RNA extraction and leptin receptor mRNA detection in bull ejaculated spermatozoa

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Abstract: **Leptin, known as a potential satiety factor, plays an important role in both metabolism and reproduction. The presence of leptin in human seminal plasma and human spermatozoa has been shown; recently, leptin receptors (Ob-R) have been localized in human spermatozoa, thus suggesting a possible action of this hormone even on these cells. Our aim was to detect leptin receptor mRNA in bull ejaculated spermatozoa by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from bull ejaculated spermatozoa and purified by different methods. Our results have revealed that sodium dodecyl sulphate (SDS) and SDS/citric acid extraction methods are superior to guanidinium isothiocyanate in terms of yield and reproducibility of RNA recovery. The mRNA for Ob-Rb was detected in all samples examined. We conclude that Ob-R mRNA is present in bull spermatozoa where seminal plasma leptin can exert its effects.**

Key words: leptin receptor, bull sperm, mRNA.

Introduction

Leptin is a hormone mainly secreted by white adipose tissue and known as a potential satiety factor (Cunningham *et al.*, 1999); it also plays an important role in immune and stress responses, neuroendocrine regulation, haematopoiesis, angiogenesis, and energy expenditure (Seidel *et al.*, 1995; Laud *et al.*, 1999; Lado-Abeal and Norman 2002; Barth *et al.*, 2005). Recent observations in female suggest that leptin is involved in relaying energy status to reproduction (Shalev *et al.*, 1986; Cunningham *et al.*, 1999; Laud *et al.*, 1999; Caprio *et al.*, 2003; Liefers *et al.*, 2005). Various evidences have also pointed out a direct role of leptin in the control of testicular function, but its actual role in the regulatory network controlling male reproduction is still a matter of debate (Shalev *et al.*, 1986; Caprio *et al.*, 2003 Aquila *et al.*, 2005; Yang *et al.*, 2006).

It has long been recognized that capacitated sperm display an increased metabolic rate and overall energy expenditure, presumably to modulate the changes in sperm signaling and function during capcitation (Ando and Aquila, 2005).There is a lack of information regarding how mammalian spermatozoa manage their energy status. In somatic cells, both leptin and insulin play a central role in regulation of energy homeostasis. Particularly, *in vitro* and *in vivo* evidences support the hypothesis that leptin can mimic insulin action on glycogen synthesis (Ando and Aquila, 2005). Sperm glycogen metabolism seems to be regulated by a modulation of glycogen synthesis in a manner similar to that

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Table 1: Purity and concentration of extracted RNA from bull spermatozoa in different experiments. *PK: Protein kinase. **Sodium dodecyl Sulfate.

	RNA µg/ml	OD 260/280	OD 260/230
Guanidium	11 ± 1	1.553 ± 0.09	0.486 ± 0.09
guanidium+PK*	12 ± .004	1.5 ± 0.006	0.44 ± 0.003
SDS**	14 ± 1.4	1.86 ± 0.3	0.81 ± 0.16
SDS+PK	6 ± 1	1.44 ± 0.07	1.07 ± 0.21
SDS+ citric acid	14 ± 1	1.55 ± 0.07	1.295 ± 0.645

observed in other tissues (Aquila *et al.*, 2005). However, evidences exist that leptin and its receptor are also important in the energy metabolism of the spermatozoa.

Leptin mediates its biological effects by interacting with several receptor isoforms that have identical extracellular and transmembrane domains but different intracellular domain. A total of six leptin receptor isoforms (OB-Ra, OB-Rb, OB-Rc, OB-Rd, OB-Re and OB-Rf) has been demonstrated in the different mouse and human tissues. These isoforms derive from a single gene by alternative splicing (Cunningham *et al.*, 1999; Lado-Abeal and Norman 2002). The long isoform (Ob-Rb) presents a long cytoplasmic region containing several motifs required for signal transduction (EL-Hefnawy and Dym, 2000; Sayed-Ahmad *et al.*, 2005). OB-Ra with truncated intracellular domain is the short isoform and presents a limited signal transduction potential only via mitogen-activated protein kinase (MAPK) (EL-Hefnawy and Dym, 2000; Ingvartsen and Boisclair, 2001; Jope *et al.*, 2003; Munzberg *et al.*, 2005; Yang *et al.*, 2006).

The aim of this work was to verify whether Ob-R is present in bull spermatozoa; moreover, we set up a protocol to extract RNA from bull spermatozoa as the human protocol is unsuitable for this species.

Materials and Methods

Semen sample preparation: Ejaculated spermatozoa from four bulls of haven fertility were obtained using an artificial vagina. A complete semen analysis was performed on each sample: The sperm concentration was $>1 \times 10^9$ /ml with motility > 65%, normal morphology > 65% and viability > 70 %. Viability was evaluated by tripan blue staining. Briefly, 50µl of undiluted semen was mixed with tripan blue solution (10 g tripan blue per liter of PBS) for 10 min then smears were prepared. The percentage of viable sperm identified by tripan blue was determined by counting 200 sperm under magnification (X 400) with bright-field microscopy. Unstained sperm were considered as viable whereas sperm showing complete blue staining were considered as dead. Motile sperm were isolated by the swim-up procedure, 250 µl of semen was layered under 1 ml of BO medium in each of 15-ml centrifuge tubes (Tajik *et al.*, 1993). After incubation for 1 h at 37°C in an atmosphere with 5% CO_2, 750 µl was removed from the top of each tube (Parrish *et al.*, 1986; Seidel *et al.*, 1995). The purity of selected spermatozoa population was checked under a light microscope. Sperm were concentrated by centrifugation at 800 X g for 10 min, the top of suspension was removed frozen in liquid nitrogen vapor and held in liquid nitrogen until RNA extraction.

RNA isolation and RT-PCR: Homogenized cattle liver tissue was used as a source of cells expressing leptin receptor. Total RNA from liver was extracted according to Chomczynski and Saachi (1986) as described below.

Total RNA was isolated from bull ejaculated spermatozoa and purified by different methods as follow:

Acid guanidinium thiocyanate-phenol-chloroform extraction method: Total RNA was extracted according to Chomczynski and Saachi (1986). Before extraction, samples were treated with or without Proteinase K (0.4 mg/ml). Briefly, 2 x 109 cells after Proteinase K treatment or directly were prepared in 0.5 ml denaturing solution containing 4M guanidinium thiocyanate. The homogenate was mixed sequentially with 50µl sodium acetate (2M pH

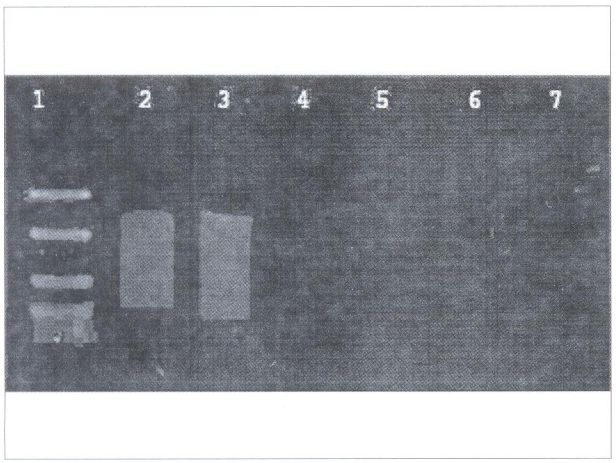

Figure 1. Analysis of RNA preparations on a 2% agarose gel and stained with ethidium bromide. Results from the initial attempt to isolate RNA from motile bull spermatozoa are shown. The numbers in the figure indicate: (1) Molecular weight standards (Fast Ruler DNA ladder, low Range, Fermentas, Germany); (2) SDS/citric acid extraction method; (3) SDS extraction method; (4) SDS/Proteinase K extraction method; (5) Guanidinium extraction method; (6) Guanidinium/PK extraction method; (7) Water.

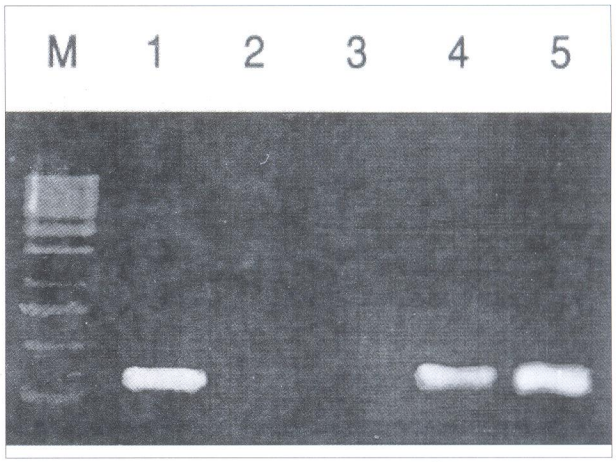

Figure 2. OB-Rb transcript in the sperm and liver. M = molecular weight standards (1 Kb DNA ladder, Fermentas, Germany). The numbers in the figure indicate: (1) DNA template was used as positive control for OB-Rb; (2) and (3) respectively RNA of sperm and hepatic cells were used as negative control; (4) sperm; (5) hepatic cells were used as control positive for OB-Rb.

4), water saturated phenol, and chloroform/isoamylalcohol (49:1). The resulting mixture was centrifuged, yielding an upper aqueous phase containing total RNA. Followed by 100% isopropanol precipitation, the RNA pellet was redissolved in 300µl denaturing solution, reprecipitated with isopropanol, and washed with 75% ethanol. The RNA samples were resuspended in 30µl DEPC-treated water and stored at -80°C.

SDS base lysis method: In this method SDS lysis solution (Tris-HCl 10mM, NaCl0.1M, EDTA 25mM and SDS 4%) with or without citric acid (25 mM) was used. Before adding SDS lysis solution, samples were also treated with or without Proteinase K (0.4 mg/ml). After 10 minutes incubation with SDS lysis solution the homogenate was mixed sequentially with 2M sodium acetate pH 4, water saturated phenol and chloroform/isoamylalcohol (49:1). The resulting mixture was centrifuged, yielding an upper aqueous phase containing total RNA. Followed by 100% isopropanol precipitation, the RNA pellet was redissolved in denaturing solution, reprecipitated with isopropanol, and washed with 75% ethanol. The RNA samples were resuspended in DEPC-treated water and stored at -80°C.

Analysis of RNA purity and concentration: Yield and purity of RNA for each method was determined by Biophotometer (Eppendorf, Germany) (OD 260/280 and 260/230 ratio) and visualized by electrophoresis after DNaseI treatment on a 2% agarose gel, stained with 0.5 ?g/ml ethidium bromide. RNA samples with no DNA bands after DNaseI treatment were used in RT-PCR reactions.

cDNA synthesis and RT-PCR: RNA samples were treated by DNAase I at 37°C for 30 min and then the enzyme was inactivated at 75°C for 15 min. Treated RNA was reverse transcribed to cDNA in a 20 µl final volume containing 1 µg of extracted RNA, 200ng random hexamer and 0.5 mM Deoxyribonucleotide triphosphate (dNTP) Mix. The mixture was heated at 65°C for 5 min. 4 U (in 1µl) RNase inhibitor, RT buffer (50 mM Tris-HCL, 75mM KCL, 3mM MgCL2), 10mM DTT and 200 units M-MuLV Reverse transcriptase (Fermentas, Germany) were added. This mixture was incubated for 60 min at 37°C. The prepared cDNA was incubated at 75°C for 15 min to denature the M-MuLV-RT and then stored at -20°C.

Aliquots of 3 µl of the first standard cDNA reaction were amplified in a 50 µl reaction volume containing a final concentration of 1 X PCR buffer, 2.5 mM $MgCl_2$ 0.2 mM dNTP mix, 2 U of

recombinant Taq DNA polymerase and 0.4 mM of each primers of leptin Ob-Rb region (F: 5-GTGCCAGCAACTACAGATGCTCTAC-3 R: 5-AGTTCATCCAGGCCTTCTGAGAACG-3) in bovine genome (GenBank AB199589). Primers were designed according to fully annotated mRNA sequences and have been published for bovine OB-Rb by Chelikani *et al* (2003). Following an initial denaturation at 94°C for 3 min, PCR was performed for 40 cycles of denaturation at 94°C for 1 min, annealing temperature 65°C for 1 min, extension at 72°C for 2 min and final extension of 72°C for 10 min in the last cycle.

15 µl of each PCR product was subjected to electrophoresis in 1.5% agarose gel and stained with 0.5 µg/ml ethidium bromide.

Results

RNA Extraction: The results of RNA yield for each extraction methods are summarized in Table1. Five methods for RNA extraction were compared. Ejaculated bull spermatozoa are reported to contain 0.0018 pg of RNA per spermatozoon (Gilbert *et al.*, 2007). Therefore, the maximum expected yield of RNA was less than 2 µg, an amount too low for accurate measurement by UV absorbance at 260 nm. To estimate the actual amount of RNA isolated by each protocol, the final precipitates were resuspended in 5µl sterile water, electrophoresed through a 2 % agarose gel and stained by ethidium bromide. When the ethidium bromide image was photographed at the concentration ≤12 µg/ml, no staining was detected. However, when the concentration for the photographic image was increased to14 µg/ml, a smear of ethidium bromide staining was observed in the lane containing the sample isolated in SDS methods (Figure 1, lane 2 and 3). No ethidium bromide staining was detectable in the lane containing the sample isolated using guanidium, guanidium /Proteinase K, guanidium citric acid and SDS/ Proteinase K.

Detection of leptin receptor mRNA in bovine spermatozoa Ob-R: RT-PCR results are shown in Figure 2. Since the primers used for the amplification of OB-Rb were designed from nucleotide sequences within one specific exon of gene, the isolated RNA samples after DNaseI treatment were directly used in RT-PCR to demonstrate the absence of genomic DNA contamination in the RNA probes (Figure 2, lane 2 and 3). The 380 bp cDNA was not detected in all RNA samples under study.

As positive control, RNA isolated from liver cells was used. RT-PCR with specific primers for OB-Rb revealed a predicted RT-PCR product of 380 bp in length (Figure 2, lane 5). The RT-PCR products were comparable in all examined sperm materials. Amplification of cDNA prepared with RNA isolated from sperm showed the same predicted RT-PCR products of 380 bp for OB-Rb (Figure 2, lane 4). The cDNA for Ob-Rb was detected in all samples examined.

Discussion

The analysis of RNA in ejaculated spermatozoa by RT-PCR can add to our understanding of the cellular and molecular events which mediate male infertility. Unfortunately there are many limitation and difficulties associated with spermatozoa RNA recovery. The results of the present study show that, following sodium dodecyl sulfate (SDS) or SDS/citric acid treatments, reproducibility of the sperm RNA extraction and the amount of RNA are more than obtained by standard guanidium extraction protocol. Goodwin *et al* (2000) have also reported the advantage of SDS/citric acid extraction method for human spermatozoa.

Spermatozoa are highly differentiated and specialized cells which transport the paternal genome to oocyte. The motility of spermatozoa is necessary for their ability to approach the oocyte, to penetrate investments surrounding it and to arrive at the egg membrane in order to fertilize it. The spermatozoa motility has been reported to depend upon adenosine triphosphate (ATP) which is produced by glycolysis when appropriate substrates (glucose or fructose) are supplied and on the utilization of endogenous substrates when external ones are not available. Seminal plasma and female reproductive tract fluids contain some amounts of fructose, glucose, sorbitol, lactate and pyruvate as a source of energy (Shalev *et*

al., 1986). The leptin receptor has been identified in spermatozoa (Ambrogi *et al.*, 2007; Jope *et al.*, 2003) and it was suggested that leptin and its receptor are involved in modulating differences of amounts of glycogen in spermatozoa for the capacitation period.

In human, Ob-R is normally expressed at high levels in hypothalamic neurons and at low levels in other cell types, including adipocytes and vascular endothelial cells (Cunningham *et al.*, 1999; Lado-Abeal and Norman, 2002). Expression of OB-Rb mRNA has been reported in various bovine tissues such as adipose depots, mammary parenchyma, semintendinosus muscle, liver, adrenal cortex, spleen, kidney, pineal gland, mesenteric lymph node, lung, aorta, abomasum, duodenum, jejunum, ileum, hypothalamus, pituitary, brain stem, cerebral cortex, cerebellar cortex, pons, and testis (but not in ejaculated sperm) (Chelikani *et al.*, 2003). To the best of our knowledge this is the first report concerning the presence of OB-Rb mRNA in bovine spermatozoa. Up to now, OB-Rb mRNA has been only identified in boar ejaculated spermatozoa (Ambrogi *et al.*, 2007).

At present, we do not have any information on the possible actions of leptin on bull spermatozoa. The presence of leptin and leptin receptors and their interactions in human spermatozoa have been demonstrated by Jope *et al* (2003) and Aquila *et al* (2005). Accordingly, the presence of mRNA of Ob-Rb in bull spermatozoa might suggest that leptin acts through leptin receptor to regulate spermatozoa energy expenditure. Moreover, since leptin interacts with insulin to regulate glycogen synthesis in mature spermatozoa, it might also affect sperm motility (Jope *et al.*, 2003). However, it could be speculated that the presence of a receptor mRNA probably indicates that even in this species leptin exerts metabolic effects, as already demonstrated in humans.

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استخراج RNA و تشخیص mRNA پذیرنده لپتین در اسپرم انزالی گاو

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چکیده

لپتین به عنوان عامل بالقوه در سیری شناخته شده و نقش مهمی در متابولیسم و تولید مثل دارد. حضور لپتین در مایع منی و اسپرم انسان مشخص شده و پذیرنده‌های آن در اسپرم انسان مورد بررسی قرار گرفته‌اند که نشاندهنده احتمال اثر این هورمون حتی بر روی سلول‌های مذکور است. هدف ما تشخیص mRNA پذیرنده لپتین در اسپرم انزالی گاو با روش RT-PCR بوده است. RNA تام اسپرم با روش‌های مختلف استخراج و خالص سازی شد. نتایج نشان داد که روش‌های SDS و SDS/Citric acid از لحاظ میزان و موفقیت در تکرار پذیری برای استخراج RNA بهتر از روش گوانیدیم ایزوتیوسیانات هستند. mRNA پذیرنده لپتین در تمام نمونه‌ها تشخیص داده شد. می‌توان نتیجه گرفت که mRNA پذیرنده لپتین در اسپرم انزالی گاو وجود دارد یا به عبارتی در جایی قرار دارد که لپتین مایع منی ممکن است اثر گذار باشد.

واژه‌های کلیدی: پذیرنده لپتین، اسپرم گاو، mRNA.

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