Developmental competence of antral follicles and their oocytes after gonadotrophin treatment of sows with gene polymorphisms for leptin and melanocortin receptors (Iberian pig)

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Abstract

Purpose To evaluate possible differences in follicle and oocyte developmental competence after gonadotrophin treatment in sows of obese and lean genotypes.

Methods Follicle dynamics, ovulation rate and oocyte developmental competence to embryo were compared between females, of obese ($n=7$) and lean genotypes ($n=10$), treated with 1,250 I.U. of eCG and 500 I.U. of hCG.

Results The obese genotype showed lower numbers of

follicles growing to preovulatory stages (12.4 ± 1.8 vs 18.6 ± 1.0 , $P < 0.05$), of corpora lutea (16.0 ± 0.9 vs 23.5 ± 0.9 , $P < 0.05$), and of recovered oocytes/embryos (8.0 ± 1.3 vs 12.9 ± 0.9 , $P < 0.05$). Thereafter, embryo viability rates also decreased when compared to lean genotypes (62.5 vs 77.6%, $P < 0.05$).

Discussion To our knowledge, this is the first study analyzing the effect of obese genotypes on the ovarian response to exogenous gonadotrophins in a non-rodent animal model, the pig. A lower efficiency of gonadotrophin treatments for stimulation of follicle development and induction of ovulation was observed.

Capsule Differences in follicle dynamics and oocyte developmental competence between obese and lean genotypes sows, submitted to exogenous gonadotrophin stimulation.

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Introduction

The Iberian pig is a breed autochthonous of the Iberian Peninsula (Spain and Portugal) which is worldwide known for the production of the Iberian ham, a highly-priced dry-cured product with a unique taste due to its abundance in intramuscular fat; in fact, Iberian pigs are obese [1]. Iberian breed is the most abundant and representative type of the Mediterranean pig (*Sus scrofa meridionalis*), which is genetically different from modern commercial breeds (*Sus scrofa ferus*).

The abundance of fat in these pigs causes an increased secretion of leptin, the hormone produced by the adipocytes that regulates appetite and food intake, when compared to lean swine breeds [2, 3]. However, Iberian pigs are homozygous for a leptin receptor gene polymorphism [4]

with effects on food intake, body weight and fat deposition [5, 6] that increases insatiability and lipogenic activity [7], giving way to *thrifty genotypes* and obesity. Such state has been also described in obese human beings and named as *leptin resistance* [8]. Moreover, an interaction between leptin and melanocortin system (mainly, melanocortin receptors 1 and 4; MC1R and MC4R) for weight regulation [9, 10] has been recently described, in obesity syndromes. Iberian pigs have also a polymorphisms for MC4R with effects on body composition [11]. Recently, a novel link between the LEPR-MC4R system controlling energy balance and the regulation of reproduction becomes apparent [12, 13]. Iberian swine are also polymorphic for genes regulating both receptors. Thus, in view of these considerations, the Iberian pig arises as a good model for obesity and leptin resistance, both in human and veterinary medicine.

In human beings, obese genotypes have been found to impair the outcome of assisted reproductive technologies [14, 15], due to insufficient follicle development, resulting in fewer oocytes and embryos [16]. In general, leptin resistance [17] and deficiencies in MC4R [13] are related to reproductive disorders; concurrently, Mediterranean pigs are also affected by a lower reproductive efficiency than modern commercial breeds [18].

Thus, the present study aimed to compare follicle growth and function, ovulatory efficiency and oocyte developmental competence to embryo, in sows of Iberian breed and lean commercial crosses (Large White×Landrace) submitted to gonadotrophin treatment. Such results may be useful for assessing possible differences in the ovarian function between obese and lean individuals of swine and other species.

Materials and methods

Animals, hormonal treatment and evaluation

Seven Iberian and 10 Large White×Landrace crossbreed nulliparous sows were used. The Iberian sows were genotyped for determining polymorphism on LEPR and MC4R genes with protocols previously described [5, 19]; all of them were homozygous for alleles LEPRc.1987 T and MC4Rc.1426 G. All the animals were housed indoors, in passively ventilated individual pens with concrete slatted floors, at the Centro La Mejorada (Olmedo, Valladolid, Spain). Throughout the experiment, gilts were fed with a standard maintenance diet and had *ad libitum* access to water.

For estrus synchronization, regardless the stage of estrous cycle, all the animals were daily treated with 20 mg of the progestagen altrenogest (Regumate®, Intervet International, Boxmeer, The Netherlands), for 18 consecutive days, by individually top-dressing over their morning

feed. Based in previous studies [20, 21], gonadotrophin treatment consisted in the administration of a single intramuscular dose of 1,250 I.U. of eCG (Foligon®, Intervet International, Boxmeer, The Netherlands) at progestagen withdrawal; ovulation was induced, 72 h later, by a single intramuscular dose of 500 I.U. of hCG (Chorulon®, Intervet International, Boxmeer, The Netherlands). All the sows were inseminated, with cooled semen of the same boar, 12 and 36 h after hCG administration.

Follicular growth and function, ovulation rate and embryo developmental competence were used as parameter to ovarian response to gonadotrophin treatment. The number and size of follicles were evaluated by ultrasound and plasma estradiol concentration was measured by enzymeimmunoassay. These evaluations were performed at the moment of eCG and hCG injection, aiming to assess eCG effect.

Ultrasonographic assessment of follicular response

Ultrasonographic studies were made using a real-time B-mode ultrasound machine (Aloka SSD 500, Ecotron, Madrid, Spain) fitted a 7.5 MHz linear array transducer, as previously described and validated in our laboratory [22]. In brief, observations were conducted with the sows maintained in dorsal recumbence by hand. Feces were removed and a hydrosoluble gel was introduced into the rectum. The probe was placed into the rectum with the transducer orientated perpendicularly to the ventral and abdominal wall. When the urinary bladder was surpassed and the uterine horns located, the probe was rotated laterally clockwise and counter-clockwise to observe both ovaries and their structures. Scans were recorded on video, for allowing animals to be released quickly, minimizing stress caused by keeping them restrained; complete examination of each female took approximately 4–5 min. The videos were examined using the Screen Calliper 4.0 image software (Iconico Inc., New York, NY). Firstly, the films were played in slow motion for identifying and counting the number of follicles. Thereafter, the image was serially frozen for measuring the largest section of the different follicles, by placing the calipers in the borderline with the ovarian stroma. All follicles ≥ 1.5 mm in size were classified by their largest diameter; five groups were categorized: total follicles ≥ 1.5 mm in size, small (1.5–2.9 mm), medium-1 (M1, 3–4.9 mm), medium-2 (M2, 5–6.9 mm) and large follicles (≥ 7 mm).

Blood sampling and hormonal assessment of follicular response

Jugular blood samples were collected concurrently with ultrasonographies, by jugular venopuncture, using 5 mL sterile blood vacuum tubes with lithium heparin (Vacutainer™

Systems Europe; Becton Dickinson, Meylan Cedex, France). Immediately after recovery, the blood was centrifuged at 4°C at 1,500 g for 15 min and the plasma was separated and stored into polypropylene vials at −20°C until assayed. Concentrations of plasma oestradiol were measured in a single analysis using enzymeimmunoassay kits (Demeditec Diagnostics GmbH, Kiel-Wellsee, Germany). The assay sensitivity was 1.4 pg/ml and the intra-assay variation coefficient was 5.7%.

Assessment of ovulation and oocyte/embryo recovery

Seven days after induction of ovulation, the presence of both ovulatory and anovulatory structures in response to gonadotrophin treatment was assessed after performing a prepubic laparotomy under general anesthesia with xylazine (Rompun™, Bayer Ag, Leverkusen, Germany) and ketamine (Imalgene 1000™, Merial, Lyon, France), after sedation with azaperone (Stresnil®, Esteve Farma, Barcelona, Spain). All the corpora lutea and follicles ≥5 mm in diameter were counted; at Day 7 after induction of ovulation, follicles may have less than 5 mm in diameter [23, 24]; thus, all follicles larger than 5 mm were considered as anovulatory follicles from the ovulatory follicular cohort. Immediately after, oocytes/embryos were recovered by flushing both uterine horns with NCSU 23 medium [25]. The collected structures were counted and evaluated under a stereoscopic microscope (Leica MZ12.5, Leica Microsystems GmbH, Wetzlar, Germany), immediately after recovery and, thereafter, viability of the embryos was determined by assessing its development to expanding or hatching blastocysts.

Indexes of the ovarian response

The following information was recorded from each female: number of corpora lutea, number of anovulatory follicles, total number of recovered oocytes/embryos, number of viable embryos, number of unfertilized oocytes and number of degenerated embryos. The rate of anovulatory follicles was obtained by dividing the total number of anovulatory follicles by the total number of corpora lutea plus anovulatory follicles. Rates of viable, unfertilized and degenerated oocytes/embryos were obtained by dividing the number of viable, unfertilized and degenerated oocytes/embryos, respectively, by the total number of recovered oocytes/embryos. All rates are expressed as percentages.

Statistical analyses

Statistical treatment of the results expressed as percentages was performed after transformation of the values for each individual percentage to the arcsine. Effects of genotype on

these variables were assessed by analysis of variance (ANOVA) and Pearson correlation procedures. Results were expressed as mean±S.E.M. and the statistical significance was accepted from $P<0.05$.

Results

Effect of genotype on follicle growth and function

The previous exposure to progestagens avoid the presence of follicles larger than 5 mm (categories M2 and L) at the day of eCG injection (Fig. 1), in sows of both genotypes. The number of follicles <3 mm (S) and 3–4.9 mm in size (M1) at day of progestagen removal and eCG injection was higher in LWxL sows, but statistically similar in both groups.

The administration of eCG induced significant changes in the follicular population in Iberian and LWxL sows. Thus, at the day of hCG injection, the number of follicles in class S had decreased in both genotypes, whilst there was a significant increase in the number of follicles in classes M2 and L; the total number of preovulatory follicles larger than 5 mm was higher in LWxL females (18.6 ± 1.0 vs 12.4 ± 1.8 in Iberian sows, $P<0.05$).

Plasma estradiol concentrations increased ($P<0.05$) in both groups throughout the follicular phase. Mean plasma concentration was not statistically different, between genotypes, in both sampling times (Fig. 2).

Effect of genotype on ovulation and embryo recovery

The Iberian sows showed a lower ovulatory efficiency (58.4 vs 87.1% in LWxL females $P<0.05$) and, therefore, a lower ovulation rate (16.0 ± 0.9 vs 23.5 ± 0.9 corpora lutea in

Fig. 1 Evolution of the number of small (1.5–2.9 mm, white section), medium-1 (M1, 3–4.9 mm, light grey section), medium-2 (M2, 5–6.9 mm, dark grey section) and large follicles (≥7 mm, black section) in lean (LWxL) and obese (Iberian) swine genotypes, at administration of eCG (0 h) and hCG (60 h). SEM values have been omitted for clarity of the figure

Fig. 2 Changes of plasma estradiol concentrations in lean (LWxL; grey bars) and obese (Iberian; black bars) swine genotypes, at administration of eCG (0 h) and hCG (60 h)

LWxL sows, $P<0.05$), as detailed in Table 1. Three of the 10 LWxL (30%) and 3 of the 7 Iberian sows (42.8%) showed anovulatory follicles. Rate of ovulatory failures in these females were 3.2, 15.4 and 57.1% for LWxL and 4.6, 100 and 100% for the Iberian breed.

The mean number of recovered oocytes/embryos in the Iberian females ovulating in response to the treatment was 8.0 ± 1.3 , which is lower than in LWxL sows (12.9 ± 0.9 , $P<0.05$). Fertilization rates were 100% in all the Iberian sows and 8 of the 9 LWxL females; one LWxL female showed no fertilization of any of the 17 recovered oocytes. In the remaining females, viability rates of fertilized embryos were 77.6 for LWxL and 62.5% for Iberian pigs ($P<0.05$).

Table 1 Differences in ovarian response after exogenous gonadotrophin administration in lean (LWxL) and obese (Iberian) swine genotypes

	LWxL	Iberian
Corpora lutea	23.5 ± 0.9^a	16.0 ± 0.9^b
Anovulatory follicles	1.9 ± 0.1^c	6.2 ± 0.4^d
Rate of anovulation (%)	7.6^c	27.9^d
Number of oocytes/embryos	12.9 ± 0.9^a	8.0 ± 1.3^b
Number of fertilized embryos	11.0 ± 0.7^a	8.0 ± 1.3^b
Fertilization rate (%)	85.3^a	100^a
Number of degenerated embryos	2.5 ± 0.2^a	3.0 ± 0.5^b
Degeneration rate (%)	22.4^a	37.5^b
Number of viable embryos	8.5 ± 0.4^a	5.0 ± 0.7^b
Viability rate (%)	77.6^a	62.5^b

Mean $\pm$ S.E.M. within rows with different superscripts differ ($^{a-b}$ $P<0.05$, $^{c-d}$ $P<0.01$)

Discussion

The present study is, to our knowledge, the first analyzing the effect of obese genotypes on the ovarian response to exogenous gonadotrophins in an animal model other than rodents. Current results indicate significant differences, between pigs of obese and lean genotypes, in follicle dynamics and oocyte developmental competence to viable embryo after exogenous gonadotrophin stimulation. Follicle population after treatment with progestagens for cycle management was similar between both genotypes, but the administration of exogenous gonadotrophins for stimulation of final follicle development and induction of ovulation showed a low efficiency in obese sows.

The ovarian response to the administration of progestagens was similar in both lean and obese genotypes. Progestagens suppressed follicular development; hence, most of the follicles had 1.5 to 5 mm in diameter at the end of the progestagen treatment. This finding is similar to results previously described after weaning in lactating lean sows (a situation depressing LH secretion in a similar way to progestagen treatments; [24, 26]). After progestagen removal and treatment with gonadotrophins, the follicles entered in a rapid growth phase to ≥ 7 mm (preovulatory diameter), increasing their estradiol secretion and, subsequently, ovulating.

Ovulation rate after gonadotrophin stimulation was lower in the Iberian than in the LWxL sows; possible causes may be related to a lower intrinsic prolificacy of the Mediterranean genotype [20, 27, 28] but also, as supported by current and previous data [29], by a lower ovulatory efficiency of the follicles stimulated to grow by the exogenous gonadotrophins. Thereafter, most of the indexes of oocyte/embryo developmental competence (excepting fertilization rate) were higher in LWxL than in Iberian sows. Embryo development may depend on either the quality of the oocyte that originated the embryo and, hence, the quality of its follicle or the adequacy of the oviduct/uterine environment.

The evidences found in the present study would support the hypothesis of a possible deficiency in final maturation of preovulatory follicles and their oocytes in obese pigs [28]; as also found in obese humans [14, 30] and diet-induced obese mice [31]. Both in humans and other mammals, the oocyte acquires gradually the ability to mature, be fertilized and finally to develop into a viable embryo, throughout final stages of folliculogenesis [32]. Furthermore, and adequate length of the final follicular growth phase was early related with the final maturation of the preovulatory follicle and its oocyte [33, 34]. The same was found in lean pigs [35]; moreover, in pigs, granulosa cells possess receptors for LH only in the later stages of follicle maturation [36]. Previous unpublished studies in

our laboratory have found that non-stimulated follicular phases are shortened in the Iberian pig when compared to lean commercial crosses. The administration of exogenous gonadotrophins may accelerate these processes and strengthen possible deficiencies in final follicle development, inducing a lack of developmental competence in oocytes and, later, in embryos of obese pigs when their growth is stimulated by exogenous gonadotrophins.

As previously stated, the Iberian sows used in this experiment had functional alteration in leptin receptors; leptin may acts both at systemic and ovarian levels [37] and deficiencies of leptin system have been associated with suppression of ovarian folliculogenesis and increasing granulosa cell apoptosis [38], ovarian failure [39–41] and alterations in oocyte developmental competence [42]. Specifically in the follicular phase, leptin acts synergistically with IGF-I and GH stimulating growth and steroidogenic activity of follicles [43–45].

The deficiency in MC4R, related in the Iberian sows used in this experiment, has been also linked to a lower ovulatory efficiency and a lower ovulation rate in mice [46]. In addition, the leptin-melanocortin system may directly stimulate LH and FSH [13, 47]. However, further studies are necessary for testing this hypothesis in the obese pig, since other recent studies in human beings also indicate that lower reproductive outputs in obese females by a possible lower oocyte/embryo developmental competence may be coincidental with alterations in oviduct/uterine environment [15, 48, 49].

Having in mind the alterations in oocyte/embryo developmental competence of current study, the Iberian pig arises as a good model for studying the relationships between reproductive failures and obesity/leptin resistance in humans and, specifically, possible alterations in endocrine function, ovulatory efficiency and/or oviduct/uterine environment. Currently, most of the studies are based in rodents since mutations in LEPR account for severe obese phenotypes both in mice (*Lepr^{db/db}* mouse; 50–53) and humans [54–56]. However, total loss of LEPR function by monogenic disorders in humans, unlike mice, are really scarce [57]; only around a 5% of the cases [58]. Functional alterations by DNA sequence variations and LEPR gene polymorphism, like in the Iberian pig, are more common [59, 60].

Conclusion

The results of the present study indicate deficiencies in preovulatory follicular growth, ovulatory efficiency and, finally, oocyte/embryo developmental competence after gonadotrophin stimulation in a pig model of leptin resistance and obesity.

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