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In situ hybridization and immunohistochemical localization of leptin hormone and leptin receptor in the seminal vesicle and prostate gland of adult rat

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ABSTRACT

The role of leptin in the regulation of male reproductive function is still a matter of debate. Knowledge about a possible source of leptin in the seminal plasma may therefore be helpful in identifying and elucidating the physiological role of leptin hormone in male reproduction. In our investigation, the expression of leptin and its long receptor isoform (Ob-Rb) was studied in adult male Wistar rats using RT-PCR, Southern blot, *in situ* hybridization and immunohistochemistry. RT-PCR analysis revealed the expression of both leptin and its Ob-Rb in the seminal vesicle and prostate gland. *In situ* hybridization also localized the mRNA transcripts of leptin and Ob-Rb in the glandular secretory epithelial cells of prostate gland and seminal vesicle. Immunohistochemistry detected the leptin hormone in the lining epithelium of both male genital glands. In conclusion, these findings suggest that the seminal vesicle and prostate gland could be the possible sources of leptin in the seminal plasma. This leptin might have a direct (paracrine, autocrine or both) effect on epithelial cells of the accessory male genital glands, on the spermatozoa via spermatozoan leptin receptors.

Introduction

Leptin, the product of obese (Ob) gene, is a 16 kDa non-glycosylated peptide hormone with a predicted tertiary structure similar to cytokine, which is essential for fertility in rodents (Zhang et al., 1994; Chehab et al., 1996). Leptin acts as an afferent satiety hormone, regulating appetite, weight gain and fat deposition (Flier, 1997; Houseknecht et al., 1998; Houseknecht and Portocarrero, 1998) via a central effect on the hypothalamic nuclei, where the long isoform of leptin receptor predominates (Tartaglia, 1997). Leptin is mainly, but not exclusively, produced and secreted by adipose tissue (Ahima and Flier, 2000; Chilliard et al., 2001). Ob-Rb, the so-called long isoform of leptin receptor, is thought to be the most important agent for transmitting the leptin signal in cells and is predominantly localized in the hypothalamus, however, it is also found at low levels in many peripheral tissues (Fei et al., 1997).

The first indication that leptin is involved in the regulation of reproduction was obtained from the observation that Ob/Ob mice are infertile and that their fertility could be rescued by exogenous leptin treatment (Chehab et al., 1996; Mounzih et al., 1997). Moreover, leptin has been reported to play a role in the timing and acceleration of the onset of puberty in several species (Cunningham et al., 1999; Almog et al., 2001; Spicer, 2001). Ob-Rb isoform is also highly expressed in the arcuate and ventromedial hypothalmic nuclei that control both sexual behavior and food intake (Fei et al., 1997; Hakansson et al., 1998). Thus it was believed that leptin may facilitate GnRH secretion via indirect mechanisms through interneurons secreting neuropeptides (Moschos et al., 2002). The endocrine effects of leptin on the peripheral reproductive organs of the male are implied by the expression of functional leptin receptor in the testicular germ cells (El-Hefnawy et al., 2000), Leydig and Sertoli cells (Tena-Sempere and Barreiro, 2002), the sperm plasma membrane (Jope et al., 2003; De Ambrogi et al., 2007) as well as male accessory genital glands (Cioffi et al., 1996; Malendowicz et al., 2006a,b). A regulatory role of leptin in the reproductive processes has been reported through the control of the hypothalamic–pituitary–gonadal axis activity in both sexes of different species (Barb, 1999; Foster and Nagatani, 1999; Ahima and Flier, 2000; Amstalden et al., 2000; Budak et al., 2006; Rago et al., 2009).

Although, the free leptin hormone has been identified in seminal plasma of different species (Camina et al., 2002; Lackey et al.,

Abbreviations: bp, base pair; BSA, bovine serum albumin; cDNA, complementary DNA; DAB, 3,3′-diaminobenzidine; DEPC, diethyl pyrocarbonate; DIG, digoxigenin-11-dUTP; dNTP, deoxynucleotide triphosphate; Ob, obese (leptin) gene; Ob-R, leptin receptor; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PEG, polyethylene glycol; RT, reverse transcription; SSC, saline sodium citrate; TBS, tris-buffered saline.

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2002; Glander et al., 2002; Rago et al., 2009) and in pig testes and epididymis, no indication of the source of this hormone in the seminal plasma has been reported. A study by Malendowicz et al. (2006a,b) has revealed the expression of leptin mRNA by RT-PCR in the seminal vesicle and prostate gland without detection of the locally reactive cells, i.e., either the stromal fat cells or the glandular epithelium of these glands. Therefore the purpose of our study was to demonstrate the expression and localization of leptin and Ob-Rb in the prostate gland and seminal vesicle of adult rats in an attempt to shed light on the locally reactive cells of leptin protein in these glands.

Materials and methods

Tissue samples

Five adult male Wistar rats (250–350 g body weight) were killed by lethal intraperitonial injection of sodium pentobarbitone. Both seminal vesicles and different lobes of the prostate gland were rapidly dissected out and samples were quickly frozen in liquid nitrogen and stored at −70 °C until RNA extraction. The remainder of the glands were fixed in diethyl pyrocarbonate (Sigma–Aldrich, St. Louis, MO, USA) treated phosphate buffered saline (PBS) containing 4% paraformaldehyde for *in situ* hybridization and immunohistochemistry. Procedures involving animals and their care were conducted in conformity with the standards for animal experiments in our university and are in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (1996).

RT-PCR

Total RNA was isolated from the prostate gland and seminal vesicle using the TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. Small pieces of the different prostatic lobes were collected together and used to isolate the RNA. RNA yields and purity were assessed by absorbency at 260 and 280 nm. Accepted ratios of absorption (260/280) were between 1.7 and 2.0. In order to eliminate the residual genomic DNA from the RNA samples, one unit DNaseI (Roche, Mannheim, Germany) was added per each microgram of RNA and incubated at 37 °C for 30 min followed by heat inactivation of the enzyme at 75 °C for 5 min.

RT reaction was performed using 2 μg of total RNA with 200 units of MMLV reverse transcriptase (Promega, Madison, WI, USA), 1 μg Oligo (dT) 15 and 10 mM dNTPs for 60 min at 42 °C in a final volume of 25 μl. To avoid residual contamination with genomic DNA, which could lead to false-positive PCR results, RNA was replaced by water for a negative control during the RT reaction. 2 μl of RT reaction product was amplified by PCR in final volume of 30 μl with 200 μM dNTPs, 25 pmol of each primer, 1.5 units Red Taq Polymerase (Sigma–Aldrich, St. Louis, MO, USA) and 10× Red Taq PCR buffer. PCR thermal cycling parameters were as follows: 1 cycle 94 °C for 3 min, followed by 35 cycles of denaturing at 94 °C for 50 s, annealing at 58 °C for 40 s, and extension at 72 °C for 1 min. The program was terminated with a final extension step at 72 °C for 5 min. To rule out the possibility of amplifying genomic DNA, in some experiments PCR was carried out without prior RT of the RNA.

Oligonucleotide primers for amplification of rat leptin and the long form of leptin receptor were selected from the specific rat sequences reported in the GenBank (Ogawa et al., 1995 and Takaya et al., 1996). Ap3 5′-gtcggtatccgccaagcagaggg-3′ and Ap14 5′-ggagtagagtgaggcttccaggac-3′ for amplification of 277 bp fragment of the rat Ob cDNA, and Ap22 5′-agggttctatttgtattagtgacc-3′ and Ap23 5′-gaaatttccctcaagtttcaaaag-3′ for amplification of 353 bp of the rat Ob-Rb. PCR products were detected by electrophoresis in a 1% agarose gel containing ethidium bromide. Southern blot analysis using water buffalo DIG labelled cDNA probes (Sayed-Ahmed et al., 2003) confirmed the specificity of the amplified products. The specific PCR products were cloned into PGEM-T vector (Promega, Madison, WI, USA) and used to construct DIG labelled RNA probes that used for *in situ* hybridization.

In situ hybridization

Paraffin embedded tissue was used to localize the mRNA transcripts for Ob, and Ob-Rb genes within the prostate and seminal vesicle glands by DIG labelled RNA probes using DIG RNA Labeling Kit (Roche). The RNA probes were generated by in vitro transcription of a linearized plasmid with NcoI or NotI (New England Biolabs, Beverly, MA, USA) for transcription with T7 or SP6 RNA polymerase to generate antisense and sense probes. Deparaffinized and hydrated sections were treated by 200 μl of 200 mM HCl for 10 min to denature the proteins then washed by DEPC water for 10 min. The sections were permeabilized for 30 min with DEPC treated PBS containing 10 μg/ml RNase free Proteinase K (Boehringer, Mannheim, Germany), followed by post-fixation for 10 min at 4 °C with DEPC treated PBS containing 4% paraformaldehyde. To reduce the non-specific binding of probes to positively charged amino acids, the slides were placed in freshly mixed 0.25% acetic anhydride in 0.1 M triethanolamine HCl, pH 8 for 10 min at room temperature. Finally, the sections were washed by DEPC water and overlaid with 30 μl of hybridization buffer containing 10 ng of DIG labelled antisense or sense RNA probe and covered by 18 × 18 coverslip. The slides were placed on a hot plate at 94 °C for 4 min then incubated at 45 °C for 20 h. The hybridization buffer consists of 50% formamide, 0.3 M NaCl, 10 mM Tris/HCl (pH 8), 1 mM EDTA, 5× Denhardt, 500 μl/ml yeast tRNA (Life Technologies, Inc., Gaithersburg, MD, USA), 10% PEG (Mw 6000; Fluka, Buchs, Switzerland), 10 mM vanadyl–ribonucleoside complex (New England Biolabs, Beverly MA, USA) and 100 μg/ml salmon testis DNA (Sigma–Aldrich) which was denatured at 99 °C for 10 min before being added to the hybridization buffer.

Post-hybridization slide processing included 2 × 10 min wash with 2× SSC at room temperature, followed by a 30 min incubation with 20 μg/ml RNase A in 2× SSC at 37 °C. The slides were then subjected to 1× SSC for 20 min at 37 °C and 2 × 15 min washes with 0.1× SSC at 46 °C. Detection of hybridization was achieved using DIG Nucleic Acid Detection Kit (Roche, Mannheim, Germany) according to manufacturer's instruction. Finally, the slides were air-dried and mounted with Entellan (Merck, Darmstadt, Germany) and photographed by light microscopy (Labomed microscope, LaboAmerica, Fremont, CA, USA; Hirocam MA88-500 digital camera eyepiece, Shanghai, China). For negative controls, parallel sections were hybridized either with the sense probe or with buffer alone.

Immunohistochemistry

Paraffin sections from prostate and seminal vesicle glands were used for immunohistochemistry using Rabbit anti-leptin polyclonal antibody (ABR-Affinity BioReagents, Golden, CO, USA). After inactivation of endogenous peroxidase, the sections were placed in 0.01 mol/l citrate buffer (pH 6) and heated in microwave oven for 10 min. The sections were blocked by PBS containing 5% bovine serum albumin for an hour, and then incubated with rabbit anti-leptin polyclonal antibody (1000× dilution in 1% BSA) at 4 °C overnight and for an hour at room temperature. The sections were washed by PBS for 5 min and incubated with biotinylated goat anti-rabbit IgG for 30 min at room temperature. The secondary antibody was detected with Vectastain ABC kit (Vector Laborato-

ries, Peterborough, UK) and the color was developed using DAB (Sigma–Aldrich). Finally, the sections were counterstained with hematoxylin, washed by distilled water, air-dried and mounted with Entellan (Merck) and photographed by light microscopy. In control experiments, preabsorption of the antibody with its respective antigen or elimination of the primary antibody was performed, which abolished all staining.

Results

RT-PCR and Southern blot

RT-PCR analysis of total RNA isolated from the different prostatic lobes and seminal vesicle of rat revealed the presence of both leptin (Ob) and the long form of leptin receptor (Ob-Rb) mRNA transcripts in these tissues (Fig. 1). Southern blot hybridization of RT-PCR products showed the specificity of the amplified products, which was hybridized with specific water buffalo DIG labelled cDNA probes for Ob, and Ob-Rb (Fig. 2).

Cellular localization of Ob and Ob-Rb

In situ **hybridization**

mRNA transcripts of leptin and its Ob-Rb were localized in the cytoplasm of the pseudostratified epithelial cells of both prostate and seminal vesicle (Figs. 3, 4, 7 and 8). No signals were, however, detected in the underling stroma. Control sections incubated with the sense probe did not show any cellular staining (Figs. 5, 6, 9 and 10).

Immunohistochemical observations. Immunohistochemical staining of leptin protein confirmed the result of *in situ* hybridization. Leptin immunostaining was only localized in the pseudostratified epithelial cells of both prostate and seminal vesicle (Figs. 11–14). Notably, neither mRNA transcripts nor protein of leptin and its Ob-Rb were detected in the coagulating gland (previously called anterior prostate) (Figs. 15 and 16).

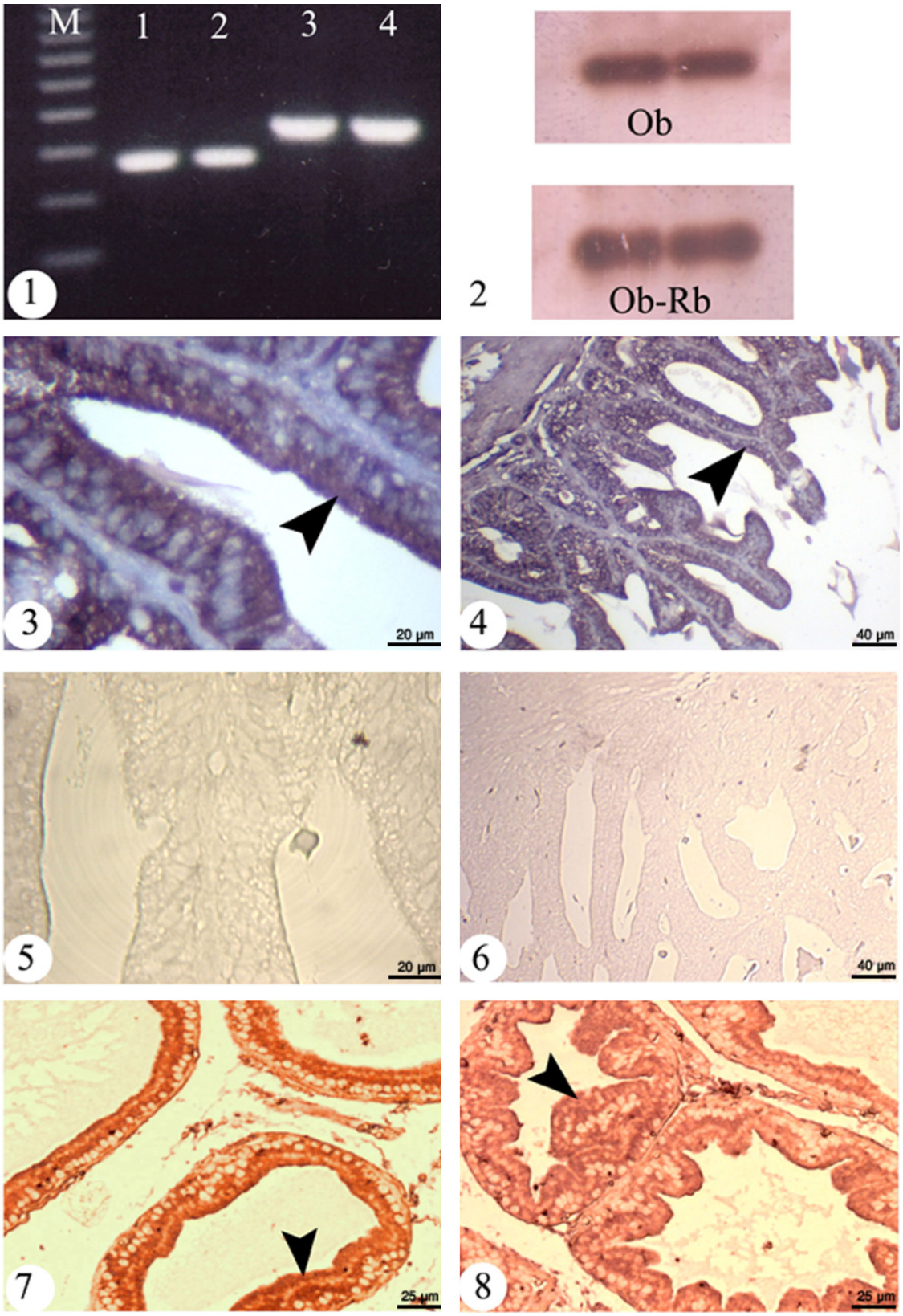
Discussion

Evidence for the potential role of leptin in influencing male fertility is provided by the fact that the ob/ob mice (lacking functional leptin) or Ob-R/Ob-R mice (lacking functional leptin receptor) are infertile and fail to undergo normal sexual maturation. Importantly, fertility of ob/ob mice is restored by leptin and not by simply reducing body weight, indicating an effect of the leptin hormone on reproductive function (Mounzih et al., 1997; Cunningham et al., 1999; Hileman et al., 2002). In addition, systemic leptin treatment or its active fragment induces FSH and LH secretion in male mice and rats (Tena-Sempere and Barreiro, 2002). In humans, the absence of endogenous leptin has been linked to hypogonadism and absence of pubertal development (Strobel et al., 1998). Also it was found that the Ob-Rb isoform, the most important form of leptin receptor, is expressed in the testis germ cells of young and adult mice (El-Hefnawy et al., 2000), ejaculated spermatozoa in human (Jope et al., 2003), boar (De Ambrogi et al., 2007) and pig testes and epididymis (Rago et al., 2009). Thus, leptin is implicated in the regulation of reproduction at the level of intratesticular (El-Hefnawy et al., 2000) or extratesticular spermatozoa (Jope et al., 2003; De Ambrogi et al., 2007; Aquila et al., 2005). Compelling evidence supports the role of leptin in the physiology of the female reproductive system in a paracrine and/or endocrine fashion (Casanueva and Dieguez, 1999; Ahima and Flier, 2000; Wauters et al., 2000; Moschos et al., 2002). In contrast, leptin involvement in the control of male reproduction is still not well defined (Rago et al., 2009).

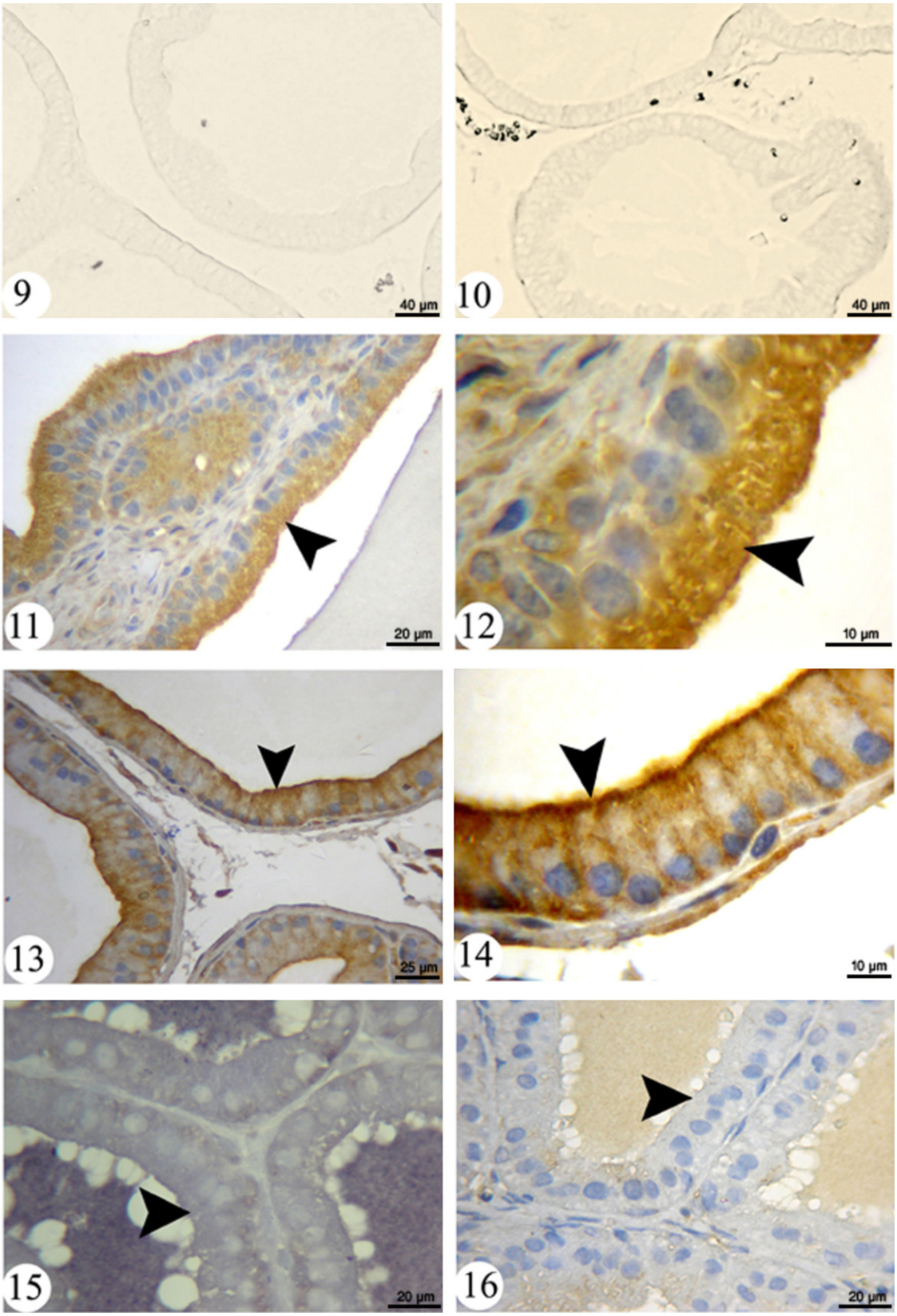
In the present study, we investigated the expression and localization of leptin hormone and it's Ob-Rb in the seminal vesicle and prostate glands of rat by means of RT-PCR, Southern blot, and *in situ* hybridization. Additionally, the immunohistochemical detection of leptin protein was carried out. Our results showed that the mRNA of leptin and Ob-Rb were expressed in the tissues collected from the different prostatic lobes and seminal vesicle. Such findings are consistent with the data of Malendowicz et al. (2006a,b). However, our results have additionally revealed that the leptin transcripts and its corresponding proteins were solely localized in the pseudostratified epithelial cells of prostate and seminal vesicles of rat. Similarly, Ob-Rb signals were detected in lining epithelium of both glands. This observation of Ob-Rb is consistent with *n* previously reported findings in humans (Cioffi et al., 1996) and rats (Malendowicz et al., 2006a,b). Although, we were unable to detect the leptin hormone in the coagulating gland, neither at the level of the mRNA transcripts nor at the level of protein, Malendowicz et al. (2006a,b) reported the expression of leptin mRNA in the coagulating gland using RT-PCR. This discrepancy may be attributed to the presence of capsular stromal fat cells in the coagulating gland. Our interpretation is substantiated by the findings that real time-semi-quantitative estimation showed the highest expression of leptin mRNA in the seminal vesicles and dorsal prostatic lobe, and the lowest expression in the coagulating, lateral and ventral lobes (Malendowicz et al., 2006b). Moreover, it is well known that the adipose tissue is the main source of leptin (Ahima and Flier, 2000). Taken together, the lining epithelium of the prostate and seminal vesicles may be one of the possible sources of the seminal plasma leptin. However, this speculation needs further studies to be undertaken.

The human prostate differs considerably from that of rodents with regard to embryological development, adult anatomy, and etiology of disease. Nevertheless, the rat has been used extensively as an animal model to study normal prostatic development and physiology and hormonal initiation and promotion of cancer (Lucia et al., 1998). The association between leptin and its functional Ob-Rb receptor in the same epithelial cells of the rat prostate and seminal vesicles glands may indicate either autocrine and paracrine action or both. This suggestion is augmented by the data of several previous reports. Leptin acts as a mitogenic and antiapoptotic factor for cancer cells enhancing the growth of cancers via MAPK and PI3-K pathways in prostate cancers (Somasundar et al., 2004). Leptin level influences cellular differentiation and progression of prostate cancer (Saglam et al., 2003). Also, high expression of leptin receptor mRNA has been observed in the prostate (Cioffi et al., 1996) and immunoreactive leptin receptor is demonstrated in normal prostate epithelial cells, in high grade PIN, and in prostate cancer cell (Stattin et al., 2001). A statistically significant positive correlation was present between serum leptin and prostate, seminal vesicle, and testis weight (Nazian and Cameron, 1999). Although obesity and leptin may not necessarily increase the risk of prostate cancer, they seem to promote it once established (Baillargeon and Rose, 2006) and to favor the development of its more aggressive forms (Amling, 2005). Moreover, men with elevated serum leptin concentration have an increased risk of prostate cancer (Chang et al., 2001; Stattin et al., 2001).

The effect of the prostatic and seminal vesicle leptin may extend (if released to the seminal plasma) to the extratesticular spermatozoa via sperm leptin receptors. Aquila et al. (2005) showed a significant increase in cholesterol efflux upon leptin treatment in uncapacitated sperm with marked increases of the sperm proteins tyrosine phosphorylation, which is an event tightly related to the capacitation and resulting downstream cholesterol efflux (Travis and Kopf, 2002). In somatic cells, both leptin and insulin play a central role in regulation of energy homeostasis, acting on PI3K/Akt pathway (Aiston and Agius, 1999). Similarly, in uncapacitated sperm, both insulin and leptin increased PI3K activity, Akt



Figs. 1–8. Fig. 1. RT-PCR of leptin and leptin receptor. Lanes 1 and 2: RT-PCR detection of rat Ob mRNA in the prostate and seminal vesicle glands, respectively. Lanes 3 and 4: detection of Ob-Rb mRNA in the same glands. M is a 100 bp molecular ladder. Fig. 2. Southern blot analysis of RT-PCR products using specific DIG labelled cDNA probes indicating the specificity of amplified fragments of leptin and long isoforms of leptin receptor in the prostate and seminal vesicle glands. Fig. 3. *In situ* hybridization of leptin in the seminal vesicle. The signals were only detected in the lining epithelium (arrowhead). Scale bar = 20 μm. Fig. 4. mRNA transcripts of Ob-Rb were also seen in epithelium of seminal vesicles (arrowhead). Scale bar = 40 μm. Fig. 5. The sense probe of leptin mRNA showed negative staining in the seminal vesicle epithelium. Scale bar = 20 μm. Fig. 6. Similarly, the sense of Ob-Rb mRNA did not show any specific signals in the seminal vesicles. Scale bar = 40 μm. Fig. 7. *In situ* hybridization of leptin in the prostate gland. The signals were only observed in the lining epithelium (arrowhead). Scale bar = 25 μm. Fig. 8. mRNA transcripts of Ob-Rb were also seen in epithelium of prostate gland (arrowhead). Scale bar = 25 μm.



Figs. 9–16. Fig. 9. The sense probe of leptin mRNA showed negative staining in the prostate gland epithelium. Scale bar = 40 μm. Fig. 10. The sense of Ob-Rb mRNA did not show any specific signals in the prostate gland. Scale bar = 40 μm. Fig. 11. Immunostaining of leptin in the lining epithelium of seminal vesicle (arrowhead). Scale bar = 20 μm. Fig. 12. Higher magnification of the leptin immunoreactive cells in the seminal vesicles (arrowhead). Scale bar = 10 μm. Fig. 13. Leptin protein reaction was seen in the lining epithelium of prostate (arrowhead). Scale bar = 25 μm. Fig. 14. Higher magnification of the leptin immunoreactive cells in the prostate glands (arrowhead). Scale bar = 10 μm. Figs. 15 and 16. Leptin was not evident in the coagulating gland either at the level of mRNA (Fig. 15) or at the level of protein (Fig. 16). Scale bar = 20 μm.

S473 and GSK-3 S9 phosphorylations (Aquila et al., 2005). Thus the secreted leptin hormone of the seminal vesicle and prostate gland might be implicated in the extratesticular sperm maturation and/or on the modulation of sperm metabolism.

In conclusion, our study elucidates the cellular source of locally synthesized leptin within the seminal vesicles and prostate gland of rat. Also this study suggests that leptin may be involved in the autocrine–paracrine regulation of the biological activity of epithelial cells of the rat seminal vesicles and prostate. Furthermore as a result we speculate that prostatic and seminal vesicle leptin may participate in sperm maturation through the interaction between seminal plasma leptin and spermatozoa via sperm leptin receptors. However, this last hypothesis still requires further investigation to be supported.

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