Introduction

In mammalian preovulatory follicles, the LH surge initially stimulates the secretion of both androgens and oestrogens (Osborn and Moor, 1983; Dieleman et al., 1983a). Oestrogen concentrations decrease about 6 h after the LH surge, which is followed by a decrease in androgens and an increase in progesterone concentrations. By 18 h after the LH surge, progesterone constitutes about 90% of intrafollicular steroid content (Osborn and Moor, 1983). These changes in intrafollicular concentrations of steroids in vivo indicate that a precise balance or sequence of steroids may be necessary for the full maturation of follicle-enclosed oocytes. This hypothesis is supported by the findings that alterations in the normal profile of intrafollicular steroids, caused by inhibitors of follicular steroidogenesis, induce both biochemical and developmental abnormalities in oocytes (Moor et al., 1980).

Although it is recognized that oocytes require a specific steroid environment to achieve full maturation and developmental competence in vivo, little information is available on the effects of steroids, in particular androgens and progesterone, on in vitro oocyte maturation. Therefore, the aim of the present study was to assess the effects of aromatizable and non-aromatizable androgens (testosterone and dihydrotestosterone, respectively) and progesterone on in vitro oocyte maturation and subsequent competence for fertilization and embryonic development. Anti-androgens...
and anti-progestins have been developed to inhibit the binding of androgens and progesterone to the respective receptors, thereby blocking their biological actions (Neri et al., 1972; Hurd and Moudgil 1988). Thus, flutamide (an anti-androgen) and mifepristone (RU486; an anti-progestin) were also used to evaluate the effects of endogenous and exogenous androgens and progesterone on oocyte maturation. Given the evidence that inhibin-related peptides may play a role in bovine oocyte maturation (Stock et al., 1997; Silva and Knight, 1998; Silva et al., 1999), concentrations of total α-inhibin, inhibin A, activin A and follistatin in cumulus–oocyte complex (COC)-conditioned medium were also measured to assess any possible influence of progesterone and androgens on the secretion of inhibin-related peptides by cumulus cells.

Materials and Methods

In vitro maturation, in vitro fertilization and embryo culture

The methods used in the present study for in vitro maturation (IVM), in vitro fertilization (IVF) and embryo culture (EC) have been described by Silva and Knight (1998) and Silva et al. (1999). Briefly, bovine ovaries were collected from an abattoir and transported to the laboratory in a sterile saline solution supplemented with 1% (v/v) antibiotic and antifungal solution (Sigma, UK, Poole). COCs were obtained by aspiration of 2–10 mm follicles and washed in washing medium (WM) and maturation medium (MM). The WM consisted of TC-199 with Earle’s salts, sodium bicarbonate and 25 mmol l⁻¹ Hepes buffer (Sigma) and supplemented with 10% heat-inactivated calf serum (Sigma), penicillin (50 iu ml⁻¹) and streptomycin (50 μg ml⁻¹). The MM consisted of TC-199 (with Earle’s salts and sodium bicarbonate; Sigma) supplemented with 10% oestrous cow serum, L-glutamine (0.4 mmol l⁻¹), pyruvate (0.2 mmol l⁻¹), penicillin (50 μg ml⁻¹), streptomycin (50 μg ml⁻¹) and EGF (Folligon: 2.5 iu ml⁻¹; Intervet, Boxmeer). Only ‘good quality’ COCs with a complete corona layer and one or more compact cumulus cell layers were selected for these experiments. Groups of 20 COCs were allocated randomly into treatment groups and cultured in 60 μl droplets of MM under mineral oil for 22–24 h, at 38.5°C in a humidified atmosphere of 5% CO₂.

After maturation, COCs were washed in IVF–TALP (Earle’s Balanced Salt Solution (EBSS; Sigma) supplemented with gentamycin (50 μg ml⁻¹), NaHCO₃ (25 mmol l⁻¹), pyruvate (0.2 mmol l⁻¹), lactate (10 mmol l⁻¹), heparin (25 μg ml⁻¹), caffeine (1 mmol l⁻¹), hypotaurine (10 μmol l⁻¹), MgCl₂ (25 μg ml⁻¹) and BSA (3 mg ml⁻¹; fraction V; Sigma) and once in the IVF–TALP. The sperm pellet was then suspended in IVF–TALP medium at a concentration of 2 × 10⁶ spermatozoa ml⁻¹ before placing into the IVF drops (final concentration: 1 × 10⁶ spermatozoa ml⁻¹). The same ejaculate from one bull was used in all experiments. Gametes were incubated at 38.5°C in a humidified atmosphere of 5% CO₂ for 22–24 h.

Presumptive embryos were washed in ECM (TCM-199 with Earle’s salts and sodium bicarbonate and supplemented with 10% (v/v) heat-inactivated calf serum (Sigma), L-glutamine (0.4 mmol l⁻¹), pyruvate (0.2 mmol l⁻¹), lactate (10 mmol l⁻¹), penicillin (50 iu ml⁻¹) and streptomycin (50 μg ml⁻¹), and placed in co-culture drops (20 per drop) with granulosa cell monolayers. Granulosa cells were cultured for 8–11 days before being used in embryo co-culture. With the exception of the first experiment (effects of testosterone and dihydrotestosterone), the same batch of cryopreserved granulosa cells was used throughout these experiments.

Experiments

Depending on the number of ‘good quality’ COCs recovered from each batch of ovaries, between one (that is, 20 COCs) and four (that is, 80 COCs) culture drops were allocated per treatment on a given day. Cumulative data from a minimum of four individual batches of ovaries contributed to each set of experimental results.

Effects of testosterone and dihydrotestosterone. Testosterone and dihydrotestosterone were used to compare the effects of aromatizable and non-aromatizable androgens on oocyte maturation. Stock solutions were prepared by initially dissolving testosterone (Sigma) or 5α-dihydrotestosterone (Koch-light Laboratories Ltd, Colnbrook, Bucks) in ethanol at a concentration of 1 mg ml⁻¹. Further dilutions (100-fold) were made with ethanol to achieve a concentration of 10 μg ml⁻¹. Aliquots of these androgen solutions were dried down aseptically in a flow cabinet and an appropriate volume of MM was added to achieve the stock concentration of 600 nmol l⁻¹. Stock solutions were stored at -70°C. Ten microlitres of stock solutions were added to MM drops (final volume of 60 μl) to give a final concentration of 100 nmol l⁻¹. This selected dose concentration was considered ‘physiological’ as it was comparable with the concentration of testosterone in the follicular fluid of preovulatory bovine follicles (Henderson et al., 1982; Dieleman, 1983b; Prevost et al., 1989).

Effects of dihydrotestosterone and flutamide. The effects of dihydrotestosterone and an anti-androgen (flutamide) on oocyte maturation were studied. The dihydrotestosterone stock solution was prepared as described above and tested at the same dose concentration as testosterone. The flutamide stock solution was prepared by initially dissolving flutamide (2-methyl-N-[4-nitro-3-(trifluoromethyl)-phenyl]propanamide; Sigma, catalogue number F9397) in absolute ethanol at a concentration of 1 mg ml⁻¹. An aliquot of 300 μl was dried
down aseptically in a flow cabinet and 5 ml of MM was added to achieve the stock concentration of 217 μmol l⁻¹ (60 μg ml⁻¹). Both stock solutions were stored at −70°C. Ten microlitres of stock solutions were added to MM drops (final volume of 60 μl) to give final concentrations of 100 nmol dihydrotestosterone l⁻¹ and 36 μmol flutamide l⁻¹. The dose of flutamide (360-fold molar excess over dihydrotestosterone) was selected on the basis of previous reports of its relative anti-androgenic potency in various in vivo and in vitro bioassays (Neri et al., 1972; Simard et al., 1986). Treatments were as follows: (1) MM only (control); (2) MM plus dihydrotestosterone (100 nmol l⁻¹); (3) MM plus flutamide (36 μmol l⁻¹); and (4) MM plus dihydrotestosterone (100 nmol l⁻¹) plus flutamide (36 μmol l⁻¹).

Effects of progesterone and mifepristone (RU486). Progesterone and an anti-progestin, mifepristone (RU486), were used to test the effect of progesterone on oocyte maturation. The progesterone stock solution was prepared by dissolving progesterone (Sigma) in absolute ethanol at a concentration of 3 mg ml⁻¹. Further dilutions (100-fold) were made with ethanol to achieve a concentration of 30 μg ml⁻¹. An aliquot of 94.4 μl was dried down in a flow cabinet and 5 ml MM was added to achieve the stock concentration of 1.8 μmol l⁻¹ (566 ng ml⁻¹). A stock solution of 600 nmol RU486 l⁻¹ in MM was prepared by diluting a preparation containing 0.1 mmol RU486 l⁻¹ in 10% ethanol (provided by P. A. Fowler, University of Aberdeen). Both stock solutions were stored at −70°C. Ten microlitres of stock solutions were added to MM drops (final volume of 60 μl) to give final concentrations of 300 nmol progesterone l⁻¹ and 100 nmol RU486 l⁻¹. The dose concentration of progesterone was compared with intrafollicular concentrations in preovulatory bovine follicles (Henderson et al., 1982; Dieleman, 1983b; Prevost et al., 1989). The concentration of RU486 was selected on the basis of its relative binding affinity for the progesterone receptor is several-fold greater than that of progesterone (Etgen and Barfield, 1986; Hurd and Moudgil 1988; Skafer 1991). Treatments were as follows: (1) MM only (control); (2) MM plus progesterone (300 nmol l⁻¹); (3) MM plus RU486 (100 nmol l⁻¹); and (4) MM plus progesterone (300 nmol l⁻¹) plus RU486 (100 nmol l⁻¹).

Hormone determinations

Secretion of total immunoreactive (ir)-α-inhibin, inhibin A, activin A and follistatin by groups of COCs during the 24 h in vitro maturation period was measured to determine whether exogenous steroids and anti-steroids affected the secretion of endogenous hormones. The concentration of each peptide was undetectable in unconditioned MM. After COC maturation, 40 μl of medium (COC-conditioned medium) was collected from each drop and frozen for hormone assay.

Total ir-α-inhibin. Concentrations of total ir-α-inhibin were measured using the competitive radioimmunoassay reported by Knight et al. (1989) and Beard et al. (1990). Purified bovine 32 kDa inhibin A was used as the standard and the sensitivity of the assay was approximately 1 ng ml⁻¹. Mean intra- and interassay coefficients of variation calculated from four assays were 8.3 and 14%, respectively.

Inhibin A. Inhibin A concentrations were determined using the two-site immunoradiometric assay (IRMA) described by Knight and Muttukrishna (1994). Purified bovine 32 kDa inhibin A was used as the standard and gave a dilution curve parallel to those for COC-conditioned media and bovine follicular fluid (bFF). The detection limit was 500 pg ml⁻¹ and mean intra- and interassay coefficients of variation calculated from four assays were 5.3 and 5.4%, respectively.

Activin A. Total activin A (free activin and follistatin-bound activin) concentrations in COC-conditioned media were determined using the two-site enzyme immunoassay (ELISA) described by Knight et al. (1996). Human recombinant (hr) activin A (gift from NHPP, Torrance, CA) was used as the standard and gave a dilution curve parallel to those for COC-conditioned media, bovine granulosa cell-conditioned culture media and bFF. Sensitivity was approximately 100 pg ml⁻¹ and mean intra- and inter-assay coefficients of variation were 6.1 and 11%, respectively (n = 5 assays).

Follistatin. Total follistatin was determined using the ELISA described by Tannetta et al. (1998). Pooled bFF was used as the working standard, since hr follistatin gave a dilution curve steeper than those for bFF- and COC-conditioned media. One millilitre of bFF working standard was found to be equivalent to 3.93 μg of hr follistatin (Tannetta et al., 1998). Consequently, follistatin concentrations are expressed here in terms of the widely available hr-follistatin preparation provided by NHPP (Torrance, CA). The sensitivity was equivalent to approximately 100 pg ml⁻¹ and mean intra- and inter-assay coefficients of variation calculated from five assays were 7.3 and 9.6%, respectively.

Statistical analysis

The proportions of oocytes or cleaved oocytes reaching defined developmental stages were transformed using the Freeman and Tukey transformation (Zar, 1996) before statistical analysis, but are presented in tables as percentages (± SEM) for clarity. The effects of each steroid and anti-steroid treatment (testosterone, dihydrotestosterone, progesterone, RU486 and flutamide) were compared and analysed by one-way ANOVA. Where a significant F ratio was obtained, post-hoc protected least significant difference (PLSD) test was used to compare individual means.

Results

Effects of testosterone and dihydrotestosterone

Both testosterone and dihydrotestosterone at the dose concentration tested (100 nmol l⁻¹) significantly (P < 0.001) increased oocyte cleavage rate (Table 1). Dihydrotestosterone
also increased the proportion of oocytes that reached at least the eight-cell stage. However, neither blastocyst yield nor the proportion of blastocysts that hatched were affected by testosterone or dihydrotestosterone (Table 1).

The addition of testosterone to the COC maturation medium significantly (P < 0.05) decreased endogenous production of follistatin, thereby increasing the activin A: follistatin ratio in COC-conditioned medium (Fig. 1). In contrast, neither endogenous follistatin nor the activin A: follistatin ratio was significantly changed by the same dose of dihydrotestosterone (100 nmol l⁻¹). Production of α-inhibin, inhibin A and activin A were not significantly changed by the addition of testosterone or dihydrotestosterone.

**Effects of dihydrotestosterone and flutamide**

As in the previous experiment, dihydrotestosterone significantly (P < 0.05) increased oocyte cleavage rate (Table 2). Flutamide, an anti-androgen, did not affect cleavage rate compared with controls but reduced the stimulatory effect of dihydrotestosterone on cleavage rate. The proportions of oocytes developing to ≥ eight-cell or blastocyst stages and the blastocyst hatching rate (the proportion of blastocysts that hatched) were not affected by the different treatments.

No significant changes in secretion of total α-inhibin, inhibin A, activin A and follistatin were found after addition of dihydrotestosterone or flutamide to COCs (Fig. 2). However, concentrations of α-inhibin and inhibin A tended to be higher in COC-conditioned medium when dihydrotestosterone was added, either alone or in combination with flutamide.

**Effects of progesterone and mifepristone (RU486)**

The addition of progesterone or the anti-progestin, mifepristone (RU486) did not significantly affect oocyte cleavage rate (Table 3). In contrast, the proportions of both total oocytes and cleaved oocytes reaching the blastocyst stage were significantly reduced (an approximately 40% reduction; P < 0.05) by the addition of progesterone (300 nmol l⁻¹) to MM (Table 3). The anti-progestin (RU486) did not significantly affect blastocyst yield when compared with controls, but partially reversed the negative effect of progesterone. Therefore, when both progesterone and RU486 were added to MM, the proportion of cleaved oocytes reaching the blastocyst stage was significantly increased when compared with the progesterone treatment group, but not when compared with controls (Table 3). Hatching rate was not significantly affected by the different treatments, although this tended to be lower with the progesterone treatment and higher with RU486 treatment.

Addition of progesterone to MM significantly (P < 0.05) increased the production of total α-inhibin by COCs (Fig. 3). No significant changes in the concentrations of inhibin A, activin A and follistatin were found after addition of progesterone or RU486 (Fig. 3).

**Discussion**

The results of the present study show that addition of androgens (testosterone and dihydrotestosterone) to MM can stimulate bovine oocyte maturation in vitro, as reflected by a subsequent increase in cleavage rate after IVF. The stimulatory effect of testosterone is unlikely to be mediated by its conversion to oestradiol by granulosa cell aromatase, since dihydrotestosterone, a non-aromatizable androgen, had a similar effect and this was partially reversed by the androgen antagonist, flutamide.

Previous studies in other species reported that testosterone exerted an inhibitory effect on oocyte meiotic maturation and embryonic development (Smith and Tenney, 1980; Ecay and Powers, 1990; Anderiesz and Trounson, 1995). However, the concentrations of testosterone that exerted such effect were ≥ 40 μmol l⁻¹, very much higher than the dose tested in the present study (100 nmol l⁻¹) which was selected on the basis of physiological concentrations reported in preovulatory bovine follicles (approximately 165 nmol l⁻¹; Dieleman et al., 1983b). Although androgens increased the cleavage rate, the proportion of cleaved oocytes reaching the blastocyst stage was not affected. Although the difference was not significant, the total number of blastocysts obtained when COCs were cultured...
in the presence of androgens was higher, supporting the hypothesis that androgens, at the dose studied (100 nmol l⁻¹), have no deleterious effect on oocyte competence for post-cleavage development.

The production of follistatin by COCs decreased when androgens were added to MM, thereby decreasing the activin A:follistatin ratio. However, it is unlikely that such an increase in endogenous free activin A was the cause of the increase in cleavage rate, since no significant increase in the activin A:follistatin ratio was observed after dihydrotestosterone treatment. Furthermore, neither activin A concentration nor the activin A:follistatin ratio in COC-conditioned MM were found to affect cleavage rate after fertilization (Silva and Knight, 1998). Likewise, the stimulatory effect of androgens on the oocyte cleavage rate is unlikely to be mediated by inhibin-related proteins, as no significant changes in total α-inhibin or inhibin A were induced by the androgens. The detection of androgen receptors in the nuclei of both granulosa cells and oocytes (Hild-Petito et al., 1991) indicates that androgens may influence both cumulus cells and oocytes. In addition, the study of Anderiesz and Trounson (1995) showed a direct effect of testosterone in denuded oocytes, demonstrating that androgens can act directly on oocytes.

The observed increase in the activin A:follistatin ratio induced by testosterone may be a consequence of the aromatization of testosterone by the cumulus cells. If this is the case, it is possible that the beneficial effect of the inclusion of oestradiol in bovine IVM medium reported by some authors (Gordon, 1994; Bevers et al., 1997) is the result of a stimulatory effect of the steroid on free activin A secretion by COCs. Unfortunately, there was insufficient volume of COC-conditioned MM available in the present study to attempt to compare oestradiol concentrations in control and testosterone-treated groups.

The stimulatory effect of androgens on cleavage rate after fertilization was confirmed in the second experiment. The anti-androgen flutamide had no effect on oocyte cleavage rate, presumably reflecting the absence of endogenous androgen secretion by the cumulus cells. However, when flutamide was added in combination with dihydrotestosterone to COC maturation medium, the stimulatory effect of dihydrotestosterone on cleavage rate was reduced. Similarly, the non-significant increase in the total number of blastocysts obtained when COCs were cultured in the presence of dihydrotestosterone was also reversed by the addition of the anti-androgen. Blastocyst quality was not

Table 2. Effects of dihydrotestosterone (DHT) and flutamide present during in vitro maturation of bovine cumulus–oocyte complexes on embryonic development

<table>
<thead>
<tr>
<th>Culture treatment</th>
<th>Number of oocytes cultured (n)</th>
<th>Percentage of oocytes developing to</th>
<th>Percentage of cleaved oocytes developing to</th>
<th>Percentage of blastocysts hatching</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≥ two-cell</td>
<td>≥ eight-cell</td>
<td>≥ eight-cell Blastocyst</td>
</tr>
<tr>
<td>Control</td>
<td>220 (11)</td>
<td>58 ± 2a 40 ± 2</td>
<td>21 ± 2</td>
<td>63 ± 7</td>
</tr>
<tr>
<td>DHT</td>
<td>219 (11)</td>
<td>68 ± 2b 45 ± 2</td>
<td>26 ± 2</td>
<td>68 ± 6</td>
</tr>
<tr>
<td>Flutamide</td>
<td>219 (11)</td>
<td>58 ± 5 36 ± 4</td>
<td>23 ± 4</td>
<td>67 ± 4</td>
</tr>
<tr>
<td>DHT + flutamide</td>
<td>220 (11)</td>
<td>62 ± 2b 42 ± 2</td>
<td>20 ± 2</td>
<td>60 ± 6</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

*Number of replicate culture drops.
Different superscripts within a column indicate significant (P < 0.05) differences between treatment groups.

Table 3. Effects of progesterone and anti-progestin (RU486) present during in vitro maturation of bovine cumulus–oocyte complexes on embryonic development

<table>
<thead>
<tr>
<th>Culture treatment</th>
<th>Number of oocytes cultured (n)</th>
<th>Percentage of oocytes developing to</th>
<th>Percentage of cleaved oocytes developing to</th>
<th>Percentage of blastocysts hatching</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≥ two-cell</td>
<td>≥ eight-cell</td>
<td>≥ eight-cell Blastocyst</td>
</tr>
<tr>
<td>Control</td>
<td>140 (7)</td>
<td>61 ± 2 43 ± 2</td>
<td>21 ± 3a</td>
<td>66 ± 10</td>
</tr>
<tr>
<td>Progesterone</td>
<td>140 (7)</td>
<td>62 ± 4 36 ± 5</td>
<td>13 ± 2b</td>
<td>62 ± 10</td>
</tr>
<tr>
<td>RU486</td>
<td>139 (7)</td>
<td>57 ± 3 37 ± 5</td>
<td>23 ± 4ab</td>
<td>72 ± 7</td>
</tr>
<tr>
<td>Progesterone + RU486</td>
<td>140 (7)</td>
<td>61 ± 3 40 ± 3</td>
<td>17 ± 4ab</td>
<td>60 ± 11</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

*Number of replicate culture drops.
Different superscripts within a column indicate significant (P < 0.05) differences between treatment groups.
affected by the different treatments, as demonstrated by the similar proportion of blastocysts that hatched in each case. Therefore, the inclusion of androgens (testosterone or dihydrotestosterone) in bovine COC maturation medium at a concentration of 100 nmol l–1 may be a way of improving fertilization and cleavage rates, in particular when cleavage rates are relatively low.

It is well established that intrafollicular progesterone concentration in the dominant follicle increases after the preovulatory surge of LH (Osborn and Moor, 1983; Gordon 1994). In the present study, addition of exogenous progesterone to MM at a concentration comparable with that occurring in preovulatory bovine follicles clearly reduced oocyte competence for development after cleavage. The reduction in both the proportion of total oocytes and cleaved oocytes that reached the blastocyst stage, induced by the addition of progesterone, was partially reversed by the addition of an anti-progestin (RU486). In a previous study that used a different anti-progestin (onapristone), Cavaco-Gonçalves et al. (1997) reported that, although anti-progestins reduced fertilization in vivo, they had no effect on oocyte nuclear maturation. This finding was supported in the present in vitro experiment, as cleavage rate and blastocyst yield were not diminished by the anti-progestin treatment.

Since the addition of progesterone to COC maturation medium induced a significant increase in endogenous secretion of total α-inhibin, it is possible that the detrimental effect of progesterone on oocyte maturation is mediated by α-inhibin. As reported by Silva et al. (1999), pro-αC and possibly

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**Fig. 1.** Effects of testosterone (□) and dihydrotestosterone (■) added to the maturation medium on endogenous concentrations of (a) total α-inhibin, (b) inhibin A, (c) activin A, (d) follistatin, and (e) the ratio of activin A:follistatin produced by each group of 20 bovine cumulus-oocyte complexes. Values are means ± SEM (n = 16–20). *P < 0.05 compared with controls (□).
other forms of free inhibin α-subunit present during COC maturation reduce oocyte development after cleavage. However, a direct action of progesterone on the oocyte cannot be excluded, as total α-inhibin concentrations induced by the presence of progesterone were not significantly different from those in the anti-progestin treatment group.

As was demonstrated in the previous experiments, blastocyst quality was not compromised by the different treatments since the proportion of blastocysts that hatched in each group was not significantly different from those in the anti-progestin treatment group.

In conclusion, the present results demonstrate that the presence of both aromatizable and non-aromatizable androgens during bovine IVM increases oocyte cleavage rate without affecting embryo development after cleavage. In contrast, physiological concentrations of progesterone reduce oocyte maturation by reducing embryo development after cleavage, an effect that is reversed by anti-progestins (RU486). These effects of steroids on oocyte maturation are unlikely to be mediated by changes in the secretion of inhibin-related proteins by cumulus cells, although progesterone did increase total α-inhibin secretion by COCs, consistent with the recent

Fig. 2. Effects of dihydrotestosterone (DHT) and flutamide (Fl) and dihydrotestosterone plus flutamide (DHT+Fl) added to the maturation medium on endogenous concentrations of (a) total α-inhibin, (b) inhibin A, (c) activin A, (d) follistatin, and (e) the ratio of activin A:follistatin produced by each group of 20 bovine cumulus–oocyte complexes. □, control. Values are means ± SEM (n = 11).
finding of Silva et al. (1999) that inhibin α-subunit also has a detrimental effect on bovine oocyte maturation in vitro.

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