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

Hormonal supplementation. Cattle. *In vitro* fertilization. *In vitro* bovine embryos.

PALABRAS CLAVE ADICIONALES

Suplementación proteica. Vacuno. Fecundación *in vitro.* Embriones de bovino *in vitro.*

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 cleavaged 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

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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 óvulos 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) mantained 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

(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 CO_2 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 CO_2 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 $1\text{-}2 \times 10^6$ total spermatozoa/ml and cultured for 24 h at 39°C under 5 percent CO_2 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 CO_2 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 mg/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).

Treatment	Nº of trials	Nº oocytes cultured	Nuclear maturation stage (percent) Germinal vesicle	Metaphase-I	Metaphase-II	Degenerated
BI	5	200	8.2±2.0	3.4±1.4	80.3±4.3[ac]	8.1±1.2
HI	5	203	7.2±1.7	5.0±1.5	78.5±4.1[ab]	9.3±2.1
Control	5	201	13.3±2.5	6.2±1.8	70.1±4.0[bd]	10.4±2.2

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 $p<0.05$, χ^2 test.

supplemented with 10 percent FCS and incubated at 39°C under 5 percent CO_2 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

***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).

Treatments	Nº of trials	Nº oocytes inseminated	Fertilization stage (percent)			
			No fertilized	Polispermic	Fertilized	Degenerated
BI	5	203	19.5±2.4	5.1±2.0	61.3±4.8^{ac}	14.1±1.3
HI	5	204	21.4±2.2	5.5±2.1	57.4±3.3^{ab}	15.7±2.3
Control	5	201	29.1±3.1	4.8±1.9	50.1±2.3^{bd}	16.0±3.5

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, χ^2 test.

were defined as normal fertilized; (3) ova with more than two pronuclei and decondensed sperm heads were considered to be polyspermic; (4) oocytes with a cytoplasm vacuolated or fragmented were considered to be degenerated. Finally, the cleavage stage of the oocytes were classified as follows: (1) oocytes without showing blastomeres were judged as no cleavage; (2) oocytes with two o more than two blastomeres were considered cleavaged; and (3) oocytes with a cytoplasm vacuolated or fragmented were considered to be degenerated.

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.

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 or human insulin resulted in higher fertilization rates (60.3 percent and 57.4 percent respectively, $p<0.05$) than that found in the control group (50.1 percent). There were not

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).

Treatments	Nº of trials	Nº oocytes inseminated	Development stage (percent) No cleavaged	Cleavaged	Degenerated
BI	5	205	24.6±2.5	55.3±3.3[a]	20.1±2.3
HI	5	201	26.5±2.6	54.8±3.1[a]	18.7±1.9
Control	5	202	32.2±2.9	42.5±2.8[b]	25.3±2.4

BI=Bovine insulin; HI=Human insulin. Data are showed as mean percentages±SEM from 5 replicates.
[a,b]Mean values in the same column with different superscripts differ significantly at $p<0.05$, χ^2 test.

significant differences between the two insulin supplemented oocyte groups.

In experiment 3, the effects of the insulin supplementation on *in vitro* cleavage rate of bovine oocytes were analysed. As shown **table III**, the cleavage rates obtained in the oocyte groups matured in the presence of bovine or human insulin (55.3 percent and 54.8 percent respectively) were significantly higher ($p<0.05$) than that observed in the control group (42.5 percent). There were not significant differences between the two insulin supplemented oocyte groups.

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 E_2. Matsui *et al.* (1995a) also reported that insulin (10 µg/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 possitive 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.

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