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17

Immunoassay – A Standard Method to Study the Concentration of Peptide Hormones in Reproductive Tissues *in vitro*

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1. Introduction

Immunoassay analysis has been widely used in many important areas of pharmaceutical analysis, such as in diagnosing diseases, monitoring therapeutic drugs, and studying clinical pharmacokinetics and bioequivalence in drug discovery and pharmaceutical industries (Findlay et al., 2000). Immunoassays are standard biochemical tests that are used to measure concentrations of various hormones, including peptide hormones, in biological fluids, such as serum/plasma, urine, follicular fluid or culture medium. In addition, immunoassays are also used to determine the level of peptide hormones in various tissues, including reproductive tissues, such as ovary, placenta, or testis.

The female reproductive system and status, such as puberty and later regular estrus cycle, are regulated by the hypothalamic-pituitary-ovary axis, which produces many hormones. In addition, the ovary produces steroid hormones, growth factors and peptides that regulate ovarian follicle development, the onset of puberty and/or ovulation. In the last decade, studies have shown that ghrelin, leptin, or resistin, which are produced and secreted mainly by the stomach or adipose tissue, are also expressed and concentrated in reproductive tissues, suggesting an autocrine or paracrine role in female reproduction.

This article focuses on one of the methods used for measuring concentrations of protein hormones, such as ghrelin, leptin, and resistin, in reproductive tissues.

2. Biological function of ghrelin, leptin and resistin

2.1 Ghrelin

Ghrelin is a 28-amino-acid peptide that is produced predominantly by the stomach, although its expression has also been demonstrated in other tissues, including the bowel, pancreas, kidneys, lung, placenta, gonads, pituitary, and hypothalamus (van der Lely et al., 2004). In its acylated form, ghrelin displays strong GH (growth hormone)-releasing activity, which is mediated by the activation of the GH secretagogue (GHS) receptor (GHS-R) type

1a. Two GHSR subtypes generated by alternative splicing of a single gene have been identified: the full-length type 1a receptor and the truncated type 1b. GHS-R1a is the functionally active, signal transduction form of the receptor. In contrast, GHS-R1b lacks transmembrane domains 6 and 7 and is unable to bind a ligand or transduce a signal. GHS-Rs are primarily expressed in the hypothalamus-pituitary unit but are also found in other central and peripheral tissues (Gnanapavan et al., 2002). Moreover, ghrelin has other endocrine and nonendocrine actions, such as stimulating lactotroph and corticotroph secretion; inhibiting the gonadal axis; controlling energy expenditure with orexigenic effects coupled; controlling gastric motility and acid secretion; and influencing endocrine and exocrine pancreatic functions, glucose metabolism, cardiovascular actions, behavior and sleep, cell proliferation, and apoptosis (Figure 1). Circulating ghrelin is primarily found in the unacylated form. Although only the acylated form of ghrelin mediates endocrine actions, the unacylated form influences gastric secretion; in fact, unacylated ghrelin is reduced by 70% after gastrectomy and gastric bypass in humans. One of the hydroxyl groups located on a serine residue of ghrelin is uniquely acylated by n-octanoic acid. Of the two circulating ghrelin forms, acylated (Ac) and unacylated (UnAc), the acylated form is essential for ghrelin biological activity (Kojima et. al., 1999). Plasma ghrelin levels are elevated after fasting and are reduced after eating. In normal-weight humans, the fasting plasma level of ghrelin is approximately 250 pg/ml (Rigamonti et al., 2002).

Circulating ghrelin levels are also increased by fasting and energy restriction and are decreased by food intake. Moreover, ghrelin secretion is negatively correlated with the body mass index (BMI). In fact, circulating ghrelin levels are increased by anorexia and cachexia, reduced by obesity with or without type 2 diabetes, and restored by weight recovery. Interestingly, these changes are the opposite of those observed with leptin, and it has been suggested that both ghrelin and leptin signal a metabolic balance and manage the neuroendocrine and metabolic response to starvation.

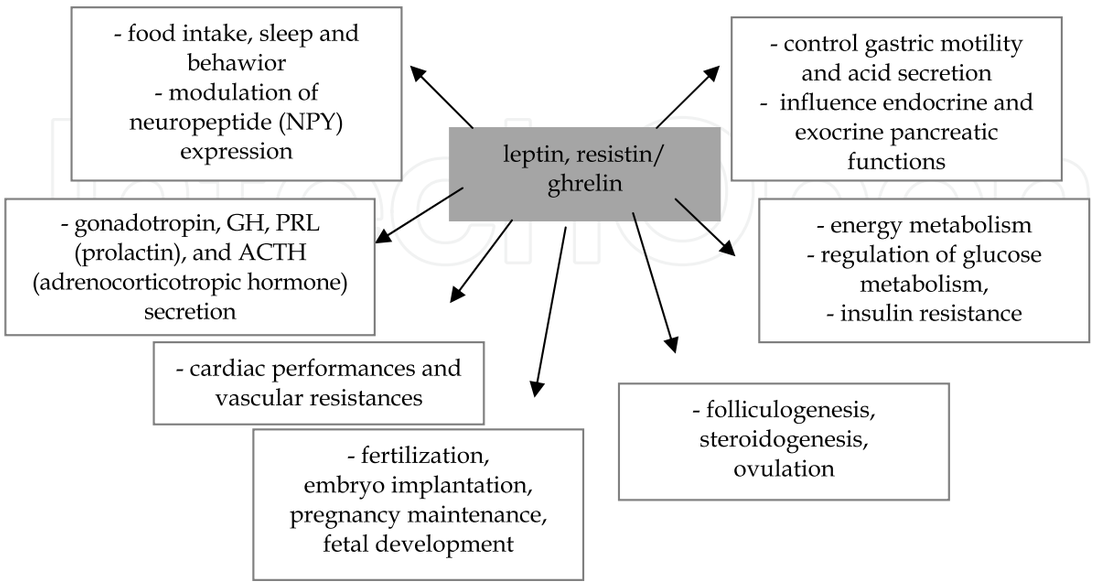


Fig. 1. Biological function of the peptide hormones.

2.2 Leptin

Leptin, the obese (ob) gene product, is a 16-kDa protein that is primarily synthesized by white adipose tissue. The secretion of this hormone is pulsatile and shows a circadian rhythm with a nocturnal rise that peaks between 1 and 2 h. Circulating levels of free/unbound leptin have been strongly correlated with both the BMI and the total amount of body fat. Plasma leptin levels were increased in overweight/obese women (37.7 ng/ml) as compared with normal-weight women (3.92-16.9 ng/ml) (Havel et al., 1996). However, in some obese individuals, the leptin levels are as high as 100 ng/ml (Knerr et al., 2006). With anorexia nervosa, severe undernutrition is associated with low plasma and cerebrospinal fluid leptin levels below 1.85 ng/ml (Mantzoros et al., 1997).

Leptin regulates energy homeostasis primarily in the brain where it might be transported by a receptor-mediated saturable transport mechanism across the blood–brain barrier. Leptin promotes weight loss by reducing appetite and increasing energy expenditure by stimulating sympathetic nerve activity in the thermogenic brown adipose tissue. Leptin induces sympathetic activation to other organs that are not usually considered thermogenic, such as the kidney, hindlimb, adrenal gland, and bone. Moreover, leptin action in the hypothalamus participates in the control of cardiovascular function, bone formation, glucose metabolism, sexual maturation and reproduction, the hypothalamic-pituitary-adrenal system, and fatty acid oxidation. The pleiotropic nature of leptin is supported by the universal distribution of OB-R leptin receptors. Leptin acts via transmembrane receptors, which show structural similarity to the class I cytokine receptor family. Ob-R has a single membrane-spanning domain and exists in different isoforms (Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Re) that are derived from the alternative splicing of mRNA. Although the white adipocytes mainly produce and secrete leptin into the bloodstream, there are other potential sources of the hormone. The placenta, gastric mucosa, bone marrow, mammary epithelium, skeletal muscle, pituitary, hypothalamus and bone also produce small amounts of leptin under certain circumstances.

2.3 Resistin

Resistin is a novel 12.5-kDa adipokine that belongs to a family of cysteine-rich C-terminal domain proteins called resistin-like molecules (RELMs) (Steppan & Lazar, 2004). The human peptide consists of 114 amino acids, which include a signal peptide of 17 amino acids, a variable region of 37 amino acids, and a conserved C-terminus. Splice variant of human resistin was characterized by the complete loss of exon 2, leading to protein truncation (Steppan et al., 2001) but this effect has not yet received further attention.

In human studies, resistin gene expression is detected in the adipocytes, and its levels are increased in morbidly obese humans as compared with lean control subjects. Whether resistin is involved in energy homeostasis is still unclear; however, its expression patterns are similar to leptin expression. Fasting reduces leptin and resistin expression, and both hormones were increased after eating. Some studies have reported a significant association between resistin and the development of obesity and insulin resistance (Ukkola, 2002). In animal experiments resistin induces insulin resistance. The human physiological effect of this cytokine is less pronounced. Increased serum resistin levels were found in obese

individuals, but some controversies exist concerning its role in type II diabetes, insulin resistance and hypertension in humans. Despite much research concerning the mechanism of role and action of resistin, the receptor mediating its biological effects has not yet been identified, and little is known about the intracellular signaling pathways that are activated by this peptide hormone.

3. Characteristics of the methods

3.1 Immunoassay

The immunoassay is based on the reaction of an antigen with a selective antibody to generate a useful product. Several types of labels have been used in immunoassay:

- Radioactivity is used in radioimmunoassay (RIA), a highly sensitive and specific assay method in which the radiolabeled and unlabeled substances compete in an antigen-antibody reaction to determine the concentration of the unlabeled substance.
- Enzymes are used in the enzyme-linked immunosorbent assay (ELISA), which is also known as an enzyme immunoassay (EIA).
- Fluorescence, luminescence or phosphorescence has also been used.

The most common label for clinical and biological analysis is the use of enzymes and colorimetric substrates.

- In capture enzyme-linked immunosorbent method, a capture antibody (Y) is passively adsorbed on a solid phase (Figure 2, Shan et al., 2002 with modification).
- The target protein contained in the sample and the enzyme-labeled reported antibody (Y-E) is added.
- Both the capture antibody and the enzyme-labeled reporter antibody bind to the target protein at different sites, thereby "sandwiching" the protein between the two antibodies.
- After a washing step, the substrate (◊) is added, and colored product (♦) is formed after reaction.
- The amount of colored product generated is directly proportional to the amount of target protein captured.

3.1.1 Sample collection and preparation

In our experiments, we used reproductive tissue, including the ovarian follicles, corpus luteum, placenta or human ovarian cell line (OVCAR-3) or breast cancer cell line (MCF-7). Independently on the sample used, tissue or cells are homogenize and then sonificated twice in appropriate ice-cold lysis buffer. For tissue sample lysis buffer I is used, why for cell line sample lysis buffer II. The lysates are clarified by centrifugation at 15,000 g at 4°C for 30 min, and supernatants are subsequently transferred to separate tubes. The protein content in the lysates are determined with Bradford reagent (Bio-Rad Protein Assay; Bio Rad Laboratories, Munchen, Germany) using bovine serum albumin (BSA) as a standard. After homogenization, the supernatants will be collected and stored at -20^{o}C until further use in the ELISA analysis.

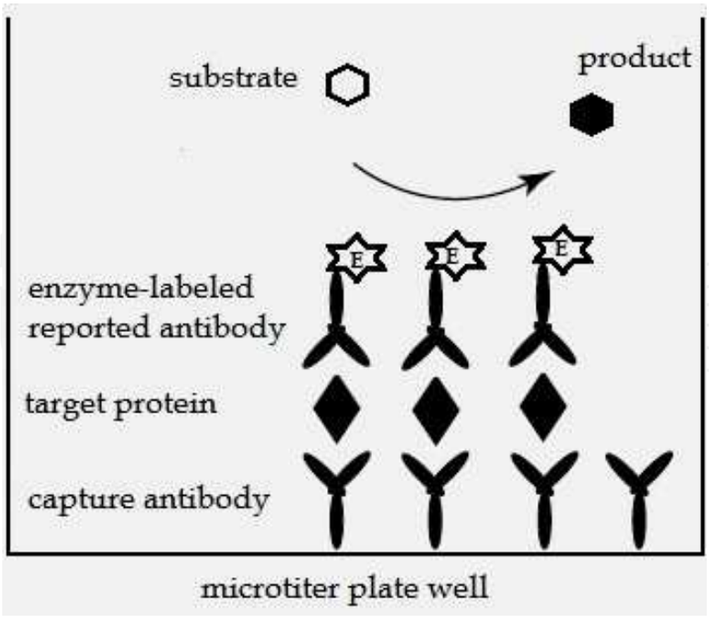


Fig. 2. Enzyme-linked immunosorbent assay (ELISA) (Shan et al., 2002 with modification).

- Lysis buffer I: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 % Na-deoxycholate, 0.5 % NP-40, 0.5 % SDS and EDTA-free protease inhibitors
- Lysis buffer II: PBS, pH 7.2, 0.5 % Tween-20, 1 nM EDTA and 1 nM phenylmethylsulfonyl fluoride (PMSF)

3.1.2 Protocol of peptide determination

It is possible prepare entire procedure described by Fujinami et al., (2004) (1) or use commercially available kits (2) to determine the peptide hormone concentration:

1. Enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies for measurement concentration of human resistin (Fujinami et al., 2004).

Preparation of the anti-human resistin antibody

In their study, Fujinami et al. (2004) used a synthetic peptide that corresponded to residues 17–44 of human resistin as deduced from the nucleotide sequence of the human resistin gene, which was generated with an additional cysteine-residue located at its N-terminus (Biologica, Nagoya, Japan).

- Following purification by reverse-phase HPLC, the synthetic peptide (purity >85 %) was coupled to a keyhole limpet hemocyanin by an N-(q-maleimidocaproyloxy) succinimide (Sigma, St. Louis, MO).
- The carrier-conjugated peptide was subsequently emulsified with Freund's complete adjuvant (Difco Laboratories, Detroit, MI), and rabbits were subcutaneously injected (0.5 mg/injection) a total of 6 times at 10-day intervals.
- Blood samples were collected at 10 days after the last injection.
- The specific antibody in the sera was purified using a resistin peptide-coupled CNBr-activated Sepharose affinity column.

- Anti-resistin antiserum was raised in a similar manner by injecting a rabbit with 50 μg of recombinant human resistin (Research Diagnostic, Flanders, NJ) into a rabbit.

Preparation of biotinylated anti-human resistin antibody

The human resistin antibody IgG fraction was isolated by chromatography on a recombinant human resistin-coupled CNBr-activated sepharose affinity column and biotinylated with 5-(N-succinimidyl-oxycarbonyl) pentyl-D-biotinamide (Dojindo, Kumamoto, Japan) (Fujinami et al., 2004).

Assay procedure

- Microtiter plates are coated with 100 μl of the affinity purified anti-resistin IgG against human (1.5 μg/ml) that is diluted with 10-mmol/l-carbonate buffer, pH 9.3, for 1 h at room temperature.
- After washing the plate twice, the nonspecific binding sites in each well were blocked with 200 μl of 10-mmol/l-carbonate buffer containing 0.5 % BSA.
- Standard solution (0.5–100 ng/ml of recombinant human resistin) and samples diluted in sample buffer are added to the wells, and then plate are incubated for 1 h at room temperature.
- After 1 h of incubation, plates are washings with BSA-free sample buffer and 100 μl biotinylated anti-recombinant human resistin IgG (5 ng/ml) was added to each well for the next 1 h of incubation.
- Next, after three more washing times, the plate are incubated for 1 h at room temperature with 100 μl of streptavidin-horseradish peroxidase diluted 10,000-fold.
- After three final washes, the plates are treated for 20 min with 100 μl of substrate solution containing 3, 3V, 5, 5V-tetramethylbenzidine (TMB) and H_2O_2.
- The reaction is stopped by the addition of 100 μl of 1 mol/l phosphoric acid, and subsequently, the absorbance is recorded at 450 nm using an ELISA plate reader.
- All washings and incubations were carried out with gentle shaking.

Sample buffer: 50 mmol/l Tris–HCl buffer, pH 7.0, containing 200 mmol/l NaCl, 10 mmol/l CaCl2, 0.1% Triton X-100 and 1% BSA

2. Much simpler and faster methods of peptide hormone determination are commercially available. Currently, many competitors provide a wide range of kits for the determination of various peptides hormones in many animal species and various biological materials.

In our study, the concentration of ghrelin, leptin or resistin in reproductive tissue was measured by ELISA using a commercially available kit (Table 1).

Standard procedure

In this assay, the immunoplate is pre-coated with anti-human peptide hormone capture antibodies, and the nonspecific binding sites are blocked. The peptides hormones in the sample or in the standard solution bind to the capture antibody immobilized in the wells. After washing, the biotinylated anti-peptide hormone detection antibody, which can bind to the peptide hormones trapped in the wells, is added. After washing, the streptavidin-horseradish peroxidase (SA-HRP), which catalyzes the substrate solution (TMB), is added (Figure 3).

	Cat. no/company	*Sensitivity*	*Inter- /intra-run precision*	*Standard curve*
Ac ghrelin	RD194062400R/ BioVendor GmbH, Heidelberg, Germany	0.3 pg/ml	8.3 %/8.1 %	0-250 pg/ml
UnAc ghrelin	RD194063400R/ BioVendor GmbH, Heidelberg, Germany	0.2 pg/ml	3.80 %/3.20 %	0-250 pg/ml
Leptin	RD191001100/ BioVendor GmbH, Heidelberg, Germany	0.2 ng/ml	4.4-6.7 %/ 4.2-7.6 %	0–50 ng/ml
Resistin	4945/DRG International Inc., USA	0.016 ng/ml	15 % / 10 %	0-10 ng/ml

Table 1. Commercially available kits used to determine the concentration of peptide hormones in reproductive tissue.

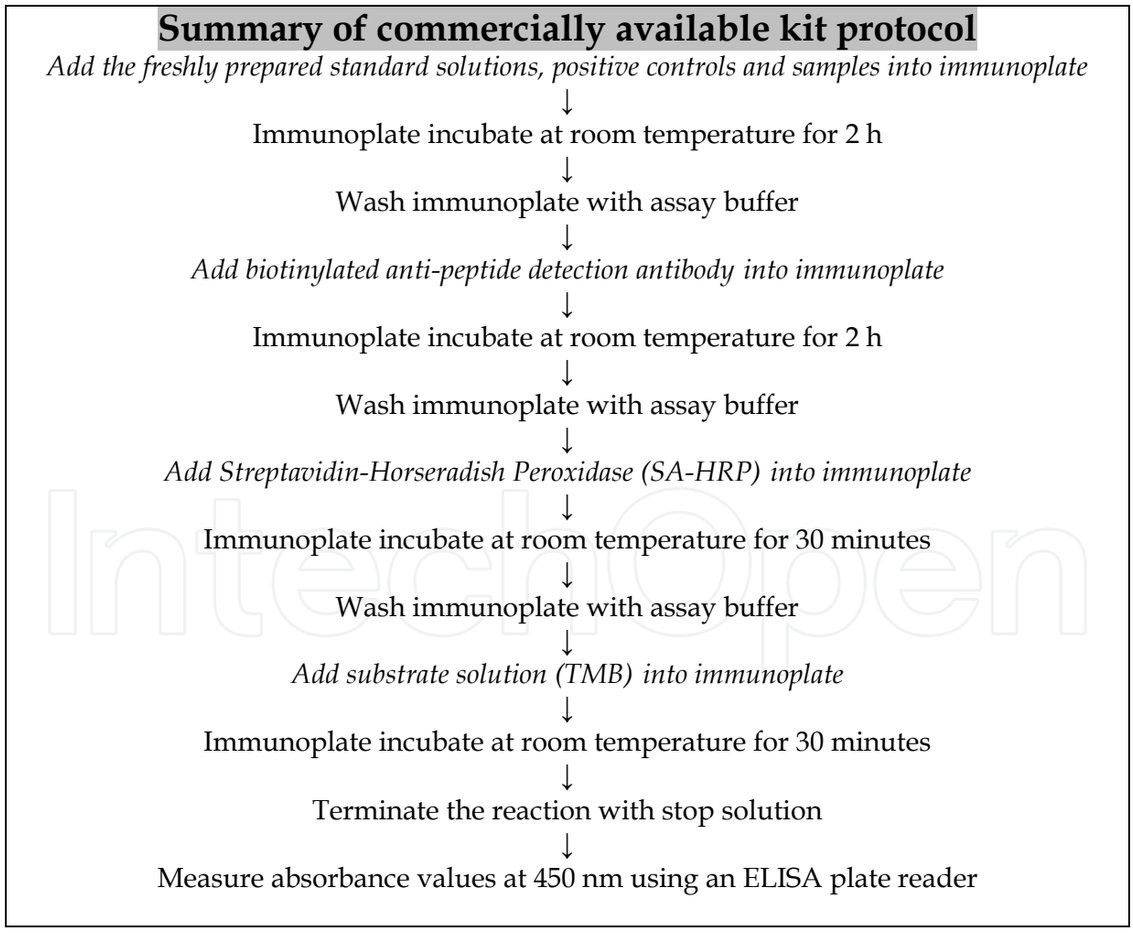


Fig. 3. Summary of commercially available ELISA assay procedure (according to the enclosed assay instructions).

The enzyme-substrate reaction is terminated by the addition of a stop solution. The intensity of the color is directly proportional to the amount of peptide hormones present in the standard solutions or samples. The standard curve is produced from the data obtained using the serial dilutions of the standards with the concentration plotted on the x-axis (log scale) versus absorbance on the y-axis (linear) (Figure 4). The absorbance values were measured at 450 nm using an ELISA plate reader.

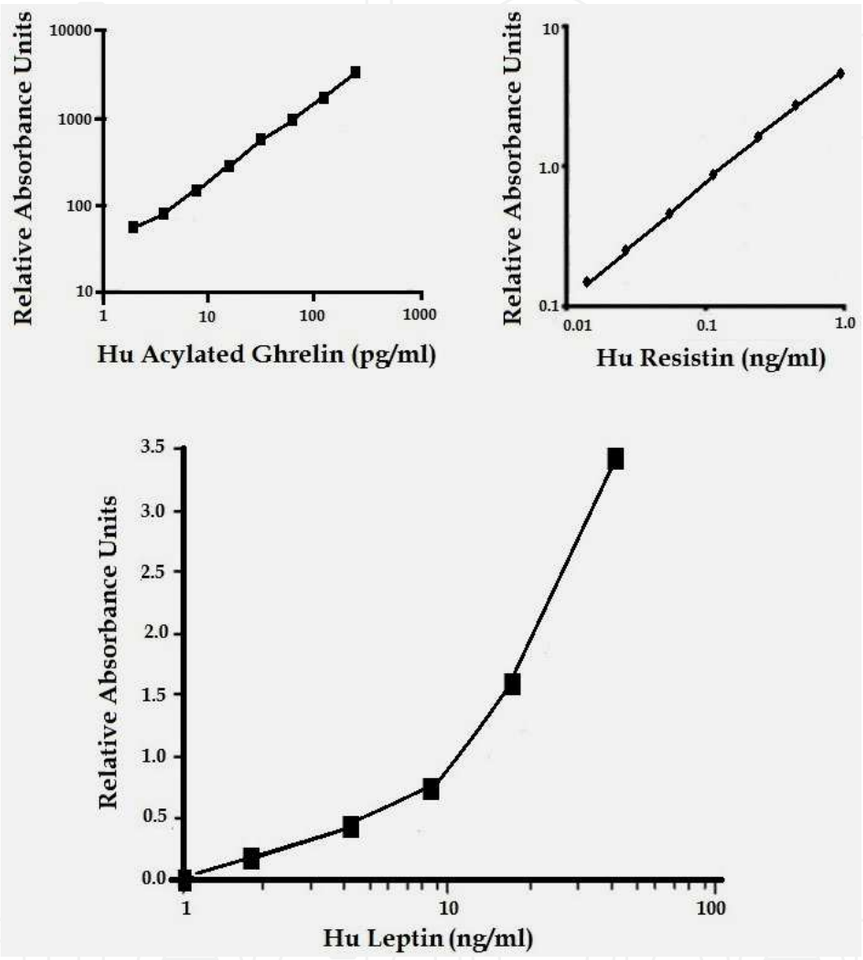


Fig. 4. Typical standard curve for peptide hormones determined by ELISA. The absorbance values were measured at 450 nm for leptin and resistin and at 414 nm for Acylated Ghrelin.

3.2 Western immunoblot assay

The Western immunoblot assay is an alternative detection method to ELISA and is used to confirm our immunoassay results. Western immunoblott is based on the detection of proteins using specific antibodies. This assay utilizes specific antibodies to proteins that have been separated according to size (molecular weight) by gel electrophoresis. The blot is a membrane, which is commonly composed of nitrocellulose or PVDF (polyvinylidene fluoride). The gel is placed next to the membrane, and an electrical current is used to transfer the proteins from the gel to the membrane where they adhere. As a result, the membrane becomes a replica of the protein gel, which can subsequently be stained with an antibody.

3.2.1 Sample collection and preparation

To prepare samples for electrophoresis, the tissues and cells are lysed to release the proteins of interest. This lysis solubilizes the proteins for their individual migration through the separating gel. There are many recipes for lysis buffers, but only a few are appropriate for Western blotting. In brief, they differ in their ability to solubilize proteins. Those containing sodium dodecyl sulfate and other ionic detergents are considered to be the harshest and are therefore most likely to generate the highest yield. In our experiments, the reproductive tissues were homogenized twice in ice-cold lysis buffer. Subsequent to sonication for 10–15 seconds to complete cell lysis, the DNA was sheared to reduce sample viscosity and centrifuged at 10000 x g for 10 minutes at 4°C. The lysate protein concentrations were determined using the Bradford assay. Equal amounts of protein were added to an equal volume of 2X sample buffer and boiled at 95-100°C for 5 minutes.

- Sample buffer: 125 mM Tris-HCl (pH 6.8 at 25°C), 4 % w/v SDS, 25 % glycerol, 20 mM DTT, 0.01 % w/v bromophenol blue or phenol red

3.2.2 Electrophoresis

Electrophoresis is a standard technique for separating proteins according to their molecular weight. Samples (10 µl) are separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis; 10 cm x 10 cm; BioRad Mini-Protean II Electrophoresis Cell), and the proteins are transferred to nitrocellulose membranes (BioRad Mini Trans-Blott apparatus). The separation of molecules is determined by the relative size of the pores formed within the gel. We used electrophoresis control markers for qualitative molecular mass determinations: tubulin (55 kDa) or β-actin (42 kDa).

- Electrophoresis buffer: 25 mM Tris base, 0.2 M glycine, 0.1 % SDS
- Transfer buffer: 25 mM Tris base, 0.2 M glycine, 20 % methanol

	Molecular weight (kDa)	*SDS-PAGE gel (%)*	*Loading control*	*PVDF membranes*	*Wet transfer conditions*
Ghrelin	3-4	20 %	tubulin	0.2 µm	1A/45 min
Ghrelin receptor (GHSR-1a)	41	10 %	tubulin	0.45 µm	1A/60 min
Leptin	16	15 %	β-actin	0.2 µm	1A/45 min
Leptin receptor (Ob-R)	100-125	7 %	β-actin	0.45 µm	1A/ 60 min
Resistin	12.5	12 %	β-actin	0.2 µm	1A/45 min

Table 2. Characteristic parameters in electrophoresis.

3.2.3 Immunoblotting

After the transfer procedure, the membranes are washed, and nonspecific binding sites are blocked with 5 % no fat milk and TBS/T (Tris-buffered saline/Tween) buffer in at room temperature for 2 h. Then, the membranes were washed three times for 5 min each with TBS/T. The membranes are incubated overnight with diluted primary antibody in 10 ml of

TBS/T/milk buffer with gentle shaking at 4°C. After incubating the membranes with the primary antibody, the membranes are washed with TBS/T and incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody diluted at 1:500 in TBS/T. The signals are detected by chemiluminescence (ECL) using Western Blotting Luminol Reagent (sc-2048, Santa Cruz Biotechnology) and visualized using a ChemidocTM Reader. All data bands visualized by chemiluminescence are quantified using a densitometer.

- TBS/T buffer: 0.2% Tween-20 in 0.02 M TBS

	Primary antibody	*Secondary antibody*
Ghrelin	Goat polyclonal antibody raised againstA peptide mapping within an internal region of ghrelin of human origin	Horseradish peroxidase-conjugated antibody
Ghrelin receptor (GHSR-1a)	Goat polyclonal antibody raised againstA peptide mapping near the C-terminus of GHS-R1a of human origin	Horseradish peroxidase-conjugated antibody
Leptin	Mouse monoclonal anti-leptin (Ob) clone LEP-13 reacts specifically with human leptin	Polyclonal goat anti-mouse/HRP
Leptin receptor (Ob-R)	Mouse monoclonal Ob-R (B-3) antibody detection of short and long forms of Ob-R of mouse, rat and human	Polyclonal goat anti-mouse/HRP
Resistin	Goat polyclonal antibody raised againstA peptide mapping within an internal region of resistin of human origin	Horseradish peroxidase-conjugated antibody

Table 3. Antibodies used in immunoblotting procedure.

4. Effect and concentration of peptide hormones in reproduction

Proper nutrition has a significant influence on fertility. In both animals and humans, appropriate weight determines the transition from puberty into maturity. Appropriate nutrition also influences the adequate amount of adipose tissue and thus the levels of hormones secreted by the adipose cells. The nutritional status is connected with reproductive efficiency; therefore, researchers have focused on the role of peptide hormones in the regulation of reproductive function and its concentration in reproductive tissue.

4.1 Ghrelin

The expression of ghrelin and its receptor has been detected in the reproductive tissues of humans, and many species such as rats, pigs, sheep and chickens (Gnanapavan et al., 2002, Caminos et al., 2003, Sirotkin et al., 2006), and in other tissues, such as the hypothalamus, pituitary, and placenta (Zhang et al., 2008, Gualillo et al., 2001). Ghrelin gene expression was consistently detected in the rat ovary throughout the estrous cycle and pregnancy, with higher levels present in the corpus luteum and during the first days of gestation (Caminos et al., 2003). The highest ghrelin gene expression levels in the pig ovary occur during the diestrous phase and that the lowest levels occur during the proestrous phase was observed

by Zhang et al., (2008). Data concerning the role of ghrelin in ovarian function are conflicting. In rats, the systematic administration of ghrelin reduces the gonadotropin-releasing hormone pulse frequency in vivo. Moreover, in the rat and human pituitary, ghrelin can suppress luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion. However, in women, the administration of ghrelin did not affect basal and GnRH-induced LH and FSH secretion during the menstrual cycle.

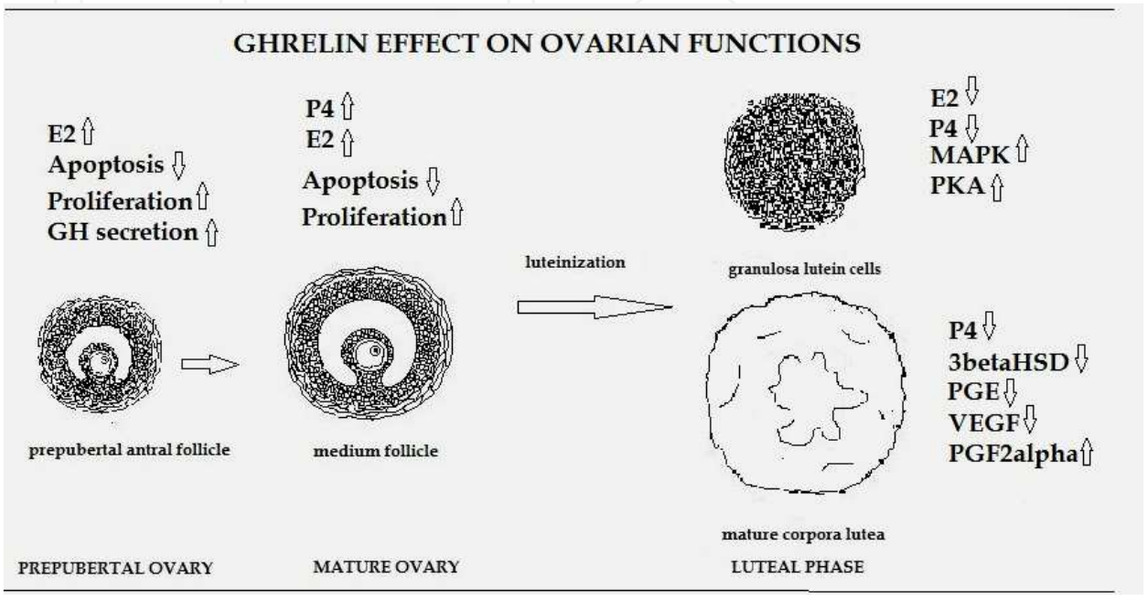


Fig. 5. Effect of ghrelin in reproduction. (↑) stimulatory and (↓) inhibitory effect, (Sirotkin et al., 2006, Rak & Gregoraszczuk, 2008, 2009, Viani et al., 2008).

Our data demonstrated that porcine ovarian follicles collected from prepubertal animals secreted ghrelin and stimulated estradiol secretion through aromatase activity (Figure 5) (Rak & Gregoraszczuk 2008, 2009). Ghrelin stimulates the secretion of progesterone, estradiol, IGF-I and arginine vasotocin in an in vitro study of chicken ovarian tissues (Sirotkin et al., 2008). However, ghrelin had an inhibitory effect on steroid synthesis in cultured human granulosa luteinizing cells (Viani et al., 2010).

An immunoassay method using a double-antibody sandwich enzyme immunoassay was used to study the levels of acylated and unacylated ghrelin throughout the normal menstrual cycle. The concentration of both forms of ghrelin remained unchanged during the entire cycle (Dafopoulos et al., 2009). Only sporadic significant correlations were found between acylated and unacylated ghrelin and E2 levels, suggesting that the magnitude of physiological changes of the sex steroids during the menstrual cycle had no major effect on ghrelin secretion from the stomach.

Using commercially available kits, our experiments showed that ghrelin was present in the porcine ovarian follicles (the follicular fluid and in follicular wall), and it is hormone that is secreted into the culture medium during the first 24 h of ovarian follicle incubation (Rak & Gregoraszczuk, 2009). Moreover, the results clearly showed that the level of ghrelin in the follicular fluid was the sum of the amounts found in the follicular wall and the culture medium (Table 4).

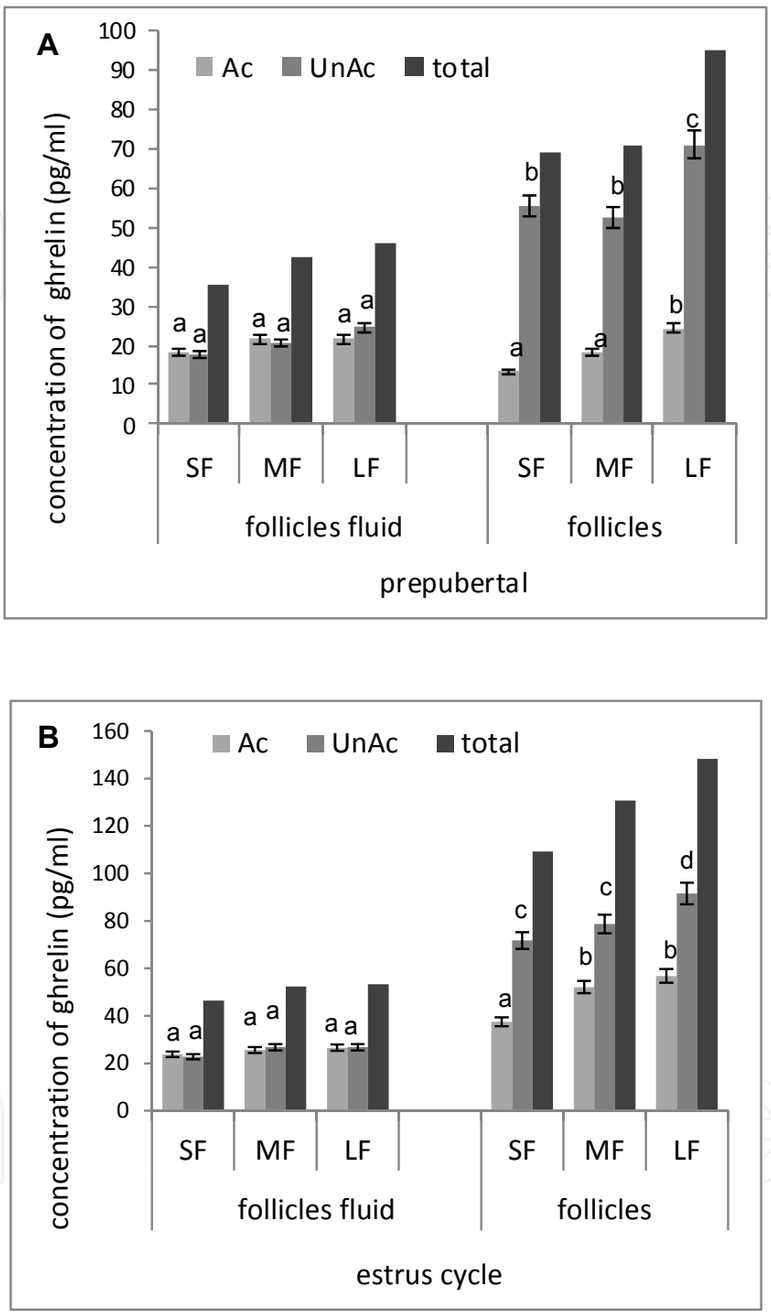


Fig. 6. Concentrations of both Ac and UnAc ghrelin in the follicular fluid of small (SF), medium (MF) and large (LF) ovarian follicles collected from (A) prepubertal animals and (B) ovarian follicles from cycling animals measured by ELISA assay. All samples were performed in triplicate in the same assay. All data are expressed as the mean ± SEM. Different letters indicate statistically significant differences among groups ($p<0.05$). (Rak-Mardyła & Gregoraszczuk, 2011, unpublished results).

	Follicular fluid	*Follicular wall*	*Medium from culture*
Ghrelin (pg/ml)	18.638 ± 5.9	13.750 ± 1.5	4.672 ± 2.0

Table. 4. Ghrelin levels in prepubertal pig ovary measured by ELISA assay (Rak & Gregoraszczuk, 2009).

Additional experiments using the ELISA method showed the concentrations of ghrelin Ac, UnAc and total (Ac plus UnAc) in ovarian follicles and the follicular fluid. In prepubertal animals, the total concentration of ghrelin was higher in the ovarian follicles than in the follicular fluid. The highest concentrations of both forms of ghrelin were observed in the large follicles. As in the case of the prepubertal animals, the total concentration of ghrelin in cycling animals was higher in the ovarian follicles than in the follicular fluid (Rak-Mardyła & Gregoraszczuk, 2011, unpublished results) (Figure 6).

The corpus luteum (CL) plays a central role in regulating the estrous cycle and in maintaining pregnancy. In addition to gonadotropins, locally produced hormones, proteins, growth factors and cytokines play crucial roles in regulating CL function. Results of our date showed, that concentration measurement by ELISA method of Ac ghrelin was unchanged during luteal phase, why levels of UnAc ghrelin significantly increased from early luteal phase to middle luteal phase (Figure 7) and was on the same levels in late luteal phase (Rak-Mardyła et al., 2012, in press). Although, first UnAc was considered an inactive form of ghrelin, accumulating evidence indicates that UnAc can modulate metabolic activities of the ghrelin system either independently or in opposition to those of Ac. Examples of UnAc actions include improvement of pancreatic β-cell function and survival and a beneficial role in cardiovascular function.

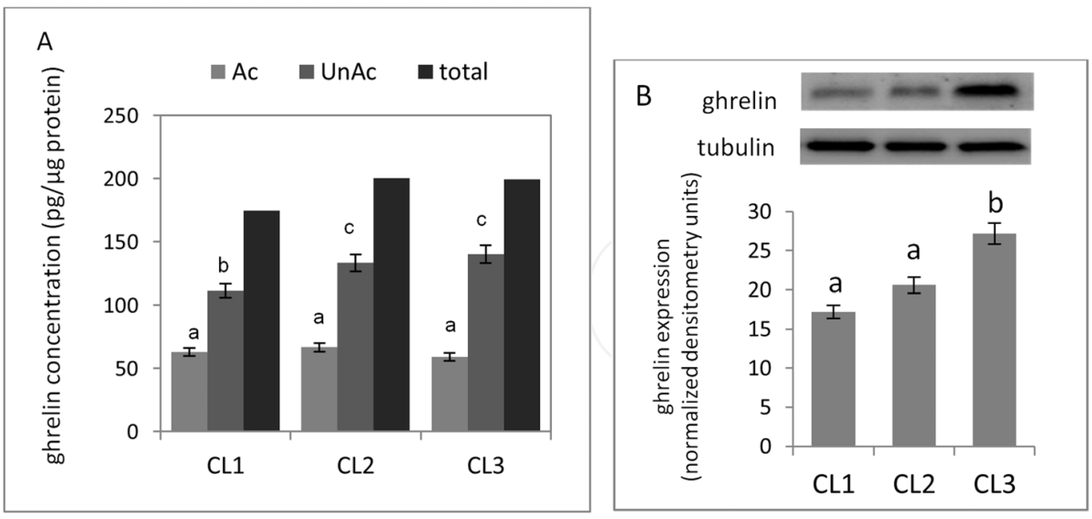


Fig. 7. Concentrations of both Ac and UnAc ghrelin in in different stages of corpus luteum development measured by ELISA assay (A) and protein expression of ghrelin measured by Western immunoblotting procedure (B). All samples were performed in triplicate in the same assay. All data are expressed as the mean ± SEM. Different letters indicate statistically significant differences among groups ($p<0.05$) (Rak-Mardyła et al., 2012, in press).

The presence of ghrelin and ghrelin receptors in the placenta clearly demonstrates that this hormone has a role in placental physiology. In humans, high placental ghrelin peptide levels are observed in the first trimester of pregnancy, primarily in cytotrophoblasts, whereas such high levels are not observed in the third trimester placental tissues (Gualillo et al., 2001). Only a few studies have reported the course of circulating ghrelin levels as measured by immunoassay during pregnancy in humans. The longitudinal changes in serum ghrelin levels during pregnancy in 11 pregnant women showed that normal nondiabetic women have maximum serum values during mid-pregnancy (Fuglsang et al., 2005). However, the serum levels declined to the lowest levels in pregnancy at the end of the third trimester with a 28 % decrease on average as compared with the mid-pregnancy values. Similar observations have been reported in a cross-sectional study measuring acylated ghrelin. The lowest circulating ghrelin levels are observed in late pregnancy and may be even lower than in nonpregnant subjects. Ghrelin is involved in the decidualization of human endometrial stromal cells. During pregnancy, ghrelin levels are at their maximum at mid-pregnancy and are at their lowest in the third trimester (at the time of increased body weight), suggesting that ghrelin is an important autocrine/paracrine factor for the growth and maintenance of the placenta during pregnancy (Fuglsang et al., 2005).

4.2 Leptin

Mice lacking leptin or the leptin receptor (Ob-R, db/db) exhibit low gonadotropin levels, incomplete development of reproductive organs and do not reach sexual maturation (Zhang et al., 1994). Leptin administration to ob/ob mice induces puberty, maturation of the gonads, gonadotropin secretion and restores fertility. Humans with congenital leptin deficiencies or leptin receptor mutations recapitulate most of the leptin-deficient reproductive phenotypes observed in the mouse models. Moreover, in young women that do not undergo puberty, leptin treatment can induce pubertal development. Plasma leptin concentrations are low after weaning in the female and progressively increase during the prepubertal and pubertal period.

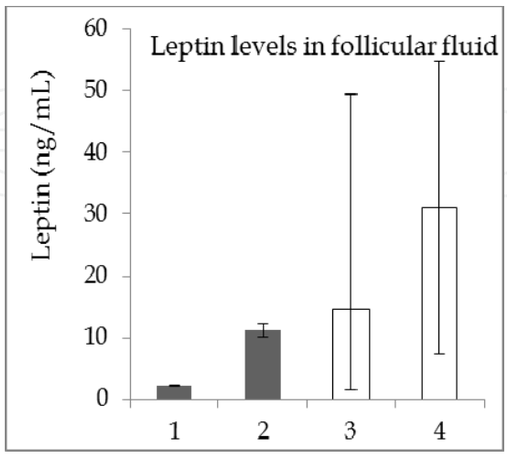


Fig. 8. Mean leptin levels in the porcine (1) and human (2-4) follicular fluid (black columns – RIA, white columns – ELISA) (1) Gregoraszczuk et al., 2004; (2) Welt et al., 2003; (3) Wunder et al., 2005, (4) Hill et al., 2007.

Leptin acts at various levels of the hypothalamic–pituitary–gonadal axis, involving different biochemical pathways. Leptin has stimulatory effects on the hypothalamic–pituitary–gonadal axis at normal serum concentrations but can have inhibitory effects when its levels are elevated, such as with obese individuals. Leptin and its receptors are expressed in the hypothalamus and pituitary, and leptin acts indirectly on the gonadotrophin-releasing hormone secreting cells (Quennell et al., 2003). Leptin also has activity at an ovarian level, and the leptin receptor expression has also been demonstrated by immunoassay in theca and granulosa cells as well as oocytes (Table 5). Moreover, the effects of leptin on ovarian cells are inhibitory and can be attributed to attenuation of gonadotropin, insulin, insulin-like growth factor 1 (IGF-I) and/or glucocorticoid-mediated steroidogenesis (Gregoraszczuk et al., 2003, 2004, 2006).

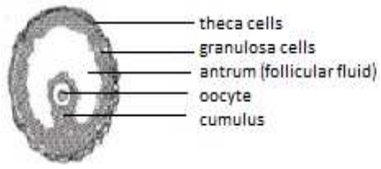
	Species/cell type	*Measure*	*References*
Ovarian follicles 	Human/gc/c/o	Leptin/ immunofluorescence staining	Cioffi et al. 1997
	Human/ff	Leptin/RIA	
	Human/gc/th/o	Leptin/ immunohistochemistry staining	Loffler et al. 2001
	Human/ff	Leptin/RIA Ob-R/ELISA	Welt et al. 2003
	Porcine/ff	Leptin/RIA	Gregoraszczuk et al. 2004
	Rat/th/o	Ob-R/ immunohistochemistry staining	Ryan et al. 2003
	Rat/th/o	Leptin/ immunohistochemical staining	Archanco et al. 2003
Corpus luteum (CL)	Human	Leptin/ immunohistochemistry staining	Loffler et al. 2001
	Porcine	Ob-R/ immunoblot	Ruiz-Cortes et al. 2000
	Porcine	Leptin/ immunoblot	Smolinska et al 2010
	Rat	Ob-R/ immunohistochemistry staining	Ryan et al. 2003
	Rat	Leptin/ immunohistochemistry staining	Archanco et al. 2003

Table 5. Location of leptin and (or) leptin receptor in the ovarian tissues of several species. gc: granulosa cells, tc: theca cells, ff: follicular fluid, o: oocyte, c: cumulus.

Consistent with these findings, our data indicated that in large porcine follicles, leptin inhibited basal and GH- or IGF-I-stimulated estradiol secretion. In contrast, leptin augmented two and four times the respective stimulatory effect of basal, IGF-I and GH on progesterone secretion. Our observations indicate that leptin's action on steroidogenesis depended on the stage of follicular development (Gregoraszczuk et al., 2003, 2004) (Fig. 9). We conclude that in preovulatory follicles, leptin is involved in the process of luteinization, which starts just before ovulation. Furthermore, the role of leptin in CL is restricted to the period of the luteal phase. Leptin protects luteal cells from excessive apoptosis and supports an appropriate cell number, which is necessary for maintenance of homeostasis in developing CL (Gregoraszczuk & Ptak, 2005).

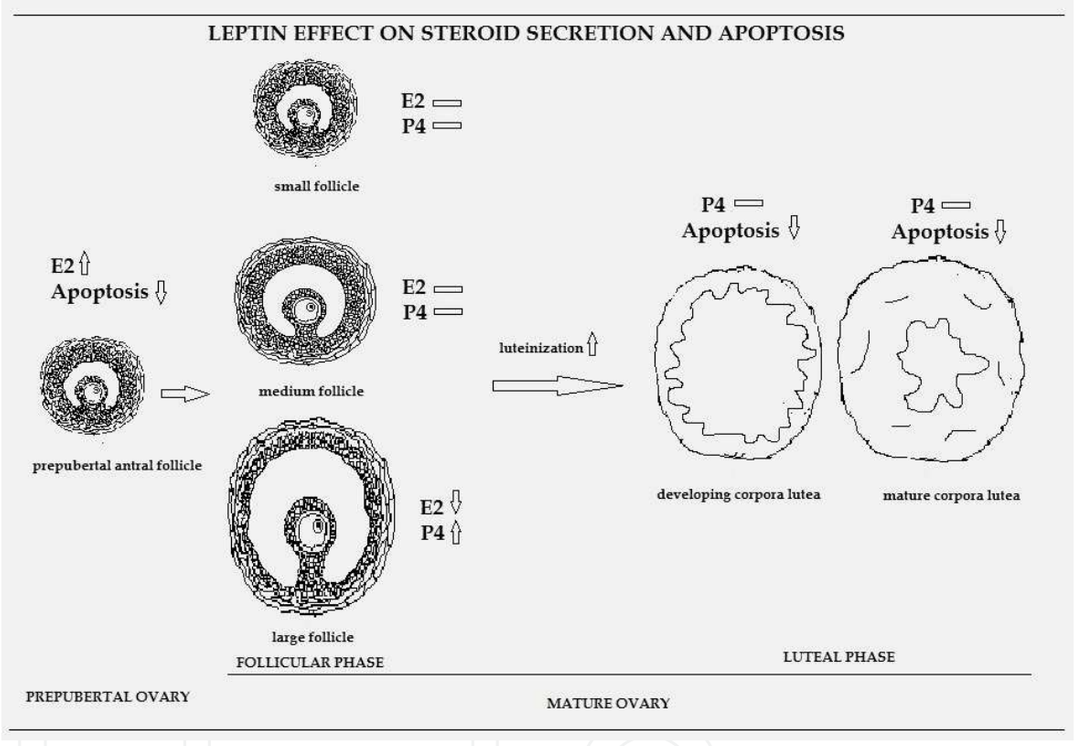


Fig. 9. Schematic summarizing leptin effect on steroid secretion in porcine ovary. (—) no effect, (↑) stimulatory effect, and (↓) inhibitory effect (Gregoraszczuk et al., 2004, 2005, 2006)

Evidence has also emerged indicating a potential direct role for leptin in the regulation of mammalian oocyte and preimplantation embryo development. Leptin and its receptor have been observed by immunoblotting and immunohistochemistry methods to be present in the secretory endometrium and preimplantation embryos. The expression of leptin and its functional receptor in the endometrium and regulation of endometrial leptin secretion by the human embryo suggests that the leptin system may be implicated in the human implantation process. Supplementation of culture medium with leptin promotes the development of preimplantation embryos from the 2-cell stage to the blastocysts, fully expanded blastocysts and hatched blastocysts (Kawamura et al., 2003). As such, perturbations in the leptin system, as observed with obese individuals, may disturb endometrial receptivity and implantation, leading to impaired fecundity. Patients with

endometriosis had significantly less serum leptin (15.6 ng/ml) than the controls (30.3 ng/ml). Perhaps, as suggested for the placenta and other tissues, leptin may regulate uterine angiogenesis and cytokine production (Matarese et al., 2000).

Thus, normal leptin secretion is necessary for normal reproductive function to proceed. In women, serum leptin levels are increased during puberty development and during pregnancy. In addition, some studies have reported higher leptin levels in the luteal phase of the cycle, while others have found no difference in leptin levels during normal menstrual cycle as measured by ELISA and or RIA assay (Table 6).

	Follicular phase	*Luteal phase*	*Methods*	*References*
Leptin (ng/ml)	25.7±3.1-28.1±3.0	28.5±3.3	RIA	Yamada et al., 2000
	14.9±2.9	20.4±4.2 *	RIA	Riad-Gabriel et al., 1998
	10.2±7.1	11.8±6.9	RIA	Teirmaa et al., 1998
	13.15±1.60	16.57±1.68 8*	ELISA	Einollahi et al., 2010
	18.14±0.28	23.75±0.64 **	ELISA	Asimakopoulos et al., 2009
Resistin (ng/ml)	4.68±0.07	5.30±0.23	ELISA	Asimakopoulos et al., 2009

Table 6. Mean serum leptin and resistin concentrations in women (normal menstrual group) (* $p<0.01$, ***$p<0.001$ *vs.* follicular phase).

Relative leptin deficiency is an emerging clinical syndrome that is associated with several clinical conditions, including exercise-induced energy deficiency, hypothalamic amenorrhea and anorexia nervosa (AN). Leptin levels in AN were first measured in 1995 using an enzyme-linked immunosorbent assay. In most anorexic patients, low leptin serum levels were detected compatible with their underweight and were associated with amenorrhea. In women with hypothalamic amenorrhea, resulting from a negative energy balance, leptin treatment increased pulse frequency and mean levels of LH, ovarian volume, the number of dominant follicles and estradiol levels.

	Human	Pig	Rat
Ovary	+, *	+, #	*
Uterus	#, *	#	no data
Placenta	+, *	#	#

Table 7. Expression of leptin protein in reproductive tissue: + ELISA or RIA detection, # Western Immunoblotting detection, * Immunohistochemical detection

Interestingly, recent studies have demonstrated that leptin stimulates growth, migration, invasion, and angiogenesis in tumor cell models, suggesting that leptin is able to promote an aggressive cancer phenotype. Several studies have indicated that serum leptin levels are positively associated with endometrial cancer, breast cancer, and ovarian cancer. Interestingly, both leptin and the leptin receptor (ObR) appear to be significantly overexpressed in the epithelium, breast and ovarian cancer tissue (Garafalo and Surmacz, 2006) as compared with noncancerous tissues.

Our data indicated that leptin, at physiological relevant concentrations, has no effect on ovarian cancer cell proliferation. Notably, leptin in obese women stimulated cell growth by the

up-regulation of genes that are responsible for inducing cell proliferation and the down-regulation of genes that are involved in the inhibition of cell proliferation. The antiapoptotic action of leptin on ovarian cancer cells was due to overexpressing anti-apoptotic factors of both extrinsic and intrinsic caspase-dependent pathways (Ptak et al., 2011, unpublished data).

4.3 Resistin

There are a few studies that indicate a connection between resistin and reproduction function. Resistin dose dependently increases both basal and human chorionic gonadotropin (hCG)-stimulated in vitro testosterone secretion from rat testicular tissue (Nogueiras et al., 2004). Additionally, the pituitary hormones LH and FSH regulate the mRNA expression of resistin in the testis. There are some data concerning resistin and female reproduction. A recent paper by Maillard et al., (2011) demonstrated that resistin is expressed in whole bovine and rat ovaries and that it can modulate ovarian steroidogenesis and proliferation. Ovarian gene expression of resistin was found throughout the estrous cycle in rats. Moreover, resistin mRNA expression in adipose tissue increases during puberty in 45-day old female rats. A recent work demonstrates that resistin mRNA is expressed in the mouse brain and pituitary gland (Morash et al., 2002). Pituitary expression of resistin is regulated in a nutritional-, age- and gender-specific manner. Furthermore, resistin gene expression increases to a peak level in the pituitary of prepubertal mice (Morash et al., 2004). In cultured theca cells, resistin enhanced 17α-hydroxylase activity, which is a marker of ovarian hyperandrogenism in polycystic ovarian syndrome (PCOS) women (Figure 10). This result suggests that resistin may play a local role in stimulating androgen production by theca cells (Munir et al., 2005). Furthermore, resistin mRNA expression in the adipocytes from PCOS women is 2-fold higher than that in the controls. However, used the ELISA method demonstrate that serum resistin levels remain unchanged in normally cycling women, suggesting that physiological changes of sex steroid levels have no effect on resistin secretion from adipocytes (Dafopoulus et al., 2009).

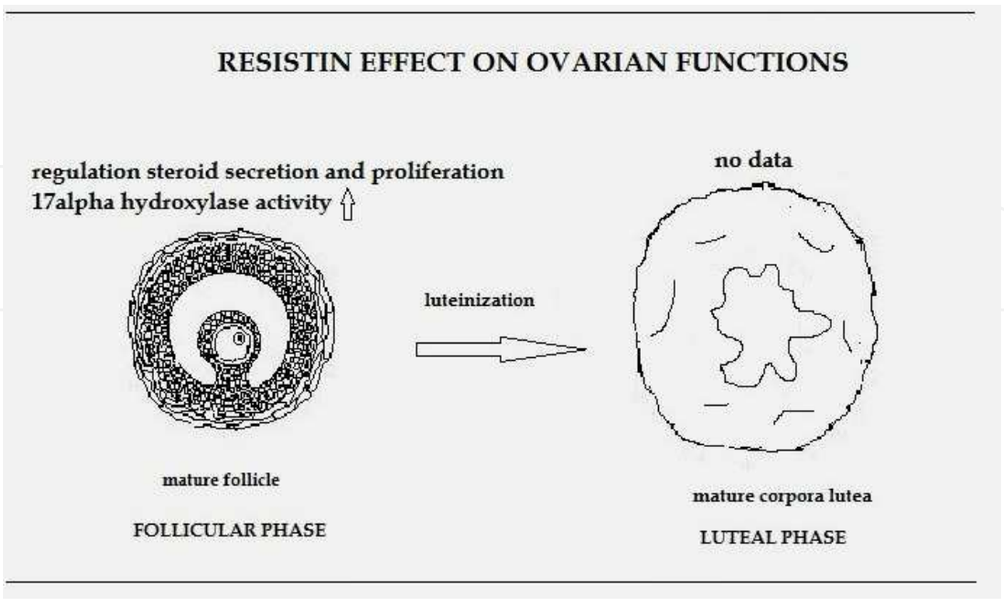


Fig. 10. Effect of resistin in ovarian reproduction. (↑) stimulatory effect, (Maillard et al., 2011, Munir et al., 2005).

Using commercially available ELISA kits, our experiments showed that the concentration of resistin increases during follicular growth, with the highest level in large follicles collected from prepubertal animals (Rak-Mardyła & Gregoraszczuk, 2011, unpublished results). We found no difference in resistin concentration in the ovarian follicles collected from animals during the estrous cycle). These results were confirmed by Western immunoblotting (Figure 11).

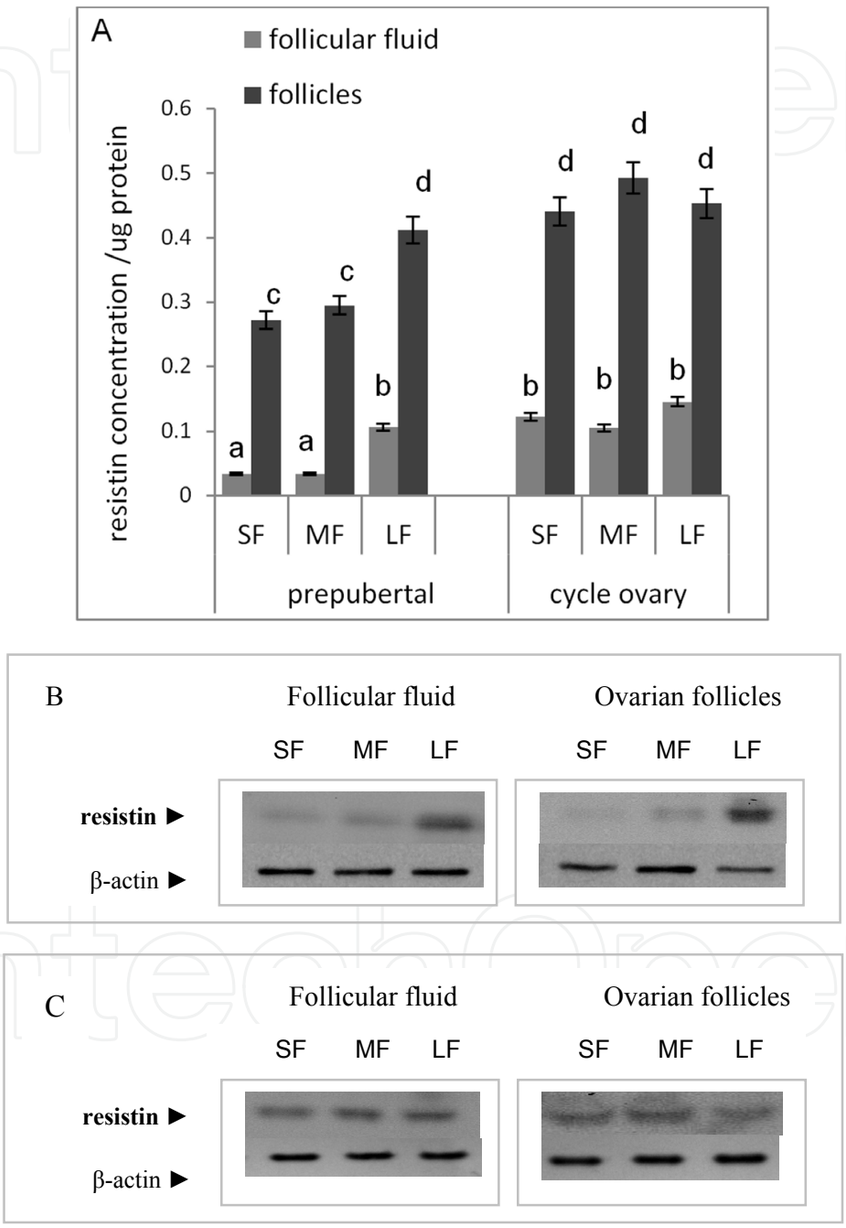


Fig. 11. (A) Concentrations of resistin in follicular fluid and ovarian tissue collected from prepubertal animals measurement by ELISA assay and (B and C) Western blotting. All samples were performed in triplicate in the same assay. All data are expressed as the mean ± SEM. Different letters indicate statistically significant differences among groups ($p<0.05$) (Rak-Mardyła & Gregoraszczuk, 2011, unpublished results).

5. Conclusion

Immunoassay methods are widely used in many studies to measure the concentration of peptide hormones, such as ghrelin, leptin or resistin in reproductive tissue. These methods are suitable for measuring a number of locally secreted peptides, such as growth hormone, insulin-like growth factor-I, insulin or the peptides hormones, ghrelin, leptin and resistin, which influence follicular development during different physiological stages.

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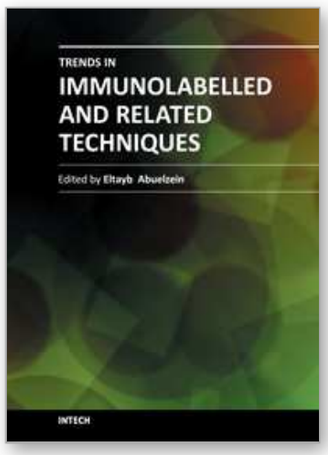

Trends in Immunolabelled and Related Techniques

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The book is coined to provide a professional insight into the different trends of immunoassay and related techniques. It encompasses 22 chapters which are grouped into two sections. The first section consists of articles dealing with emerging uni-and-multiplex immunolabelled methods employed in the various areas of research. The second section includes review articles which introduce the researchers to some immunolabelled techniques which are of vital significance such as the use of the conjugates of the Staphylococcus aureus protein "A" and the Streptococcus Spps. protein "G" in immunolabelled assay systems, the use of bead-based assays and an overview on the laboratory assay systems. The book provides technological innovations that are expected to provide an efficient channel for developments in immunolabelled and related techniques. It is also most useful for researchers and post-graduate students, in all fields, where immunolabelled techniques are applicable.

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