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Acute fasting before conception affects metabolic and endocrine status without impacting follicle and oocyte development and embryo gene expression in the rabbit

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Abstract. Food deprivation affects female reproduction. The goal of the present study was to elucidate in the rabbit model the effects of acute energy restriction on ovarian function (follicle development, atresia rate and *in vitro* oocyte maturation) and embryonic development and gene expression of some candidate genes. Serum metabolic parameters (non-esterified fatty acids (NEFA), triglycerides, glucose, insulin and leptin concentrations) and endocrine markers (oestradiol-17β and progesterone concentrations) were also studied. A control group of nulliparous does fed *ad libitum* and a 72-h fasted group were used. At the end of the nutritional treatment, the ovaries of half of the animals were retrieved while the other animals were re-fed and artificially inseminated to recover embryos at 84 h after insemination, during the luteal phase. At the end of fasting, increased serum NEFA and decreased leptin concentrations were observed in the fasted group, but no differences appeared in serum steroid concentrations, follicle population and atresia rate or nuclear and cytoplasmic oocyte maturation. In the luteal phase, insulin concentrations increased notably in the fasted group. The number of recovered embryos per female and the speed of embryo development were reduced in the food-deprived group. Acute fasting altered both metabolic and endocrine markers and embryo development, but follicle and oocyte development and embryo gene expression were not affected.

Additional keywords: apoptosis, maternal–embryo interaction.

Introduction

The reproductive function requires balanced nutrition. Energy restriction is largely known to negatively affect reproduction, but the mechanisms are not yet clearly defined. In several species, long-term (chronic) and short-term (acute) undernutrition has been observed to suppress female reproduction through suppression of gonadotrophin-releasing hormone (GnRH) secretion, inhibition or delay of the pre-ovulatory surge release of LH, decrease in oestradiol-17β (E_2) discharge (Boland *et al.* 2001) and increase in serum progesterone (P_4) concentrations (Kiyma *et al.* 2004). Also, undernutrition affects ovarian follicle development (Alexander *et al.* 2007), ovulation (Yan *et al.* 2008) and fertility rates (Brecchia *et al.* 2006). Short-term fasting also results in reduced oocyte quality in terms of *in vitro* fertilisation (IVF) rates and embryonic development (Papadopoulos *et al.* 2001), presumably through alterations in the expression levels of genes related to the oocyte metabolism (Pisani *et al.* 2008).

Metabolic hormones, such as leptin and insulin, and nutrients affect the reproductive system through hypothalamic or ovarian

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regulation (Scaramuzzi *et al.* 2006). In this way, when oxidisable fuel is scarce the release of GnRH and LH is inhibited, thereby altering steroidogenesis, reproductive cyclicity and sexual behaviour. A rapid increase in plasma non-esterified fatty acid (NEFA) concentrations occurs during starvation (Chelikani *et al*. 2004; Brecchia *et al*. 2006) reporting to the hypothalamus the negative energy balance situation (Dallman *et al.* 1999). Increased NEFA concentrations in follicular fluid affect oocyte quality (Leroy *et al.* 2005), whilst *in vitro* culture in hyperlipidaemic conditions reduces embryo quality and developmental potential (Leroy *et al.* 2010). Glucose, insulin and leptin seem to act directly at the ovarian level affecting follicle development (Viñoles *et al.* 2005). In this sense, glucose was shown to influence the process of *in vivo* and *in vitro* oocyte development (Muñoz-Gutiérrez *et al.* 2002; Viñoles *et al.* 2005). Insulin acts directly on antral follicular growth and function (Hamilton *et al.* 1999). Finally, leptin has been proposed as a metabolic signal for the reproductive system and it affects the expression of steroid receptors (Catalano *et al.* 2004) and steroidogenesis (Karamouti *et al.* 2003; Zerani *et al.* 2004). On the other hand, follicular concentrations of E_2 and P_4 could be implicated in normal meiotic and cytoplasmic oocyte maturation (Kaji *et al.* 1987; Younis *et al.* 1989).

The above mentioned metabolic effects of fasting have been studied mostly in ruminants, but little is known about the effect of food deprivation on these parameters in other species. Chronic undernutrition has been extensively investigated although the effects of acute food restriction have received less attention. Models in acute fasting could be useful to understand the alterations of reproductive physiological mechanisms in response to the negative energy balance (NEB) that occurs not only in fasting but also in some physiological situations (i.e. end of gestation and early lactation period), and is associated with high infertility rates in high-yielding females, including rabbits. In this species, artificial insemination can be performed at Day 4 postpartum in intensive rhythms leading to a similar metabolic conflict to that observed in high-yielding dairy cows. The model is also interesting for understanding human metabolic alterations, such as the NEB observed in patients suffering anorexia nervosa, which impairs their reproductive function. Moreover, restriction of nutrient supply during the periconceptional period, when oocyte maturation and embryo differentiation occurs, may alter the health and development of the offspring (Chavatte-Palmer *et al.* 2008). On the other hand, rabbits are a suitable *in vitro* model in experimental procedures using mammalian oocytes and embryos due to moderate size, short gestation period, high number of oocytes and embryos available, easy *in vitro* manipulation of early embryos and precise timing of ovulation (Duranthon 2009). Previously, the influence of acute caloric shortage for 24 h or 48 h in metabolic status was studied and related to some aspects of reproduction (receptivity, ovulation and fertility rate) in this species (Brecchia *et al.* 2006). With the aim of deepening the knowledge of the physiological causes of the alterations in reproductive processes like ovulation and fertility, we studied the impact of severe food deprivation during the periconceptional period of nulliparous rabbits on follicle development and atresia, *in vitro* oocyte maturation, embryo development and gene expression of some selected genes. Also, we determined the effects on bodyweight and estimated body composition as well as serum concentrations of glucose, insulin, triglycerides, NEFA and leptin to evaluate the metabolic status. Oestradiol-17β and progesterone serum concentrations were also analysed to evaluate the ovarian endocrine response.

Materials and methods

Unless otherwise stated, all the chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA). Experimental procedures were approved by the Animal Ethics Committee of the Polytechnic University of Madrid (Spain), and were in compliance with the Spanish guidelines for care and use of animals in research (BOE 2005).

Animals and experimental design

To avoid potentially confounding effects of lactation and doe–litter interactions, only young unmated female rabbits were used. A total of 22 Californian × New Zealand White rabbit does aged 4 months were housed at the experimental farm in the Animal Production Department, Polytechnic University of Madrid (Spain), in individual metal cages, under a constant photoperiod of 16 h light per day, a temperature of 18–22°C and a relative humidity of 60–75% maintained by a forced ventilation system. During the period before the experiment, all females were fed *ad libitum* a commercial diet containing 16.0% crude protein, 15.7% crude fibre, 2.5% fat and 2650 kcal kg^{-1} digestible energy (NANTA SA, Madrid, Spain). Water was provided *ad libitum*.

Animals were randomly distributed into the experimental groups ensuring that both groups had similar weights at the start of the experiment. A total of 12 rabbits were assigned to the control group (C), which continued to be fed *ad libitum* the standard diet indicated before. The rest of the animals ($n = 10$) were deprived of food completely for 72 h (fasted group; F). Before and after the food deprivation period, blood samples were obtained for the analysis of metabolic and endocrine parameters. Also, live bodyweight (LBW) and bioelectrical impedance analysis (body content of water, ash, protein, lipids and energy) were recorded. At the end of fasting, seven does from the control group and five does from the fasted group were killed and ovaries were collected. Morphological ovarian features (number of preovulatory follicles) and ovary weight were recorded. Follicular categorisation and apoptosis rate was analysed in one of the ovaries; the other was processed to study *in vitro* oocyte nuclear and cytoplasmic maturation. The other animals (five per group) were immediately re-fed *ad libitum* with the same diet, inseminated after a GnRH injection and killed 84 h later (luteal phase). Corpora lutea were counted and embryos were flushed from the reproductive tract, morphologically classified and immediately snap-frozen in liquid nitrogen for gene expression analysis. Also, blood samples were obtained to study metabolic and endocrine parameters at that point.

Blood sampling. Serum metabolic and hormone assays

Blood samples were collected from the margin ear vein into non-heparinised tubes at 0900 hours to avoid circadian variations. Serum was obtained after centrifugation at 1200*g* for 10 min at 4°C and stored at −32°C until analysed.

Glucose

Serum glucose was determined by the GOD-PAP method from Randox Laboratories (Antrim, UK) according to Barham and Trinder (1972). This method is linear up to a glucose concentration of 400 mg dL^{-1}.

Triglycerides

Serum triglycerides were determined by the GPO-PAP method from Randox Laboratories according to Tietz (1995). The test is linear up to a triglyceride concentration of 1000 mg dL^{-1}.

Non-esterified fatty acids (NEFA)

Serum NEFA determinations were performed in duplicate samples using a two-reaction enzymatic-based colourimetric assay from Wako Pure Chemical Industries (Osaka, Japan) according to Brecchia *et al.* (2006). The method is linear over the range 0.0–2.0 mmol L^{-1}.

Insulin

Insulin concentrations were determined by radioimmunoassay (RIA). Plasma insulin was determined by the double antibody–PEG technique using a porcine insulin RIA kit (Linco Research Inc., St Charles, MO, USA) according to Brecchia *et al*. (2006). The antiserum was guinea-pig anti-porcine insulin, while the standards used purified recombinant human insulin. The limit of sensitivity was 2 μU mL^{-1} and intra- and inter-assay coefficients of variations were 6.8 and 9.2%, respectively.

Leptin

Leptin concentrations were measured by double-antibody RIA using the multi-species leptin kit (Linco Research Inc.) according to Brecchia *et al.* (2006). The intra- and inter-assay coefficients of variation were 3.3 and 7.8%, respectively.

Oestradiol-17β (E_2) and progesterone (P_4)

Serum E_2 and P_4 concentrations were measured in duplicate samples by specific chemiluminescence methods (CMIA; Abbott laboratories, Abbott Park II, IL, USA) according to Arias-Álvarez *et al.* (2009). For E_2 detection, purified rabbit anti-oestradiol monoclonal antibodies were used. P_4 detection was achieved by using rabbit anti-progesterone polyclonal antibodies. Intra- and inter-assay coefficients of variation were 6.6 and 7.0%, respectively, for E2, and 5.8 and 6.3%, respectively, for P_4. The detection limit was 10 pg mL^{-1} for E_2 and 0.2 ng mL^{-1} for P_4.

Estimated body composition

Body composition was estimated by means of bioelectrical impedance analysis (BIA) using a four-terminal body composition analyser (Model Quantum II; RJL Systems, Detroit, MI, USA) that reports reactance and resistance between two sets of two electrodes. In our case, we used standard 21-gauge needles (Terumo Europe N.V. 3001, Leuven, Belgium) as electrodes inserted in the skin of the animal. One pair of needles were held 1 cm apart in the neck and the other pair in the rump. These components have been correlated with BIA measurements through regression equations validated by Pereda (2010).

Tissue preparation. Study of follicular population and follicular atresia assessment by TUNEL

The number of preovulatory follicles >1 mm in size in the ovarian surface of both ovaries was first recorded. Then, the left ovary of each doe was placed into a 4% (w/v) buffered neutral paraformaldehyde solution (pH 7.2 to 7.4). All samples were gradually dehydrated with increasing concentrations of ethyl alcohol (50–100% v/v), embedded in paraffin, prepared by sectioning at 5 μM and stained with hematoxylin-eosin. To study follicle populations, four histological sections of each ovary separated by 30 μm were examined with a light microscope (Olympus BX40; Olympus, Hamburg, Germany). Rabbit ovarian follicles were categorised into four specific developmental stages related to the number of layers of granulosa cells according to Rebollar *et al*. (2008): primordial, primary, secondary and antral follicles.

Examination of apoptosis was performed following the protocol described by Arias-Alvarez *et al*. (2009). Briefly, sections were deparafinised by a standard protocol; slides were doubly stained for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL; *In Situ* Cell Death Detection Kit, POD; Roche Diagnostics S.L., Applied Science, Barcelona, Spain) and 4,6-diamino-2-phenylindole (DAPI; Vector Laboratories Ltd, Peterborough, UK) for DNA staining as follows. Positive control sections were treated with DNAse I (Roche Diagnostics) for 10 min at room temperature in a humidified chamber before incubation with the TUNEL reaction mixture. Microscopic examination of the specimens was carried out using a fluorescent microscope (F550; Leica, Wetzlar, Germany). Green fluorescence could be visualised only in TUNEL-positive cells. Follicles in early or late stages of atresia were examined according to Kasuya (1995). Follicles in late atretic phase showed more than 50% of the granulosa cells labelled. Early atretic follicles were those in which apoptosis occurred in less than 50% of the granulosa cells. The percentage of apoptosis expressed is the number of TUNEL-positive follicles in each section divided by the total number of recorded follicles.

Oocyte collection. In vitro maturation (IVM) and study of nuclear and cytoplasmic oocyte maturation by confocal microscopy

The counterpart ovaries were placed in PBS at 37°C and transported to the laboratory. Cumulus–oocyte complexes (COC) were obtained by aspiration with a 2-mL syringe and 25-gauge needle from ovarian follicles ≥1 mm in size under a stereoscopic microscope. COCs with compact cumulus were washed and placed in 500 μL maturation medium in four-well dishes (Nunc, Roskilde, Denmark). They were cultured for 16 h at 38°C under an atmosphere of 5% CO_2 in air with maximum humidity. The maturation medium consisted of tissue culture medium (TCM-199) with 2 mM L-glutamine, 0.1 mg mL^{-1} sodium pyruvate supplemented with 10% (v/v) fetal calf serum (FCS), 10 ng mL^{-1} epidermal growth factor (EGF) and 100 ng mL^{-1} insulin growth factor (IGF) according to previous reports (Garcia-Garcia *et al.* 2009).

After the maturation period, COCs were treated for the confocal study as previously described by Arias-Alvarez *et al*. (2009).

Table 1. Details of primers used for qRT–PCR

Gene	GenBank accession number	Primer sequence (5′–3′)	Fragment size (bp)
H2AFZ	NM_016750	F: AGGACGACTAGCCATGGACGTGTG R: CCACCACCAGCAATTGTAGCCTTG	212
TP53	NM_001082404.1	F: GTGCTGACCAGGGACACGGC R: CTGCACCAGGGCAGACCAGC	223
SHC1 SHC	NM_001075305	F: GGTTCGGACAAAGGATCACC R: GTGAGGTCTGGGGAGAAGC	335
SCL2A4	NM_001089313.1	F: GGCGGCATGATTTCCTCC R: GAAGGGCAGCAGGATCAGCT	396
IGF2R	XM_002714965.1	F: AGCCCTCTGCTGCGTGCTTG R: TCACGTCGTGCTGCTGGCTG	349

Briefly, denuded zona pellucida-free oocytes were treated with permeabilisation solution then incubated for 30 min at room temperature with 100 μg mL^{-1} fluorescein isothiocyanate of *Lens culinaris* (FITC) to stain for cortical granules (CG). This was followed by a 15-min incubation at 39°C with 10 μg mL^{-1} propidium iodide for nuclear staining. After that, oocytes were mounted between a coverslip and a glass slide and examined under a confocal-laser scanning microscope (TCS SP5; Leica). Complete nuclear maturation was measured in terms of MII rate. CG distribution was classified as follows: (*a*) peripheral; CG were distributed adjacent to the plasma membrane, since they were cytoplasmically matured, (*b*) cortical; most of the CG were localised in the cortical area of oocytes, thus being considered as partially matured, (*c*) homogeneous: CG were scattered throughout the cytoplasm, since they did not show cytoplasmic maturation, and (*d*) non-homogeneous or abnormal; anomalous distribution of CG compatible with poor-quality or degenerated oocytes.

Artificial insemination (AI) and embryo recovery

AI was carried out as previously reported by Rebollar *et al*. (1992) using a pool of fresh heterospermic semen with more than 20 million spermatozoa in 0.5 mL of commercial diluent (Magapor S.L., Zaragoza, Spain). Ovulation was induced by intramuscular injection of 1 μg buserelin (Suprafact; Hoechst Marion Roussel SA, Madrid, Spain).

Embryos were recovered by flushing the reproductive tract with PBS + 0.1% BSA. Morphology of morulae and blastocysts were evaluated immediately after recovery with a stereomicroscope (SMZ800; Nikon, Tokyo, Japan), blastocysts were snap-frozen in liquid nitrogen and stored at −80°C for transcript-abundance analysis.

RNA extraction, reverse transcription and quantification of mRNA transcript abundance

Poly(A) RNA was prepared as previously described (Bermejo-Álvarez *et al.* 2010) from three groups of 10 blastocysts from each experimental group using the Dynabeads mRNA Direct Extraction KIT (Dynal Biotech, Oslo, Norway) following the manufacturer's instructions with minor modifications. Immediately after mRNA extraction, the RT reaction was carried out following the manufacturer's instructions (Bioline; Ecogen, Madrid, Spain) using poly(T) primer, random primers and Moloney murine leukaemia virus reverse transcriptase enzyme in a total volume of 40 μL to prime the RT reaction and to produce cDNA. Tubes were heated to 70°C for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 100 units of Superscript RT enzyme. They were then incubated at 42°C for 60 min to allow the reverse transcription of RNA, followed by 70°C for 10 min to denature the RT enzyme.

The quantification of all mRNA transcripts was carried out by real-time qRT–PCR (Bermejo-Álvarez *et al.* 2008). For qRT–PCR, three groups of cDNA per experimental group were used with two repetitions for all genes of interest. Experiments were conducted to contrast relative levels of each transcript and histone *H2AFZ* in every sample. PCR was performed by adding a 2-μL aliquot of each sample to the PCR mix containing the specific primers to amplify histone H2AFZ (*H2AFZ*), and several transcripts that have been associated with aspects of embryo quality including apoptosis (tumour protein 53 (*TP53*) and Src homology 2 domain-containing transforming protein 1 (*SHC1 SHC*)), glucose metabolism (solute carrier family 2 (facilitated glucose transporter), member 4 (*SCL2A4*, previously known as *GLUT4*)) and mitogenic, anti-apoptotic and anabolic effects (insulin-like growth factor 2 receptor (*IGF2R*)). Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 1. PCR conditions were optimised to achieve efficiencies close to 1 and then the comparative cycle threshold (CT) method was used to quantify expression levels as described in Schmittgen and Livak (2008). Quantification was normalised to the endogenous control, *H2AFZ*, and fold-changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$.

Statistical analysis

Trial data were analysed as a completely randomised design using the Statistical Analysis Systems (SAS 1999–2000). Mean differences of LBW, estimated body composition, metabolic and endocrine parameters between control and fasted groups were analysed with a MIXED procedure of the SAS program. The main effect studied was the treatment (fasted or non-fasted) and the doe was considered a random effect nested in the treatment. Means were compared using a protected *t*-student test. Means of ovary weight, number of follicles >1 mm, follicular categorisation, number of corpora lutea and embryos

Table 2. Live bodyweight, serum metabolic and endocrine parameters at start and end of 72-h fasting in rabbit does

Data are mean ± s.e.m. Live bodyweight (LBW), metabolic (glucose, triglycerides and NEFA concentrations) and endocrine (insulin, leptin, E_2 and P_4 concentrations) parameters were recorded in Group F rabbits (fasted 72 h) or Group C rabbits (control group, fed *ad libitum*) at the start and end of the experiment. Within the same row, values with different superscript letters differ significantly ($P < 0.05$). NEFA, non-esterified fatty acids; TG, triglycerides; E_2, oestradiol-17β; P_4, progesterone; n.s., not significantly different

	Start fasting	End fasting		*P* value
		Group F	Group C	
Number of does	22	10	12	
Bodyweight (kg)	3.96 ± 0.06^{b}	3.97 ± 0.09^{ab}	4.03 ± 0.09^{a}	0.0003
Metabolic parameters				
NEFA (mmol L^{-1})	0.17 ± 0.03^{b}	0.29 ± 0.04^{a}	0.09 ± 0.04^{b}	0.003
Glucose (mg dL^{-1})	117 ± 8.92	118 ± 12.6	115 ± 8.67	n.s.
TG (mg dL^{-1})	55.9 ± 7.34	49.3 ± 10.4	74.4 ± 9.95	n.s.
Endocrine parameters				
Insulin (μIU mL^{-1})	11.8 ± 2.95	8.72 ± 4.46	12.1 ± 3.86	n.s.
Leptin (ng mL^{-1})	5.06 ± 0.41^{a}	2.28 ± 0.63^{b}	4.63 ± 0.55^{a}	0.05
E_2 (pg mL^{-1})	33.8 ± 1.20	31.7 ± 1.58	35.0 ± 1.44	n.s.
P_4 (ng mL^{-1})	0.55 ± 0.28	0.23 ± 0.39	0.38 ± 0.35	n.s.

Table 3. Live bodyweight, serum metabolic and endocrine parameters in the luteal phase (84 h after end of fasting) in rabbit does

Data are mean ± s.e.m. Live bodyweight (LBW), metabolic (NEFA, glucose and triglycerides concentrations) and endocrine parameters (insulin, leptin, E_2 and P_4 concentrations) were recorded in Group F rabbits (fasted 72 h) or Group C rabbits (control group, fed *ad libitum*) 84 h after the end of food deprivation and GnRH injection (named luteal phase). Within rows, values followed by different superscript letters differ significantly ($P < 0.05$). NEFA, non-esterified fatty acids; n.s., not significantly different

	Group F	Group C	*P* value
Number of does	5	5	
Bodyweight (kg)	4.20 ± 0.09	4.10 ± 0.09	n.s.
Metabolic parameters			
NEFA (mmol L^{-1})	0.09 ± 0.06	0.13 ± 0.06	n.s.
Glucose (mg dL^{-1})	112 ± 18.6^{a}	$245 \pm 4\ 6.8^{b}$	0.001
Triglycerides (mg dL^{-1})	86.5 ± 15.3	89.2 ± 15.3	n.s.
Endocrine parameters			
Insulin (μIU mL^{-1})	41.3 ± 5.97^{c}	16.7 ± 5.96^{d}	0.02
Leptin (ng mL^{-1})	5.06 ± 0.83	4.58 ± 0.83	n.s.
Oestradiol-17β (pg mL^{-1})	33.9 ± 2.20	28.1 ± 2.19	n.s.
Progesterone (ng mL^{-1})	1.53 ± 0.44	1.38 ± 0.42	n.s.

were analysed with a GLM procedure of the SAS program with treatment as the main effect. Chi-squared test was carried out to compare nuclear maturation and the CG migration index of IVM oocytes between experimental groups. Differences in mRNA expression between experimental groups were tested using one-way repeated-measures ANOVA with arcsine transformation by the SigmaStat software package (Jandel Scientific, San Rafael, CA, USA). Differences were considered to be significant at $P < 0.05$.

Results

Circulating metabolites

At the end of fasting, after 72 h of food deprivation, NEFA values of the fasted group were significantly higher ($P < 0.003$) than those of the control group at both the start and end of the experiment (Table 2). Glucose concentrations were similar between groups. After re-feeding (Table 3), NEFA concentrations returned back to normal serum concentrations and they were similar between groups. However, glucose concentrations were significantly higher in the control than in the fasted group ($P < 0.001$). Triglycerides were not affected by treatment in the two periods studied (mean 59.1 ± 5.2 mg dL^{-1}).

Circulating hormones

After food deprivation (Table 2) the serum concentration of insulin was not significantly different between groups, but leptin concentrations were lower ($P < 0.05$) in the fasted group than in the control group. Circulating levels of E_2 and P4 were not significantly different at the end of fasting. After re-feeding (Table 3), insulin concentrations increased significantly

Table 4. Ovary weight, follicular histological categorisation and atresia rate for control and fasted rabbit does after 72 h of fasting and in luteal phase
Data are mean ± s.e.m. Ovarian parameters were analysed from retrieved ovaries of Group F rabbits (fasted 72 h) or Group C rabbits (control group, fed *ad libitum*) at the end of fasting experiment and in the re-feeding period (luteal phase; 84 h after end of fasting and GnRH injection). Within rows, values followed by different superscript letters differ significantly ($P < 0.05$). n.s., not significantly different

	End of fasting		Luteal phase		*P* value
	Fasted	Control	Fasted	Control	
Number of does	5	7	5	5	
Ovary weight (mg)	179 ± 17.8	191 ± 36.4	212 ± 22.9	211 ± 19.2	n.s.
Follicles ≥1 mm	10.8 ± 2.44[a]	12.7 ± 1.17[a]	5.60 ± 1.72[b]	8.60 ± 1.54[b]	0.01
Mean of follicular population/ovary (*n*)					
Primordial follicles	276 ± 88.6	192 ± 52.9	260 ± 85.9	141 ± 22.3	n.s.
Primary follicles	45.5 ± 7.81[a]	36.7 ± 6.63[a]	42.1 ± 7.92[a]	19.0 ± 2.28[b]	0.03
Secondary follicles	54.1 ± 14.6	37.1 ± 7.55	31.8 ± 4.63	26.7 ± 4.20	n.s.
Antral follicles	22.3 ± 2.23	21.9 ± 2.10	23.8 ± 6.80	21.2 ± 2.75	n.s.
Follicular apoptosis rate (%)					
Healthy follicles	57.4	53.2	60.5	58.7	n.s.
Early atretic follicles	17.2	15.4	12.4	19.1	n.s.
Late atretic follicles	25.4	31.4	27.1	22.2	n.s.

($P < 0.05$) in fasted rabbits; in these animals leptin levels rose, reaching values similar to those of the control group. No differences were found in serum E_2 and P_4 concentrations between groups after re-feeding. However, when comparing the ovulation phase (at the end of fasting) with the luteal phase (after re-feeding), E_2 concentrations significantly decreased in the control group (35.0 ± 1.44 vs 28.1 ± 2.19 pg mL^{-1}; $P < 0.04$), but remained high in the fasted group (31.7 ± 1.58 vs 33.9 ± 2.20 pg mL^{-1}). Besides, in both groups progesterone levels increased significantly in the luteal phase compared with the ovulation induction time (control group, 1.38 ± 0.42 vs 0.38 ± 0.35 ng mL^{-1}; fasted group, 1.53 ± 0.44 vs 0.23 ± 0.39 ng mL^{-1}; $P < 0.001$).

Live bodyweight and estimated body composition

LBW at the beginning of the experiment was comparable for both groups (control group, 3962 ± 95.8; fasted group, 3972 ± 96.0 g). As shown in Table 2, LBW of fasted does was similar at the end of starvation to before it, and comparable to the control group; however, non-fasted animals showed a higher LBW than at the start of fasting ($P < 0.0003$). Bodyweight increased from the beginning to Day 6.5 in the fasted group (3.96 ± 0.06 vs 4.20 ± 0.09 kg; $P < 0.05$) reaching that of control animals (4.10 ± 0.09 kg) at the end of the experiment.

No differences were observed in estimated body composition between control and restricted animals at the end of the fasting period; all animals contained on average: 61.0 ± 0.27% water, 2.96 ± 0.02% ash, 18.6 ± 0.31% lipids, 18.4 ± 0.03% protein and 1206 ± 12.6 kJ 100 g^{-1} of energy. After re-feeding, no differences were found in LBW between groups (Table 3).

Ovarian parameters, follicular population and atresia rate

Mean ovarian weight was similar throughout the experiment in both groups (see Table 4). Mean number of follicles >1 mm in the ovarian surface were not different after fasting and in the luteal phase between groups, but significant differences were found between the two experimental times within the groups ($P < 0.01$), as expected. After fasting, the follicular population was similar for primordial, primary, secondary and antral follicles between the two groups. In the luteal phase, there were no differences in follicle population, except for primary follicles that decreased in the control group ($P < 0.03$). The percentage of healthy, early and late atretic follicles did not vary between fasted and control rabbits.

Oocyte maturation assay

Both the nuclear maturation rate and the percentage of migrated cortical granules in oocytes were similar in the two groups (63.6 vs 53.7% and 12.0 vs 4.76%, respectively). However, partially migrated oocytes were higher in fasted rabbits than in non-fasted ones (20.0 vs 47.6%; $P < 0.04$). No differences were found between groups in non-migrated and degenerated oocyte rates (56.0 vs 47.6% and 12.0 vs 0%, respectively).

Mean number of corpora lutea, embryo recovery rate and embryo gene expression

The mean numbers of corpora lutea were not significantly different between the control and fasted groups (13.4 ± 1.7 vs 12.8 ± 2.3), but the mean number of recovered embryos by female was lower in fasted animals than in controls (4.4 ± 1.7 vs 9.0 ± 1.1; $P < 0.05$). Speed of embryo development was affected by fasting; most of the embryos in the control group were recovered in blastocyst stage compared with fasted rabbits (60 vs 36.6% of blastocysts; $P < 0.05$).

As shown in Fig. 1, acute fasting did not alter the expression levels of *TP53*, *SHC1 SHC*, *SCL2A4* or *IGF2R* in blastocysts.

Discussion

The interconnection between reproduction and metabolic status is still unclear in several aspects, particularly those bound to the earliest events of the reproductive process such as follicle development, oocyte maturation and embryo development, which have been poorly studied. In the present work a severe

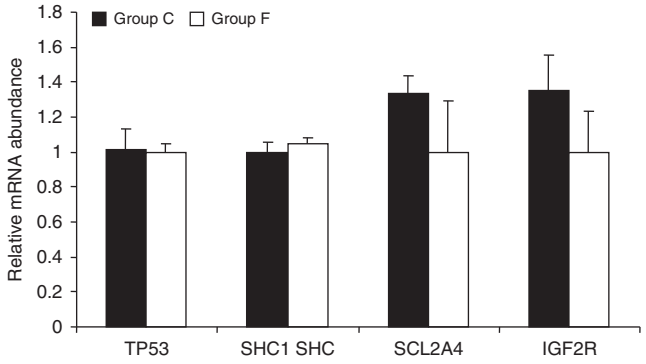

Fig. 1. Relative poly(A) mRNA abundance of the genes TP53, SHC1 SHC, SCL2A4 and IGF2R in blastocysts recovered from rabbits deprived of food during 72 h (group F) or fed *ad libitum* (group C). Embryos were recovered after 84 h from the end of the fasting period and GnRH injection for both groups. Error bars, SEM.

food restriction extended over 72 h before ovulation was imposed to elucidate the impact of maternal nutritional status on reproductive function. Thus, we investigated the effect of fasting on the development and quality of follicles, oocytes and embryos and their relationship with several metabolic and endocrine markers. In this sense, the most important observations were that severe fasting affected serum metabolic signals to the reproductive system like NEFA and leptin concentrations, ovarian endocrine parameters, such as E_2 levels in the luteal phase, and embryo development and embryo developmental speed. However, events related to ovarian physiology (follicular development and oocyte maturation) and embryonic gene expression of some candidate genes related to metabolism, apoptosis and embryo development remained almost unaltered in our rabbit model, independent of the acute food deprivation.

In the present work, some metabolic parameters were affected by nutritional deprivation. Fasting elevated serum NEFA concentrations, which suggests an increase in lipolytic activity, as described by several authors (Jorritsma *et al*. 2003; Brecchia *et al*. 2006; Sosa *et al*. 2006). After re-feeding, NEFA concentrations returned to normal, in agreement with other studies (Chelikani *et al.* 2004; Korbonits *et al.* 2007). It is possible that the increase in NEFA concentrations was caused by a decrease in plasma insulin levels. Dietary restriction reduces circulating concentrations of insulin in rabbits (Brecchia *et al.* 2006), ruminants (Vizcarra *et al.* 1998) and humans (Larivière *et al.* 1994). In our work, insulin concentrations were lower for the fasted group but significant differences were not observed. This may be the cause of the lower NEFA concentrations in this study compared with those reported in other species. Insulin levels increased markedly in the fasted group at the luteal phase, thus maintaining the glycaemia in the normal range and much lower than in control rabbits. Previous studies showed that plasma insulin levels rose 1 h after re-feeding to the same levels in fasted rabbits (Brecchia *et al.* 2006).

Glucose concentrations remained unchanged during the restriction period in fasted rabbits, according to the findings reported by Brecchia *et al.* (2006) in 24- and 48-h food-restricted rabbits. A possible explanation for the absence of differences between the control and fasted groups in this parameter is that during food deprivation, gluconeogenesis occurs favoured by lower insulin levels (8.72 vs 12 $\mu IU\ mL^{-1}$) and higher glucagon levels (not determined in this study), according to a well known mechanism described in many species. In the re-feeding period, we observed a notable increase in serum glucose levels in the control group; these elevated values were obtained from several animals in the group. In addition, two animals in the fasted group showed low glucose concentrations coincident with a low number of embryos recovered. It has been reported that low glucose levels can affect the oocyte's developmental competence (Leroy *et al.* 2006). In accordance with NEFA findings, leptin concentrations were lower in restricted than in control rabbits after food deprivation, but increased during the re-feeding period. These findings are coincident with those of other authors (Chelikani *et al.* 2004; Brecchia *et al*. 2006; Sosa *et al*. 2006). Furthermore, serum triglyceride content was maintained in fasted animals during food deprivation as well as LBW, estimated body condition and ovary weight. However, bodyweight increased slightly in control animals in the first part of the experimental period. This fact is not amazing since these animals are 4-month-old rabbits and the average body growth when feeding a standard commercial diet has been established at 37.7 $g\ day^{-1}$ (Rommers *et al.* 2001). Chelikani *et al*. (2004) reported no differences in bodyweight between control and fasted heifers but they observed decreased bodyweight in the fasted group by 24 h of fasting. On the other hand, Yan *et al*. (2008) found that food restriction significantly diminished body and even ovary weight in fasted mice.

Serum P_4 concentrations were similar in both groups after 72 h of fasting and in the luteal phase. As expected, this parameter increased significantly in both groups in the luteal phase. Food restriction in ruminants increases serum progesterone concentrations and delays the pre-ovulatory surge release of LH (Kiyma *et al*. 2004; Alexander *et al.* 2007). Nevertheless, in rabbit does the timing of the LH surge is known since they are induced ovulators. Manal *et al*. (2010) did not find any effect of fasting on P_4 serum concentrations in rabbits during pregnancy, when values are physiologically elevated. On the other hand, we observed comparable E_2 concentrations among groups at the end of fasting and in the luteal phase according to the data reported by Alexander *et al*. (2007) in sheep. It has been suggested that the immediate effect of nutrition is not acting via the regulation of follicular steroidogenesis in supplemented animals (Viñoles *et al.* 2005). When E_2 concentration during the ovulation phase (at the end of fasting) was compared with the concentration in the luteal phase, distinct patterns were observed among groups: it decreased in the control group, following the expected physiological tendency, but in the fasted group E_2 concentrations remained higher. Other authors have also observed increased E_2 concentrations post-oestrus in undernourished ewes (Sosa *et al.* 2006) and altered follicular dynamics, because large follicles persisted in the ovary and exhibited a lack of dominance (cow, Boland *et al.* 2001; ewe, Sosa *et al*. 2011). In the rabbit, follicular dynamics are not well established, but continuous follicular waves have been described; Kranzfelder *et al*. (1984) showed preovulatory follicles 40–50 h after the LH surge. In our work, we did not observe significant differences in the mean number of macroscopic

preovulatory follicles, which are usually responsible for E_2 concentrations. A plausible explanation may be that we observed follicles from different waves in the ovaries of animals in the luteal phase; there could still be some antral follicles of large size, which should have been ovulated in a normal ovary in the food-deprived group, whereas follicles recorded in the control group could be new antral follicles of slightly smaller size belonging to a new follicular wave. Yan *et al*. (2008) reported that persisting larges follicles usually contain dead oocytes. We have evaluated the atresia rate in antral follicles, but we did not find any differences between groups; in any case, oocytes were never marked by the TUNEL assay. The atresia rates observed were consistent with physiological atresia described in previous reports in rabbit does (Arias-Álvarez *et al*. 2009).

It has been described that the effect of nutritional treatments directly affects the follicles undergoing recruitment (Muñoz-Gutiérrez *et al*. 2002). We observed similar follicle populations among deprived and control rabbits after fasting and in the re-feeding period; only changes in the mean number of primary follicles in the control group during the luteal phase were observed. Insulin may be involved in the activation of primordial follicles (for review, see McLaughlin and McIver 2009), and thus, the elevated concentrations of insulin in the luteal phase in fasted rabbits may be responsible for the increase in the mean number of primary follicles compared with control rabbits. Taken together, these findings could be relevant for human health (i.e. anorexia nervosa patients), since recruitment of primary follicles from the primordial pool decreases the original follicle pool that serves as a ticking clock to the onset of menopause (da Silva Faria *et al*. 2010).

Few studies have reported the effects of fasting on oocyte quality, but it is believed that this feature is influenced by dietary intake. In the present work, acute fasting did not inhibit oocyte nuclear and cytoplasmic maturation, in contrast to the situation described in mouse (Yan *et al*. 2008), where food deprivation impaired germinal vesicle breakdown and polar body extrusion. However, other reports have shown no effect on oocyte developmental competence (Vazquez *et al*. 2010) or oocyte quality (Kendrick *et al*. 1999; Tripp *et al*. 2000). To our knowledge, there are no other studies in the literature about the impact of acute fasting on *in vitro* oocyte maturation.

Although the number of corpora lutea was not affected by food restriction, in accordance with the studies of Novak *et al*. (2003) in gilts and Sosa *et al*. (2009) in ewes, the number of recovered embryos was lower and their development was slower in fasted than in control animals. The last findings agree with other works (Abecia *et al*. 1995; Novak *et al*. 2003) that reported a gradual decrease in the percentage of embryos reaching successive stages of early development. The uterine environment could have been affected by fasting, since changes in the ability of endometrial tissue to respond to P_4 and a reduction of receptors in the early stages of pregnancy have been reported (Sosa *et al.* 2006). Alterations in the follicular environment could be manifested at this point resulting in the lower speed of development of embryos from fasting rabbits found in spite of adequate oocyte maturation.

The expression of genes involved in physiological functions such as glucose and lipid metabolism can be altered by environmental factors (Bermejo-Álvarez *et al.* 2010). However, in this study we did not observe a significant effect of acute food deprivation on the expression of the chosen candidate genes related to metabolism, apoptosis and embryo development. The insulin-regulated facilitated glucose transport *SCL2A4* provides energy substrates for periods of rapid growth or high metabolic demand (Williams *et al.* 2001) and its expression was previously reported in rabbit morulae and blastocysts (Navarrete Santos *et al.* 2004). *TP53* is a principal apoptotic gene that plays a role in hyperglycaemia-induced apoptosis in the mouse preimplantation embryo (Keim *et al.* 2001), whereas and *SHC1 SHC* is an important apoptosis regulator. The imprinted gene *IGF2R* has a role in embryonic growth and regulation in the mammalian uterus (Wang *et al.* 1994), and its expression is known to be altered by insults occurring during preimplantation development (Young *et al*. 2001). The lack of differences among groups suggests that the quality of the recovered embryos in terms of gene expression was not affected by maternal fasting.

In conclusion, acute fasting affects some metabolic and endocrine markers, as well as embryo development and speed of development. However, follicular atresia, oocyte maturation and blastocyst gene expression of selected genes implicated in metabolism, apoptosis and embryo development were not impaired in the rabbit model studied. It may be that the length of restricted feeding in this study was not severe enough to induce a negative impact on long-term events such as oocyte maturation and embryo gene expression.

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