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BRIEF REPORT

Maternal diet-induced obesity in swine with leptin resistance modifies puberty and pregnancy outputs of the adult offspring

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The assessment of reproductive features (puberty, fertility and prolificacy) in female Iberian pigs indicates that exposition to intrauterine maternal malnutrition, either by deficiency or excess, is associated with juvenile obesity and a significantly earlier age of puberty onset. At adulthood, prenatal exposition to undernutrition affects reproductive outputs by diminishing prolificacy, an effect that was not found in females exposed to prenatal overnutrition.

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Introduction

There is abundant evidence supporting the hypothesis of the foetal origin of some non-transmissible alterations such as obesity, metabolic syndrome, diabetes and cardiovascular diseases, the so-called Developmental Origin of Health and Disease (DOHaD).[1,2] On the other hand, the role of DOHaD in the occurrence of other emerging non-transmittable diseases, such as reproductive dysfunctions (earlier menarche and menopause, lower fertility and fecundity), is not so well known and controversial,[3–5] as effects from prenatal nutrition are commonly masked by the influence of the postnatal nutrition.

The study of the DOHaD phenomena is mostly based on epidemiological studies on humans. The application of interventional studies for boosting the current knowledge makes necessary the use of animal models. The main animal models are based on laboratory rodents; the rodents need little space, are relatively inexpensive to maintain, have a sequenced genome and are easily modified by genetic engineering. However, the use of large animals (rabbit, sheep, pig) offers numerous profitable characteristics, mainly, in translational studies. Briefly, body size allows application of imaging techniques[6] and serial sampling of large amounts of blood and tissues. Afterwards, pathways regulating appetite, energy balance and adipogenesis in large animals are more similar to humans.[7,8] Moreover, in large animal species, the study of prenatal programming effects has a dual purpose, not only from the viewpoint of biomedical models but also from the viewpoint of animal production, health and welfare.[9]

Specifically, the pig is considered an outstanding animal model for studies linking nutrition, metabolism, reproduction and development from long time ago.[10] Pigs share many more anatomical and physiological similarities with humans than any other animal species,[11–14] including proportional organ sizes, omnivorous habits, propensity to sedentary behaviour and obesity and characteristics of metabolism and cardiovascular system. Moreover, there are swine breeds (Mediterranean pigs) with a severe obese phenotype.

The most representative Mediterranean swine is the Iberian pig (*Sus scrofa meridionalis*). Iberian pigs, like the other Mediterranean breeds, are genetically different from the modern commercial breeds (*Sus scrofa ferus*)[15,16] and have been reared in semi-feral conditions for centuries. These animals have coped with seasonal cycles of feasting and famine by storing excess fat during food abundance, which enables survival during periods of scarcity.[17,18] In fact, Iberian pigs have a higher voluntary food intake and a higher trend towards adiposity than lean swine breeds.[19–21] The abundance of fat increases secretion of leptin,[22] which should diminish food intake. However, the Iberian pig has a gene polymorphism for the *leptin receptor* (*LEPR*), similar to the syndrome of leptin resistance described in human medicine,[23–25] with effects on food intake, body weight and fat deposition.[20,21] Thus, Iberian pigs are prone to obesity.

The Iberian swine is currently characterized as a robust, amenable and reliable translational model for studies on obesity, metabolic syndrome and nutrition-associated diseases in humans.[26,27] Moreover, malnutrition during pregnancy modifies the hypothalamic expression of anorexigenic and orexigenic peptides in the neonates (Ovilo, unpublished results) and their subsequent postnatal development.[28] Thus, an additional advantage of the Iberian pig model is its usefulness in translational studies assessing DOHaD in populations adapted for surviving in harsh environments but currently exposed to nutrient excess, such as China, India and Middle East countries.

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In view of these considerations, the present study used the Iberian pig as a model for determining the effects of prenatal programming by maternal undernutrition or overnutrition on the female reproductive features (attainment of puberty, fertility and pregnancy outcomes). Possible effects of postnatal programming were avoided by maintaining all the animals in the same conditions of housing and nutrition.

Material and methods

This experiment was carried out at the facilities of the INIA Animal Laboratory Unit (Madrid, Spain), which meets the requirements of the European Union for Scientific Procedure Establishments. The experimental design and procedures were approved by the INIA Scientific Ethic Committee, and the animal manipulations were performed according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 about the protection of animals used in experimentation.

A total of 25 Iberian gilts of the Torbiscal strain were used. These gilts were born from females that were fed, during the entire pregnancy, with the same standard diet but fulfilling either their daily maintenance requirements (Control group, seven gilts), or 1.6 or 0.5 folds such requirements (Overfed and Underfed groups, nine gilts in each one). At the beginning of pregnancy, the maternal mean weights were similar between groups (152.6 ± 6.7, 151.8 ± 6.6 and 153.3 ± 7.1 kg in the Overfed, Control and Underfed groups, respectively). At the end of pregnancy, the maternal mean weights were 199.8 ± 9.2, 173.1 ± 5.2 and 143.7 ± 8.1 kg in the Overfed, Control and Underfed groups, respectively ($P < 0.05$ between groups). However, there were no differences between groups in the litter size (9.0 ± 0.6 *v.* 8.4 ± 0.5 and 8.5 ± 1.0 piglets in the Overfed, Control and Underfed groups, respectively) and sex ratio.

The gilts used in this experimental study were selected as representative of the litters, having a mean weight around 1.4 kg, for avoiding effects from too large or too small individuals. These gilts were housed, after weaning and throughout the experimental procedure, in collective pens and, independently from maternal nutrition, were fed the same diets for avoiding effects of postnatal nutrition. At the first month after weaning, all the piglets were fed with a standard diet with mean values of 18% of crude protein, 2.5% of fat and 3.35 Mcal/kg of metabolizable energy. Afterwards, 60-day-old gilts were fed with a diet containing mean values of 15.1% of crude protein, 2.8% of fat and 3.08 Mcal/kg of metabolizable energy; the amount of food offered was recalculated with age for fulfilling daily maintenance requirements. In order to induce obesity, 120-day-old gilts had *ad libitum* access to a diet with similar composition as the previous one but enriched in fat (6.3%) and having 3.36 Mcal of metabolizable energy/kg. All the animals had *ad libitum* access to water.

Evaluation of changes in body weight and fatness was performed every 2 weeks from starting the obesogenic diet at 120 days of age. The gilts were weighed, and concurrently, back-fat depth was measured at 4 cm from the midline, at the level of the head of the last rib, with a SonoSite S-Series ultrasound machine equipped with a multifrequency (5–8 MHz) lineal array probe (SonoSite Inc., Bothell, WA, USA).

Determination of puberty onset was carried out from 120 days of age. The criterion used for determining the occurrence of puberty was an increase in plasma progesterone levels above 2.0 ng/ml for at least two consecutive samples; onset of puberty was identified with the first of these two samples. Thus, blood samples were drawn from the jugular vein or from the orbital sinus, coincidentally with weighing, by venopuncture with 5 ml sterile heparin blood vacuum tubes (Vacutainer^{TM} Systems Europe, Becton Dickinson, Meylan Cedex, France). Immediately after recovery, the blood was centrifuged at 4°C at 1500 ***g*** for 15 min and the plasma was separated and stored into polypropylene vials at −20°C until assay. Plasma progesterone concentrations were measured in a single analysis using an enzyme immunoassay kit (Demeditec Diagnostics GmbH, Kiel-Wellsee, Germany); assay sensitivity was 0.045 ng/ml and intra-assay variation coefficient was 5%.

Evaluation of fertility and pregnancy parameters was performed when the animals were around 300 days of age. Seven gilts in the Control group and six gilts in each one of the nutritionally treated groups were randomly selected for cycle synchronization and artificial insemination. Synchronization consisted on the daily administration, for 18 consecutive days, of 20 mg of the progestagen altrenogest (Regumate®, MSD, Boxmeer, The Netherlands), by individually top-dressing over their morning feed; the treatment was initiated irrespective of the stage of the cycle. Oestrus detection was performed twice daily, from 24 h after progestogen removal, both by inspection of the vulva for reddening and swelling (pro-oestrus) and by control of the standing reflex (oestrus) in contact with a mature boar. Sows were inseminated 12 and 24 h after the onset of oestrus with cooled semen doses from the same tested Iberian boar. Fertility was evaluated at Day 35 after insemination, when pregnancy diagnosis was performed by ultrasonography with a real-time B-mode ultrasound machine SonoSite S-Series (SonoSite Inc.), and at delivery when reproductive outputs, in terms of prolificacy and viability (number of living and stillborns piglets), were also evaluated.

Statistical analysis

The effects of prenatal diet on age, weight and back-fat depth at the onset of puberty were assessed by one-way analysis of variance (ANOVA) or by a Kruskall–Wallis test after logarithmic transformation of the values when a Levene's test showed non-homogeneous variables. Afterwards, ANOVA was also used for evaluating the effect of prenatal diet on possible differences in fertility (estimated as differences in the percentage of pregnant females), number of total newborns and number of stillborns. Statistical treatment of the data

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expressed as percentages was performed after transformation of the values for each individual percentage to the arcsine. All the results were expressed as mean ± S.E.M. and the differences were considered to be statistically significant at $P < 0.05$.

Results

Attainment of puberty

The results found in the current study indicate that the maternal diet during the pregnancy period affects juvenile development of their daughters. At the first assessment of weight and fatness at 120 days of age, Overfed and Underfed females were significantly heavier than Control piglets and have higher values of back-fat depth ($P < 0.005$ for both groups and parameters; Fig. 1). Afterwards, both Overfed and Underfed females continued to be significantly heavier and fattened than Control gilts ($P < 0.0005$). On the other hand, there were no significant differences when comparing Overfed and Underfed females at any stage of development.

Therefore, maternal diet influences the age, weight and fatness of the gilts at puberty attainment. In the Control group, mean age and weight at puberty onset were 224.2 ± 1.9 days old and 64.3 ± 1.6 kg, respectively. On the other hand, the gilts affected by prenatal programming, either by overnutrition or undernutrition during pregnancy period, showed an earlier puberty than the control group (Fig. 2, $P < 0.005$). In fact, two gilts (one of them in each group) were cycling at first blood sample taken at 120 days of age, whereas the first gilt to be cycling in the Control group reached puberty at 190 days of age. At that age, absolutely all the gilts from both overfed and underfed pregnancies were cycling, excepting one female in the Overfed group that was found to be affected by hypogonadism. There were no significant differences between Overfed and Underfed groups in the mean age at puberty (175.7 ± 1.9 and 180.0 ± 1.6 days old, respectively).

There were no significant differences between Overfed and Underfed groups in the body weight at puberty attainment, despite a higher weight in the Underfed than in the Overfed gilts (87.0 ± 1.5 *v.* 77.4 ± 1.6 kg, ns). The mean weight at

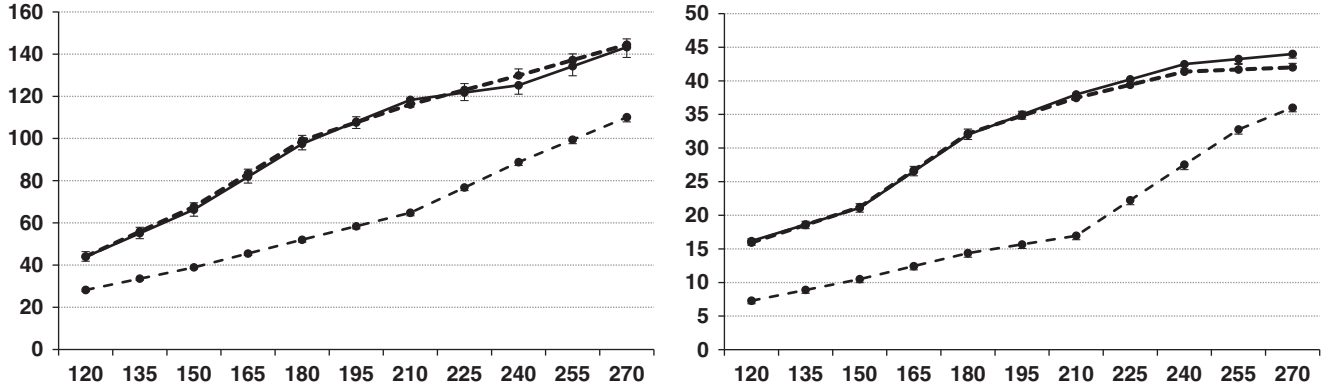

Fig. 1. Mean ± S.E.M. values of body weight (left panel) and back-fat depth (right panel) over time in control gilts (discontinuous line) and gilts from sows that were either underfed (pointed line) or overfed during pregnancy (continuous line).

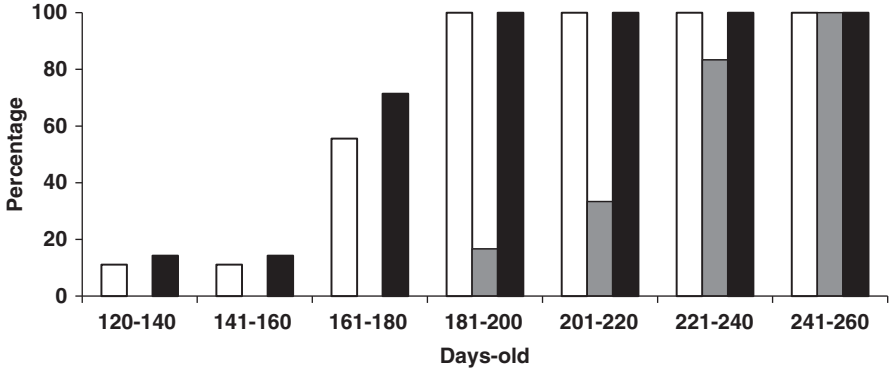

Fig. 2. Cumulative percentage of individuals attaining puberty over age, in control gilts (grey bars) and gilts from sows that were either underfed (white bars) or overfed during pregnancy (black bars).

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puberty of both groups was higher than in the Control gilts, although differences were only found to be statistically significant between animals of Underfed and Control groups ($P < 0.05$). However, measurements of the back-fat depth at puberty showed a significantly higher fattening in both Overfed and Underfed groups (26.7 ± 3.3 and 27.0 ± 2.8 mm, ns) than in the control females (17.1 ± 3.2 mm, $P < 0.05$ for both groups).

Evaluation of fertility and pregnancy outcomes

In the same way, the maternal diet during the pregnancy period affects reproductive features of the daughters at adulthood.

Six females in the Control group (6/7; 85.7%), four females in the Overfed group (4/6; 66.7%) and five females in the Underfed group (5/6; 83.3%) became pregnant, respectively; differences were not statistically significant. At delivery, there were no significant differences in the number of newborns between Control females and females from sows that were overfed during pregnancy (7.5 ± 0.7 *v.* 7.3 ± 0.5 piglets, respectively). On the other hand, females born from Underfed mothers showed a significantly lower number of offspring than both Control and Overfed groups (4.2 ± 1.1 piglets, $P < 0.05$). The percentage of stillborns was similar in females from Overfed and Underfed pregnancies (25.3% and 24.3%, respectively) and, higher in both groups than in Control females (10.5%), although differences were not statistically significant ($P = 0.08$). Finally, there were no differences between groups in the mean body weight (around 1.4 kg in all the groups) and sex ratio of the litters.

Discussion

The results of the present study indicate that the exposition to intrauterine maternal malnutrition, either by deficiency or excess, is associated with significant differences in the female reproductive function. First, prenatal malnutrition is associated with a significantly earlier age of puberty onset. At adulthood, prenatal exposition to undernutrition affects reproductive outputs by diminishing prolificacy, an effect that was not found in females exposed to prenatal overnutrition.

The effects of prenatal nutritional deficiency or excess on the onset of puberty found in the current trial are reinforcing previous evidences in human medicine, showing that women with low birth weight (indicating intrauterine nutritional deficiency) have a reduced age of pubertal onset and menarche.[29–31] In the same way, our results are supporting previous studies in a different animal model (rat).[32] Our work has the added value that, in rodents, follicular differentiation is accomplished at postnatal stages[33,34] and, thus the external environment can directly influence reproductive function, limiting these species as a model for prenatal programming of human reproductive function[35]; in pigs, like in human, the follicular pool is entirely established prenatally.

However, it is controversial to establish whether advanced menarche is a direct consequence of prenatal malnutrition or an indirect consequence through the postnatal occurrence of obesity by catch-up growth,[4] as an earlier age of menarche in obese girls, independently of prenatal origin, has been previously described.[36–38] Our results, indicating significant differences in juvenile growth – in terms of weight and fatness – between gilts exposed to prenatal malnutrition and control gilts, may support a prominent causative role of juvenile obesity. In the current study, the females exposed to uterine malnutrition evidence an increased adiposity in relationship to body development. The observational design of the current experiment does not allow establishing possible causal mechanisms of advanced puberty, but, unequivocally, relates obesity and earlier puberty, setting the basis for future translational studies aimed at increasing the knowledge on the factors determining the adult phenotype. The increased fat content in gilts exposed to prenatal malnutrition may be identified as the cause for advanced puberty onset, in agreement with previous knowledge.[36,37,39]

Attainment of puberty, in all the mammals, is the result of a complex dynamic interaction between genetic, endocrine and environmental factors. The two main factors are metabolic status and body composition (e.g. absolute and relative amounts of fat). The influence of body composition on puberty attainment was first demonstrated in animals,[40] and thereafter in humans,[41] with females having higher body fat being more likely to have an earlier pubertal onset. The key link between earlier puberty and body fat is the hormone leptin. Fat, the adipose tissue, secretes leptin, a hormone that has been related to the puberty onset in several species and, specifically, in pigs.[42,43] Excess adiposity may influence the timing of pubertal initiation by high leptin levels. Serum leptin levels increase with age and body fat reserves; changes in concentrations of leptin have been found to be associated with puberty attainment.[37,39] However, there are evidences supporting the emerging role of insulin as a key factor in pubertal maturation.[44] Reductions of insulin sensitivity and compensatory hyperinsulinemia are physiological during puberty, and increased insulin secretion may facilitate puberty onset by promoting bioavailability of sex steroids.[45]

At adulthood, the reproductive outcomes of the females in the current study were affected by prenatal nutrition, results that are opposite to previous hypothesis indicating that reproductive function would be less affected than other body features in cases of nutritional prenatal insult.[4] Females that were exposed to intrauterine undernutrition had a significantly lower number of piglets than control females, whereas such an effect was not observed in females exposed to intrauterine overnutrition. These results are opposite to early described in humans, suggesting no effects[46] or even increased lifelong fertility in women affected by undernutrition in the womb (although the increase in fertility reported, from 1.7 to 2.0 children, is very small).[47]

However, although direct effects of undernutrition during pregnancy on fertility of the offspring have not been previously fully explored, as elegantly revised by Dupont and co-workers,[48] there is evidence of reduced litter size in rodents,[49]

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which may be related to deficiencies in folliculogenesis and ovulation, either by changes in the expression of genes acting on for follicle maturation and ovulation or by an increased oxidative stress in the ovary.[50] In large animals, such as sheep, it has also described a reduction in the ovulation rate of the adult females exposed to by maternal undernutrition.[51,52] In this species, there has been described a curvilinear relationship between fecundity and early-life events, with apparent decreasing litter size at either end of the birth weight range.[4] In this study, increases in body fatness during adolescence have a main role than prenatal origin on pregnancy outcomes. In contrast, for the first time to the best of our knowledge, our study indicates a significant effect of the degree of prenatal nutrition, independently of postnatal obesity, on pregnancy outcomes. Thus, having in mind the lack of previous information in the effects of prenatal nutrition, in experimental designs avoiding influences from postnatal nutrition,[48] our results set the basis for further research on the causes and intrinsic mechanisms of these effects, as well as for studies on the prevention and treatment of these disorders.

In conclusion, intrauterine exposition to maternal malnutrition, both by deficiency and excess, induces juvenile obese phenotype and precocious puberty attainment. At adulthood, prenatal exposition to undernutrition may affect reproductive outcomes by diminishing prolificacy, an effect that was not found in females exposed to prenatal overnutrition.

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Conflicts of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (Spanish Policy for Animal Protection RD1201/05) and has been approved by the institutional committee (INIA Scientific Ethic Committee).

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