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PROGESTERONE IMPROVES PORCINE *IN VITRO* FERTILISATION SYSTEM

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In an effort to improve the quality of *in vitro* produced porcine embryos, the effect of progestagens – progesterone analogues – on the *in vitro* developmental competence of porcine oocytes was studied. A total of 1421 *in vitro* matured oocytes, from 4 replicates, were inseminated with frozen-thawed spermatozoa. Progestagens were added to late maturation and embryo cultures (10 IU/ml). Fertilisation success (pre-maturation, penetration, monospermy and efficiency) and nuclear maturation were evaluated. There were no differences among pre-maturation rates between groups ($P = 0.221$). Penetration rates were higher ($P < 0.001$) in the presence of progestagens (75.0%) as compared to the control (51.7%). However, no differences were observed in monospermy percentages ($P = 0.246$). The results indicated that supplementation with progestagens increased the efficiency of the *in vitro* fertilisation system ($P < 0.001$). An additional beneficial effect was observed in nuclear maturation with progestagens ($P = 0.035$). In summary, progestagen supplementation is an important factor to improve the *in vitro* fertilisation procedure.

Key words: *In vitro* fertilisation, oocyte maturation, progesterone, porcine

The overall efficiency of *in vitro* porcine embryo production and the quality of embryos are still low when compared with *in vivo* results (Funahashi, 2003). One of the major problems includes improper *in vitro* maturation (IVM) of oocytes, in both the nuclear and cytoplasmic compartments (Niwa, 1994; Marchal et al., 2001). While a large percentage of oocytes reached metaphase II after maturation, inadequate cytoplasmic and molecular maturation occurred (Sirard et al., 2006), leading to a lack of subsequent embryo development success. There is a lack of information on ovarian factors that may be important for the maturation and subsequent fertilisation of oocytes.

The production of sex steroids by follicular cells is proposed to be influenced by the maturity of oocytes. Oestradiol, progesterone and testosterone are the main steroid hormones that play an essential role during the follicular and

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luteal phases of the menstrual cycle (Wen et al., 2008). Many researchers have attempted to increase *in vitro* penetration percentages of porcine oocytes by adding PMSG (Funahashi and Day, 1993), hCG hormone (Raziel et al., 2006) or both (Funahashi et al., 1994; Wang and Niwa, 1995; Faerge et al., 2006; Silvestre et al., 2007). However, no studies have been conducted on the effects of progesterone on *in vitro* fertilisation (IVF) of porcine oocytes yet. Based on *in vivo* observations, high concentrations of progesterone are present in porcine follicular and utero-tubal fluids (Hunter et al., 1988), but the possible actions of progesterone during early embryo development are still undetermined. Hunter (1991) hypothesised that progesterone may modify the interaction of gametes. Raychoudhury and Suárez (1991) as well as Peters and Wells (1994) affirmed that this hormone is the signal to affect oviduct epithelial cells to release spermatozoa. There have been reports to suggest that progesterone plays an important role in capacitation, inducing acrosome reaction (Katayama et al., 2002).

Progesterone has also been identified as a hormone of pregnant mammals. Many structural and functional changes of the reproductive tract during pregnancy have been ascribed to progesterone. The present study was undertaken with the objectives of investigating if progesterone improves the commonly used porcine oocyte maturation and culture medium under the conditions of *in vitro* fertilisation.

Materials and methods

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich Chemicals (Alcohobendas, Madrid, Spain). The medium used for the collection and washing of cumulus-oocyte complexes (COCs) was Dulbecco's phosphate buffered saline medium (DPBSm) composed of 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 and 1.46 mM $CaCl_2 \times 2\ H_2O$, 0.34 mM sodium pyruvate, 5.4 mM glucose and 70 μg/ml kanamycin, adding 4 mg/ml bovine serum albumin (BSA; fraction V) just before use. The basic medium used for oocyte maturation was a modified North Caroline State University (NCSU)-23 supplemented with 10% (v/v) porcine follicular fluid, 0.8 mM cysteine and 10 ng/ml epidermal growth factor (EGF). The fertilisation medium was a modified Tris-buffered medium (mTMB) consisting of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \times 2\ H_2O$, 20 mM Tris, 11 mM glucose and 5 mM sodium pyruvate, supplemented with 2 mM caffeine and 0.2% BSA. The embryo culture medium was glucose-free NCSU-23 supplemented with sodium lactate, pyruvic acid and 0.4% BSA.

Oocyte collection and in vitro *maturation*

Ovaries of prepuberal gilts were collected at a slaughterhouse and transported to the laboratory in DPBSm at 33 °C. COCs were collected from follicles 2–6 mm in diameter. They were washed and matured for 22 h in 500 μl matura-

tion medium supplemented with 10 IU /ml of eCG and 10 IU/ml of hCG at 39 °C in an atmosphere of 5% CO_2 in air and 70% humidity. As the second step, they were subsequently cultured for 22 h in the chorionic gonadotropin free maturation medium, supplemented with 0 or 10 IU/ml of progestagens (Sinestro Neosan®, Pfizer, Spain). After maturation, oocytes were collected and the surrounding cumulus cells were removed by vortex in 0.1% hyaluronidase for 2 min. Matured oocytes were washed in fertilisation medium. Groups of 30 oocytes were placed into 50 µl droplets of the same medium for about 30 min until addition of spermatozoa. Resting oocytes were fixed after 48 h of maturation to determine nuclear maturation.

In vitro *fertilisation and culture*

For fertilisation, straws of ejaculated semen, previously cryopreserved using an adapted Westendorf et al. (1975) method as modified (Carvajal et al., 2004), were thawed at 37 °C for 21 sec and then resuspended in BTS (1:4, v/v; 37 °C). One hundred µl of thawed semen were washed by centrifugation at 1900 × *g* for 3 min in mDPBS. The pellet was resuspended in fertilisation medium and sperm concentration was adjusted to produce a final optimum ratio of 2000:1 spermatozoa/oocyte previously demonstrated for boars used in the experiments. Sperm and oocytes were co-incubated at 39 °C under 5% CO_2 in air for 6 h. Presumptive zygotes were removed from the fertilisation medium, washed three times in embryo culture medium and then transferred to a 500 µl drop of the same medium under mineral oil, and cultured at 39 °C in 5% CO_2 in air for 16–18 h to evaluate the fertilisation parameters.

Assessment of in vitro *fertilisation*

To evaluate *in vitro* fertilisation, presumptive zygotes and resting oocytes were then fixed for at least 72 h in slide fixing solution (1:3; acetic acid : ethanol) and then stained with Lacmoid (1%). Pre-maturation rate (oocytes in MII after 44 h maturation/total inseminated), penetration rate (presence of female pronucleus and one or more swollen sperm heads and/or male pronuclei with their corresponding head and two polar bodies/matured inseminated), monospermy rates (oocytes containing only one sperm head or male pronuclei/total penetrated) and efficiency of fertilisation (monospermic oocytes/total inseminated) were measured. Nuclear maturation was measured in resting oocytes.

Experimental design

In this experiment, the effects of progestagens on *in vitro* late maturation and fertilisation were examined in different *in vitro* systems. Control oocytes were matured with PMSG (eCG) and hCG for 22 h and subsequently matured without hormones for 22 h. After fertilisation, presumptive zygotes were cultured

in hormone-free media for 16–20 h. Oocytes from the test group were matured with PMSG and hCG for 22 h and subsequently matured with progestagens for 22 h. After fertilisation, presumptive zygotes were cultured in media supplemented with progestagens for 16–20 h. A total of 1421 oocytes from four replicates were used.

Statistical analysis

All analyses were performed using SPSS 15.0 for Windows. Percentage data were assessed using Pearson's chi-squared test. Differences were considered significant at $P < 0.05$.

Results

Effects of progestagens added to late maturation and culture media on fertility parameters

As shown in Table 1, the pre-maturation percentages (after a 44-h maturation period) did not differ significantly between the two groups. However, sperm penetration per oocyte was significantly higher ($P < 0.001$) in progestagen-cultured oocytes (75%) as compared in monospermy ($P = 0.246$). The addition of progestagens remarkably increased the efficiency of the IVF system ($P < 0.001$).

Table 1

Effect of progestagen supplementation during *in vitro* late maturation of porcine cumulus-oocyte complexes (COCs) and embryo culture on fertility parameters

Treatment	Number of oocytes	Pre-maturation (%)	Penetration (%)	Monospermy (%)	Efficiency (%)
Control	583	78.6	51.7[a]	87.8	35.7[a]
Progestagens	636	80.5	75.0[b]	89.9	51.7[b]
P value		0.221	< 0.001	0.246	< 0.001

[a, b]Values with different superscript letters within a column differ significantly ($P < 0.05$)

Effects of progestagens added to late maturation media on nuclear maturation

The rates of nuclear maturation of porcine oocytes after a 48-h maturation period are summarised in Table 2. The metaphase II formation was significantly ($P < 0.035$) higher in COCs that were matured in the presence of progestagens during the late maturation period (last 22 h) compared with the control COCs without progestagens (72.1% vs. 58.8%, respectively).

Table 2

Effects of progestagen supplementation during *in vitro* late maturation of porcine cumulus-oocyte complexes (COCs) on maturation rates

Treatment	Number of oocytes	Maturation (%)
Control	80	58.8[a]
Progestagens	122	72.1[b]
P value		0.035

[a, b]Values with different superscript letters within a column differ significantly ($P < 0.05$)

Discussion

In vitro production of porcine embryos by IVF is still inefficient compared with *in vivo* produced embryos (Abeydeera et al., 1998). This inefficiency is due to various causes such as a high rate of polyspermy and a low quality of blastocysts. The main factors affecting the yield and quality of pig blastocysts obtained *in vitro* are the culture conditions. The production of sex steroids by follicular cells is proposed to be influenced by the maturity of oocytes. The study demonstrated that progesterone had a marked effect on maturation and fertilisation *in vitro*. Supplementation of late maturation and culture media with 10 IU of progesterone enhanced penetration and efficiency of the IVF system. In addition, progesterone increased MII formation.

Several reports have investigated improving two commonly used porcine embryo culture media, NCSU-23 and 37 (Raychoudhury and Suárez, 1991; Kikuchi et al., 2002; Karja et al., 2004; Beebe et al., 2009). Very few experiments have studied the use of hormones in the maturation of porcine oocytes (Eroglu, 1993; Bing et al., 2001; Dode and Graves, 2002). The addition of a suitable hormone supplement to maturation media enhances nuclear maturation and cumulus expansion (Minato and Toyoda, 1982). However, subsequent studies demonstrated that the removal of hormones improved cytoplasm maturation (Funahashi and Day, 1993).

Dode and Graves (2002) have indicated that no sexual steroids need to be added to the maturation medium of pig oocytes because COCs secrete steroids during maturation. Moreover, the addition of steroids reduced the proportion of oocytes that reached MII (Singh et al., 1993; Bing et al., 2001). However, Kim et al. (2011) observed a positive effect of oestrogens on oocyte maturation and early embryo culture during the first half of the IVM period. Funahashi and Day (1993) demonstrated that 20 h of maturation with no hormone supplementation would be necessary to improve cytoplasmic maturation. Based on the regulated

sequence of hormonal changes during *in vivo* maturation, some reports established a two-step IVM system consisting of 22-h incubation with hCG and eCG followed by 22-h incubation with hormone-free media (Marques et al., 2007; Gil et al., 2010).

The discovery of the inhibitory effects of oestrogens by the addition of progesterone in previous reports (Li et al., 2004) was the basis of the present study. Progesterone is required for the maturation of rat (Zhang and Armstrong, 1989) and pig (Shimada and Terada, 2002) oocytes. In porcine (Eroglu, 1993) and bovine (Sirotkin, 1992) oocytes, the addition of progesterone to a maturation medium stimulated meiotic resumption. Our results are also consistent with these findings. The competence of monospermic fertility is dependent on the degree of cytoplasmic maturation in MII oocytes (Han et al., 1990). In the present study, the incidence of polyspermic penetration was very low in both groups. The mechanism of action of progesterone is not clear at present. Hunter (1991) hypothesised that progesterone may modify the interaction of gametes. Furthermore, progesterone reduces the expression of connexin 43, a gap protein, in the outer layer of cumulus cells, resulting in meiotic resumption (Shimada and Terada, 2002). In a previous study, Shimada and Terada (2001) demonstrated that the total amount of connexin 43 in cumulus cells was reduced after 12 h of culture, concomitantly with the activation of meiosis. In conformity with our results, Katayama et al. (2002) showed that progesterone induced acrosome reaction, promoting male pronucleus formation. Although progesterone is produced in media in which COCs were cultured with LH and FSH, supplementation in the last period of maturation improved penetration and the total efficiency of IVF. In contrast, recently Larson et al. (2011) have confirmed that the use of progesterone during the culture of *in vitro* produced embryos does not improve embryo characteristics.

In conclusion, the addition of progesterone to late maturation and culture media caused an improvement in maturation and an increase in penetration and efficiency of the IVF system. These results showed that a high level of progesterone is involved in the cytoplasmic maturation of porcine embryos.

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