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Leptin and queen ovary: New insights about ovulation

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ABSTRACT

Leptin has been proven to be crucial in the ovulatory process. Aims of this study are to assess the expression of leptin receptor (Ob-R) in the ovaries of queens at estrus and to evaluate the capability of leptin in modulating ovarian contractility in vitro. Right ovaries underwent immunoblot analysis. Left ovaries were mounted in an organ bath under physiological condition and exposed to murine leptin (10^{-6} M). Immunoblot analysis showed that the queen ovary expresses leptin receptor at estrus. Leptin at the dose of 10^{-6} M significantly reduced the contractile activity of the ovary. The presence of ovarian Ob-R and leptin inhibitory effects on ovarian contractility suggest leptin implication in the modulation of ovarian activity, as well as in ovulatory disorders.

Ovulation is a central event in the reproductive cycle and follicular contraction is important for follicle rupture. In fact, smooth muscle cells have been localized in the follicular wall and their capability to contract under the stimulus of different drugs has been observed (Espey, 1978; Miyamoto et al., 2001; Madekurozwa et al., 2010; Rizzo et al., 2010). Coordinated actions of the multiple hormonal and cellular components of the hypothalamic-pituitary-ovarian axis control this process (Richards, 1994), even if the exact mechanism underlying ovulation is still unknown.

Leptin, a hormone mainly synthesized and secreted by the adipocytes, has been shown to play an important role in reproduction (Ricci et al., 2006). The expression of leptin receptors has been demonstrated in ovaries of different species (Karlsson et al., 1997; Ryan et al., 2002; Ryan et al., 2003; Zerani et al., 2004; Smolinska et al., 2007; Sarkar et al., 2010). The effects of leptin in reproduction are contradictory, since this hormone displays both an inhibitory and stimulatory action on the ovarian activity (Ricci et al., 2006). Since the mechanism through which leptin controls ovulation is not well defined, the aims of our study are to evaluate by immunoblotting the expression of leptin receptor (Ob-R) on the ovaries of queens at estrus, and to test, in vitro, the capability of leptin to modulate the contractility of the ovaries of the same queens. The study was performed on fifteen owned queens in estrus (2–4 years; 3–4 kg) housed at the Obstetric Clinic section of the Veterinary Medicine Hospital of the University of Bari "Aldo Moro" (Italy), for ovariectomy. Owner consent was obtained for all cats. In this study, all procedures were conducted in accordance with EU directive 2010/63/EU on animal protection used for scientific purpose.

All cats underwent a complete clinical exam aiming at assessing their health status. Estrus, was identified based on the individual medical history, vaginal cytology stained with a rapid modified Wright–Giemsa method (Diff Quick®, Dyaset, Ferrara, Italy), in association with the 17β-Estradiol assay (Estradiol ELISA®, DRG Instruments GmbH, Marburg, Germany, specificity 100%, sensitivity 9.714 pg/ml, inter-assay 6,72%, intra-assay 2,71%). Each queen was anesthetized by medetomidine 80 μg/kg i.m. (Dorbene® 1 mg/1 ml, Fortdodge Animal Health spa, Italy) and ketamine 5 mg/kg i.m. (Imalgene 1000, Merial Spa, Milan, Italy), intubated and maintained with Isoflurane (Isoba, Intervet, Milan, Italy) 1.5% in oxygen. All animals underwent ovariectomy by median laparotomic technique. Each queen received antibiotic therapy, amoxicilline–clavulanic acid association 12.5 mg/kg (Synulox® injection suspension-Pfizer, Italy) subcutaneously at the end of the surgery. A non-steroidal anti-inflammatory drug, (Robenacoxib 2 mg/kg; Onsior® injectable solution, Novartis, Italy), was administred subcutaneously, before the surgery. Antibiotic and anti-inflammatory therapy was carried out by oral administration for further 5 days by the owners (Synulox® tablets 50 mg-Pfizer, Italy; Onsior® tablets 6 mg, Novartis, Italy).

After excision, the right ovary of each queen was frozen in liquid nitrogen and stored at −80 °C until molecular analysis. The left ovary was placed in a shallow dish containing Kreb's solution at pH 7.4. The detection of preovulatory follicles was achieved by the observation of the ovaries, and those bearing follicles with a diameter of 2–3 mm (Bristol-Gould and Woodruff, 2006) were 13 and chosen for the study.

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Each right ovary was pulverized in liquid nitrogen and protein extracted according to Cosola et al. (2006). Proteins from *Pisum sativum* leaves were also extracted and utilized in the immunoblot as control for antibody (Ab) specificity. Forty micrograms of proteins from each sample together with a size standards (10–250 kDa) (Bio-Rad, Milan, Italy) were submitted to PAGE (Cosola et al., 2006). Polypeptides were electrotransferred to an Immobilon-P membrane (Millipore, Milan, Italy) by the Trans-Blot semi-dry system (Bio-Rad).

Filter was stained with a coomassie blue solution (0.2% coomassie blue, 7.5% acetic acid, 50% ethanol) destained with an acqueous solution of 1:5 (v/v) acetic acid/ethanol and saturated with 5% non-fat dry milk in blotto 1× (20 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 1% (v/v) Triton-X 100) for 1 h at room temperature. The membrane was then incubated overnight at 4 °C with a respectively 1:800 and 1:200,000 diluted 1× blotto solution of a primary Ab anti-mouse Ob-R SC-8391 (Santa Cruz Biotechnology) and anti-mouse GAPDH (Sigma Aldrich, Milan, Italy). After washing in blotto 1× to remove unbound Ab, The membrane was incubated for 1 h in the presence of an anti-mouse secondary Ab IgG HRP coniugated (SC-2005, Santa Cruz Biotechnology) diluted 1:20,000. The immunocomplex was detected using the Enhanced Chemiluminescence (ECL)-kit (Amersham International, Buckinghamshire, UK) and revealed on Hyperfilm ECL (Amersham).

Left ovaries contractility was studied utilizing the technique described by Rizzo et al. (2011). After the equilibration period, under 2 g tension 6 μg/ml of acetylcholine in water (Sigma–Aldrich) were added to the organ bath, in order to test the ovarian capability to contract. After 20 min a wash out was performed allowing the reestablishment of basal contractility, then murine leptin (10^{-6} M) (Sigma–Aldrich) dissolved in 15 mmol/l of hydrochloric acid and 7.5 mmol/l of sodium hydroxide was added to the organ bath. Preliminary studies (unpublished data) allowed us to determine the minimal efficacious dose of leptin. The effect of this drug was evaluated for a 20 min period. Moreover it is tested the effects of the vehicle (hydrochloric acid and of sodium hydroxide) on ovarian contractility. All chemicals were purchased from Sigma Aldrich unless otherwise indicated.

Tension and frequency were expressed as mean ± standard deviation and analyzed by Student's *t*-test, using SPSS software (PASW statistics 19). The results were considered significant for $p < 0.05$.

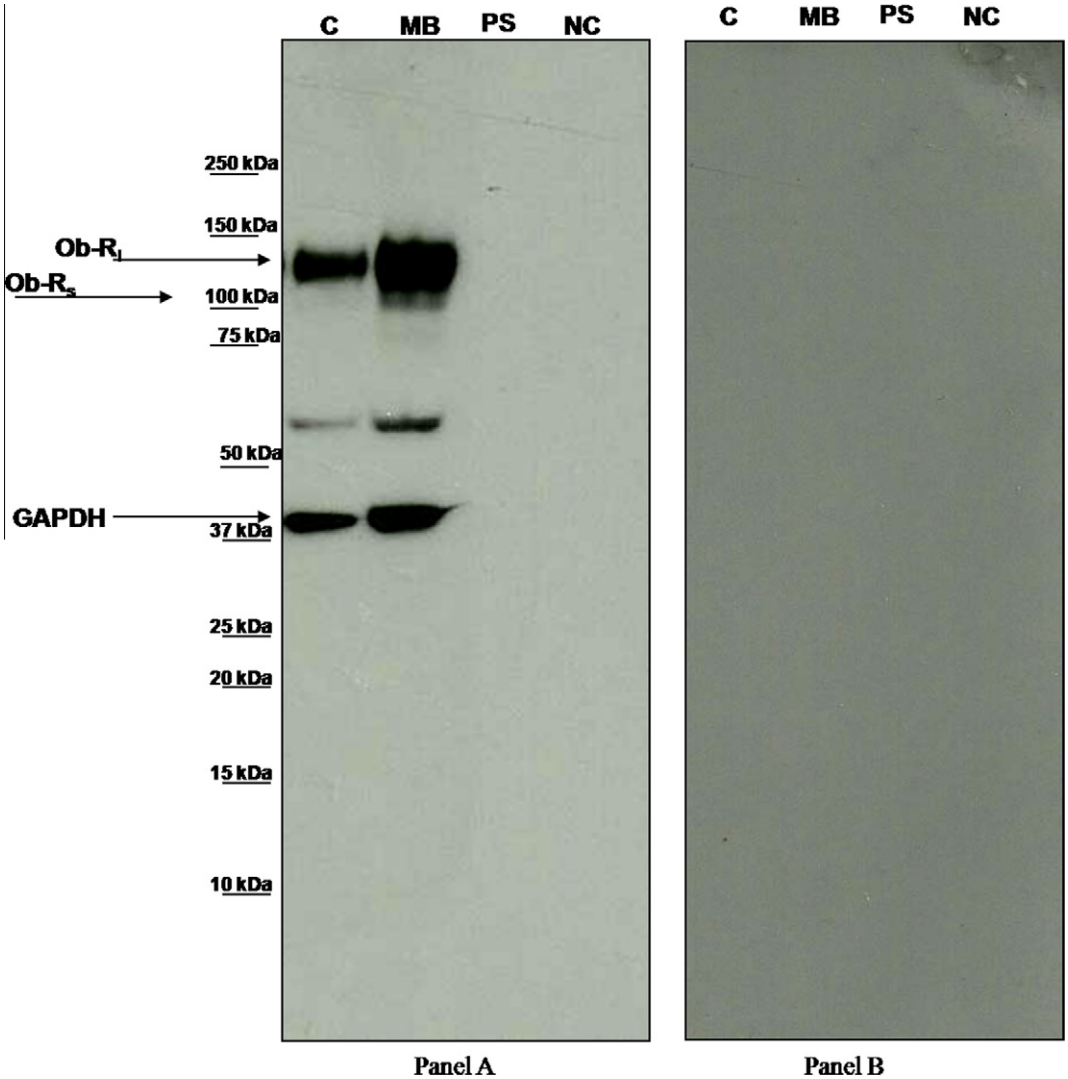

Fig. 1. Representative western blot of queen ovary homogenate proteins (lane C, panel A) with antibody to leptin receptor (Ob-R) and to GADPH. Lanes MB, PS and NC are respectively proteins from mouse brain, *Pisum sativum* and the negative control (no proteins loaded into the gel). The queen ovary expresses only the 120 kDa band while mouse brain expresses both forms of Ob-R, the long 120 kDa band (Ob-Rl) and the short 100 kDa band (Ob-Rs), an extra band of ~50 kDa is also visible as reported in the data sheet of the monoclonal anti Ob-R. Panel B is the control experiment in which the two primary antibodies were omitted.