

THE EFFECT OF DIFFERENT SERA AND BOVINE SERUM ALBUMEN FRACTION (BSA) ON *IN VITRO* MATURATION OF IMMATURE BOVINE OOCYTES

EFFECTO DE DIFERENTES SUEROS Y DE LA FRACCIÓN ALBUMINOSA DEL SUERO BOVINO (BSA) SOBRE LA MADURACIÓN *IN VITRO* DE OVOCITOS DE BOVINO

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ADDITIONAL KEYWORDS

Serum supplementation.

PALABRAS CLAVE ADICIONALES

Suplementación sérica.

SUMMARY

This research was designed to study the effects of oestrous cow serum (OCS), fetal calf serum (FCS), bovine serum albumin fraction (BSA), oestrous gilt serum (OGS) and anoestrous cow serum (ACS), on *in vitro* oocyte maturation of immature bovine oocytes. Complete and unexpanded cumulus oocytes from follicles (1-5 mm in diameter) were recovered from ovaries obtained at slaughter and cultured for 24 h in TCM-199 maturation medium supplemented with 20 percent (v/v) OCS, FCS, OGS, ACS or 0.6 percent (w/v) BSA. The results obtained in the present experiment indicate that oocyte maturation rates at the second metaphase stage obtained in OCS, FCS and BSA treatments (75 percent, 77 percent and 85 percent respectively) were significantly higher ($p < 0.001$) than those

obtained in control treatment (40 percent). In the same way, when bovine oocytes were cultured in OGS-supplemented medium (58 percent), maturation rate obtained was significantly higher ($p < 0.05$) than that found in control treatment. The maturation percentages obtained with ACS-serum supplement (50 percent) were lightly higher than to those found in control treatment, though such differences were not statistically significant.

In conclusion, the serum supplementation (mainly with OCS or FCS), or BSA supplementation added to the maturation medium of immature bovine oocytes, contributes for stimulating the *in vitro* maturation of such oocytes.

RESUMEN

El objetivo de este trabajo fue estudiar los efectos del suero de vaca en celo (OCS), suero

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de ternero fetal (FCS), fracción albuminosa de suero bovino (BSA), suero de cerda en celo (OGS) y suero de vaca en anoestro (ACS), sobre la maduración *in vitro* de ovocitos inmaduros de bovino. Ovocitos procedentes de folículos con un diámetro entre 1-5 mm de ovarios de animales sacrificados fueron cultivados durante 24 h en medio TCM-199 suplementado con un 20 p.100 de OCS, FCS, OGS, ACS ó 0,6 p.100 de BSA. Los resultados obtenidos indican que los índices de maduración ovocitaria, hasta el estadio de metafase-II, observados en presencia de OCS, FCS y BSA (75, 77 y 85 p.100 respectivamente) fueron superiores significativamente ($p < 0,001$) que los obtenidos en el grupo control (40 p.100). Del mismo modo, cuando los ovocitos fueron cultivados en medio suplementado con OGS (58 p.100), obtuvieron un índice de maduración superior significativamente ($p < 0,05$) que el encontrado por el grupo control. Los porcentajes de maduración obtenidos con el suplemento ACS (50 p.100) fueron ligeramente superiores a los encontrados por el tratamiento control, aunque tales diferencias no fueron estadísticamente diferentes.

En conclusión, la suplementación sérica (principalmente con OCS o FCS), o con BSA añadida al medio de maduración de ovocitos inmaduros de bovino, contribuye a la estimulación de la maduración *in vitro* de tales ovocitos.

INTRODUCTION

The cell cycle in mammalian oocytes is arrested during fetal life at the diplotene stage of the first prophase. Meiotic arrest is then maintained until immediately before ovulation, when the oocyte resumes maturation. During this maturation process, important changes occur at the nuclear and cytoplasmic levels which prepare the oocyte for fertilization and early embryo development. In the same period, the

intercellular communications between cumulus cells and oocytes, fundamental for oocyte maturation, undergo a progressive reduction. *In vivo*, all these changes are believed to be triggered by the gonadotropin surge. Generally these hormones are used by the majority of the authors in their methods of maturation, fertilization and embryo culture, for favouring the oocyte development *in vitro* (Johnston *et al.*, 1989; Schellander *et al.*, 1990; Saeki *et al.*, 1990).

The oestrous-female serum is the most widely and economic form to supply hormones to the maturation medium. This serum, besides to contain a high level of hormones, also contains certain proteins and growth factors which stimulate the *in vitro* oocyte maturation. Kane and Headon, (1980) reported that the effect of serum albumin appeared to be due to the presence of a relatively high molecular weight protein. Since the presence of serum macromolecules were found to be necessary for culture of oocytes, FCS, BSA, OCS, and maternal serum have been added to the culture media.

The aim of this work was to evaluate the effect of different serum supplements, such as: OCS, FCS, OGS, ACS and BSA, added to the culture medium, on *in vitro* maturation of bovine immature oocytes.

MATERIALS AND METHODS

PREPARATION OF THE OCS, OGS AND ACS SUPPLEMENTS

Blood was collected by venepuncture from oestrous cows and gilts (in

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the first 8-20 h of oestrous), and anoestrous cows. The blood was centrifuged at 1000 g for 10 min. The serum was inactivated by heating at 56°C for 30 min and stored at -20°C until required for oocyte culture.

COLLECTION AND MATURATION OF BOVINE FOLLICULAR OOCYTES

Ovaries were collected at slaughter, placed into physiological saline (0.9 percent, w/v, NaCl) with antibiotics (100 IU penicillin, 100 µg/ml streptomycin) maintained at 30-37°C, and transported to the laboratory within 1-2 h of slaughter. Cumulus-oocyte complexes were aspirated from small (1-5 mm diameter) antral follicles with an 18-G 1-inch needle attached to a 5 ml disposable syringe containing modified phosphate buffered saline (PBS) supplemented with 5 percent (v/v) heat-inactivated FCS (Sigma) and antibiotics. Oocytes were washed three times with Hank's balanced salt solution supplemented with antibiotics (100 IU penicillin, 100 µg/ml streptomycin), and 5 percent (v/v) FCS (washing medium). The basic maturation medium was TCM-199 with Earle's salts (Sigma) supplemented with 2.5 mM HEPES (Sigma) and antibiotic-antimycotic solution (Sigma). Oocytes with an intact cumulus cell were cultured in 1 ml TCM-199 maturation medium and incubated at 39°C, 5 percent CO₂ in air for 24 h. The supplements were added as described below.

EXPERIMENTAL DESIGN

To evaluate the effects of different supplements added to the maturation

medium on *in vitro* oocyte maturation, the oocytes were matured for 24 h according to the following procedures; 1) the oocytes were cultured in the culture medium TCM-199, supplemented with 20 percent OCS; 2) the oocytes were matured in the basic maturation medium supplemented with 20 percent FCS; 3) the oocytes were matured for 24 h in the maturation medium supplemented with 0.6 percent BSA; 4) the oocytes were cultured in the culture medium TCM-199 supplemented with 20 percent OGS; 5) the oocytes were matured in the basic maturation medium supplemented with 20 percent ACS; 6) the oocytes were cultured in TCM-199 medium without supplements, as control treatment.

CHROMOSOME PREPARATION OF THE OOCYTES

At the end of the culture period for maturation, the oocytes were transferred to 3 ml conical tubes and vortex-agitated for 2 min in trisodium citrate (0.88 percent) and trypsin (0.02 percent) hypotonic solution. After a slight agitation to remove the cumulus oophorus cells (COC), denuded oocytes were transferred to a culture plate containing 2 ml of the same hypotonic solution without trypsin for one hour. The oocytes were fixed in an initial fixing solution of 1:1 methanol:acetic acid for 5 min, followed by 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.

Table I. Effect of different types of serum supplements and bovine albumin serum on in vitro maturation of bovine oocytes after 24 h of culture in TCM-199 medium. (Efecto de diferentes tipos de suplementos séricos y fracción albuminosa del suero bovino sobre la maduración ovocitaria *in vitro* después de 24 h de cultivo en medio TCM-199).

Supplements	Nº of trials	Nº of oocytes	Germinal Vesicle	Nuclear Stage		
				Metaphase-I	Metaphase-II	Degenerated
OCS	5	200	14±1.2	8±0.8	75±4.3 ^a	3±0.3
FCS	5	210	18±1.5	3±0.4	77±4.4 ^a	2±0.2
BSA	5	198	5±0.5	9±0.8	85±4.9 ^a	1±0.1
OGS	5	203	20±2.0	19±1.7	58±3.6 ^{bc}	3±0.2
ACS	5	200	25±2.2	18±1.6	50±3.4 ^b	6±0.5
Control	5	201	43±3.0	12±1.1	40±2.8 ^d	5±0.4

^{a-d}Different superscripts in the same column denote significant differences by Chi-square analysis.

^{b,d}Values differ significantly ($p < 0.05$).

^{a,c}Values differ significantly ($p < 0.01$).

^{a,d}Values differ significantly ($p < 0.001$).

Data are showed as mean percentages±SEM from 5 replicates.

CRITERIA FOR MATURATION AND STATISTICAL ANALYSIS

Oocytes were cytogenetically evaluated for stage of maturation after culture. The meiotic progress of the oocytes was classified as follows: 1) germinal vesicle stage; an intact nuclear membrane with the chromatin meiotically inactive; 2) Metaphase I; the nuclear membrane broken and a chromatin pattern characteristic of an oocyte resuming meiosis; 3) Metaphase II; a polar body present within the perivitelline space with maternal chromatin complement identified in the oocyte; and 4) degeneration oocyte; showing obvious degenerative changes such as vacuolated or fragmented cytoplasm or scattered chromatin complement. Data

were statistically analysed by Chi-square test (S.A.S Institute Inc., 1982).

RESULTS

The results of the maturation rates of the oocytes matured in the TCM-199 medium and supplemented with different serum supplements are shown in **table I**. When immature oocytes were matured in the presence of 20 percent OCS (75 percent), 20 percent FCS (77 percent), 0.6 percent BSA (85 percent) or 20 percent OGS (58 percent), the percentage reaching metaphase II stage was significantly higher ($p < 0.001$ and $p < 0.05$, respectively) to that found in the control group (40 percent). The oocyte matura-

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tion rate obtained when maturing oocytes in the presence of 20 percent ACS (50 percent) was higher to that found in the control group. However, significant differences between ACS and control treatments were not observed.

The oocytes matured with OCS, FCS or BSA showed a homogeneous cytoplasm without sign of degeneration. However, when cultivating the oocytes in medium to which OGS or ACS had been applied, an important change in the cytoplasm of the oocytes was observed with clear signs of degeneration such as intensive cytoplasmic vacuolization and chromatin fragmentation.

DISCUSSION

The results obtained in this study showed that serum or BSA supplementation had an important role on both nuclear and cytoplasmic maturation *in vitro* of immature bovine oocytes. It is generally believed that the beneficial effects of serum are due to cyclic adenosine monophosphate, catecholamines, vitamins, putative growth factors, lipids and albumin. Similar advantageous results of the serum supplementation were reported by other authors in several animal species such as cattle (Sanbuissho and Threlfall, 1990; Newcomb *et al.*, 1978), porcine (Nakanishi *et al.*, 1990), and equine (Del Campo *et al.*, 1992). When bovine follicular oocytes were cultured in TCM-199 medium supplemented with serum or BSA, the percentage of oocyte maturation was higher than that found in control treatment in which serum supplement

was not added. However, the best results on both rates and quality of maturation, were obtained when oocytes were cultured in presence of OCS, FCS or BSA supplements.

The OCS supplement is widely used on *in vitro* oocyte maturation of bovine oocytes (Sanbuissho and Threlfall, 1990; Ocaña *et al.*, 1994), *in vitro* fertilization (Kim *et al.*, 1990) and subsequent embryonic development (Lambert *et al.*, 1986; Xu *et al.*, 1987). In our study, the results indicated that TCM-199 medium enriched with OCS supplement supported the *in vitro* oocyte maturation of bovine follicular oocytes. These results were similar to that reported by Schellander *et al.* (1990), who postulated that the beneficial effect of OCS might be due to relatively high LH (Luteinizing Hormone) content. This hormone is known for being responsible for breaking the COC-oocyte interaction and for maintaining the oocyte at germinal vesicle stage whose breaking immediately starts the maturation (Mattioli *et al.*, 1991). Kim *et al.* (1990) reported that 10 percent OCS supplementation to the culture medium provides higher maturation and fertilization rates than when the medium was supplemented with 15 percent and 20 percent of OCS supplement. However, in the present study when 20 percent OCS was used in the maturation medium, the oocyte maturation rates were higher than that reported by these authors. Therefore, we suggest that both 10 percent and 20 percent OCS added to the culture medium constitute an excellent supply of maturation-stimulating components such as LH.

High maturation rates were also obtained when FCS supplement without hormones was added to the maturation medium. These percentages of oocyte maturation were similar to those obtained in OCS treatment. FCS supplement is also routinely added to the *in vitro* maturation and fertilization medium of several animal species such as bovine (Saeki *et al.*, 1990), porcine (Zheng and Sirard, 1992), equine (Willis *et al.*, 1990), hamster (Leibfried-Rutledge *et al.*, 1986) etc. Presumably, the beneficial effect of FCS supplement might be due to the fact it contains maturation-stimulating components such as hormones, proteins and growth factors, similar to those found in OCS supplement, which are active on germinal vesicle breakdown and inducing the oocyte meiosis resumption (Saeki *et al.*, 1990; Sanbuissho and Threlfall, 1990). However, it has been reported that FCS contains some undefined growth-promoting components that are absent in the serum of adult animals (Mochizuki *et al.*, 1991; Allen *et al.*, 1982). In this sense, Schroeder *et al.* (1991) found a major glycoprotein in FCS (Fetuin) that inhibits zona pellucida hardening during mouse oocyte maturation and increase the rates of maturation and fertilization *in vitro*.

The BSA supplementation without hormones provided the highest maturation rates of all treatments used. Though, when oocytes were cultured in TCM-199 medium supplemented with BSA, OCS and FCS significant differences were not found. It has been demonstrated that the beneficial effect of BSA supplement is due to the presence of a relatively high molecular

weight protein which contributes to maturation of bovine oocytes (Kane and Headon, 1980; Kane, 1985). However, results similar to those obtained in the present work were not found, neither by Leibfried *et al.* (1986), in bovine, nor by Zheng and Sirard, (1992) in porcine.

In our study the FCS and BSA supplements were only added to the maturation medium without hormones because our aim was to use a economic protocol to mature bovine oocytes *in vitro* and comparing with others protocols in the literature which use FSH and LH.

Finally, OGS and ACS supplementation provided oocyte maturation rates lightly higher than to that observed in control treatment and significantly lower than those found in OCS, FCS or BSA treatments. We have not references about OGS and ACS supplements used in the maturation medium of bovine oocytes. Though, we suggest that these sera might to contain hormonal and growth factors levels lower than that found in OCS, FCS and BSA supplements. In this sense, Zheng and Sirard, (1992) also reported that OGS supplement added to the culture medium of porcine oocytes did not provide high maturation rates. These results obtained by these authors are similar to that obtained by us in bovine.

In conclusion, serum supplements (specially OCS or FCS) and BSA added to the maturation medium contributed to support the meiotic maturation *in vitro* of immature bovine oocytes. However, other works are needed to improve the maturation conditions leading to normal fertilization.

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