Expression of leptin and leptin receptor transcripts in ovine corpus luteum

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Abstract:

BACKGROUND: Leptin, the product of the obesity (ob) gene, acts as a signaling adipokine for modulating food intake, energy metabolism and reproductive functions in mammals. Leptin's effects on the reproductive system at various levels of the hypothalamic-pituitary-gonadal axis have been established. Moreover, the direct and local effect of leptin on bovine oocyte maturation and corpus luteum function has been determined. **OBJECTIVES:** Due to species differences, this study was designed to investigate expression of leptin mRNA as well as its long isoform receptor (Ob-Rb) mRNA in sheep corpus luteum. **METHODS:** Ovaries of sheep containing mature corpus luteum were collected in the reproductive season from abattoirs. Total RNA of corpus luteum was extracted, cDNA synthesis was carried out subsequently and PCR reaction was performed using primers which were designed specifically for each gene. Beta-actin was used as housekeeping gene to verify reactions, and adipose tissue was selected as positive sample for expression of leptin and leptin receptor. **RESULTS:** Gel electrophoresis of PCR products showed the amplification of 162 and 121-bp amplicons in all samples for leptin and leptin receptor respectively. Moreover, sequencing the amplified fragments and blasting them confirmed the accuracy of results. **CONCLUSIONS:** Our findings confirm the expression of leptin and its functional receptor transcripts in ovine corpus luteum. More studies for determining leptin effects on corpus luteum are guaranteed.

Introduction

Leptin as a product of the obesity (Ob) gene (Zhang et al., 1994) is involved in appetite control, metabolism and the regulation of reproduction (Barash et al., 1996; Cervero et al., 2006). In addition to fat tissue which is the main source of leptin, its expression (protein or mRNA) has been shown in many organs and cells, such as the placenta (Señarís et al., 1997), ovaries (Ryan et al., 2003), testes (Abavisani et al., 2008), ovarian follicles (Cioffi et al., 1997), sperm (Abavisani et al., 2011) and oocyte (Taheri and Parham, 2016) of many different species. Leptin effects are mediated through leptin receptor (LEPR) on plasma membrane. There are at least six splice

variants of leptin receptor named Ob-Ra to Ob-Rf, with Ob-Rb being the long or functional form of receptor. The Ob-Rb includes an intracellular domain containing specific motifs for intracellular signal transduction (Snodgrass, 1996; Tartaglia, 1997). Other variants (short forms) are involved in regulation of leptin signal transduction and transport of leptin (Ghilardi and Skoda, 1997; Tartaglia, 1997). The long form of leptin receptor (mRNA or protein) has been reported in many tissues, such as ovary (Karlsson et al., 1997), testis (Roostaei-Ali Mehr et al., 2013), follicles (Cioffi et al., 1997), oocyte (Taheri and Parham, 2016) and oviduct (Xi et al., 2017).

Evidence shows that leptin deficiency and resistance to leptin in animal and human models results in serious fault in the reproductive system function (Caprio et al., 2003). Interaction of leptin and leptin receptor (Ob-Rb) causes the activation of Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT). Meanwhile leptin interaction with Ob-Rb and the truncated Ob-Ra trigger Mitogen-Activated Protein Kinase (MAPK) pathway (Bjørbæk et al., 1997). The function of leptin in humans and rodent species is well established, however, it has been less investigated in ruminants. Leptin acts as a metabolic signal to the reproductive system which establishes a link between adipose tissue and the brain (Barash et al., 1996). Leptin's effect has been reported in the onset of puberty and the regulation of reproductive cycle (Ryan et al., 2002; Schneider, 2004), follicular development, implantation (Castellucci et al., 2000; Craig et al., 2005), and pregnancy (Hardie et al., 1997; Linnemann et al., 2000).

The corpus luteum plays an important role in controlling the ovarian cycle and the creation and maintenance of pregnancy (Hansel and Blair, 1996). The identification of genes affecting various aspects of corpus luteum function is the first step in understanding the biology of these organs (Casey et al., 2004). Leptin or leptin receptor mRNA expression have been shown in the corpus luteum of some animal species such as cow (Nicklin et al., 2007), rat (Ryan et al., 2003), and buffalo (Kumar et al., 2012). Kumar et al. (2012) showed that leptin has positive effects on corpus luteum steroidogenesis.

Although some studies have also shown the effect of leptin on the reproductive neuroendocrine axis, other studies have revealed that leptin has a direct action on the reproductive organs (Barash et al., 1996; Spicer and Francisco, 1998; Castellucci et al., 2000; Cervero et al., 2006; Nicklin et al., 2007). However, the leptin signaling pathway in the reproductive organs such as ovary and its structures in different species still remain to be fully understood. Therefore, the first step to clarify the local effects of leptin in the ovine corpus luteum is investigating gene expression of leptin and its receptor. Therefore, the aim of the present study was to assess the expression of leptin and its functional receptor (Ob-Rb) mRNA in ovine corpus luteum.

Materials and Methods

Sample collection: Subcutaneous adipose tissue and ovaries containing mature corpus luteum of the healthy adult and non-pregnant ewes (Kurdish breed) were collected immediately after slaughter in a local slaughterhouse (Mashhad, Iran) in the reproductive season (September). The

tissues were frozen in liquid nitrogen and subsequently stored at -80 °C until RNA extraction. Blood samples of ewes were collected and progesterone levels were measured by radioimmunoassay (RIA) method in order to verify that the present corpus luteum in the ovaries is active and mature. Thorburn et al. (1969) reported progesterone levels to be greater than 1 ng/ml for ewes in diestrous period with active corpus luteum (Thorburn et al., 1969). So, only the ovaries of those ewes whose progesterone level was more than 2 ng/ml were selected for RNA extraction and molecular analysis, and finally 4 ovaries (repeats) were chosen.

RNA extraction and cDNA synthesis: Total RNA of all samples was isolated by RNA isolation kit according to manufacturer's instructions (Denazist, Iran). Briefly, the samples were lysed and homogenized in 1 ml G1 buffer of isolation kit using homogenizer (Heidolph, Germany). According to manufacturer's protocol, the homogenate was incubated at room temperature (20 ˚C) for 10 minutes. In the case of the fat samples, insoluble material was removed using pipette tip from the homogenate after centrifuging. After that, 200 µl chloroform per milliliter of G1 buffer was added. Samples were centrifuged at 12,000 g for 15 min at 4 ˚C and upper phase containing RNA was precipitated with equivalent of half the volume aqueous phase of the isopropyl alcohol and the same volume from G2 buffer. Then, samples were washed with 75% ethanol (diluted with diethyl pyrocarbonate-treated water), dried in contact with air, and resuspended in 50 µl DEPC treated water. In order to remove any possible genomic DNA from the RNA samples, five unit RNase free DNAse I (Roche, Germany) was added per each 20 µg of RNA and incubated at 34 ˚C for 20 min, followed by adding 0.8 µl of 0.5 M EDTA and heat inactivation of the enzyme at 75 °C for 10 min. The quantity and quality of all extracted RNAs were verified using Nanodrop Spectrophotometer and agarose gel (2%) electrophoresis respectively.

For cDNA synthesis, an AccuPower kit (Bioneer, South Korea) was used. First, 1 µg of total RNA and 1 µl Oligo(dT) 18 (Thermo Scientific) were mixed and incubated at 70 °C for 5 minutes. Then, the mixture was transferred to tube containing reverse transcriptase, dNTP mix and reaction buffer. Diethylpyrocarbonate (DEPC) treated water was added up to final volume of 20 µl and was followed by heating for 60 min at 42 °C. Incubation at 70 °C for 10 min was performed and was followed by 94 °C for 5 min to end the reaction. The reaction was carried out in a DNA thermocycler (Eppendorf personal, Germany).

Polymerase chain reaction (PCR): Oligonucleotide primers for amplification of ovine leptin and functional leptin receptor as well as beta actin were designed from GenBank (NCBI) information (Table 1) using Primer Premier V.5 (Premier Biosoft International, Palo Alto, CA, USA). The forward and reverse functional leptin receptors were designed to span the junction of two exons to be RNA specific. So, the amplification of the cDNAs and DNAs of the same gene resulted in the different lengths of PCR products. One µl of cDNA was amplified by PCR in a final volume of 25 µl with12.5 µl Master Mix (Ampilicon, Denmark), 10.5 µl water and 0.5 µl of each primer. The program was set for PCR thermal cycling as follows: 1 cycle 94 °C for 5 min, followed by 40 cycles of denaturing at 94 °C for 45 s, annealing at specific temperature of each

Table 1. Primers used in PCR reactions.

Gene name	GenBank Acc. No.	Forward	Reverse	Annealing temperature	Fragment size
Beta actin	NM_001009784	CGGGAAATCGTCCGTGAC	CCGTGTTGGCGTAGAGGT	58	277
Leptin	AF310264.1	CAGCAGAACAAAGGAGGA	CAGCCCATAGCACCAGT	50	162
Functional leptin receptor	NM_001009763.1	GAAGGAGTAGGGAAACCGAAGA	CAAGCAATAAGATTGAGGAGGAGAT	57.4	121

gene for 45 s (Table 1) and extension at 72 °C for 45 s. The program was terminated with a final extension step at 72 °C for 10 min.

Fat tissue was used as the positive control for leptin and leptin receptor expression. The housekeeping gene beta actin was used as an internal control to verify successful isolation of RNA and preparation of cDNA. 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 100 bp molecular ladder as a standard size marker. In addition, PCR products of amplified fragments of 3 genes were sequenced (Macrogen, South Korea), and the sequencing results were edited by CLC bio software. The edited sequences were finally aligned in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to verify the results.

Results

As the mRNA for ovine beta actin acted as an internal positive control, the amplification of beta-actin was first assessed in all samples. Gel electrophoresis results showed that only a 277-bp fragment was amplified in all samples (Figure 1). This finding confirms RNA purification as well as proper RT-PCR conditions.

Sheep corpus luteum expresses leptin mRNA: Gel electrophoresis results showed that the predicted PCR product fragment (162 bp) of leptin has been amplified in both corpus luteum and adipose tissue (positive control) (Figure 2). Thus, the RT-PCR analysis confirmed that leptin mRNA is expressed in the ovine mature corpus luteum.

Sheep corpus luteum expresses leptin receptor mRNA: Observation of predicted amplified fragment of 121 bp on gel electrophoresis using leptin receptor primer pair confirmed that functional leptin receptor mRNA is expressed in the ovine corpus luteum (Figure 3). Adipose tissue was used as the positive control for the expression of Ob-Rb mRNA.

Finally, the amplified products were sequenced. Then the questionable sequences (amplified fragments) were aligned with their recognized sequences in NCBI. The correct alignment of sequences in NCBI verified the results.

Discussion

The identification of genes affecting various aspects of corpus luteum function is the first step in understanding the biology of this organ. An understanding of these mechanisms is potential knowledge for development of new methods to regulate or manipulate the ovarian cycle, and finally to improve fertility in animals (Casey et al., 2004). To our knowledge, in this study for

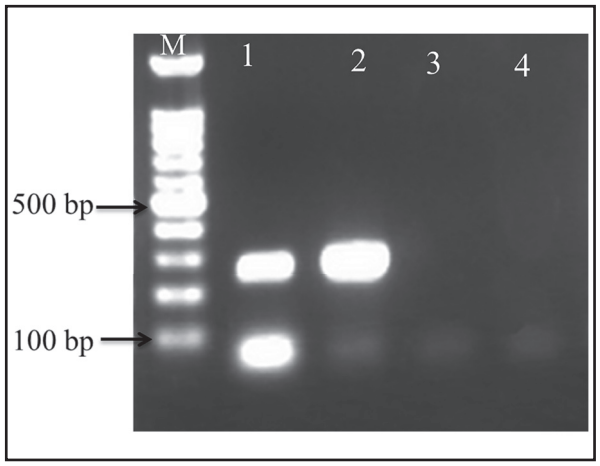

Figure 1. Beta actin was used as positive internal controls for samples to verify that the RT-PCR reactions were successful and confirm the absence of genomic contamination. Representative agarose gel demonstrating amplification of 277-bp bovine beta actin cDNA from fat (lane 1) and corpus luteum (lane 2). Lane 3 was negative control; lane 4 was RT minus (extracted RNA from corpus luteum was used as template); and M was 100-bp DNA marker.

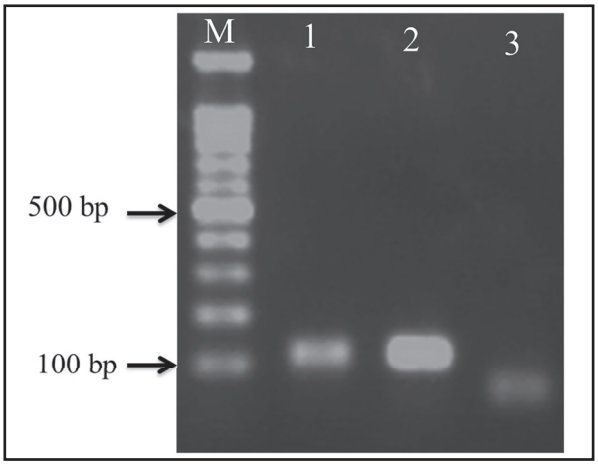

Figure 3. Detection of ovine leptin longform receptor (Ob-Rb) mRNA. Representative agarose gel demonstrating amplification of 121-bp Ob-Rb cDNA from ovine fat (lane 1) and ovine corpus luteum (lane 2). Lane 3 was negative control and M was 100-bp DNA marker.

the first time we succeed to show the expression of leptin and its receptor (Ob-Rb) mRNA in ovine corpus luteum, which is in agreement with results from other species such as cow (Nicklin et al., 2007), canine (Balogh et al., 2012), buffalo (Kumar et al., 2012), and porcine (Smolinska et al., 2007).

Evidence suggests that leptin has a role in follicular development and subsequent

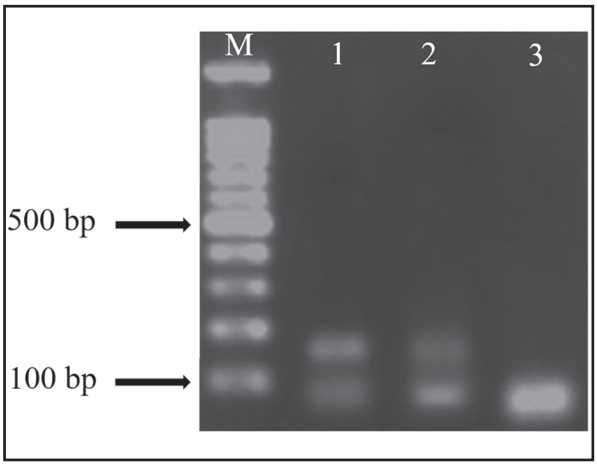

Figure 2. Representative ethidium bromide-stained gel electrophoresis of leptin cDNA fragments (162-bp) amplified by RT-PCR from ovine fat (lane 1) and corpus luteum (lane 2). Lane 3 was negative control and M was 100-bp DNA marker.

luteal function (Castracane and Henson, 2006). Leptin regulates reproductive function through several mechanisms such as altering the sensitivity of the pituitary gland to GnRH and the regulation of luteal steroidogenesis (Hausman et al., 2012). It has been shown that an excessive amount of leptin in bovine theca and granulosa cells inhibits steroid synthesis (Spicer and Francisco, 1998). In buffalo, expression of leptin and its receptor increase during the formation of the corpus luteum and decrease over disappearance of corpus luteum. The expression pattern of leptin and its receptor is associated with a pattern of progesterone secretion which refers to positive effect of leptin on corpus luteum steroidogenesis function (Kumar et al., 2012).

Angiogenesis is an essential process for the developing corpus luteum. Angiogenic factors such as vascular endothelial growth factor-A (VEGF-A) and basic fibroblast growth factor (FGF-2) have a central role in stimulating cell proliferation, angiogenesis and the regulation of the stability of blood vessels in the growing corpus luteum (Tamanini and De Ambrogi, 2004; Miyamoto

et al., 2013). Leptin adjusts the production of VEGF and Angiopoeitin-1 and FGF-2 in growing luteal tissue and results in the formation of the corpus luteum and progesterone production synthesis (Kumar et al., 2012). In goat, Leptin stimulates the expression of these growth factors in the early luteal phase which may lead to luteal angiogenesis (Wiles et al., 2014). Aberrant production of angiogenic factors may result in vascular dysfunction and the development of ovarian disorders in women (Hazzard and Stouffer, 2000). In horse's corpus luteum, leptin can enhance progestrone, protaglandin E2, MIF (macrophage migration inhibitory factor), TNF-α (tumor necrosis factor-alpha), nitric oxide, and angiogenesis in the early stages of the corpus luteum activity (Galvão et al., 2014). Studies on pig ovary have shown that leptin and leptin receptor increase during formation of the corpus luteum and then decrease as the corpus luteum regresses. Therefore, it is thought that the expression of the leptin receptor is associated with the highest levels of progesterone (Smolinska et al., 2007).

In conclusion, our findings in this study confirmed the mRNA expression of leptin and functional leptin receptor in ovine corpus luteum. So, we are sure that the existence of leptin ligand and its receptor mRNA may indicate the involvement of leptin in the physiology of ovine corpus luteum, which should be investigated in further studies.

Acknowledgments

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بیان رونوشت لپتین و گیرنده آن در جسم زرد گوسفند

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چکیده

زمینه مطالعه: لپتین که پروتئین حاصل از بیان ژن چاقی است به عنوان یک آدیپوکاین حاوی پیام در تنظیم اخذ غذا، متابولیسم انرژی و فعالیت های تولیدمثلی پستانداران عمل می کند. اثرات تولیدمثلی لپتین در سطوح مختلف محور هیپوتالاموس-هیپوفیز-گناد مورد تأیید قرار گرفته است. همچنین، اثر مستقیم و موضعی لپتین در بلوغ اووسیت و جسم زرد گاو مشخص شده است. **هدف:** با توجه به تفاوت‌های احتمالی بین گونه‌ای، این مطالعه به منظور بررسی بیان رونوشت (mRNA) لپتین و گیرنده عملکردی آن در جسم زرد گوسفند طراحی گردید. **روش کار:** تخمدان‌های حاوی جسم زرد از گوسفندان بالغ کشتار شده در فصل تولیدمثلی جمع آوری گردید. RNA تام از نمونه های جسم زرد استخراج شد و سنتز cDNA انجام گرفت. در ادامه، واکنش زنجیره‌ای پلیمراز با استفاده از پرایمرهای اختصاصی هر ژن انجام شد. بتا اکتین به عنوان یک ژن خانه دار برای تأیید صحت واکنش‌ها استفاده شد و بافت چربی نیز به عنوان نمونه کنترل مثبت برای بیان لپتین و گیرنده آن مورد استفاده قرار گرفت. **نتایج:** الکتروفورز محصولات واکنش زنجیره‌ای پلیمراز نشان داد که قطعات موردانتظار یعنی قطعه ۱۶۲ جفت بازی لپتین و قطعه ۱۲۱ جفت بازی گیرنده آن تکثیر شده‌اند. علاوه بر این، تعیین توالی قطعات به دست آمده تأیید کننده نتیجه اولیه بود. **نتیجه گیری نهایی:** یافته‌های ما بیان رونوشت لپتین و گیرنده آن در جسم زرد گوسفند را تأیید کرد. این یافته می‌تواند مبنایی برای انجام مطالعات بعدی جهت بررسی اثرات مستقیم لپتین بر روی جسم زرد گوسفند باشد.

واژه های کلیدی: جسم زرد، رونوشت لپتین، رونوشت گیرنده لپتین، گوسفند، نسخه برداری معکوس-واکنش زنجیره ای پلیمراز

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