Praca oryginalna ***Original paper***

Effect of immunocastration using Improvac on the regulation of adiponectin and leptin in the testes of Landrace boars*

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Summary

Immunological castration of male pigs is an attractive alternative to surgical castration used in many countries to reduce the production of the main androgen, androstenone, and skatole, responsible for meat taint. To understand the effect of gonadoliberin (GnRH) vaccination with Improvac on the functional status of Landrace pig testes at the molecular level the role of adipokines was studied. Using western blotting, immunohistochemistry, and enzyme-linked immunosorbent assay, we explored adipokine and leptin signaling and evaluated cholesterol concentrations. In control testis, adiponectin and its receptors were localized to interstitial Leydig cells and spermatogenic cells (especially adiponectin and its receptor 2) while in immunocastrates spermatogenic cells were negative. In control, leptin was exhibited by spermatids and Leydig cells while its receptor only by the later cells. In immunocastrates, leptin immunosignal was not found in spermatogenic cells. In addition, for all studied proteins, the immunosignal was of moderate or weak intensity when compared to the control. Concomitantly, decreased expression of all proteins ($p < 0.5$) was detected. Similarly, in immunocastrates, cholesterol concentrations were increased ($p < 0.01$).

In summary, we showed for the first time the coincidence of disturbed adiponectin signaling and leptin signaling together with increased cholesterol concentration and attenuated spermatogenesis as a result of halted androstenone production. Altered GnRH signaling affects the adipokine system in testes of Landrace castrates which may impact further functional changes leading to complete spermatogenesis alteration, as well as lipid homeostasis and fattening perturbances.

Keywords: adiponectin, pig, cholesterol, immunocastration, leptin, testis

One of the alternatives for surgical castration of piglets is an immunocastration with a modified gonadoliberin (GnRH) component (26). The GnRH vaccine (Improvac) induces an endogenous immune reaction (immunization) leading to a high level of anti-GnRH antibodies (40). The high titer of antibodies against GnRH reduces lutropin secretion and decreases testicular androgen secretion (androstenone – responsible for

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a specific urine odor) and skatole production (causes a fecal odor in meat often referred to as "boar taint"). In the boar, these compounds accumulate in muscle tissue, fat tissue, and salivary glands. (31). However, other main effects of immunocastration include disturbed testicular development and functioning, testis immaturity correlated with anti-Mullerian hormone expression, and alteration in testicular length and histomorphometry have been demonstrated (23, 36). Immunocastrated animals show a lower carcass yield and lower meat percentage compared to surgically castrated animals; although these parameters also depend on the animal's age at castration (16). Increased aggressive behavior has also been reported in these males. It should be added here that the molecular effects of immunocastration on the testes and other tissues are not fully known and thus raise concerns regarding pork consumption (35).

In pigs, fat deposition plays a key role in both the quality and the nutritional value of the meat (39). Adipose tissue is not only a site of lipid storage, but it is also an active endocrine organ (21). It is the main tissue involved in the regulation of lipid metabolism including sex steroid hormone production and is also the site of *de novo* fatty acid synthesis in pigs (28). Moreover, it releases adipokines, which are hormones involved in metabolism regulation *via* paracrine and autocrine interactions (19). For instance, adipokines regulate lipid and glucose metabolism (20). In mammals, the action of adipokines is associated with body mass index (BMI), food intake, and reproductive functions (4, 11). Adiponectin and leptin are the best-known adipokines in pigs (2). Adiponectin signals in tissues by binding to seven-transmembrane domain adipokine receptors 1 and 2 (ADIPOR1 and ADIPOR2, respectively) whereas leptin exerts its action through the leptin receptor (Ob-R), a member of the class I cytokine receptor family. Adipokine signaling and leptin signaling have been described in various pig tissues (15, 24, 25, 33).

Our recent studies have demonstrated that inflammation of testicular tissue occurs together with incomplete spermatogenesis ablation in immunocastrated Landrace boars (29). The adiponectin/leptin ratio is a functional biomarker of adipose tissue dysfunction, inflammation, metabolic syndrome, or obesity (12). Therefore, here the adiponectin signaling and leptin signaling were studied in the testes of Landrace boars immunized with Improvacto develop a deeper understanding of the molecular aspects of their role in spermatogenesis as well as fat metabolism.

Material and methods

Tissue collection. Testes were collected from n = 40 Polish Landrace boars at the Pig Slaughter Utility Control Station Chorzelów, Poland. Ten boars were kept untouched and used as control animals. Thirty boars were injected with Improvac (Zoetis, Louvain-la-Neuve, Belgium) according to the manufacturer's recommendations [one dose (2 ml) after 8 weeks of age, the second one 4-6 weeks before slaughter at the age of the 117 days]. Improvac is a solution for injection for pigs containing one dose (2 ml): active substance: synthetic peptide analog of GnRH conjugated with diphtheria toxoid min. 300 μg; adjuvant: diethylaminoethyl (DEAE) – dextran, 300 mg water-based non-mineral oil adjuvant; excipient: chlorocresol 2.0 mg. The animals at the Pig Slaughter Utility Control Station Chorzelów were kept in individual pens, and the control fattening was performed from 30 to 120 kg (data in preparation). The feeding program was based on two mixtures I: proper fattening feed used from 30 kg to 80 kg (13.5 Mj/kg energy, 17-19% total protein, 2.4-4.0% crude fiber) and mixture II: feed at the end of fattening from 80 up to 120 kg (13 Mj/kg energy, 16-18% total protein, 3.0-5.0% fiber). Nutrition took place in the *ad libitum* system with a finely granulated mixture of a known and constant composition fed from vending machines. Feed intake was strictly controlled. The remaining conditions of keeping and handling the animals were in line with the current methodology of evaluation in Pig Slaughter Utility Control Station Chorzelów according to the Directive 2010/63/EU on the Protection of Animals Used for Scientific Purposes. The use of boar tissues was approved by the Local Ethics Committee in Krakow, Poland (permission number: 144b/2015).

All animals vaccinated with Improvac showed a response. The skin, behavior, and breeding conditions were monitored twice a day. After animals (uncastrated boars and immunocastrated ones) were slaughtered testicular tissues were obtained and cut into pieces and next fixed in 10% formalin or immediately frozen for laboratory analyses.

Western blot. The testicular tissues were homogenized and sonicated with a cold Tris/EDTA buffer (50 mM Tris, 1 mM EDTA, pH 7.5) and supplemented with acid proteases and aminopeptidases as well as inhibitors of serine and cysteine (Sigma-Aldrich). The protein concentration was estimated using the Bio-Rad DC Protein Assay Kit with BSA as a standard (Bio-Rad Labs, GmbH, München, Germany). Equal amounts of protein were resolved by SDS-PAGE under reducing conditions, transferred to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany), and then analyzed by Western blotting with antibodies (Tab. 1). The presence of the primary antibody was

Tab. 1. Primary antibodies used for Western blot and immunohistochemistry

Antibody	Host species	Vendor	Dilution
Adiponectin	rabbit	Invitrogen – PA1-054	1 : 100 (IHC) 1 : 1000 (WB)
AdipoR1	rabbit	Invitrogen – PA5-35347	1 : 100 (IHC) 1 : 1000 (WB)
AdipoR2	rabbit	Invitrogen – PA1-12759	1 : 100 (IHC) 1 : 1000 (WB)
Leptin	rabbit	Invitrogen – PA5-1051	1 : 200 (IHC) 1 : 700 (WB)
Ob-R	rabbit	Santa Cruz – Sc-8391	1 : 200 (IHC) 1 : 200 (WB)
β-actin	mouse	Sigma-Aldrich – A5316	1 : 1000 (WB)

revealed with horseradish peroxidase-conjugated secondary antibodies diluted 1 : 3000 (Vector Laboratory, Burlingame, CA, USA) and visualized with an enhanced chemiluminescence detection system as previously described (27). The specificity of the antibodies was assessed using blocking peptide and/or antibody dilutant. All immunoblots were stripped with a stripping buffer containing 62.5-mM Tris-HCL, 100-mM 2-mercaptoethanol, and 2% SDS (wt:v; pH 6.7) at 50°C for 30 min. and then incubated in antibody against β-actin (loading control). Protein abundance within the control group was arbitrarily set as 1, against which the statistical significance of each of the experimental groups was analyzed. To obtain quantitative results, the bands (representing each data point) were densitometrically scanned using public domain ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The results of often separate measurements were expressed as mean ± SD.

Separation of protein preparations was performed by SDS-PAGE under reducing conditions and then proteins were transferred to polyvinylidene difluoride membranes. Nonspecific binding sites were blocked with non-fat dry milk containing Tween® 20. Next, the membranes were incubated with antibodies (Tab. 1) as were those for immunohistochemistry at 4°C overnight, followed by a horseradish peroxidase-conjugated secondary antibody (Vector Laboratories) at room temperature. Proteins were detected by chemiluminescence and documented with a ChemiDocTM XRS+ System (Bio-Rad Laboratories). The specificity of antibodies was assessed using blocking peptides and/or positive controls (not shown). All immunoblots were stripped and re-probed with an anti-β-actin antibody as the loading control. The molecular weights of target proteins were estimated by reference to standard proteins (Sigma-Aldrich). To obtain quantitative results, immunoblots were analyzed densitometrically using the ImageLab software (Bio-Rad Laboratories) by an independent observer.

Immunohistochemistry. Testicular tissues were cut into 4 µm thin sections. Sections were immersed in a 10 mM citrate buffer (pH 6.0) and heated in a microwave oven (2 × 5 min, 700 W). Thereafter, sections were incubated sequentially in H_2O_2 (3%; v/v) for 10 min and normal goat serum (5%; v/v) for 30 min as described previously (21). Thereafter, the sections were incubated overnight at 4°C with primary antibodies (Tab. 1). Next, biotinylated goat anti-rabbit IgG (Vector, Burlingame CA, USA) and avidin-biotinylated horseradish peroxidase complex (ABC/HRP; Dako, Glostrup, Denmark) were applied in the succession. The staining was developed using 3, 3'-diaminobenzidine (DAB). Positive controls and negative controls in the absence of primary antibodies were performed for each immunostaining. Thereafter, sections were slightly counterstained with Mayer's hematoxylin and mounted using DPX mounting media (Sigma-Aldrich). Serial sections were examined with a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany).

Cholesterol concentration measurement. Cholesterol concentration was measured by ELISA Assay (cat. no. ELK8282; Bio-Connect, Huissen, the Netherlands) using the manufacturer's instructions. Testicular lysates were obtained by homogenization and sonication of fresh tissues with a cold Tris/EDTA buffer (50 mM Tris, 1 mM EDTA, pH 7.5). Before the assay, the sample amount was calculated based on the predicted concentration of the hormone. For analysis, 50 µl standard (prepared according to instructions) was used. The blank was set with a standard diluent buffer. Concentrations were calculated from mean ± SD (from three separate measurements). The assay sensitivity was 2.48 µg/mL.

Statistics. Each variable was tested using the Shapiro-Wilk W-test for normality. The homogeneity of variance was assessed with Levene's test. The distribution of the variables was normal and the values were homogeneous in variance. All statistical analyses were performed using a one-way analysis of the variance (ANOVA) followed by Tukey's *post hoc* comparison test to determine which values differed significantly from the controls. The analysis was made using Statistica software (StatSoft, Tulsa, OK, USA). Data were presented as a mean ± SD. Data were considered statistically significant at $p < 0.05$. All experimental measurements were performed in triplicate from material derived from different animals.

Results and discussion

The efficiency and quality of pork production are strongly dependent on the metabolic processes involved in lipid synthesis and deposition (30). Consequently, to optimize production traits, it is crucial to better understand the biological processes involved in the regulation of adipogenesis and lipid metabolism (2). Knowledge of these processes in pigs is relevant for the study of obesity and metabolic disorders in humans. The testis steroidogenic function is maintained through the availability of lipids and their metabolic homeostasis (9). It should be added here that the anatomic location of the reproductive system and adipose tissue allow for their interactions with cross-secreting molecules e.g. adipokines (5). Interestingly, our prior studies have reported the presence of adipocytes in human pathological testicular interstitial tissue (7). Moreover, adiponectin and leptin levels were correlated with patient BMI. Links between the role of adipokine in obesity and in fertility are quite well-studied in females. In males, existing data are scarce (10). In this study, we demonstrated, for the first time, the presence of adiponectin and its receptors 1, 2, as well as leptin and its receptor in the testes of Landrace pigs. Moreover, after vaccination with Improvac, significant changes in both the distribution and expression of these proteins were found. In these pigs, the lack of adiponectin and its receptors 2 in spermatids of the decreased immunosignalin Leydig cells of interstitial tissue were observed (Fig. 1A-F). Similarly, the immune signal for leptin receptor was not present in highly differentiated spermatogenic cells (Fig. 1G-J). However, the presence of leptin and its receptor in Leydig cells was not affected when compared to controls. It was shown that an increase in adiponectin levels positively affects sperm parameters e.g. concen-

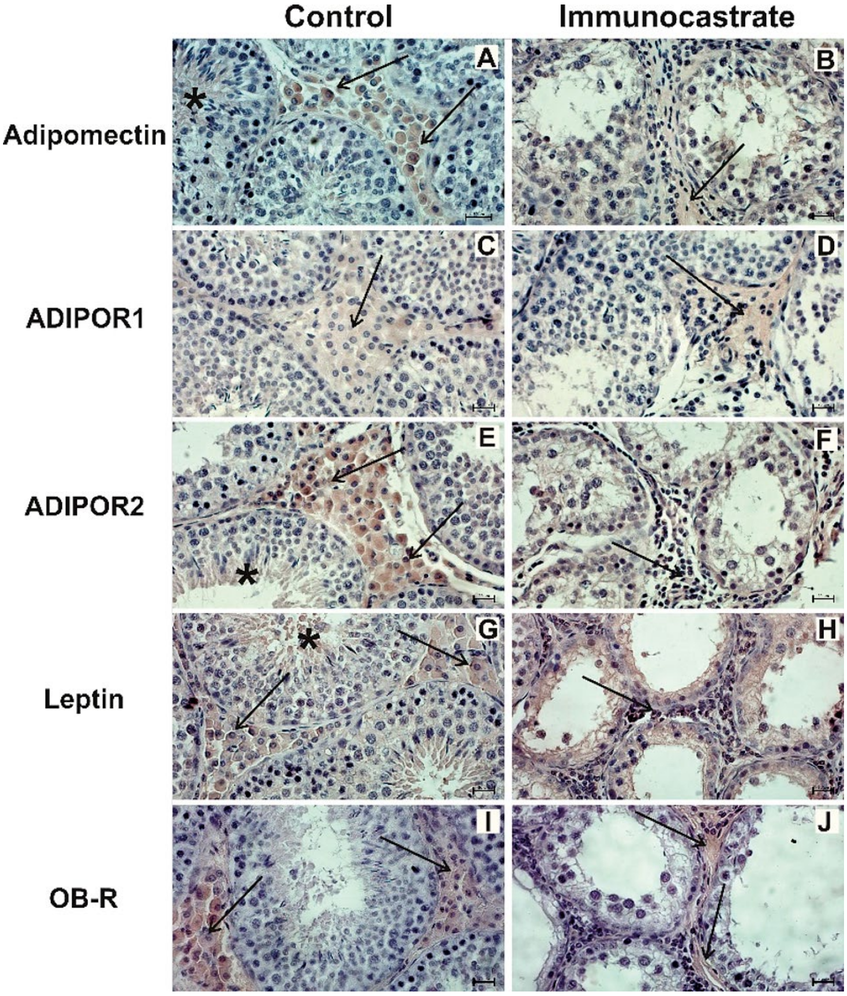

Fig. 1. Representative microphotographs of immunohistochemical localization of adiponectin (A, B), receptor 1; adipo R1 (C, D), receptor 2; adipo R2 (E, F), leptin (G, H), and leptin receptor; Ob-R (I, J) in control and immunocastrate testes. Counterstaining with hematoxylin. Bar 20 µm. Immunostaining was performed at three serial sections from each animal. Arrows and asterisks depict positive immunosignal in interstitial tissue (Leydig cells; arrows) and spermatogenic epithelium (spermatids; asterisks)

tration, motility, or morphology (8). Therefore, in non-vaccinated males, spermatogenesis was not fully halted (29). This is possibly partially due to the disturbed expression of adiponectin signaling in both interstitial tissue and the spermatogenic epithelium (mainly in elongated spermatids). The most studied adipokine – leptin has a dual effect: a positive effect on sperm fertility parameters when its level is physiological and a negative effect when its concentrations are high in the semen (10). In infertile patients, leptin was located in spermatocytes, while its receptor was found in Leydig cells (17). In obese patients, increased leptin expression correlated with dysfunction of spermatogenesis (34). Herrid et al. (13, 14) found expression of leptin and its receptor transcript in rodent Leydig cells. Our previous studies have revealed that leptin expression in Leydig cells of seasonal breeders is independent of their reproductive activity (27). Our additional findings demonstrated that in GnRH vaccinated Landrace pigs, the replacement of fetal and perinatal Leydig cell populations with the adult cells seems to be impaired with significant Leydig cell shrinkage (29). All of these events are closely linked to the blockage of sex steroid secretion by the GnRH vaccine. However, in light of the present data, it is possible that changes in adiponectin signaling and leptin signaling may also affect morpho-functional Leydig cell status. Here we also found that protein expression of adiponectin and its receptors and leptin and its receptor were significantly decreased in response to GnRH immunization ($p < 0.5$) (Fig. 2A, B). In contrast, cholesterol concentrations were markedly increased ($p < 0.01$) (Fig. 3). This suggests alterations in lipid metabolism possibly due to perturbation in adipokine functioning and/or the direct effect of altered lutropin action and then lowered androgen levels. It should be added that leptin action is strongly correlated with cholesterol metabolism (18). Leptin was found to directly inhibit lipid synthesis, activate lipolysis, and reduce levels of low-density lipoprotein receptors. Immunocastration of boars is usually performed during the fattening phase of pigs, which is sometimes uneconomical for pig fatteners (38). Herein, a slight increase in fat thickness was noted (data and own observations by M. Małopolska; unpublished). Our results show that the regulatory action of both adiponectin and leptin in the testes of Landrace pigs is altered and may then be involved and/or may then be a *vice versa* consequence of fat growth perturbations. Comparative studies in Large White and Meishan pigs have reported differences in the adipokine expression correlated positively or negatively, depending on adipokine type, with the fat thickness (2). Moreover, the decreased levels of androstenone in immunocastrated Landrace pigs (29) tend to confirm the decreased adiponectin and leptin signaling and increased cholesterol levels observed in the present study. Adipokines and cytokines of the adipose tissue are regulated by androgens and estrogens. Leptin was demonstrated to block only basal but not a gonadotropin-stimulated synthesis of testosterone in rats and sheep (13, 37). Our prior findings have shown that in patients with Leydig cell tumors, increased estrogen levels are associated with increased adiponectin and leptin expressions (7). Moreover, after blocking estrogen signaling in the testes of seasonally breeding rodents, testicular levels of leptin were decreased indicating the involvement of sex steroid hormones interplay with adipokines (27). In obese people, an increase in estrogen levels is correlated with a high accumulation of adipose tis-

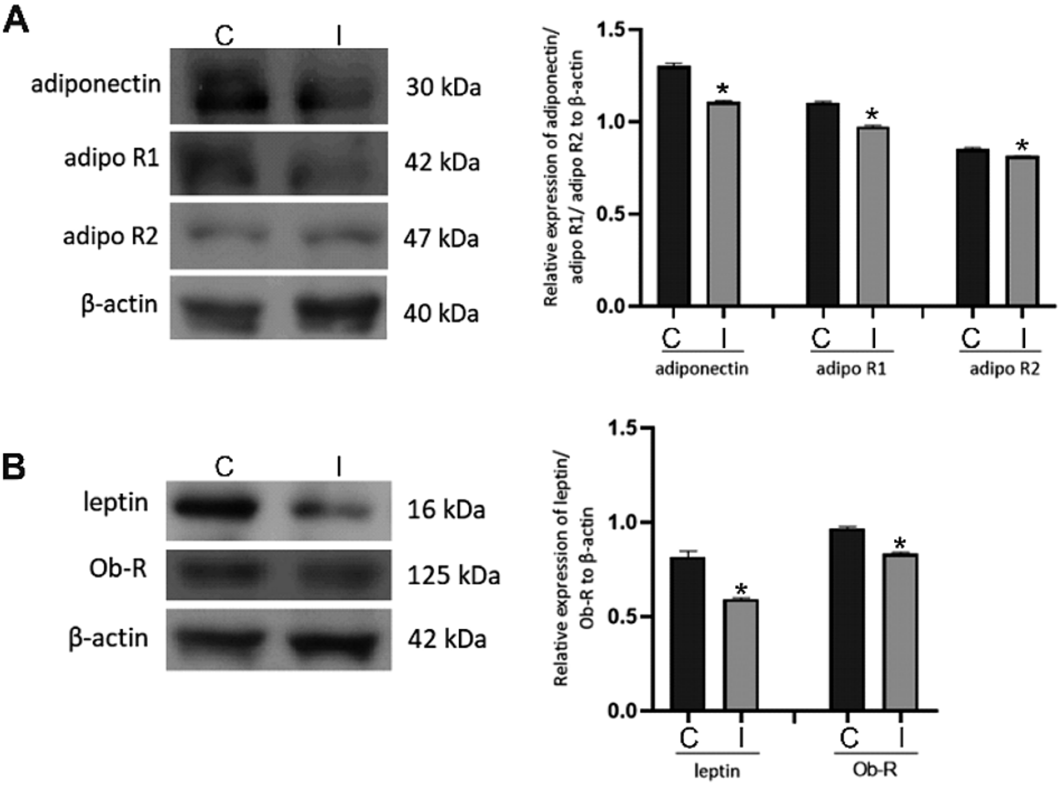

Fig. 2. Representative blots of qualitative expression and relative expression (arbitrary units) of adiponectin, receptor 1; adipoR1, receptor 2; adipoR2 (A), leptin and leptin receptor; Ob-R (B) in control and immunocastrate testes. The relative amount of respective proteins normalized to β-actin. Relative intensity from three separate analyses is expressed as means ± SD. Asterisks show significant differences between control and immunocastrate. Significant differences are denoted as * $p < 0.05$. Analysis was performed in triplicate

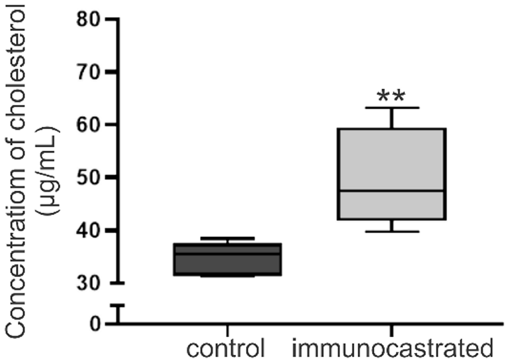

Fig. 3. Cholesterol concentrations in control and immunocastrate testes. Boxplots represent medians ± quartile range. Asterisks show significant differences between control and immunocastrate. Significant differences are denoted as ** $p < 0.01$. Analysis was performed in triplicate

sue (32). Higher circulating leptin levels observed in obese or overweight men lead to decreased testosterone production by Leydig cells and spermatogenesis disruption (1). On the other hand, a modulatory effect of progesterone on adiponectin signaling in the porcine uterus during early pregnancy has been confirmed (6). Therefore, the interaction of altered sex steroid hormone levels with altered adipokine expression and cholesterol metabolism after GnRH vaccination affects the functioning of both the reproductive system and adipose tissue.

Taken together, our results report for the first time the expression pattern of adiponectin and leptin and their receptors as well as cholesterol concentrations in the testes of Landrace pigs with a special emphasis on GnRH vaccinated ones. We revealed that adiponectin and leptin are regulated by GnRH. Furthermore, we postulate that signaling and concentration of these adipokines affect cholesterol metabolism which is reflected in spermatogenesis efficiency and fattening. Further research is needed to better understand the role of adipokine in fertility but especially in the lipid metabolism of fattening boars vaccinated with Improvac.

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