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DETECTION OF LEPTIN IN MUSCLE TISSUES AND ORGANS OF PIGS

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ABSTRACT

The aim of this study was detection of gene leptin in muscles, liver and kidneys from pigs of breed Large White. Using Real time PRC method, we determined the Ct values of leptim gene in muscle, liver, kidney. The body weight of pigs ranged from 100 kg to 103 kg. The average body weight was 101.6 kg. The thickness of backfat ranged from 10 to 20 mm, average backfat thickness was 16 mm. The minimal Ct value of leptin gene in liver was 24.05 and the maximal value was 25.79. Average Ct value of leptin gene was 24.84. The minimal Ct value of leptin gene in muscle tissue was 25.83 and the maximal value was 27.05. Average Ct value of leptin gene was 26.41. The Ct value of leptin gene in liver ranged from 24.05 to 25.79. Average Ct value of leptin gene was 24.84. Leptin gene is expressed by porcine preadipocytes and leptin gene expression is highly dependent on dexamethasone induced preadipocyte differentiation. Hormonally driven preadipocyte recruitment and subsequent fat cell size may regulate leptin gene expression in the pig.

Keywords: Leptin gene, backfat thickness, liver, kidney

INTRODUCTION

Real time PCR is the best and fastest method to assess the level of DNA because it is not time consuming, not labour intensive, sufficiently sensitive, quantitative, does not require the use of radioactivity and has no substantial probability of cross contamination (**Reischl *et al.*, 2002**).

Leptin (Ob) is a protein hormone, produced primarily by cells of adipose tissue that acts mainly on the hypothalamus, where it causes profound effects on food intake and energy balance. Leptin exerts its activity through a receptor (ObR) of which a variable number of isoforms have been reported in different animal species (six in rodents and four in humans). All these variants have an identical extracellular domain and the same binding affinity for leptin, but differ in amino acid composition and in length of the trans-membrane and intracellular domains (**Cammisotto and Bendayan, 2012**). There is an isoform defined as "long" that is considered the only active part in the signal transduction (**Marroquí *et al.*, 2012**). The other isoforms are commonly referred to as "short" forms and are probably very active in promoting the transport of leptin in the plasma, the crossing of the blood-brain barrier, *etc*.

Recently, other tissue sites that can produce leptin have been identified and that may be the target of the action of this peptide. This has led to the hypothesis that there are other possible functional roles for this molecule. The presence of the Ob-gene and its related protein have been detected in salivary glands (**De Matteis *et al.*, 2002**), in the gastric mucosa (**Cammisotto and Bendayan, 2012**), in trophoblast cells of the placenta (**Dall'Aglio *et al.*, 2012**) and in some endocrine glands, including the pituitary gland (**Sone and Osamura, 2001**) and the pancreas (**Reddy *et al.*, 2004** and **Ehrström *et al.*, 2005**), mainly in humans and laboratory animals. ObR was visualized outside the brain in the spleen, testes, kidney, liver, lung and adrenal glands of the rat (**Hoggard *et al.*, 1997**) and in mouse and human glucagon-cells of the pancreas (**Tudurí *et al.*, 2009**). Regarding the data on the presence and localization of leptin and its receptor in the endocrine pancreas (**Reddy *et al.*, 2004**) it has been suggested that leptin may influence the functionality of the endocrine pancreas by possible autocrine action, in particular by modifying the electrical and secretory activity of the alpha and beta cells (**Marroquí *et al.*, 2012**). In domestic animals, the presence and distribution of leptin and its receptor have been widely studied in ruminants (**Chilliard *et al.*, 2005** and **Abavisani *et al.*, 2012**) and pigs (**Lin *et al.*, 2000**). In both cases, wide distribution of the protein and its related receptor have been reported thus indicating the possibility that leptin may have multiple physiological functions.

The aim of present study was detection of leptin gene in tissue, liver and kidney of pigs and monitoring of the correlation between leptin, backfat thickness and live weight of pigs.

MATERIAL AND METHODS

Biological material

Internal organs (liver, kidney) and thigh muscles of pigs (breed Large White) were used as biological material. The samples were taken from five pigs.
These indicators were evaluated for each pig:

- body weight (kg),
- backfat thickness (mm),
- leptine gene in liver (Ct value),
- leptine gene in thigh muscle (Ct value),
- leptine gene in kidneys (Ct value).

Information on live weight and backfat thickness were obtained from supporting documents the animals.

Isolation of DNA from tissues

- there were added 10 mg of the sample into a 1.5 mL eppendorf tubes,
- lysis solution T (180 µl) was added to the samples,
- proteinase K (20 µl) was added,
- incubation was carried out in a water bath at 55 °C for 2 - 4 hours

Composition and preparation of lysis solution

Lysis solution C (200 µl) was added into eppendorf tubes after incubation. The samples were vortexed 15 seconds. Samples were Incubated at 70 °C for 10 minutes.
Column preparation - there were added 500 µL of the Column Preparation Solution to each pre-assembled GenElute Miniprep Binding and centrifuge at 12.000 × g for 1 minute. Flow-through liquid was discarded. The Column Preparation Solution maximizes binding of DNA to the membrane resulting in more consistent yields.
Prepare for binding - there were added 200 µL of ethanol (95–100 %) to the lysate; mix thoroughly by vortexing 5–10 seconds. A homogeneous solution was essential.
Load lysate - the entire contents of the tube was transfered into the treated binding column. Samples were centrifuged at 6500 × g for 1 minute. The tube

containing the flow-through liquid was discarded and placed the binding column in a new 2 mL collection tube.
First wash - the Wash Solution Concentrate was dilute with ethanol prior to first use. There were added 500 mL of Wash Solution to the binding column and samples were centrifuged for 1 minute at 6.500 × g. The tube containing the flow-through liquid was discarded and placed the binding column in a new 2 mL collection tube.
Second wash - there were Added another 500 µL of Wash Solution to the binding column. samples were centrifuged for 3 minutes at maximum speed (12.000-16.000 × g) to dry the binding column. The tube containing the flow-through liquid was discarded and placed the binding column in a new 2 mL collection tube.
Elute DNA - the Elution Solution (200 mL) was pipetted directly into the center of the binding column. Samples were centrifuged for 1 minute at 6.500 × g to elute the DNA. To increase the elution efficiency, incubation for 5 minutes at room temperature after adding the Elution Solution, then centrifugation were performed.

Real-time PCR analysis
These primers were used at PCR analysis:
Forvard (FOR) 5 ATGCGCTGTGGACCCCTGTAT C 3',
Revers (REV) 5' TGGTGTCATCCTGGACCTTCC 3'.
PCR was carried out in a thermocycler StepOne.

Procedure for Real-time PCR
1. Incubation - 95 °C, 2 minutes - is used to activate of the polymerase.
2. Denaturation - 95 ° C, 5 seconds - 40 cycles.
3. Annealing - 60 °C , 15 seconds.

Increase in fluorescence was observed in all examinated samples, which indicates positivity of tested samples for the presence of leptin. In a real time PCR assay a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (ie exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (ie the lower the Ct level the greater the amount of target nucleic acid in the sample).
Statistical Analysis
Mathematical and statistical analysis (arithmetic mean, standard deviation, standard error, coefficient of variation) were performed using the program system Statgraffic.

RESULTS AND DISCUSSION

Determination of body weight

The adipose tissue produces various cytokines that are involved in inflammatory and thrombotic pathways, such as leptin, IL-6, TNF-α, adiponectin, and PAI-1 (**Kershaw and Flier, 2004** and **Tongjian *et al.*, 2005**). Regulation of energy distribution entails a balance among several factors such as feeding behavior, adipose tissue mass and activation of catabolic processes. A change in body mass or nutritional status leads to alterations in concentration of many hormones and growth factors in serum that regulate adipocyte function and leptin secretion (**Barb *et al.*, 2001)**.

The lowest body weight of pigs was 100 kg (sample no. 1). The highest body weight was 103 kg (sample no. 3) (Figure 1). The average body weight was 101.6 kg (Tab. 1).

The results obtained in pigs with regard to the association between the C3469T Lep polymorphism and fat-related traits are contradictory in terms of both magnitude and direction. Divergence among studies, as well as to the experimental design and the statistical methods used, are usually attributed to population-specific linkage disequilibrium of the polymorphisms with causal mutations (**Villaba *et al.*, 2009**).

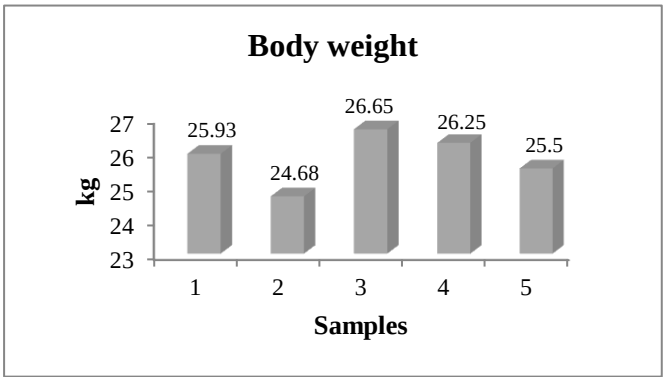


Figure 1 Body weight of pigs (kg)

Determination of backfat thickness (mm)

The lowest value of backfat thickness was 10 mm (sample no. 1). The highest value of backfat thickness was 20 mm (sample no. 3 and sample no. 5) (Figure 2). The average backfat thickness was 16 mm (Tab. 1).

Backfat thickness has been used as a selection trait to increase the carcass lean percentage of pigs. However, a conflicting relationship exists between pork quality and increased lean meat production.

Villaba *et al.* (2009) found that genotype effects are not constant throughout the growing period and therefore estimated effects can be influenced by age of measurement and/or the fattening state. Using measurements taken at different time-points throughout the fattening period, they proved that the association of the C3469T Lep polymorphism with some traits increases with age (i.e. longisimus dorsi muscle thickness) or even changes to the opposite direction (i.e. intramuscular fat - IMF content). This result may help to explain contradictory results from studies using either pigs tested at different ages or pigs tested at the same age but which are from genetic types differing in their fat deposition pattern. There have found a differential association of the C3469T Lep polymorphism with subcutaneous backfat (SB) and IMF. It is known that there is also a differential expression of genes and biochemical constituents, including leptin, in adipocytes from different tissues (**Harper and Pethick, 2004**). In particular, **Gardan *et al.* (2006)** found that the Lep mRNA levels differed between IMF adipocytes at 160 and 210 days of age but not in SB adipocytes. It can be then hypothesized that the C3469T polymorphism is associated with a differential expression of leptin across fat tissues and throughout time, either because it has a regulatory role or because it is linked with another mutation affecting gene expression.

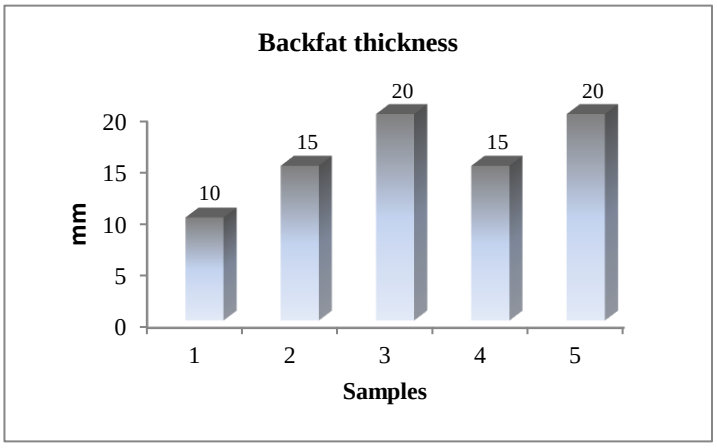
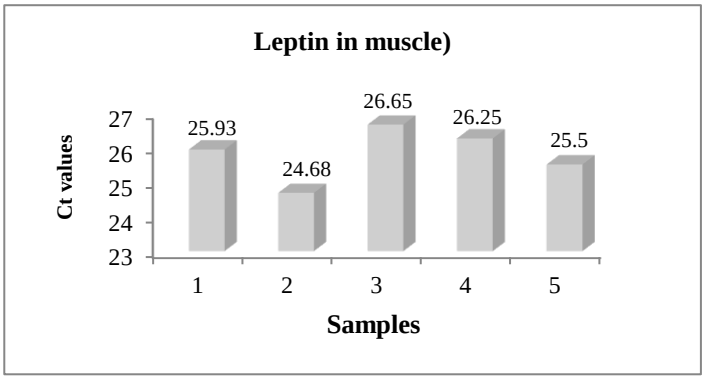


Figure 2 Backfat thickness (mm)

De Rensis *et al.* (2006) have determined relationships between sow backfat depth, plasma leptin concentrations, and reproductive performance after weaning. On the day of farrowing (day 0), and at weaning (day 21), single blood samples were obtained from 120 mixed-parity sows and their backfat depth (P2) measured. Based on backfat depth at day 0, sows were classified as FAT (>24 mm, n = 16), MEDIUM (16-24 mm, n = 54), or THIN (<16 mm, n = 14). Sows were further classified on the basis of P2 backfat changes during lactation of <2 mm, 2-4 mm, or >4 mm. Reproductive performance was measured as weaning-to-oestrous intervals (WOI) of <6 d, 6-9 days, or > or =10 d, and pregnancy rates. There was a positive relationship ($P < 0.0001$) between backfat depth at day 0 and backfat loss during lactation.

Leptin gene in thigh muscle

Suzuki *et al.* (2009) found that backfat thickness (BF) and leptin are good indicators of selection for decreasing fat deposition. Increased genetic correlation of the SFS with intermuscular fat area at 56TV (0.74) suggests that seam fat score (SFS) is an effective indicator for decreasing intermuscular fat. The genetic correlation between the leptin concentration and feed conversion ratio was high (0.75 ± 0.04). Results of this study indicate that the combination of BF and serum leptin concentration is a valuable indicator that can be incorporated into selection programs to improve carcass quality and feed efficiency in pigs.
The minimal Ct value of leptin gene in muscle tissue was 25.83 and the maximal value was 27.05 (Figure 3). Average Ct value of leptin gene was 26.41 (Tab. 1).



Figre 3 Leptin gene in muscle (Ct value)

Berg *et al.* (2003) found that differences exist between breeds of pigs in a manner consistent with breed-specific traits for growth, leanness, and quality; thus, leptin may serve as a useful marker for selection or identification of specific growth and carcass traits.

Leptin gene in liver

Leptin exerts direct effects on liver, a major site of glucose metabolism. Several lines of evidence indicate that leptin mimics some of the anabolic actions of insulin on liver. Leptin enhances the inhibitory effects of insulin on glycogenolysis and hepatic glucose production in liver in vivo (**Nemecz *et al.*, 1999**).

The minimal Ct value of leptin gene in liver was 24.05 and the maximal value was 25.79 (Figure 4). Average Ct value of leptin gene was 24.84 (Tab. 1).

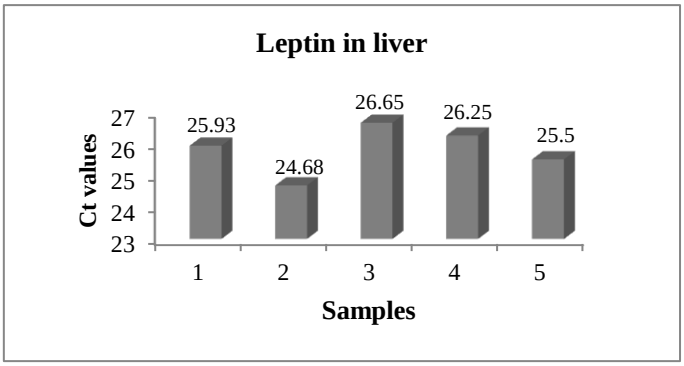


Figure 4 Leptin gene in liver (Ct value)

Raman *et al.* (2004) studied the effects of leptin on gluconeogenesis in rat hepatocytes. Direct effects of leptin on gluconeogenesis in rat hepatocytes are equivocal, and model systems from other species have not been extensively explored in assessing the regulation of glucose metabolism by leptin. Therefore, the goal of their study was to compare the effects of leptin on gluconeogenesis in pig and rat hepatocyte cultures as well as to investigate an underlying mechanism of action at the level of phosphoenolpyruvate carboxykinase (PEPCK). In rat hepatocytes, leptin exposure (3 h, 50 and 100 nM) attenuated glucagon-stimulated hepatic gluconeogenesis by 35 and 38 % ($P < 0.05$), respectively. However, leptin did not produce any significant acute effect in pig hepatocytes. Leptin exposure for 24 h failed to produce any significant effect on gluconeogenesis in either rat or pig hepatocytes cultured in the presence of glucagon or dexamethasone. Mechanistically, there was a 25-35% decrease ($P < 0.05$) in glucagon-induced PEPCK mRNA levels in rat but not pig hepatocytes cultured with leptin.

Leptin gene in kidney

Chronic hyperleptinemia can increase blood pressure through the sympathetic nervous system and renal salt retention. The concept of selective leptin resistance in obesity is emerging, whereby leptin's effect on appetite and energy expenditure is blunted, with a concomitant increase in leptin's other effects as a result of the accompanying hyperleptinemia. The divergence in response likely is explained by different receptors and post-receptor activating mechanisms. Chronic kidney disease is a known cause of hyperleptinemia. There is an emerging view that the effect of hyperleptinemia on the kidney can contribute to the development and/or progression of chronic kidney disease in selective resistance states such as in obesity or type 2 diabetes mellitus. The mechanisms of renal injury are likely the result of exaggerated and undesirable hemodynamic influences as well as profibrotic effects (**Nasrallah *et al.*, 2013).**

The minimal Ct value of leptin gene in kidney was 24.68 and the maximal value was 26.65 (Figure 5). Average Ct value of leptin gene was 25.80 (Table 1).

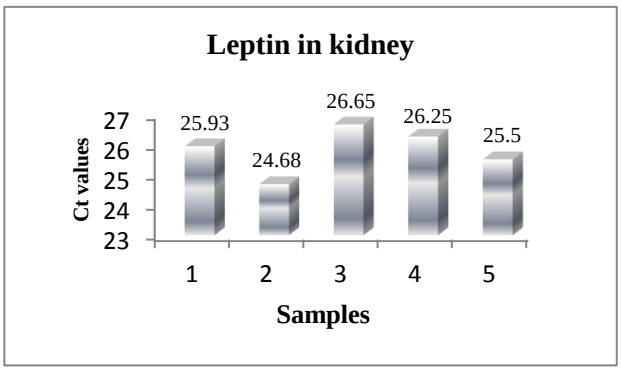


Figure 5 Leptin gene in kidney (Ct value)

Sharma and Considine (1998) investigated, whether leptin is cleared by the kidney and is elevated in hemodialysis patients. In patients with intact renal function there was a net renal uptake of 12 % of circulating leptin, whereas in patients with renal insufficiency there was no renal uptake of leptin. In a separate cohort of 36 patients with end-stage renal disease (ESRD), peripheral leptin levels factored for body mass index was increased by fourfold as compared to a group of healthy controls (N = 338). The leptin receptor exists in a long and short form, with the long form primarily expressed in the hypothalamus but also in the lungs and kidneys of the mouse. Further studies are necessary to clarify the role of leptin in regulating appetite in patients with ESRD and the role of leptin in directly affecting kidney function via its receptors.

Table 1 Basic statistical characteristics of pigs

Indicator	n	x	s	S_x	v%
BW(kg)	5	101.6	1.02	0.46	1.00
BT (mm)	5	16	3.74	1.67	23.3
Lep. in muscle	5	26.41	0.43	0.19	0.16
Lep. in liver	5	24.84	0.72	0.32	0.29
Lep. in kidney	5	25.80	0.68	0.30	0.26

BW (kg) – body weight, n – number of animals, x – arithmetic mean, s – standard deviation, S_x – standard error, v – coefficient of variation (%).

CONCLUSION

The pig is emerging rapidly as an important biomedical research model, and whereas genetic influences may impact the degree of similarity between pig and human systems, the regulation of some endocrine and metabolic processes in the pig may be more similar to humans than are rodents. Various cellular functions of leptin in different organs of pigs are summarized in several researches. Our results showed the presence of higher concentrations of leptin and in the kidneys. There are still relatively few results about function of leptin in kidneys.

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