Detection of leptin and leptin receptor mRNA expression in the bovine testis

Abavisani, A.[1], Shayan, P.[2], Baghbanzadeh, A.[1]*, Tajik, P.[3]

[1]*Department of Physiology, Faculty of Veterinary Medicine, University of Tehran, Tehran-Iran.*

[2]*Department of Pathophysiology, Faculty of Veterinary Medicine, University of Tehran, Tehran-Iran.*

[3]*Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran-Iran.*

(Received 9 March 2008 , Accepted 29 July 2008)

Abstract: **Leptin, hormonal product of the ob gene, is known to regulate food intake, energy metabolism and reproductive functions in mammals. The mechanism by which leptin affect male reproductive system, in contrast to its well proven effects in female fertility, has been a matter of debate. Expression of leptin and its receptor in some reproductive organs suggest that leptin has both endocrine and paracrine/autocrine effects on reproduction. Various evidences have pointed to a direct role of leptin in the control of rodent testicular function such as steroidogenesis and spermatogenesis. So, detection of leptin and leptin receptor mRNA in bovine testis will be the first crucial step to an understanding of its paracrine/autocrine effect on testes in cattle. In the present study, we showed the expression of leptin mRNA as well as its functional receptor (Ob-Rb) mRNA in whole testis of Holstein cattle using reverse transcription and polymerase chain reaction (RT-PCR) analysis. To confirm the first results, RT-PCR products were amplified with Nested PCR using inner leptin primer pairs designed on different exons. Based on our results, although we could not determine the exact cell source of leptin in testis, it suggests that besides its primary actions at the hypothalamic-pituitary level, leptin can also involved in autocrine and/or paracrine mechanisms in testicular physiology in cattle.**

Key words: cattle, leptin, leptin receptor, testis, RT-PCR.

Introduction

Leptin, the hormone encoded by obesity (Ob) gene, is a 146 aa protein with a tertiary structure similar to cytokines (Zhang *et al.,* 1994). Leptin with diverse biological functions has been shown to contribute to the regulation of energy, metabolism, feeding behavior, and reproduction in mammals by a large variety of neuroendocrine, paracrine, and autocrine actions (Messinis and Milingos, 1999; Baratta, 2002; Williams *et al.,* 2002; Zeiba *et al.,* 2005; Cervero *et al.,* 2006).

This hormone acts through five receptor isoforms. Among these isoforms, only the long form (Ob-Rb) is functional and is responsible for the physiological effects of leptin (Tartaglia, 1997; Kawachi *et al*., 2007). Signal transduction pathways affected by leptin/leptin receptor interaction are the activation of janus kinase/signal transducer and activator of transcription (JAK/STAT) via the full length Ob-Rb and mitogen-activated protein kinase (MAPK) via Ob-Rb and also via the truncated Ob-Ra (Bjorbaek *et al*., 1997).

Although the expression of functional leptin receptor is highest in the central nervous system, the widespread distribution of the receptor in bovine various tissues has been demonstrated over the last decade, including fat, mammary parenchyma, liver, muscle, adrenal cortex, testis, lung, spleen, kidney, abomasum, small intestine, and this could be understand as an evidence for multiple peripheral

*Corresponding author's email: abaghban@ut.ac.ir, Tel: 021-61117081, Fax: 021-66933222

effects of leptin (Frunbeck, 2001; Silva *et al*., 2002; Smith and Sheffield, 2002; Chelikani *et al.,* 2003; Sayed-Ahmed *et al*., 2004; Bartha *et al*., 2005; Kawachi *et al*., 2007).

In comparison, the expression of bovine leptin has been only detected in adipose tissue, mammary parenchyma (Silva *et al*., 2002; Smith and Sheffield, 2002; Chelikani *et al.,* 2003; Sayed-Ahmed *et al*., 2004; Bartha *et al*., 2005) and calf rumen, abomasum, and duodenum (Yonekura *et al*., 2002). Based on these findings, it is believed that leptin mainly acts through endocrine route. Nevertheless, based on an extensive growing numbers of recent publications, leptin also expresses in some other tissues such as mouse testis (Herrid *et al*., 2007), mouse placenta and fetal tissue (Hoggard *et al*., 1997), stomach (Bado *et al*., 1998), skel *et al* muscle (Wang *et al*., 1998), brain and pituitary (Morash *et al*., 1999), and human spermatozoa(Jope *et al.,* 2003; Aquila *et al*., 2005). So, it increases the probability of paracrine and/or autocrine role of leptin, at least in some organs.

In normal animals leptin serves as a metabolic signal to the reproductive system (Barash *et al*., 1996; Cunningham *et al*., 1999). This appears to be a tightly regulated action, carried out at different levels of the hypothalamic-pituitary-gonadal system (Tena-Sempere, 2002). Although the participation of leptin in female reproduction is well established, the contribution of leptin to the proper functioning of the male reproductive system has been less clear (Camina *et al*., 2002; Tena-Sempere, 2002). The leptin deficient Ob/Ob mouse, exhibit atrophied testes with hollow seminiferous tubules (Barash *et al*., 1996; Mounzih *et al*., 1997); increased apoptosis (Bhat *et al.,* 2005); elevated steroidogenesis (Tena-Sempere, 2002); and small ovaries with reduced numbers of primary and Graafian follicles (Barash *et al*., 1996). However, the underlying mechanisms are not still clear as to how leptin acts through the hypothalamic-pituitary axis to exhibit effects on the reproductive organs or whether leptin can directly affect their development or either the combination of direct and indirect effects (Baratta, 2002; Tena-Sempere, 2002).

As a first step to identify the paracrine or autocrine effects of leptin in cattle, especially in reproduction, it is necessary to demonstrate gene expression and its receptor in reproductive organs. Thus, the aim of the present study was to determine the presence of leptin mRNA and functional leptin receptor (Ob-Rb) mRNA in whole bovine testis using RT-PCR.

Materials and Methods

Tissue samples: Non-lactating mammary gland tissue of female adult Holstein cows and Pericardial adipose tissue, liver and testis tissues of male adult Holstein cows were collected immediately after slaughtering. Samples were transferred to the laboratory next to ice cold at 5 °C and subsequently about 30 mg of each sample was used directly for RNA isolation or kept in 1.5ml tubes and stored at -80 °C until RNA extraction.

RNA isolation and DNAse I treatment: Total RNA was isolated by TRIzol reagent (Invitrogen). Briefly, the samples were first lysed and homogenized in 1 ml TRIzol using vortexing and repeatedly drawing up and down through a 26 gauge needle. The homogenates were incubated at room temperature (20 °C) for 5 minutes. In the case of the fat samples, insoluble material was first removed from the homogenate before incubation. After that, 200 µl chloroform per milliliter of TRIzol was added. Samples were centrifuged at 12,000 × g for 15 min at 4 °C and upper phase containing RNA was precipitated with 500 µl isopropyl alcohol per milliliter of TRIzol, washed with 75% ethanol (diluted with diethyl pyrocarbonate-treated water), air dried and resuspended in 50 µl DEPC treated water. In order to eliminate the possible residual genomic DNA from the RNA samples, one unit RNasefree DNAse I (Fermentas) was added per each 8 µl of RNA sample and incubated at 37 °C for 30 min followed by adding 1 µl 25 mM EDTA (Fermentas) and heat inactivation of the enzyme at 65 °C for 10 min.

Reverse Transcription -Polymerase Chain Reaction: The reverse transcription reaction was performed using 10 µl of total RNA as template with 200 units of MMLV reverse transcriptase, 0.5 µg Oligo(dT) 18, 1 mM dNTP mix, 4 µl 5X reaction

Table 1: Nucleotide sequences of the primer sets used for RT-PCR. *For nested PCR, Leptin R.= Leptin Receptor.

Primers	Gene Bank Acc. No.	Forward	Reverse	Fragment size (cDNA), bp	Fragment size (DNA), bp
leptin (P1/P2)	U50365, AB003143	gtgcccatccgcaaggtcca	tcagcacccgggactgaggt	441	2194
Leptin (P3/P4)*	U50365, AB003143	tcatcaagacaattgtcaccagg	cagctgccgcaacatgtcctg	384	2131
Leptin R.(P5/P6)	NM-001012285, AJ580801	gtgccagcaactacagatgctctac	agttcatccaggccttctgagaacg	380	380
Beta actin(P7/P8)	NM-173979, NC007326	atcactgccctggcacccag	cttagagagaagcggggtggc	509	639

buffer and 20U Ribonuclease Inhibitor for 60 min at 42 °C in a final volume of 20 µl followed by heating at 70 °C for 10 min to stop the reaction. All used reagents were from Fermentas. The RT-PCR were carried out in a DNA thermocycler (MWG, Germany).

Oligonucleotide primers for amplification of bovine leptin and leptin receptor as well as beta actin (forward and reverse primers) were designed from GenBank sequence information (table 1). The forward and reverse leptin as well as the beta-actin primer pairs 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 PCR products in length (as shown in table 1). For the control of DNA contamination, 1 µl DNase I treated RNA from each sample was amplified directly with the primer pairs listed in table 1.

One µl of RT reaction product was amplified by PCR in a final volume of 50 µl with 0.2 mM dNTP mix, 20 pmol of each primer, 1.25 units SmarTaq DNA Polymerase, 1.5 mM MgCl2 and 5 µl 10X PCR buffer. PCR thermal cycling parameters were as follows: 1 cycle 94 °C for 5 min, followed by 40 cycles of denaturing at 94 ?C for 45 s, annealing at 62 °C for 45 s, and extension at 72 °C for 45 s. The program was terminated with a final extension step at 72 °C for 10 min. For nested PCR, using inner leptin primers, 1 µl of PCR reaction product amplified with leptin primers was amplified by second PCR similar to above mentioned condition. Fat and mammary gland tissues were used as positive control for leptin and liver was positive control for leptin receptor. The housekeeping gene beta actin was used as a control for successful isolation of RNA and preparation of cDNA.

All PCR products were run on a 1.5% agarose gel in Tris-borate EDTA buffer, stained with ethidium bromide and visualized under UV transillumination. The location of the predicted products was confirmed using 100 bp or 200 bp molecular ladder as a standard size marker.

Results

Expression of leptin in bovine testis: The amplification of the cDNA prepared with the RNA isolated from fat tissue and non-lactating mammary gland revealed a predicted PCR product with the nucleotide length of 441 bp with the primer pair P1/P2, whereas the RT-PCR with the cDNA from testes (P1/P2) showed no detectable amount of PCR product on agarose gel (Fig.1A, lane 1, 2 and lane 3). Amplification of 1 µl of the above mentioned PCR products with primer pair P3/p4 (nested-PCR) resulted in a predicted PCR product of 384 bp in length for all three tissues (Fig. 1B, Lane 1, 2 and 3). These results confirmed that leptin mRNA expressed in adult testis as well but with low expression level compared to the fat and mammary gland tissues. The fragment size of PCR products using bovine DNA as template for PCR reaction with leptin primer pairs (P1/P2 and P3/P4) was detected as 2194 and 2131-bp band, respectively (data not shown). The mRNA for bovine beta actin acted as an internal positive control and was detected at all samples (Fig.1C lane 2, 3 and 4). No amplification was observed in the negative controls (Fig.1A4, 1B5 and 1C5).

Expression of functional leptin receptor (Ob-Rb) in bovine testis: The RT-PCR analysis using isoform-specific primer pairs confirmed that Ob-Rb

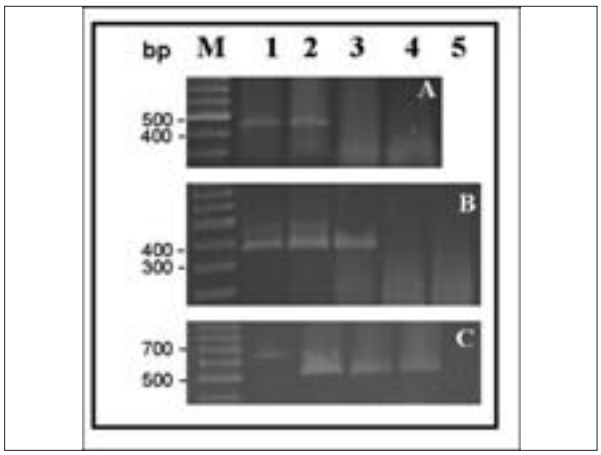

Fig. 1. A- Representative ethidium bromide-stained gel electrophoresis of leptin cDNA fragments (441 bp) amplified by RT-PCR using P1/P2 primer pairs, from fat (lane 1) and non-lactating mammary gland (lane 2). cDNA prepared from testis showed no detectable PCR product (lane 3). Lane 4 was as negative control and M was 100-bp DNA marker. B) Nested PCR analysis of leptin cDNA fragments (384 bp) amplified using P3/P4 primer pairs, from fat (lane 1) and non-lactating mammary gland (lane 2), testis (lane 3) and negative control (lane 4) of first PCR product as template. Lane 5 was as negative control and M was 100-bp DNA marker.C) Representative agarose gel demonstrating amplification of cDNA from above mentioned tissues with specific primer derived from bovine beta actin mRNA. Lane 1= genomic DNA, lane 2= fat, lane 3= non-lactating mammary gland, lane 4= testis and lane 5 = negative control. M was 100-bp DNA marker.

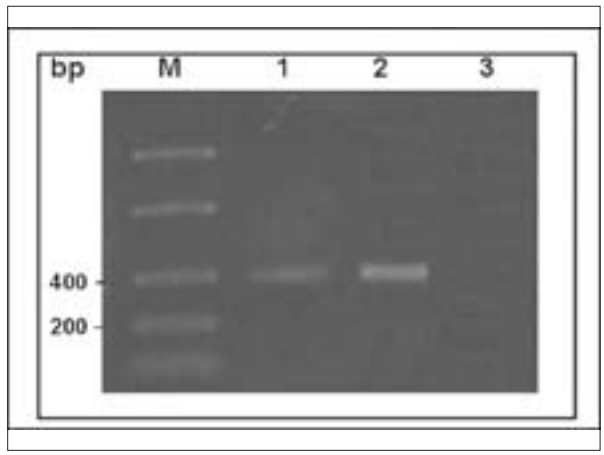

Fig. 2- Detection of bovine leptin longform receptor (Ob-Rb) mRNA. Expression of bovine Ob-Rb mRNA was analyzed by reverse transcription (RT) PCR of RNA isolated from bovine total testis. Representative agarose gel demonstrating amplification of 380-base pair Ob-Rb cDNA from bovine liver (lane 1), bovine testis (lane 2) and isolated RNA after DNase I treatment as template (lane3). M was 200-bp DNA marker.

mRNA is expressed in the adult bovine testis (Fig. 2, lane 2). The 380-bp fragment was not detected following PCR using total RNA incubated without reverse transcriptase as the template, demonstrating the absence of genomic DNA contamination (Fig.2, lane 3). Liver was used as positive control for expression of Ob-Rb (Fig.2, lane 1).

Discussion

This is the first study revealed the expression of leptin in bovine testis. Additionally, the present investigation also confirmed the expression of longform leptin receptor (Ob-Rb) in bovine testis in agreement with other reports in bovine (Chelikani *et al*., 2003; Kawachi *et al*., 2007) and in rodents (Caprio *et al*., 1999; El-Hefnawy *et al*., 2000; Tena-Sempere *et al*., 2001). The specific amplified leptin fragment was not detectable in the first PCR using specific primer pairs but it was clearly amplified in nested PCR.

Although Leptin is able to pass the blood-testis barrier (Banks *et al*., 1999), there is no correlation between leptin concentration in blood and seminal plasma (Camina *et al*., 2002). Thus, it is postulated to be considered an independent production of leptin in testis. However, based on our results, the origin cells of leptin mRNA is not exactly defined.

The Ob-R isoforms are produced by different alternative splicing of the common transcript. Therefore, the patterns of alternative splicing of Ob-R transcript must differ among bovine tissues (Kawachi *et al*., 2007). Ob-Rb has full function because this isoform contains boxes 1-3, which are implicated in signal transduction pathways involving both Janus kinase (JAK) and signal transducers and activators of transcription (Tartaglia, 1997). The presence of Ob-R typical signal transduction, such as phosphorylation of STAT3, were shown in mouse germ cells and phosphorylation of extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) and STAT3 in mouse interstitial cells (El-Hefnawy *et al.,* 2000).

In male animals leptin acts at different levels of the hypothalamic-pituitary-testicular system (Tena-Sempere, 2002). It also does participate in functional regulation of the male gonadal axis (Caprio *et al*., 1999; Tena-Sempere, 2002). The predominant

stimulatory effects of leptin, primarily at the hypothalamus, are observed at its physiological levels above a minimal threshold. In contrast, direct inhibitory actions at the testicular level may take place in the presence of a significantly elevated leptin concentration, as detected in obesity (Tena-Sempere, 2002). Furthere, leptin has a novel direct negative action on LH/hCG-stimulated androgen production from Leydig cells in culture. It is consistent with the reduced testicular function in obese male rats (Caprio *et al*., 1999). leptin has also inhibitory actions on hormonally-stimulated ovarian steroidogenesis in vitro (Spicer and Francisco, 1997).

Leptin treatment rescued the sterility of genetically obese ob/ob males (Mounzih *et al*., 1997) and observation of testicular histology indicated that leptin stimulate cellular activity in the seminiferous tubules (Barash *et al*., 1996). The existence of leptin in human testicular tissue and a negative correlation between leptin concentrations in seminal plasma and the motility of human spermatozoa were described (Glander and Kratzsch, 2000).

It has been shown that expression of Ob-Rb in testis is less than liver in cattle (Kawachi *et al*. 2007). Similarily, the low level of expression of leptin receptor in mouse Leydig cells has been detected but it was highly efficient and functional (Caprio *et al*., 1999).

Our results showed the presence of leptin and leptin receptor in the bovine testis. These results together with above mentioned findings create the condition for an autoregulative mechanism (autocrine or paracrine leptin loop) at this level, in addition to an endocrine effect. Leptin secretion suggests that the bovine testis has the ability to modulate its desired function such as metabolism, relatively independent of the plasma leptin levels.

Acknowledgments

This work was supported by Research Council of University of Tehran under No. 7506004/6/2. We would like to thank Investigating group of Molecular Biological System Transfer (MBST, Iran) for scientific support. Our special thanks to Dr. Gholam-Reza Nikbakht Borojeni and Dr. Mohammad Roostaee Alimehr (Faculty of Veterinary Medicine, University of Tehran, Iran) for the fruitful discussions and Mrs Narges Amini for technical help.

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مجله بین المللی تحقیقات دامپزشکی، ۱۳۸۷، دوره ۲، شماره ۱، ۱۲۵-۱۱۹

شناسایی بیان mRNA لپتین و گیرنده آن در بیضه گاو

عباس ابویسانی[۱] پرویز شایان[۲] علی باغبانزاده[۱*] پرویز تاجیک[۳]

۱) بخش فیزیولوژی دانشکده دامپزشکی دانشگاه تهران، تهران-ایران.
۲) گروه پاتوبیولوژی دانشکده دامپزشکی دانشگاه تهران، تهران-ایران.
۳) گروه علوم درمانگاهی دانشکده دامپزشکی دانشگاه تهران، تهران-ایران.

(دریافت مقاله: ۱۹ اسفند ماه ۱۳۸۶، پذیرش نهایی: ۸ مرداد ماه ۱۳۸۷)

چکیده

لپتین، هورمون حاصل از ژن Ob بوده و در تنظیم مصرف خوراک، متابولیسم انرژی و اعمال تولیدمثلی پستانداران نقش دارد. با وجود اثرات شناخته شده لپتین در باروری جنس ماده، مکانیسم عمل آن در سیستم تولید مثلی نر هنوز مورد سوال است. بیان لپتین و گیرنده آن در برخی از ارگانهای تولیدمثلی پیشنهادکننده اثرات اندوکرینی و هم اثرات پاراکرینی/اتوکرینی آن بر روی سیستم تولید مثل است. شواهد زیادی مؤید اثر مستقیم لپتین در کنترل عملکرد بیضه جوندگان نظیر تولید هورمون های استروئیدی و اسپرماتوژنز می باشند. بنابراین، ردیابی mRNA لپتین و گیرنده آن در بیضه گاو اولین مرحله از شناسایی اثرات پاراکرینی/اتوکرینی آن در این بافت خواهد بود. در مطالعه اخیر، mRNA لپتین و گیرنده عملکردی آن (Ob-Rb) با روش رونوشت برداری معکوس و واکنش زنجیره ای پلیمراز در بیضه گاو شناسایی شدند. به منظور تایید نتایج اولیه، محصولات حاصل از RT-PCR با روش nested PCR و با استفاده از زوج پرایمرهای اختصاصی طراحی شده بر روی اگزونهای متفاوت تکثیر شدند. اگرچه در مطالعه اخیر منشأ دقیق سلولی لپتین در بیضه شناسایی نشد، لیکن بر اساس یافته های حاصله پیشنهاد می شود که لپتین علاوه بر اثرات اولیه در سطح هیپوتالاموس- هیپوفیز در مکانیسم های اتوکرینی و یا پاراکرینی فیزیولوژی بیضه گاو نیز دخالت دارد.

واژه‌های کلیدی: گاو، لپتین، گیرنده لپتین، بیضه، RT-PCR.

Code 1587

*) نویسنده مسؤول: تلفن: ۰۲۱-۶۱۱۱۷۰۸۱ نمابر: ۰۲۱-۶۶۹۳۳۲۲۲ Email:abaghban@ut.ac.ir