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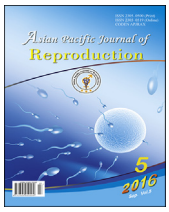

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Sheep oocyte expresses leptin and functional leptin receptor mRNA

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ABSTRACT

Objective: To investigate the expression of leptin and its functional receptor mRNA by reverse transcription-polymerase chain reaction analysis in sheep oocytes.
Methods: Sheep ovaries were collected in reproductive season from abattoir and immediately were transported to the lab. Cumulus–oocyte complexes were aspirated from antral follicles and were denuded to obtain denuded oocytes. Total RNA of denuded oocytes was extracted, cDNA synthesis was carried out subsequently and cDNA was subjected to PCR amplification using specific designed primers.
Results: Gel electrophoresis of PCR products showed the amplification of 162 and 121-bp amplicons for leptin and functional leptin receptor respectively. Sequencing the amplified fragments also confirmed the results.
Conclusions: The result of present study reveals that leptin and its functional receptor (Ob-Rb) mRNA are expressed in sheep oocyte and further studies should investigate the role(s) of leptin on sheep oocyte physiology and embryo development.

1. Introduction

Leptin, a 167-amino acid hormone, is secreted mainly by fat tissue [1]. Leptin belongs to helical cytokines class 1 which affect food intake, energy expenditure and reproductive functions [2]. Physiological effects of leptin on reproduction including puberty, estrous cycle, pregnancy, lactation, and even the early stages of embryo development have been proven [3].

It is known that leptin and its receptor in addition to adipose tissue, express in other tissues which confirm role of leptin in various cells, organs and systems [4]. Several forms of the leptin receptor have been identified including several short isoforms (Ob-Ra, c, d and e) and a long functional isoform, Ob-Rb. It is thought that only the long form of the receptor (Ob-Rb) is able to activate the JAK-STAT signaling pathway and is responsible for most of the biological effects of leptin [5]. Leptin and Ob-Rb are expressed in the goat oocyte and follicular cells [6], sheep granulosa and theca cells [7], human follicles [8], bovine testis [9] and bovine ovary [10]. Nevertheless, the expression of leptin and Ob-Rb in ovine oocyte has not yet been reported.

It seems that leptin is essential for the maturation of the reproductive axis, because it has the ability to induce puberty and restores fertility in ob/ob mice [11]. Leptin stimulates endocrine system of reproduction in both sexes and is a signal to the reproductive system in normal animals [12]. In addition to secretion control of gonadotropins through the axis hypothalamus-pituitary, leptin has a direct effect on the function of gonads. Source of leptin for this effect could be blood or their organs and tissues. Due to presence of leptin receptors on the surface of reproductive organs, autocrine/paracrine effects of leptin are probable [2].

Leptin participates in the regulation of ovarian folliculogenesis by influencing the proliferation of granulosa cells, steroidogenesis and apoptosis [13]. In human, leptin secretion caused monthly period and is required at certain amount for maintenance of natural cycle and the appearance of oocyte in ovaries [14]. Moreover, the beneficial effects of leptin on oocyte maturation and embryo development have also been observed [15,16]. As a first step towards understanding the effects of leptin in sheep oocyte physiology, it is necessary to demonstrate the expression of leptin and its functional receptor in oocyte. The objective of this study was to determine whether leptin and Ob-Rb mRNA is expressed in the sheep oocyte.

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2. Materials and methods

2.1. Collection of oocytes

Ovaries from adult sheep (Kurdish breed) were obtained from a local slaughterhouse (Mashhad, Iran) in reproductive season and transported to the laboratory in saline solution at 37.5 °C. Ovaries were used to obtain cumulus–oocyte complexes (COCs) by aspiration of antral follicles. After collecting the COCs, the cumulus cells were separated by treating COCs with sodium citrate 3% for 5 min, vortex and pipetting. Then denuded oocytes (DOs) were observed under a stereomicroscope to confirm deletion of cumulus cells. DOs were immediately subjected to total RNA extraction.

2.2. RNA extraction

Total RNA was extracted from oocyte pool using High Pure RNA Isolation Kit (Roche, Germany) according to the manufacturer's instructions. To delete any probable genomic DNA contamination, DNase treatment was done during RNA extraction protocol based on manufacturer's instructions. Total extracted RNA was quantified by a Nanodrop Spectrophotometer and quality of RNA was determined using gel electrophoresis. Only appropriate samples were selected for next stages.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

For each sample, 1 μg of total RNA was reverse transcribed (RT) into complementary DNA (cDNA) using the AccuPower RT-PCR PreMix (Bioneer, South Korea) according to the manufacturer's instructions.

PCR reactions were performed using *Taq* DNA Polymerase 2× Master Mix RED (Amplicon, Denmark) and specific designed primers of beta-actin, leptin and functional leptin receptor (Table 1). The forward and reverse functional leptin receptor primer pairs were designed to span the junction of two exons to be RNA specific. So, the amplification of the cDNA and DNA resulted in the different PCR products in length. One microliter of each RT reaction product was used as template for PCR reactions in a final volume of 25 μL with 12.5 μL Master Mix of "Amplicon", 10.5 μL distilled water, 0.5 μL of forward and 0.5 μL of reverse primer. The following amplification conditions were utilized: 1 cycle 94 °C for 5 min, followed by 40 cycles of denaturing at 94 °C for 45 s, annealing at 58 °C (for beta-actin), 50 °C (for leptin), 57.4 °C (for leptin receptor), and extension at 72 °C for 45 s. The program was terminated with a final extension step at 72 °C for 10 min. Beta-actin (a housekeeping gene) was used as positive internal controls for all samples to verify accuracy of RT-PCR reactions.

As the expression of leptin and functional leptin receptor has been previously shown in ovine fat tissue [17], fat tissue was used as positive control. Negative control reactions were also performed similarly with addition of 1 μL distilled water instead of template. All PCR products were run on a 1.5% agarose gel in Tris-acetate EDTA buffer, stained with ethidium bromide and visualized under UV transillumination. The size of the predicted products was confirmed using a 100 bp standard molecular ladder. In addition, PCR products of amplified fragments of three genes were sequenced (Macrogen, South Korea), and the results were edited by CLC bio, and finally checked in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to verify the results.

3. Results

The sheep beta-actin was selected as an internal positive control. So, the amplification of beta-actin was first assessed in all samples. Amplification of beta-actin from cDNA resulted in only 277-bp product, while amplification of bet-actin DNA produced 274 and 366-bp products. Detection of only 277-bp fragment at samples showed proper RNA purification and RT-PCR conditions along with no genomic contamination (Figure 1).

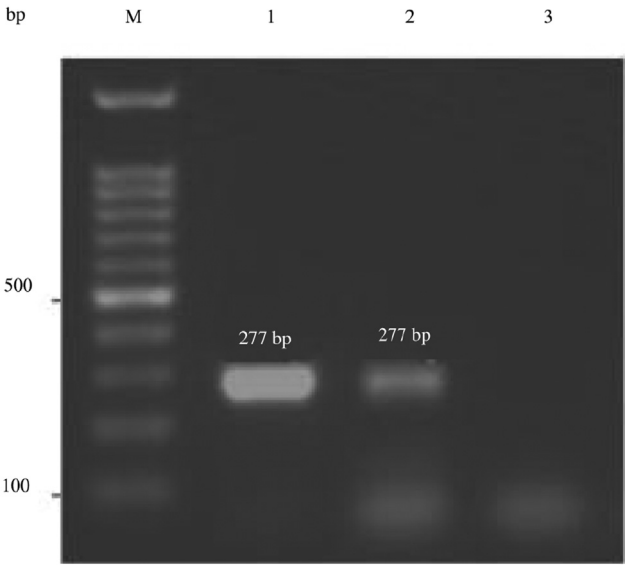

Figure 1. Beta-actin was used as positive internal controls for all samples to verify that the RT-PCR reactions were successful, and representative agarose gel demonstrated amplification of only 277-bp sheep beta-actin cDNA from fat (lane 1) and oocyte (lane 2). Lane 3 was negative control, and M was 100-bp DNA marker.

Table 1
Primers sets which were used in PCR reactions.

Primer	GenBank Acc. No.	Forward	Reverse	Fragment size (cDNA) (bp)	Fragment size (DNA) (bp)
Beta-actin	NM_001009784	CGGGAAATCGTCCGTGAC	CCGTGTTGGCGTAGAGGT	277	366 274
Leptin	AF310264.1	CAGCAGAACAAAGGAGGA	CAGCCCATAGCACCAGT	162	162
Functional leptin receptor	NM_001009763.1	GAAGGAGTAGGGAAACCGAAGA	CAAGCAATAAGATTGAGGAGGAGAT	121	1 984

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