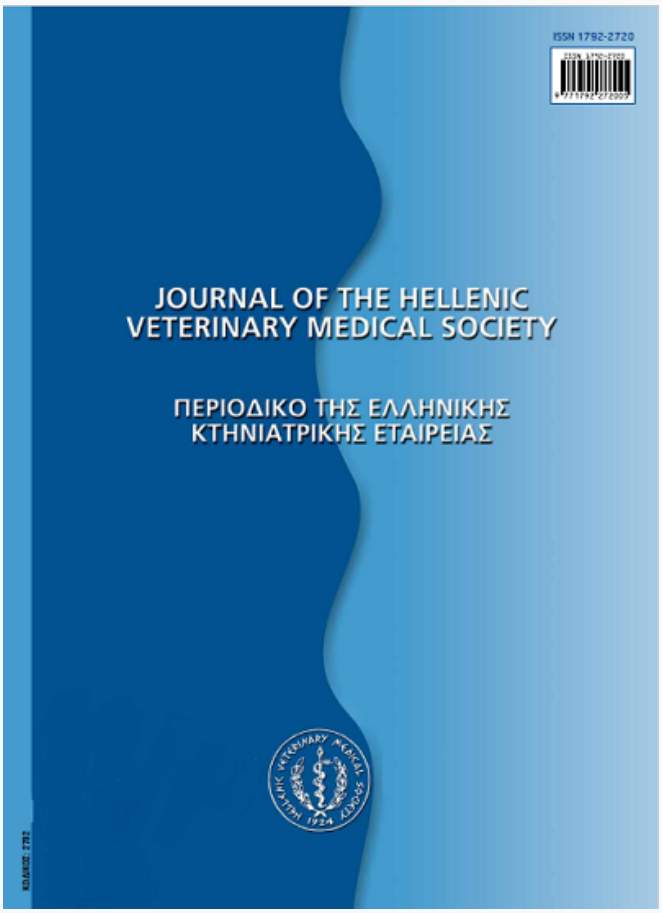
Journal of the Hellenic Veterinary Medical Society

Vol 68, No 3 (2017)



Fetal bovine serum is associated with polar body degeneration after in vitro maturation of bovine oocytes

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doi: 10.12681/jhvms.15469

To cite this article:

MACÍAS-GARCÍA, B., MACEDO, S., ROCHA, A., & GONZÁLEZ-FERNÁNDEZ, L. (2018). Fetal bovine serum is associated with polar body degeneration after in vitro maturation of bovine oocytes. *Journal of the Hellenic Veterinary Medical Society*, *68*(3), 279–284. https://doi.org/10.12681/jhvms.15469

J HELLENIC VET MED SOC 2017, 68(3): 279-284
ΠΕΚΕ 2017, 68(3): 279-284

Research article
Ερευνητικό άρθρο

Fetal bovine serum is associated with polar body degeneration after *in vitro* maturation of bovine oocytes

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ABSTRACT. *In vitro* fertilization (IVF) in cattle is commonly used worldwide. Although extensive research has been conducted using different additives in the different IVF steps, little is known regarding how protein type may affect bovine oocytes during the fertilization period. In addition, unlike Tissue Culture Medium 199 (TCM), fertilization medium may induce oocytes' chromatin degeneration during prolonged incubation in the horse (Modified Whitten's medium). Thus, in the present work TCM-199 supplemented with either 7 mg/ml of Bovine Serum Albumin (TCM+BSA) or 10% Fetal Bovine Serum (v/v; TCM+FBS) was used. Bovine oocytes were matured *in vitro* and placed in the previously mentioned media for further 18 hours, in the absence of added sperm (sham fertilization) and their chromatin conformation was evaluated. After IVM, 78.9% of the initial oocytes had reached the MII stage. After sham fertilization, 58.6% of the oocytes in TCM+BSA while just 28.3% in TCM+FBS maintained the MII chromatin conformation ($p < 0.05$). Subsequent experiments run using PB extruded oocytes and incubated in TCM+BSA and TCM+FBS during sham fertilization, demonstrated that FBS was consistently associated with polar body dissolution or degeneration.

Keywords: bovine, oocyte, polar body, FBS, BSA

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Date of initial submission: 4.5.2016
Date of revised submission: 20.6.2016
Date of acceptance: 7.7.2016

INTRODUCTİON

In vitro fertilization (IVF) is routinely used in cattle in conjunction with intrauterine embryo transfer resulting in successful calving (Hasler, 2014). The sperm-oocyte co-incubation step has been related to an increase in the production of reactive oxygen species (ROS) and other toxic compounds that potentially damage the oocyte (Gianaroli et al., 1996; Agarwal et al., 2006). In addition, the type and source of protein used during IVM has shown to exert a marked effect on DNA degeneration in canine oocytes (Lopes et al., 2011) and also influence the developmental rate of bovine oocytes (Gomez and Diez, 2000).

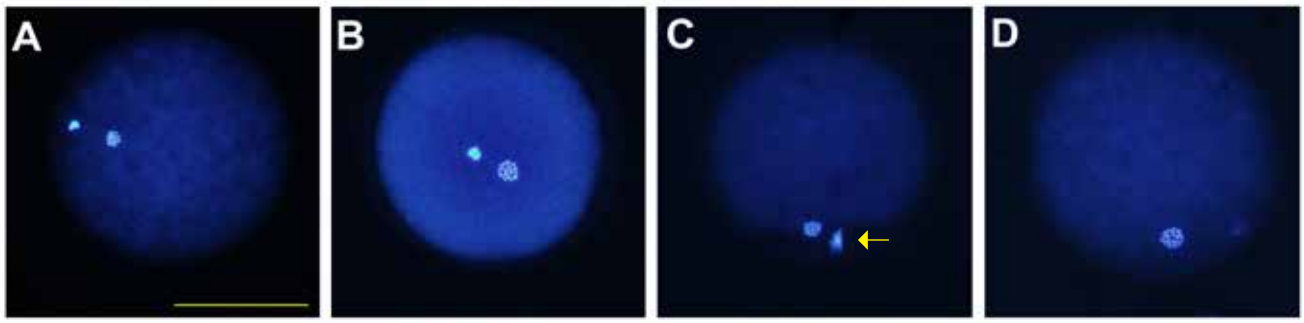
A recent study demonstrates that Modified Whitten's medium may induce polar body (PB) degeneration during prolonged incubation of *in vitro* matured (IVM) equine oocytes, while this effect was reduced when TCM was used, especially when it was combined with FBS (González-Fernández et al., 2015). Addition of FBS or BSA has been extensively studied in bovine IVF during maturation or embryo culture (Fukui and Ono, 1989; Gandhi et al., 2000), but little is known regarding how protein type may affect bovine oocytes during fertilization.Thus, in the present study, we wanted to elucidate if prolonged incubation of IVM bovine oocytes in TCM supplemented with 10% FBS or 7 mg/ml of BSA differently affected the oocyte´s chromatin in the bovine species.

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma-Aldrich Inc. (Barcelona, Spain) unless otherwise stated.

Figure 1. *Bovine oocytes showing PB extrusion before and after sham fertilization.*
Bovine oocytes were subjected to IVM, separated based on PB extrusion and cultured in TCM-FBS or TCM-BSA for 18 hours (sham fertilization). Representative micrographs of oocytes showing PB extrusion after IVM are shown. A) Oocyte at the end of 24 h of IVM; B) TCM+BSA; C) and D) TCM+FBS. Section C shows a degenerating PB marked with a yellow arrow head. Scale is provided in section A for 100 µm.



Oocyte incubation media

The incubation medium was TCM-199 with 25 mM HEPES (M2520; Sigma) supplemented with 25 mM bicarbonate. The pH was raised to 7.4 and osmolarity was ≈ 280 mOsm/Kg. TCM-199 supplemented with 7 mg/ml of BSA or 10% FBS was designated as TCM+BSA or TCM+FBS respectively. Media were supplemented with 10 U/ml of penicillin and 10 µg/ml of streptomycin. All media were placed in an incubator with an atmosphere of 5%CO_2/95% air at 100% humidity at 38.5°C, covered with mineral oil and allowed to equilibrate for at least 3 hours prior the beginning of the experiment.

Oocyte harvesting and in vitro maturation

Bovine ovaries were collected at an abattoir and were maintained at 22 ºC in 0.9% NaCl solution during transport (2 hours total). After arrival, the ovaries were washed with 0.9% NaCl at 37 ºC and the oocytes were aspirated from 2-8 mm follicles. Oocytes with 5 or more layers of compact cumulus cells and homogeneous cytoplasm were selected and matured in vitro for 24 hours in TCM-199 supplemented with 10% FBS (v/v), 10 mU/ml of follicle-stimulating hormone (FSH; Life Technologies Corporation) and 10 mU/ml of luteinizing hormone (LH; Life Technologies Corporation).

Chromatin evaluation

Bovine oocytes were evaluated at two time points: immediately after IVM and after IVM and subsequent incubation for 18 further hours (sham fertilization) in TCM+FBS or TCM+BSA. Oocytes used in Table 3 were visualized after IVM using bright field microscopy to discriminate the presence of PB and placed in TCM+BSA or TCM+FBS; after sham fertilization their chromatin configuration was analyzed. The protocol used for chromatin evaluation was a well-established protocol in our laboratory (Macias-Garcia et al., 2015). Briefly, bovine oocytes were denuded by 5-minutes vortexing in a 15-ml conical tube in PBS supplemented with 0.2% polyvinyl alcohol (PBS+PVA) and 0.4% hyaluronidase. Then, oocytes were fixed in 4% formaldehyde in PBS+PVA overnight at 4°C, washed in PBS+PVA and stained with Hoechst 33342 (1 μg/ml, 37°C, 10 minutes in the dark). Oocytes were mounted on slides using glycerol and a cover slip, sealed using nail polish and allowed to dry. Chromatin conformation was assessed and classified as germinal vesicle (GV), metaphase I (MI) or metaphase II (MII) following the classification by Hinrichs et al. (1993). Fluorescence was evaluated using an Olympus BX41 microscope (New Hyde Park, NY, USA) equipped with 40× objective. Oocytes were considered as degenerated when no DNA was visualized or when abnormal chromatin configurations were found.

Table 1. DNA conformation of bovine oocytes after IVM.

GV (%)	MI (%)	MII (%)	DEG (%)	n
2 (1.4)	22 (15.5)	112 (78.9)	6 (4.2)	**142**

Values are represented as total number and (percentage). GV: Germinal vesicle; MI: Metaphase I; MII: Metaphase II; DEG: Degenerated or absent chromatin; n: total oocyte number.

Statistical Analysis

Proportions of oocytes showing different chromatin configurations were compared among groups by Chi-square test with the Yates correction for continuity using Sigma Plot software version 11.0 for Windows (Systat Software, Chicago, IL, USA). A level of $p < 0.05$ was considered significant.

RESULTS

Effect of protein type on bovine oocytes subjected to sham fertilization

Oocyte maturation and chromatin conformation were evaluated immediately after IVM and the percentage of oocytes reaching MII were 78.9%, as shown in Table 1. After sham fertilization, oocytes incubated in TCM+BSA showed a higher percentage of oocytes that reached MII (58.6%) compared to TCM+FBS (28.3%). The TCM+FBS medium significantly increased the proportion of bovine oocytes showing aligned chromosomes in a metaphase plate (in MI; 60.2%) compared to TCM+BSA (29.7%). Degeneration rates were also significantly higher in TCM+FBS compared to TCM+BSA (Table 2).

Effect of protein type on polar body degeneration.

To determine whether the decreased percentage of oocytes in MII after sham fertilization was due to a degeneration of the PB, we decided to study the oocytes with PB separately. IVM bovine oocytes were denuded and the PB extrusion was checked under a dissection microscope. Oocytes with unmistakable PB were placed in separate droplets of TCM+FBS or TCM+BSA. When PB extruded oocytes were examined, the ones incubated in TCM+BSA showed higher proportion of MII chromatin conformation (88%) than the TCM+FBS group (39%) and had a lower PB degeneration rate (8.9%; Table 3). In TCM+FBS treatment, the majority of PBs had disappeared (Fig. 1D). In some cases a Hoechst positively-stained "blur" was present between the ZP and the membrane (Fig. 1C); those oocytes were considered as having undergone PB degeneration and are referred as oocytes showing a degenerated PB (Table 3).

Table 2. DNA conformation and degeneration after sham fertilization.

	GV (%)	MI (%)	MII (%)	DEG (%)	n
TCM+BSA	11 (9.9)	33 (29.7)[a]	65 (58.6)[a]	2 (1.8)[a]	**111**
TCM+FBS	0 (0)	68 (60.2)[b]	32 (28.3)[b]	13 (11.50)[b]	**113**

Values are represented as total number and (percentage). Within columns, values bearing different superscripts differ statistically ($p < 0.05$). GV: Germinal vesicle; MI: Metaphase I; MII: Metaphase II; DEG: Degenerated or absent chromatin; n: total oocyte number.

DISCUSSION

Our work demonstrates that *in vitro* maturated bovine oocytes experience PB degeneration after prolonged culture in TCM supplemented with FBS or BSA (78.9% of oocytes had MII configuration after IVM vs. 58.6% after sham fertilization in TCM+BSA or 28.3% in TCM+FBS; $p < 0.001$). FBS addition during sham fertilization results in a lower proportion of MII DNA conformation and a higher DNA degeneration compared to TCM+BSA (Table 2). To confirm that the observed effect was related to FBS addition, oocytes showing an extruded PB were separated after IVM and subjected to sham fertilization; significant differences were found between TCM+BSA and TCM+FBS in PB extruded groups in MI and MII chromatin configuration percentages (Table 3). Therefore, our study demonstrates that TCM+BSA was the medium that best preserved MII conformation, PB intactness and decreased PB chromatin degeneration (Table 3 and Fig. 1B). Our results suggest that BSA helps to maintain oocytes' chromatin. In the oocytes in which PB degeneration was demonstrated, the remaining chromosome plates appeared to be normal (Fig. 1D), thus FBS seems to affect only PB's chromatin. In addition, different batches of FBS were used during the experiments, and the results remained unchanged (data not shown). In mice, PB degeneration has been associated to oocyte's ageing (Miao et al., 2004) and apoptosis (Jiao et al., 2012). In our conditions, FBS was consistently associated with higher PB degeneration over BSA; as all the experimental procedures were performed in parallel and following the same timelines, we can ensure that the marked differences observed in PB degeneration due to the protein source added and not just to oocyte's ageing. Recently, in humans, first and second PB conformation has been associated with enhanced embryo quality (Zhou et al., 2015). Zhou et al. (2015) classified the PBs, 16 h - 18 h after insemination, into two groups (intact or fragmented) and they demonstrated that the group initially showing intact PB yielded better quality embryos. It is well established that ROS are produced during *in vitro* maturation (Morado et al., 2009) inducing oocytes' DNA degeneration (Chaube et al., 2005). We therefore hypothesize that BSA could have a ROS scavenger effect, as demonstrated in neurons (Vega et al., 2009) and stallion sperm (Macias-Garcia et al., 2015), helping to protect oocyte's DNA conformation. To the best of our knowledge this is the first report describing PB degeneration during (sham) fertilization in the presence of FBS, in oocytes of any species. It remains to be seen if PB dissolution is associated with any effect on bovine embryo quality. In addition, any experimental work involving FBS addition during bovine IVF or prolonged oocyte incubation in media containing FBS would need to take in account the vivid PB dissolution that occurs, as demonstrated in the present paper.

Table 3. Effect of protein type on proportions of PB degeneration and DNA conformation after sham fertilization of bovine oocytes with verified PB at the end of IVM.

Treatment	GV (%)	MII (%)	PB DEG (%)	DEG (%)	n
TCM+BSA	0	59 (88)[a]	6 (8.9)[a]	2 (2.9)	**67**
TCM+FBS	0	32 (39)[b]	48 (58.5)[b]	2 (2.4)	**82**

Values are represented as total number and (percentage). Values bearing different superscripts in a column differ significantly ($p < 0.05$). GV: Germinal vesicle; PB DEG: Polar body degeneration; MII: Metaphase II; DEG: Degenerated or absent chromatin; n: total oocyte number.

ACKNOWLEDGMENTS

BM-G and LG-F hold a postdoctoral grant of the Fundação para a Ciência e a Tecnologia (Portuguese Ministry of Education and Science). Both grants are co-funded by Programa Operacional Potencial Humano (POPH) financed by European Social Fund (ESF) and Portuguese national funds from Ministry of Education and Science (MEC). Grant references: SFRH/BPD/84354/2012 (BM-G) and SFRH/BPD/85532/2012 (LG-F). The authors thank CECA/ICETA (University of Porto) for funding the abattoir dislocations. The collaboration of IZICAR abattoir is highly appreciated. The authors wish to thank the Laboratory of Applied Physiology (Department of Aquatic Production) of the ICBAS (University of Porto) and especially to Mariana Hinzmann for allowing us to use their fluorescence microscope. The assistance of Mr. Paulo Ferreira is also appreciated.

REFERENCES

Agarwal A, Said TM, Bedaiwy MA, Banerjee J, Alvarez JG (2006) Oxidative stress in an assisted reproductive techniques setting. Fertil Steril 86:503-512.

Chaube SK, Prasad PV, Thakur SC, Shrivastav TG (2005) Hydrogen peroxide modulates meiotic cell cycle and induces morphological features characteristic of apoptosis in rat oocytes cultured in vitro. Apoptosis 10:863-874.

Fukui Y, Ono H (1989) Effects of sera, hormones and granulosa cells added to culture medium for in-vitro maturation, fertilization, cleavage and development of bovine oocytes. J Reprod Fertil 86:501-506.

Gandhi AP, Lane M, Gardner DK, Krisher RL (2000) A single medium supports development of bovine embryos throughout maturation, fertilization and culture. Hum Reprod 15:395-401.

Gianaroli L, Fiorentino A, Magli MC, Ferraretti AP, Montanaro N (1996) Prolonged sperm-oocyte exposure and high sperm concentration affect human embryo viability and pregnancy rate. Hum Reprod 11:2507-2511.

González-Fernández L, Macedo S, Lopes JS, Rocha A, Macías-García B (2015) Effect of different media and protein source on equine gametes: potential impact during in vitro fertilization. Reprod Domest Anim 50:1039-1046.

Gomez E, Diez C (2000) Effects of glucose and protein sources on bovine embryo development in vitro. Anim Reprod Sci 58:23-37.

Hasler JF (2014) Forty years of embryo transfer in cattle: a review focusing on the journal Theriogenology, the growth of the industry in North America, and personal reminisces. Theriogenology 81:152-169.

Hinrichs K, Schmidt AL, Selgrath JP (1993) Atlas of chromatin configurations of germinal vesicle stage and maturing horse oocytes. Equine Vet J 25 (issue S15):60–63.

Jiao ZX, Xu M, Woodruff TK (2012) Age-associated alteration of oocyte-specific gene expression in polar bodies: potential markers of oocyte competence. Fertil Steril 98:480-486.

Lopes G, Alves MG, Carvalho RA, Luvoni GC, Rocha A (2011) DNA fragmentation in canine oocytes after in vitro maturation in TCM-199 medium supplemented with different proteins. Theriogenology 76:1304-1312.

Macias-Garcia B, Gonzalez-Fernandez L, Loux SC, Rocha AM, Guimaraes T, Pena FJ, Varner DD, Hinrichs K (2015) Effect of calcium, bicarbonate, and albumin on capacitation-related events in equine sperm. Reproduction 149:87-99.

Miao Y, Ma S, Liu X, Miao D, Chang Z, Luo M, Tan J (2004) Fate of the first polar bodies in mouse oocytes. Mol Reprod Dev 69:66-76.

Morado SA, Cetica PD, Beconi MT, Dalvit GC (2009) Reactive oxygen species in bovine oocyte maturation in vitro. Reprod Fertil Dev 21 608-614.

Vega L, Arroyo AA, Tabernero A, Medina JM (2009) Albumin-blunted deleterious effect of amyloid-beta by preventing the internalization of the peptide into neurons. J Alzheimers Dis 17:795-805.

Zhou W, Fu L, Sha W, Chu D, Li Y (2015) Relationship of polar bodies morphology to embryo quality and pregnancy outcome. Zygote 1-7.