Influence of leptin on *in vitro* maturation and steroidogenic secretion of cumulus–oocyte complexes through JAK2/STAT3 and MEK 1/2 pathways in the rabbit model

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Abstract

Extreme body mass indexes may impair reproductive outcome in assisted reproductive technologies. Leptin reflects the amount of body fat and could act as a modulator of oocyte quality through activation of specific transcription factors. The aim of this work was to establish whether: 1) leptin influences meiotic and cytoplasmic oocyte maturation; 2) STAT3 and MAPK mediate the effects of leptin and 3) leptin modulates steroid secretion by cumulus–oocyte complexes (COC) during *in vitro* maturation (IVM). We confirmed immunolocalisation of leptin receptor in oocytes, cumulus/granulosa cells during the peri-ovulatory period. The confocal study showed that COC supplemented with 1, 10 and 100 ng/ml leptin had a significantly higher metaphase II (MII) percentage than those IVM without leptin ($P < 0.05$) and a similar MII index compared to the group supplemented with 10% FCS. Leptin did not increase the percentage of cytoplasmically matured oocytes in terms of cortical granule migration rate, whereas a significantly higher index was found in the FCS group ($P < 0.001$). Oestradiol concentrations in spent media were higher in the FCS group compared to other treatments ($P < 0.001$). Leptin-stimulated nuclear oocyte maturation was significantly impaired when leptin-induced JAK2/STAT3 and MEK 1/2 activation was suppressed by the inhibitors ($P < 0.001$). Steroid secretion of COC was not affected by leptin activation of JAK2/STAT3 or MEK 1/2 pathways. In conclusion, JAK2/STAT3 and MEK 1/2 pathways mediate the enhancement of nuclear oocyte maturation by leptin; however, neither cytoplasmic oocyte maturation nor steroidogenic response of COC were improved in the present rabbit model.

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Introduction

Obesity is a rising health problem commonly associated with infertility (Denison 2009) which may impair the outcome of assisted reproductive technologies. Studies performed with infertile women and animals indicate that the optimisation of ovary function is fundamental in the fertility prognosis (Bellver 2006). Different endocrine factors related to the body mass index may affect oocyte quality and therefore reproductive success. Thus, leptin, a 16 kDa peptide hormone encoded by *LEP* (previously known as the obese gene, *OB*) and secreted mainly by the adipose tissue, plays an important role in the regulation of food intake, energy expenditure and reproductive success (Keim *et al.* 1998, Cervero *et al.* 2006). In fact, a minimum level of leptin is required for maintenance of the reproductive function (Cheung *et al.* 1997), but high leptin concentrations associated with

obesity itself or polycystic ovarian syndrome have been reported to explain partially the negative impact of these pathologies on fertility (Bellver 2006).

Leptin influence on reproduction is mediated by the regulation of the hypothalamus–pituitary axis and the ovarian function through its receptor (Chehab *et al.* 1996, Brecchia *et al.* 2006). Earlier studies showed that mice lacking either leptin (*OB/OB*) or leptin receptors (*DB/DB*) are both obese and infertile (Zhang *et al.* 1994, Lee *et al.* 1996), whereas administration of exogenous leptin has the ability to restore fertility in these animals (Chehab *et al.* 1996). In the ovary, leptin receptor (LEPR, previously known as OBR) has been detected in granulosa cells of follicles, cumulus cells and oocytes in several species (human: Cioffi *et al.* 1997; mouse: Antczak & Van Blerkom 1997; mouse: Ryan *et al.* 2002; pig: Craig *et al.* 2004; rabbit: Zerani *et al.* 2004; ewe: Muñoz-Gutiérrez *et al.* 2005; cattle: Paula-Lopes *et al.* 2007). The LEPR is a

member of the class-I cytokine receptor superfamily with six known isoforms (Tartaglia *et al.* 1995, Lee *et al.* 1996). Both long (LEPR_b) and short transmembrane isoforms (LEPR_{a,c,d,f}) contain identical extracellular and transmembrane domain, but the cytoplasmic region is different in length (Frühbeck 2006). LEPR usually activates cytoplasmic JAK2 to transmit leptin signals (Frühbeck 2006). LEPR_b is highly expressed in the hypothalamus and mediates leptin signalling through activation of both the STAT3 and MAPK (Tartaglia *et al.* 1995, Lee *et al.* 1996, Bjørbaek *et al.* 1997). The short isoforms of the receptor are more widely expressed in peripheral tissues and are capable of signalling through the MAPK pathway (Bjørbaek *et al.* 1997, Tartaglia 1997). Specifically, MAPK kinases 1–2 (MEK1 and MEK2) are dual-specific protein kinases involved in a MAPK cascade and appear as predominant MAPK in cumulus–oocyte complexes (COC) during *in vitro* maturation (IVM) in heifers (Van Tol *et al.* 2008). Although leptin may be integrated in a complex intracellular cross-talk system that regulates specific cell functions (Zhang *et al.* 1994), there is scarce information about the role of leptin through these pathways in the oocyte of humans and other animal species. In this sense, it is known that human COC are capable to respond to leptin during pre-ovulatory period (Cioffi *et al.* 1997), and its potential direct effects on oocyte maturation, mainly as a modulator of the oocyte fertility potential, could be through activation of specific transcription factors.

Additionally, interactions between leptin and cumulus cell-enclosed oocytes could modulate steroid production in cumulus cells (Karamouti *et al.* 2003, Ruiz-Cortés *et al.* 2003, Swain *et al.* 2004). It has been reported that follicular cells control oocyte physiology by secreting steroids (Mingoti *et al.* 1995). Therefore, concentrations of oestradiol (OE₂) and progesterone (P₄) during the peri-ovulatory period could be implicated in normal meiotic and cytoplasmic oocyte maturation by paracrine or autocrine pathways (Kaji *et al.* 1987, Younis *et al.* 1989, Andersen 1993, Tesarik & Mendoza 1997). However, elucidation of leptin's role in such processes has been challenging, with conflicting results reported by several investigators. Understanding these mechanisms is essential because the influence of leptin on oocyte maturation ability has important implications for ovulation induction and could be helpful to find optimal hormone combinations to apply in animal and human infertility therapies.

Rabbits are widely used as an *in vitro* model in experimental procedures on mammalian oocytes and embryos. As a consequence, taking all these findings together and for the first time in the rabbit oocyte, the objective of this study after the immunolocalisation of the leptin receptor (LEPR) in the ovary throughout the peri-ovulatory period was to investigate: 1) the direct role of different leptin concentrations on meiotic and cytoplasmic *in vitro* oocyte maturation;

2) the JAK2/STAT3 and MEK 1/2 leptin-dependent intracellular mechanisms by specific pathway inhibition; 3) the steroidogenic response of COC to leptin during the oocyte maturation process.

Results

Immunolocalisation of leptin receptor (LEPR)

Positive immunostaining for LEPR was found in oocytes, cumulus cells and granulosa cells of the follicles at all stages of development in all time points measured during the peri-ovulatory period (Fig. 1). Stroma and corpora lutea exhibited high immunoreactivity for LEPR as well. Theca interna cells showed moderate immunostaining, whereas LEPR was not immunolocalised in theca externa cells. Control tissue samples showed no positive staining.

Effect of leptin on nuclear and cytoplasmic oocyte maturation

As shown in Table 1, nuclear oocyte IVM was significantly improved ($P < 0.005$) when leptin was added to the culture media at all concentrations tested (1, 10, 100 ng/ml) compared with the group without leptin. Groups 1 Lep, 10 Lep and 100 Lep showed similar percentages of metaphase II (MII) compared with the group cultured with FCS. Groups supplemented with 1 and 10 ng/ml leptin showed a lower rate of metaphase I (MI) compared with 0 Lep and FCS groups ($P < 0.05$).

Figure 1 Immunolocalisation of leptin receptor (LEPR) in ovarian sections of rabbit does during the peri-ovulatory period. (A) Negative control. (B) Positive staining of LEPR in oocytes and granulosa cells of primordial, primary and secondary follicles. (C) Antral follicle with staining of LEPR in granulosa cells. (D) Immunodetection detail of LEPR in granulosa and theca cells. Theca externa cells show negative immunostaining (o, oocyte; g, granulosa cells; t, theca cells).

Table 1 Meiotic and cytoplasmic configuration percentages, in terms of cortical granule (CG) migration, observed in rabbit oocytes at 16 h maturation time following the use of different leptin concentrations.

	0 ng/ml leptin (0 Lep)	1 ng/ml leptin (1 Lep)	10 ng/ml leptin (10 Lep)	100 ng/ml leptin (100 Lep)	10% FCS (FCS)
Nuclear maturation (%)					
Metaphase II	54.05 ^a	79.35 ^b	75.19 ^b	66.38 ^b	71.62 ^b
Metaphase I	19.8 ^c	5.4 ^d	7.8 ^d	11.2 ^{c,d}	17.6 ^c
Germinal vesicle	17.1	15.2	14.0	25.0	18.9
Abnormal chromosome configuration	9.0 ^d	0.0 ^c	3.1 ^{c,d}	7.8 ^d	9.5 ^d
Cytoplasmic maturation (%)					
Peripheral distribution of CG	7.9 ^c	9.3 ^c	10.0 ^c	2.9 ^c	25.7 ^d
Cortical distribution of CG	6.3 ^{c,d}	23.3 ^c	8.3 ^{c,d}	20.0 ^{c,d}	2.9 ^d
Homogeneous distribution of CG	82.5	60.5	81.7	77.1	68.6
Abnormal distribution of CG	3.2	7.0	0.0	0.0	2.9

Values are mean $\pm$ S.E.M. Means in rows with different letters differ: ^{a,b}($P < 0.005$) ^{c,d}($P < 0.05$).

Peripheral and cortical distribution of cortical granule (CG) migration (Table 1) was similar for all experimental leptin groups. However, the group supplemented with FCS showed a significantly higher number of cytoplasmically matured oocytes compared to the treatments without serum ($P < 0.05$). No significant differences were found in the percentage of oocytes with homogeneous and abnormal CG distribution between groups.

STAT3 and MAPK leptin-associated signal transduction during IVM

Leptin-stimulated nuclear oocyte maturation in terms of MII rate was significantly impaired ($P < 0.001$) when specific JAK2/STAT3 and MEK 1/2 inhibitors at all concentrations tested were added to the maturation media (Tables 2 and 3 respectively). The percentage of MI and germinal vesicle (GV) was increased in the 10 ng/ml leptin groups supplemented with both the inhibitors. The addition of both inhibitors in the 0 Lep group did not modify MII, MI, GV and abnormal nuclear configuration proportion. The percentage of cytoplasmically matured oocytes was similar in both the leptin IVM media

supplemented with JAK2/STAT3 and MEK 1/2 inhibitors (Tables 2 and 3 respectively) and the respective control groups (0 Lep and 10 Lep). The groups supplemented with leptin and 100 μ M JAK2/STAT3 or 50 μ M MEK 1/2 inhibitor concentrations showed a significant increase in the number of oocytes with abnormal distribution of CG migration ($P < 0.05$).

Steroidogenic response of COC

As shown in Table 4, mean OE₂ secretion by COC was significantly higher in the FCS group compared to those oocytes cultured in media supplemented with 0, 1, 10 and 100 ng/ml leptin ($P < 0.005$). No significant differences were found in P₄ concentrations at the end of the IVM period between treatments.

Mean OE₂ and P₄ secretions by COC were similar when JAK2/STAT3 inhibitors were added to the IVM media compared to the respective control groups. When MEK 1/2 inhibitors were added, a significant increase in the mean OE₂ secretion by COC was observed in the 0/10, 0/50 and 10/10 groups as well as similar increases in P₄ secretion in 10/10 and 10/50 groups compared to the treatment with 0 and 10 ng/ml leptin ($P < 0.05$; Table 5).

Table 2 Meiotic and cytoplasmic configuration percentages, in terms of cortical granule (CG) migration, observed in rabbit oocytes matured *in vitro* with 0 or 10 ng/ml leptin media plus JAK2/STAT3 inhibitors (AG490) at different concentrations.

	0 ng/ml leptin (0 Lep)	0 ng/ml leptin/10 μ M AG498 (0/10)	0 ng/ml leptin/100 μ M AG490 (0/100)	10 ng/ml leptin (10 Lep)	10 ng/ml leptin/10 μ M AG490 (10/10)	10 ng/ml leptin/100 μ M AG490 (10/100)
Nuclear maturation (%)						
Metaphase II	54.1	40.0	45.5	75.2 ^a	38.7 ^b	45.9 ^b
Metaphase I	19.8	24.0	30.3	7.8 ^a	25.3 ^b	17.6 ^{a,b}
Germinal vesicle	17.1	24.0	18.2	14.0 ^a	33.3 ^b	27.0 ^b
Abnormal chromosome configuration	9.0	12.0	6.1	3.1	2.7	8.1
Cytoplasmic maturation (%)						
Peripheral distribution of CG	7.9	11.8	17.6	10.0 ^{c,d}	17.1 ^c	2.2 ^d
Cortical distribution of CG	6.3	23.5	14.7	8.3	17.1	17.8
Homogeneous distribution of CG	82.5	64.7	67.6	81.7 ^c	58.5 ^d	64.4 ^{c,d}
Abnormal distribution of CG	3.2	0.0	0.0	0.0 ^c	7.3 ^{c,d}	15.6 ^d

Values are mean $\pm$ S.E.M. Means in rows with different letters differ: ^{a,b}($P < 0.005$) ^{c,d}($P < 0.05$).

Table 3 Meiotic and cytoplasmic configuration percentages, in terms of cortical granule (CG) migration, observed in rabbit oocytes matured *in vitro* with 0 or 10 ng/ml leptin media plus MEK 1/2 inhibitors (PD98059) at different concentrations.

	0 ng/ml leptin (0 Lep)	0 ng/ml leptin/10 µM PD98059 (0/10)	0 ng/ml leptin/50 µM PD98059 (0/50)	10 ng/ml leptin (10 Lep)	10 ng/ml leptin/10 µM PD98059 (10/10)	10 ng/ml leptin/50 µM PD98059 (10/50)
Nuclear maturation (%)						
Metaphase II	54.1	51.4	54.4	75.2 ^a	44.1 ^b	49.3 ^b
Metaphase I	19.8	20.3	14.7	7.8 ^a	22.6 ^b	26.8 ^b
Germinal vesicle	17.1	23.0	29.4	14.0 ^c	29.0 ^d	22.5 ^{c,d}
Abnormal chromosome configuration	9.0	5.4	1.5	3.2	4.3	1.4
Cytoplasmic maturation (%)						
Peripheral distribution of CG	7.9	7.1	7.5	10.0	10.5	9.1
Cortical distribution of CG	6.3	12.5	2.5	8.3	13.2	11.4
Homogeneous distribution of CG	82.5	69.6	87.5	81.7	76.3	68.2
Abnormal distribution of CG	3.2	10.7	2.5	0.0 ^a	0.0 ^a	11.4 ^b

Values are mean $\pm$ S.E.M. Means in rows with different letters differ: ^{a,b}($P < 0.005$) ^{c,d}($P < 0.05$).

Discussion

The current work shows for the first time the effect of leptin on nuclear and cytoplasmic *in vitro* oocyte maturation, its underlying intracellular mechanisms and the steroidogenic response using the rabbit oocyte as a model. The results obtained demonstrate that the beneficial influence of leptin on meiotic oocyte maturation is mediated by JAK2/STAT3 and MEK 1/2 pathways.

The presence of leptin and its receptor in the body is essential for reproductive function (Chehab *et al.* 1996). The universal distribution of leptin receptors between species and their different physiological status are due to the pleiotropic nature of leptin and the highly preserved structure of the LEPR (Tartaglia *et al.* 1995, Bjørbaek *et al.* 1997). In the present study, long and short isoforms of the LEPR were immunolocalised in the rabbit oocyte, cumulus and granulosa cells of the ovarian follicles at all stages of development, as well as in theca interna, estroma and corpus luteum cells during the peri-ovulatory period. These findings corroborate those obtained in ovaries of several species (human: Karlsson *et al.* 1997; rat: Zamorano *et al.* 1997; mouse: Matsuoka *et al.* 1999; gilt: Lin *et al.* 2000, Craig *et al.* 2004; cattle: Thorn *et al.* 2007), including pseudo-pregnant rabbits (Zerani *et al.* 2004), and contribute to the significant body of evidence around the physiological relevance of this hormone, especially in the ovary and during the pre-ovulatory period (Cioffi *et al.* 1997, Duggal *et al.* 2002, Archanco *et al.* 2003).

Oocyte maturation is an essential physiological event for species survival; thus, leptin influence in oocyte quality is of keen clinical interest and potentially

important for IVF protocols. In the present work, we have demonstrated that leptin enhances *in vitro* meiotic oocyte maturation. However, controversial results have been reported in the literature. In some species, leptin seems to promote rearrangement of cytoskeletal elements (Suzuki *et al.* 2010) that are involved in chromosome segregation and in processes of organelle movement (Sun & Schatten 2006). Other studies have reported that leptin enhances an appropriate spindle assembly during metaphase (Jin *et al.* 2009) and stimulates meiotic oocyte maturation in mouse (Matsuoka *et al.* 1999), pig (Craig *et al.* 2004, Kun *et al.* 2007, Zhang *et al.* 2007) and cattle (Paula-Lopes *et al.* 2007, Van Tol *et al.* 2008). Nevertheless, Ryan *et al.* (2002) showed that leptin only induced meiotic resumption when the oocyte was cultured within its normal follicular environment. Other authors have even reported that leptin had no effect on meiotic resumption and development to MII (mouse: Swain *et al.* 2004; pig: Suzuki *et al.* 2010).

Regarding cytoplasmic maturation, studies by Runner & Gates (1954) observed that oocytes from OB/OB leptin-deficient mice were able to undergo normal fertilisation and embryo development *in vivo*, although these animals were obese and showed reproductive failures (Coleman 1978, Zhang *et al.* 1994). This led to postulate that the sterility defect due to leptin was not in the oocytes (Swain *et al.* 2004). According to these findings, in the present work, leptin supplementation did not improve oocyte quality in terms of CG migration rate at the concentrations tested compared to the FCS group, which achieved the best results of cytoplasmic maturation, similar to those previously reported in rabbits by our group (Garcia-Garcia *et al.* 2009). This finding is

Table 4 Steroid production in rabbit cumulus–oocyte complexes (COC) at 16 h maturation time following the use of different leptin concentrations.

	0 ng/ml leptin (0 Lep)	1 ng/ml leptin (1 Lep)	10 ng/ml leptin (10 Lep)	100 ng/ml leptin (100 Lep)	10% FCS (FCS)
OE ₂ /COC (pg/ml)	0.28 $\pm$ 0.14 ^a	0.22 $\pm$ 0.02 ^a	0.32 $\pm$ 0.14 ^a	0.34 $\pm$ 0.11 ^a	4.13 $\pm$ 0.77 ^b
P ₄ /COC (ng/ml)	0.03 $\pm$ 0.01	0.02 $\pm$ 0.004	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01	0.03 $\pm$ 0.003

Values are mean $\pm$ S.E.M. Means in rows with different letters differ: ^{a,b}($P < 0.005$) ^{c,d}($P < 0.05$).

Table 5 Steroid production in rabbit cumulus–oocyte complexes (COC) at 16 h maturation time following the use of different concentrations of JAK2/STAT3 (AG490) and MEK 1/2 (PD98059) leptin-induced pathway inhibitors.

	0 ng/ml leptin (0 Lep)	0 ng/ml leptin/10 µM AG498 (0/10)	0 ng/ml leptin/100 µM AG490 (0/100)	10 ng/ml leptin (10 Lep)	10 ng/ml leptin/10 µM AG490 (10/10)	10 ng/ml leptin/100 µM AG490 (10/100)
OE ₂ /COC (pg/ml)	0.18 ± 0.04	0.23 ± 0.11	0.10 ± 0.05	0.39 ± 0.17	0.44 ± 0.24	0.14 ± 0.05
P ₄ /COC (ng/ml)	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.06 ± 0.03	0.02 ± 0.00

	0 ng/ml leptin (0 Lep)	0 ng/ml leptin/10 µM PD98059 (0/10)	0 ng/ml leptin/50 µM PD98059 (0/50)	10 ng/ml leptin (10 Lep)	10 ng/ml leptin/10 µM PD98059 (10/10)	10 ng/ml leptin/50 µM PD98059 (10/50)
OE ₂ /COC (pg/ml)	0.05 ± 0.01 ^a	0.10 ± 0.02 ^b	0.11 ± 0.01 ^b	0.04 ± 0.01 ^a	0.13 ± 0.02 ^b	0.07 ± 0.01 ^a
P ₄ /COC (ng/ml)	0.02 ± 0.00	0.03 ± 0.01	0.05 ± 0.00	0.01 ± 0.00 ^a	0.04 ± 0.00 ^b	0.05 ± 0.01 ^b

Values are mean ± s.e.m. Means in rows with different letters differ: ^{a,b}($P < 0.05$).

consistent with that obtained by Paula-Lopes *et al.* (2007). These authors found a similar proportion of polyspermic oocytes when bovine COC were supplemented with leptin or not in IVM media. However, other studies have suggested that leptin only may exert its effect when it is added *in vitro* at very high concentrations (500–1000 ng/ml), inhibiting microfilament reorganisation in pigs (Suzuki *et al.* 2010) or improving cytoplasmic maturation in cattle (Van Tol *et al.* 2008). In turn, these results are not in agreement with the findings obtained by other authors, in which much lower leptin concentrations (1–100 ng/ml) resulted in an increase of developmental competence related to an improvement of oocyte cytoplasmic maturation (bovine: Boelhauve *et al.* 2005; pig: Craig *et al.* 2004, Jin *et al.* 2009). These apparently controversial IVM results reported in the literature are probably dependent on the culture medium used or the species studied. The experiments described in the present work and in Van Tol *et al.* (2008) were performed in a serum-free medium in absence of any growth factor or hormone other than leptin. However, the other studies usually added gonadotropins, growth factors or follicular fluid to culture media; these additives could have enhanced the sensitivity of COC to leptin or its pathways, showing different responses at lower concentrations. Thus, possible interactions between them and oocyte maturation remain unclear. Further standardised studies are warranted, since leptin may affect oocyte quality, mediating the action of other drugs used in the stimulation protocols of fertility treatments.

The development potential of oocytes is also influenced by the synthesis and release of cumulus- and oocyte-derived factors as steroids (Andersen 1993). Measurements of steroids at the end of the maturation period are commonly used to assess the steroidogenic capacity of COC (Lorenzo *et al.* 1997, Mingoti *et al.* 2002, Shirazi & Moalemian 2007). In this sense, the present work shows that leptin, in a serum-free culture medium, did not exert a short-term effect on the final OE₂ and P₄ concentrations of COC in a dose-dependent manner. Therefore, under our experimental conditions,

in rabbit oocytes, leptin stimulation of meiotic maturation is not mediated by an increase of steroid secretion by COC *in vitro*. Our results agree with previous reports showing that OE₂ and P₄ do not seem to play a decisive role in oocyte meiotic maturation *in vitro* (Dode & Graves 2002, Lucidi *et al.* 2003, Wang *et al.* 2006). However, contradictory findings exist about stimulatory or inhibitory effects of leptin on steroidogenesis (Zachow & Magoffin 1997, Tsai *et al.* 2002, Catalano *et al.* 2003, Ruiz-Cortés *et al.* 2003, Swain *et al.* 2004). OE₂ secreted by cumulus cells could induce a rapid increase in free intracellular calcium concentration, a mechanism that improves cytoplasmic maturation (bovine: Younis *et al.* 1989; human: Tesarik & Mendoza 1997). Also, in bovine oocytes, OE₂ probably participates in the formation of the polyspermic block via CGs (Karlach 1987). This finding is consistent with the increase of the CG migration rate in the FCS group showed in this study, but not in the rest of the leptin groups.

Leptin acts as modulator of oocyte maturation through activation of specific transcription factors. The current work provides the first evidence that leptin uses both JAK2/STAT3 and MEK 1/2 pathways to improve meiotic IVM of rabbit oocytes in agreement with previous results in other species (mouse: Matsuoka *et al.* 1999; pig: Craig *et al.* 2004). Matsuoka *et al.* (1999) first suggested that the function of leptin during nuclear oocyte maturation was due to the activation of the STAT3 signal transduction pathway. In heifers, cumulus cells seem to be more influential in leptin activity than the oocyte itself, mediating the leptin-STAT3 signals to improve oocyte meiotic maturation by the long isoform LEPR (Paula-Lopes *et al.* 2007). Of the known LEPR isoforms, only the full length one contains the intracellular domains necessary to mediate JAK2/STAT3 and MAPK signal transduction pathways. In contrast with the findings reported by Matsuoka *et al.* (1999) and Paula-Lopes *et al.* (2007), other authors showed that leptin did not increase phosphorylated STAT3, but more phosphorylated MEK1 and MEK2 proteins were predominant in COC during IVM (Van Tol *et al.* 2008). In this sense, Craig *et al.* (2004)

also showed that leptin activates MAPK pathway in the pig oocyte. MAPK enhance maturation promoting factor activity and are involved in post-meiotic resumption events, such as spindle formation, MI to MII transition and MII arrest in several species, including rabbits (Yu *et al.* 2002), as it was also suggested in the current work.

As we expected, this study also showed the non-suppression of leptin-induced oocyte CG migration by JAK2/STAT3 and MEK 1/2 inhibitors. However, the increase of abnormal distribution when media were supplemented with leptin and the higher JAK2/STAT3 and MEK 1/2 inhibitor concentrations (100 and 50 μ M respectively) imply that the oocyte may be a more sensitive cell to higher concentrations of both inhibitors widely used in other cell studies (thymus cells: Mansour *et al.* 2006; endometrial cancer cells: Sharma *et al.* 2006; hepatocarcinoma cells: Chen *et al.* 2007; breast cancer cells: Saxena *et al.* 2007) or a non-specific effect. In agreement with our cytoplasmic maturation results, it has been reported that although leptin signalling through the STAT3 pathway is beneficial for meiotic maturation (Matsuoka *et al.* 1999), it does not seem essential for reproduction, at least at the oocyte level (Takeda *et al.* 1997). Bates *et al.* (2003) even showed that mice with disrupted LEPR_b-STAT3 signalling were obese yet fertile. However, MAPK proteins seem to be necessary for pronuclear formation in parthenogenetically activated oocytes (Posada & Cooper 1992, Liu *et al.* 1998, Ito *et al.* 2004) for microfilament formation and thus for organelle distribution and polarity establishment in the oocyte (Verlhac *et al.* 1993, Sun & Schatten 2006). This discrepancy with our results points to a possible species-specific mode of action of the leptin system on oocyte maturation, or that the MAPK involved in some aspects of cytoplasmic maturation were other than those inhibited in this study.

On the other hand, steroid concentrations were not altered in the 0 or 10 Lep groups with or without inhibitors. This allows us to determine that, in rabbit oocytes, stimulation of meiotic maturation by leptin was not mediated by an increase of steroid secretion induced by activation of JAK2/STAT3 and MEK 1/2 proteins. The higher secretion of steroids found only in some groups treated with leptin and MEK 1/2 inhibitors was unclear. Maybe some compensatory mechanisms could increase steroid secretion when leptin-induced MEK 1/2 pathway was inhibited. However, the reason for this and the potential cross-talk between JAK2/STAT3 and MAPK pathways and steroid secretion in COC units need further research.

In conclusion, the current work provides the first evidence that leptin enhances meiotic oocyte maturation through activation of both JAK2/STAT3 and MEK 1/2 pathways in the rabbit oocyte model. This beneficial effect is not observed on cytoplasmic maturation in terms of CG migration nor it is mediated by an increase of OE₂ and P₄ secretion by COC. These findings contribute to

enhance the knowledge about the relationship between obesity/lean-associated pathologies, leptin and oocyte quality to continue optimising IVF techniques in humans and animals.

Materials and Methods

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company. Ovaries were obtained from slaughtered adult animals from the abattoir (Jumogar, SL, Tarancón, Spain). Additionally, twelve 18-week-old New Zealand $\times$ California white rabbit does (*Oryctolagus cuniculus*) were used for immunolocalisation of LEPR in different time points during the peri-ovulatory period. 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 were housed in individual flat-deck cages under a constant photoperiod of 16 h of light per day, a temperature of 18–22 °C and a relative humidity of 60–75% maintained by a forced ventilation system in the Polytechnic University of Madrid (Spain). Ovulation was induced by i.m. injection of 1 μ g buserelin (Suprafact, Hoechst Marion Roussel, SA, Madrid, Spain). Does were killed with 30 mg/kg of i.v. pentobarbital sodium (Dolethal, Vetoquinol, Alcobendas, Spain) and subjected to laparotomy in order to collect their ovaries at different time points during the peri-ovulatory period: 1) before ovulation induction (0 h, $n=4$); 2) 8 h after ovulation induction ($n=4$); 3) 16 h after ovulation induction ($n=4$).

Immunohistochemistry of leptin receptor (LEPR)

After slide deparaffinisation and rehydration in graded alcohol, ovarian sections of each experimental group described above were heat-treated in sodium citrate solution (pH 6) to unmask antigen epitopes. Endogenous peroxidase activity was blocked by incubating the slides with 3% v/v hydrogen peroxide in methanol solution for 30 min. Non-specific binding of immunoglobulins was blocked with normal goat serum (1:10, sc-2043, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS at room temperature (RT) for 30 min. Primary antibody against LEPR (1:10, sc-8391, Santa Cruz Biotechnology) was incubated overnight at room temperature in a humidified chamber. The antibody used in this study specifically recognises long and short forms of human LEPR. In the negative control sections, incubation with primary antibody was replaced by PBS solution. After that, samples were incubated with biotinylated secondary antibody (1:200, biotinylated anti-mouse IgG (H+L) made in goat, Vector Laboratories, Ltd, Peterborough, UK) for 30 min, and subsequently with the avidin-biotin complex (ABC Vector Elite kit, Vector Laboratories, Burlingame, CA, USA) at RT for 30 min. After chromogen incubation (Vector Nova RED substrate Kit for Peroxidase, Vector Laboratories), the sections were counterstained with haematoxylin, photographed and assessed under a light microscope (Olympus BX40, Hamburg, Germany).

Figure 2 Experimental design scheme of oocyte *in vitro* maturation studies. (A) *In vitro* maturation with different concentrations of leptin (ng/ml). (B) *In vitro* maturation with 0 and 10 ng/ml leptin plus JAK2/STAT3 (AG490) and MEK 1/2 (PD98059)-pathways inhibitors.

Oocyte collection and IVM of COC

Ovaries of slaughtered adult animals were placed in PBS at 37 °C and transported to the laboratory. COC were obtained by aspiration with 2 ml syringe and 25 G needle from ovarian follicles ≥ 1 mm in size under a stereoscopic microscope. COC with compact cumulus were washed and placed in 500 μ l maturation medium in four-well dishes (Nunc Surface, Nunc, Roskilde, Denmark). They were cultured for 16 h at 38 °C under an atmosphere of 5% CO₂ in air with maximum humidity.

Role of leptin on meiotic and cytoplasmic *in vitro* oocyte maturation

Six replicates were made ($n=750$ oocytes) to assess the influence of leptin on the oocyte maturation process (Fig. 2A). The maturation medium consisted of tissue culture medium (TCM-199) with 2 mM L-glutamine, 0.1 mg/ml sodium pyruvate, 0.3% w/v BSA supplemented with 0, 1, 10, 100 ng/ml leptin or 10% v/v FCS (0 Lep, 1 Lep, 10 Lep, 100 Lep and FCS groups respectively).

Assessment of JAK2/STAT3 and MEK 1/2 leptin-mediated pathways during IVM

In order to assess if leptin acts on oocyte maturation activating STAT3 and MAPK pathways, we have performed a dose-response study in five replicates ($n=578$ oocytes). For maturation, COC were randomly divided into two groups: control with 0 ng/ml leptin (0 Lep group) and leptin group with 10 ng/ml leptin (10 Lep group). As shown in Fig. 2B, during the whole maturation period, each group (0 Lep or 10 Lep) was further treated with different concentrations of inhibitors for JAK2/STAT3 (Tyrphostin AG490) or MEK 1/2 (PD98059, MEK1 inhibitor, Cell Signalling Technology, Inc., Beverly, MA, USA) pathways. Tyrphostin AG490 is a JAK2 protein tyrosine kinase inhibitor that inhibits leptin action, ranging from 10 to 100 μ M in different cell types (Mansour *et al.* 2006, Sharma *et al.* 2006, Chen *et al.* 2007, Saxena *et al.* 2007). PD98059 [2-(2-amino-3-methoxyphenyl)-4h-1-benzopyran-4-one] has shown to act as a highly selective inhibitor of MEK1 activation and the MAPK cascade (Crews *et al.* 1992). PD98059 binds to MEK1 and

prevents activation by upstream activators such as c-Raf (Rosen *et al.* 1994). Concentrations for inhibitory activity against MEK1 and MEK2 are 10 and 50 μ M respectively.

Study of nuclear and cytoplasmic oocyte maturation by confocal microscopy

After the maturation period, COC were treated for the confocal study. First, cumulus cells were removed in PBS with 2 mM hyaluronidase by gentle pipetting. Next, oocytes were treated with 0.5% w/v pronase in PBS to digest the zonae pellucidae, fixed in PBS containing 4% w/v buffered neutral paraformaldehyde solution (pH 7.2–7.4) and stored in PBS. Oocytes were washed with permeabilisation solution (0.02% v/v Triton X-100 in PBS) and treated for 40 min with blocking solution (7.5% w/v BSA in PBS). They were then incubated for 30 min

Figure 3 Cortical granule distribution in rabbit oocytes after *in vitro* maturation period under confocal microscopy. (A) Peripheral CG distribution. (B) Cortical distribution. (C) Homogeneous distribution. (D) Abnormal distribution. Oocyte diameter is around 80 μ m.

at room temperature with 100 µg/ml FITC of *Lens culinaris* for CG staining and for 15 min at 39 °C with 10 µg/ml propidium iodide for nuclear staining. After that, oocytes were mounted between a coverslip and a glass slide supported by columns of paraffin and examined under a confocal laser scanning microscope (Leica, TCS SP5, Wetzlar, Germany). Complete nuclear maturation was measured in terms of MI rate. The rest of nuclear configurations were classified as oocytes that reinitiated the meiosis process (MI index) or not (GV rate). According to a previous work (Arias-Álvarez *et al.* 2009), CG distribution was classified as follows (Fig. 3): 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; D) non homogeneous or abnormal: anomalous distribution of CG compatible with poor quality or degenerated oocytes.

Determination of steroids in spent maturation media

Spent maturation media were collected after completion of oocyte maturation period and stored at –32 °C until analysed. Steroid concentrations were measured by ELISA based on the principle of competitive binding using a specific kit (Demeditec Diagnostics GmbH, Kiel, Germany) for OE₂ (OE₂-sensitive ELISA) and for P₄ (P₄ ELISA) quantification. Purified anti-OE₂ and anti-P₄ polyclonal antibodies were used. Intra- and inter-assay coefficients of variation were 5.5 and 6.8% for OE₂, and 5.4 and 9.9% for P₄, respectively. The assay ranges were 0–200 pg/ml (OE₂) and 0–40 ng/ml (P₄). Results are expressed as average OE₂ (pg/ml) and P₄ (ng/ml) concentrations produced by each COC after the IVM period.

Statistical analysis

Data were analysed using the SPSS program for Windows (SPSS 15.0, Inc., Chicago, IL, USA). χ^2 -test was carried out to compare different categories of nuclear maturation and CG migration rate between experimental groups. Mean OE₂ and P₄ concentrations in spent media by each COC were analysed with the ANOVA test (*post-hoc* was Duncan test) for the different IVM groups. All the results are expressed as the mean $\pm$ S.E.M., and statistical significance was accepted for $P < 0.05$.

Declaration of interest

The authors declare that there is no potential conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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