACGATGACAAGAAGTAGC	183
FGF2	F:TGTGTCTATCAAAGGAGTGTGTG R:GCCCAGTTCGTTTCAGTGC	182
FGFR1	F:GCTACAAGGTCCGTTATGC R:TTGATGCTGCCGTATTCG	106
FGFR2	F:GTCAGTGAGAACAGTAACAG R:ACCTTATAGCCTCCAATGC	197
FGFR3	F:CGACTACTACAAGAAGACAAC R:CGAAGGACCACACATCAC	107
SOD2	F:TGACTGCTGTATCTGTTGG R:AGACCTGTTGTTCCTTGC	124
HIF-α	F:TGGCAGCAATGACACAGAG R:GGTCGGCACTACTTCTAAGC	164
CAT	F:CGGTTAAGAACTTCAGTGATGTC R:GGTTTCTCCTCATTGTATTTGTCC	82
β-actin	F:CCAAGGCCAACCGTGAGAAG R:CCATCTCCTGCTTCGAAGTCC	350