INFLUENCE OF HUMAN AND BOVINE INSULIN ON IN VITRO MATURATION, FERTILIZATION AND CLEAVAGE RATES OF BOVINE OOCYTES

INFLUENCIA DE LA INSULINA BOVINA Y HUMANA SOBRE LOS ÍNDICES DE MADURACIÓN, FECUNDACIÓN Y DIVISIÓN IN VITRO DE OVOCITOS DE BOVINO

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ADDITIONAL KEYWORDS

SUMMARY
The present study was carried out to determine the effects of supplementation of the maturation media with human or bovine insulin on in vitro maturation, fertilization and cleavage rates of bovine oocytes. Cumulus-intact bovine oocytes were cultured in a maturation medium (TCM-199 containing 10 percent fetal calf serum) with or without human or bovine insulin supplementation (10 μg/ml). For the bovine insulin supplement, the maturation (80.3 percent), fertilization (61.3 percent) and cleavage (55.3 percent) rates were significantly higher (p<0.05) than those obtained in the control group (70.1; 50.1 and 42.5 percent respectively). Thus, the percentages of cleaved ova obtained in presence of human or bovine insulin (54.8 and 55.3 percent respectively) were significantly higher (p<0.05) than those observed in control group (42.5 percent). No difference was found among human and bovine insulin treatments. These results demonstrate that the addition of human or bovine insulin to the maturation medium increased the percentages of matured oocyte, increasing the subsequent fertilization and cleavage rates of bovine oocytes in vitro.

RESUMEN
Se realizó un estudio para determinar los efectos de la suplementación del medio de maduración con insulina humana o bovina sobre los índices de maduración, fecundación y división in vitro de ovocitos de bovino. Ovocitos de bovino se cultivaron en medio de maduración (TCM-199

conteniendo 10 p.100 de suero de ternero fetal) con o sin insulina humana o bovina (10 µg/ml). Para la insulina bovina, los índices de maduración (80,3 p.100), fecundación (61,3 p.100) y división (55,3 p.100) fueron superiores significativamente (p<0,05) que aquellos obtenidos por el grupo control (70,1; 50,1 y 42,5 p.100 respectivamente). Además, los porcentajes de ovulos divididos obtenidos en presencia de insulina humana o bovina (54,8 y 55,3 p.100 respectivamente), fueron superiores significativamente (p<0,05) en comparación con los obtenidos por el grupo control (42,5 p.100). No se apreciaron diferencias significativas entre los tratamientos con insulina humana y bovina. Estos resultados ponen de manifiesto que la adición de insulina humana o bovina al medio de maduración incrementa los porcentajes de ovocitos maduros, mejorando posteriormente los índices de fecundación y división de ovocitos de bovino in vitro.

INTRODUCTION

Embryos may be obtained from a wide range of mammalian species by flushing the reproductive tract or produced from the recovery of mature or immature oocytes from antral follicles in the ovary. Once that oocytes are fully grown, they may be matured, fertilized and cultured in vitro, producing viable embryos with full developmental potential.

It is known that follicular development is controlled by various factors (Gonadotrophins, steroids, growth factors) of endocrine and paracrine origin (Ireland, 1987; Webb et al., 1994). Several studies have indicated that insulin and insulin-like growth factor-I (IGF-I) stimulate the proliferation of granulosa cells and the production of progesterone (Gong et al., 1993; Spicer et al., 1993). Therefore, it is expected that insulin and the growth factor IGF-I have some beneficial effects on in vitro maturation of bovine oocytes. Some studies showed that supplementation of the maturation medium with insulin improved cumulus expansion and oocyte fertilizability in vitro (Lorenzo et al., 1994; Webb et al., 1994), but other reports showed that insulin had no significant effect on the fertilization rate or morula formation (Stubbings et al., 1990). The role of insulin on in vitro maturation, fertilization and cleavage of bovine oocytes has not been fully understood.

The aim of the present study was to obtain information about the effects of supplementation of the maturation media with human or bovine insulin on in vitro maturation, fertilization and cleavage rates of bovine oocytes.

MATERIALS AND METHODS

Collection and preparation of oocytes for maturation

Ovaries were collected from cows at a local slaughterhouse, put into physiological saline (0.9 percent, w/v, NaCl) with antibiotics (100 µg/ml streptomycin, 100 IU/ml penicillin and 0.25 µg/ml anphotericin) maintained at 30 to 37°C, and transported to the laboratory within 1 to 2 h of slaughter. The cumulus-oocyte complexes were aspirated from small antral follicles of 1 to 5 mm of diameter with an 18-ga needle attached to a 5 ml syringe containing PBS (phosphate buffered saline supplemented with 5 percent (v/v) heat-inactivated fetal calf serum
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(FCS) and antibiotics. After washing once in Hank's salt solution and twice in maturation medium, oocytes with compact cumulus cells were placed in a Falcon plastic culture dish (3.5 x 1.0 cm) containing 2 ml of maturation medium and cultured for 24 h at 39°C under 5 percent CO₂ in air. The basic maturation medium was TCM-199 with Earle's salts and Hepes (Sigma Chemical Company, St. Louis, MO, USA) plus 0.2 percent L-glutamine, 0.02 percent of an antibiotic-antimycotic solution (100 μg/ml streptomycin, 100 IU/ml penicillin and 0.25 μg/ml anphotericin; Sigma) and supplemented with 10 percent fetal calf serum (FCS).

SPERM CAPACITATION AND IN VITRO FERTILIZATION

For washing spermatozoa, 30 and 45 percent isotonic Percoll solutions were prepared by diluting of 90 percent isotonic Percoll solution (Percoll, Pharmacia LBK Biotecnology AB, Uppsala, Sweden). Then, 2 ml of 30 percent Percoll solution was placed on 2 ml of 45 percent Percoll in a 10 ml test tube. For the preparation of capacitated spermatozoa, two straws of frozen semen were thawed for 5 seconds in 39°C water, and 2 ml of semen were deposited on the upper layer of the Percoll gradient solution. The tube was centrifuged for 15 min at 700 x g. The sedimented spermatozoa displaying good motility in the bottom of tube were resuspended in 1 ml of H-TALP medium containing 0.6 percent BSA (Sigma), 5 mM caffeine and 100 μg/ml heparin (Sigma) and were incubated for 15 min in 5 percent CO₂ incubator for capacitation. 300 μl of the capacitated sperm suspension were added to 1 ml of freshly prepared fertilization medium (TCM-199 supplemented with 10 percent FCS), containing 20-40 matured oocytes at a concentration of 1-2x10⁶ total spermatozoa/ml and cultured for 24 h at 39°C under 5 percent CO₂ in air.

IN VITRO ZYGOTE CULTURE

Twenty-four hours after gamete co-culture, the oocytes were gently washed twice in Hank's solution in a 10 ml plastic tube in order to remove the cumulus cells and free spermatozoa, and then transferred to a culture dish containing 1 ml of TCM-199 supplemented with 10 percent FCS and incubated for 96 h at 39°C under 5 percent CO₂ in air. The culture was observed every 12 h by microscope in order to evaluate the cleavage stage.

EXPERIMENTAL DESIGN

The influence of human and bovine insulin added to the maturation medium on the in vitro nuclear maturation rate and subsequent fertilization and cleavage rates of matured oocytes was evaluated. TCM-199 containing 10 percent FCS was used as maturation medium. COCs were matured in the maturation medium with or without 10 µg/ml of human (Humulina NPH 40 IU, Eli Lilly and Company, Indianapolis, USA) or bovine (Sigma) insulin supplementation. After maturation period, some oocytes were cytogenetically processed to evaluate the nuclear maturation stage, the remaining oocytes were fertilized and cultured for developing. Finally, the presumptive zygotes were cultured for 96 h in TCM-199 medium.
**Table I. Effect of supplementation of the maturation medium with human or bovine insulin on in vitro maturation rates of bovine oocytes.** (Efecto de la suplementación del medio de maduración con insulina humana o bovina sobre los índices de maduración in vitro de ovocitos de bovino).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nº of trials</th>
<th>Nº oocytes cultured</th>
<th>Nuclear maturation stage (percent)</th>
<th>Germinal vesicle</th>
<th>Metaphase-I</th>
<th>Metaphase-II</th>
<th>Degenerated</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI</td>
<td>5</td>
<td>200</td>
<td>8.2±2.0</td>
<td>3.4±1.4</td>
<td>80.3±4.3</td>
<td>8.1±1.2</td>
<td></td>
</tr>
<tr>
<td>HI</td>
<td>5</td>
<td>203</td>
<td>7.2±1.7</td>
<td>5.0±1.5</td>
<td>78.5±4.1</td>
<td>9.3±2.1</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>201</td>
<td>13.3±2.5</td>
<td>6.2±1.8</td>
<td>70.1±4.0</td>
<td>10.4±2.2</td>
<td></td>
</tr>
</tbody>
</table>

BI=Bovine insulin; HI=Human insulin. Data are showed as mean percentages±SEM from 5 replicates. 

*C,D* Mean values in the same column with different superscripts differ significantly at p<0.05, x² test.

supplemented with 10 percent FCS and incubated at 39°C under 5 percent CO₂ in air. After 96 h in culture, the zygotes were cytogenetically analysed to evaluate the cleavage stage.

**CHROMOSOME PREPARATION OF THE OOCYTES AND ZYGOTES**

At the end of the culture period for maturation, fertilization and cleavage, oocytes or zygotes were transferred to 3 ml conical tubes and vortex-agitated for 2 min in trisodium citrate (0.88 percent) and a hypotonic-trypsin (0.02 percent) solution. After a slight agitation to remove the cumulus oophorus cells, denuded oocytes were transferred to a culture plate containing 2 ml of the same hypotonic solution without trypsin for 45-60 min. The oocytes were fixed in an initial fixing solution of 1:1 methanol:acetic acid for 5 min. Then, they were fixed in a second solution of 3:1 methanol:acetic acid for 24 h. Finally, the oocytes were mounted on slides, stained with 5 percent Giemsa and examined with the light microscope at 400 and 1500 x magnification for evaluation of maturation.

**CRITERIA FOR MATURATION, FERTILIZATION, CLEAVAGE AND STATISTICAL ANALYSIS**

Oocytes were morphologically evaluated for stage of maturation after culture. The meiotic progress of oocytes was classified as follows: (1) germinal vesicle stage, an intact nuclear membrane with meiotically inactive chromatin; (2) Metaphase I, the nuclear membrane was broken and a chromatin pattern characteristic of an oocyte resuming meiosis was present; (3) Metaphase II, a polar body present within the perivitelline space and the maternal chromatin complement was identified in the oocyte; and (4) Degenerative, oocytes with a cytoplasm vacuolated or fragmented. Fertilization stage oocytes were classified as follows: (1) oocytes without both male and female pronuclei were judged as unfertilized; (2) ova with both male and female pronuclei and with residual sperm-tail...
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**Table II.** Effects of supplementation of the maturation medium with bovine or human insulin on in vitro fertilization rates of bovine oocytes. (Efectos de la suplementación del medio de maduración con insulina humana o bovina sobre los índices de fecundación in vitro de ovocitos de bovino).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Nº of trials</th>
<th>Nº oocytes inseminated</th>
<th>No fertilized</th>
<th>Polyspermic</th>
<th>Fertilized</th>
<th>Degenerated</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI</td>
<td>5</td>
<td>203</td>
<td>19.5±2.4</td>
<td>5.1±2.0</td>
<td>61.3±4.8</td>
<td>14.1±1.3</td>
</tr>
<tr>
<td>HI</td>
<td>5</td>
<td>204</td>
<td>21.4±2.2</td>
<td>5.5±2.1</td>
<td>57.4±3.3</td>
<td>15.7±2.3</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>201</td>
<td>29.1±3.1</td>
<td>4.8±1.9</td>
<td>50.1±2.3</td>
<td>16.0±3.5</td>
</tr>
</tbody>
</table>

BI=Bovine insulin; HI=Human insulin. Data are showed as mean percentages±SEM from 5 replicates. c,d Mean values in the same column with different superscripts differ significantly at p<0.05, χ² test.

In experiment 2, the effects of the insulin supplementation of the maturation medium on in vitro fertilization rate of bovine oocytes were studied. Data obtained in this experiment and shown in table II indicated that the oocytes group matured in presence of bovine insulin (80.3 percent) was significantly higher (p<0.05) than those obtained in the control group (70.1 percent). In the same way, the oocyte group matured in presence of human insulin obtained a maturation rate slightly higher (78.5 percent) than that of control group, although there was no statistically significant difference among both groups. No significant differences (p>0.05) were observed between the two insulin supplemented oocyte groups.

The results are expressed as the mean percentage ± SEM from five replicates. Unrelated two-way ANOVA was used to compare the individual supplements and the appropriate control for significance of difference.

**RESULTS**

As shown in table I, after 24 h of culture, the maturation rate obtained in the group of oocytes cultured in presence of bovine insulin (80.3 percent) was significantly higher (p<0.05) than those obtained in the control group (70.1 percent). In the same way, the oocyte group matured in presence of human insulin obtained a maturation rate slightly higher (78.5 percent) than that of control group, although there was no statistically significant difference among both groups. No significant differences (p>0.05) were observed between the two insulin supplemented oocyte groups.
**Table III. Effect of the supplementation of the maturation medium with bovine or human insulin on in vitro cleavage rates of bovine oocytes.** (Efectos de la suplementación del medio de maduración con insulina humana o bovina sobre los índices de división in vitro de ovocitos de bovino).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Nº of trials</th>
<th>Nº oocytes inseminated</th>
<th>Development stage (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No cleaved</td>
</tr>
<tr>
<td>BI</td>
<td>5</td>
<td>205</td>
<td>24.6±2.5</td>
</tr>
<tr>
<td>HI</td>
<td>5</td>
<td>201</td>
<td>26.5±2.6</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>202</td>
<td>32.2±2.9</td>
</tr>
</tbody>
</table>

BI=Bovine insulin; HI=Human insulin. Data are showed as mean percentages±SEM from 5 replicates.

**DISCUSSION**

There are several reports of pregnancies from IVF of in vitro matured bovine oocytes (Lu et al., 1987; Utsumi et al., 1988). However, the yield of preimplantation embryos from oocytes matured, fertilized, and cultured in vitro is still quite low.

In our study, the addition of human and bovine insulin to the maturation medium showed a positive effect on in vitro maturation, fertilization, and cleavage of bovine oocytes matured in vitro, when oocytes were cultured in TCM-199 medium supplemented with FCS. Our results are in disagreement with previous reports. Stubbings et al. (1990) reported that bovine insulin (1-1000 ng/ml) has no effect on in vitro maturation when bovine oocytes are cultured in TCM-199 medium containing FCS, gonadotrophins and E2. Matsui et al. (1995a) also reported that insulin (10 mg/ml) had no effect on the maturation, fertilization and cleavage rates of bovine oocytes. However, Zhang et al. (1991) reported that the addition of insulin (1 μg/ml) to the maturation medium which did not contain gonadotrophins or estradiol improved in vitro fertilization rate of bovine oocytes.

It is known that bovine insulin (0.1-10 mg/ml) enhances the mitosis of bovine granulosa cells (Spicer et al., 1993). Gong et al. (1993) reported that this stimulating effect of insulin on the proliferation of bovine granulosa cell
is synergistic with gonadotrophins. Furthermore, Spicer et al. (1993) found that insulin increases progesterone and estradiol production by bovine granulosa cells in the presence of FSH.

In experiment 2, human and bovine insulin increased the in vitro fertilization rates. Similar results were found by Matsui et al. (1995a). These authors suggest that the supplementation of the maturation medium with bovine insulin improves the fertilization rate by the effect of insulin on the cumulus cells. Ball et al. (1982) reported that cumulus cells surrounding bovine oocytes synthesize glycosaminoglycans (GAGs) such as hyaluronic acid. This acid has been reported to induce the acrosome reaction of bovine sperm (Handrow et al., 1982). Matsui et al. (1995a) postulated that the addition of insulin to the maturation medium stimulates GAGs secretion from cumulus cells and improves the fertilization rate as a result of the promotion of cumulus-induced sperm capacitation.

In experiment 3, human and bovine insulin had a positive effect on in vitro cleavage rate of bovine oocytes matured in vitro. Similar results were reported by Matsui et al. (1995b) and Liu et al. (1995). Insulin has been shown to enhance in vitro development of bovine embryos (Matsui et al., 1995b). It has been reported that the synergistic beneficial effect of insulin and amino acids on the development of bovine embryos into the morula stage may be caused by facilitating the transport of amino acids by insulin (Kaye et al., 1986; Matsui et al., 1995b). Insulin and IGF-I may be related to the quantitative and qualitative changes in protein synthesis or to the onset of new RNA synthesis between 8- and 16-cell stage which may arise by transcriptional shifts. Insulin stimulated amino acids transport (Kaye et al., 1986), mitosis (Gardner and Kaye, 1991), and protein synthesis in mouse embryos (Harvey and Kaye, 1988). It also stimulated development of pig (Saito and Niemann, 1991), Guinea pig (Travers et al., 1992), and bovine (Matsui et al., 1995b) embryos. It is suggested that insulin might support cell proliferation of bovine embryos by promoting the transport of amino acids and the synthesis of proteins as in mouse embryos.

In conclusion, the present study has demonstrated that the supplementation of the maturation medium with human or bovine insulin improves the in vitro maturation, fertilization, and cleavage rates of bovine oocytes when cumulus-intact oocytes are cultured in a defined maturation medium with serum.

REFERENCES


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