DOES THE FOLLICULAR FLUID OBTAINED FROM BOVINE FOLLICLES OF DIFFERENT DIAMETERS INFLUENCE OOCYTE DEVELOPMENT?

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ABSTRACT

The effect of follicular fluid (FF) of small (SFF= ≤8mm) and large (LFF= >8mm) follicles in the maturation medium on bovine oocyte maturation, fertilization and subsequent development was evaluated. Oocytes were randomly assigned into 5 groups. The oocytes of Estrous Mare's Serum (EMS) group were treated with 10% of EMS. In the remaining four groups 20% of bovine FF was added. These groups received FF from different follicle diameter. In the group EMS (n=147) oocytes were maintained for 24h in 10% EMS; Group SFF (n=149) for 24h in SFF; Group LFF (n=147) for 24h in LFF; Group SFF+LFF (n=149) for 12h in SFF plus 12h in LFF; Group LFF+EMS (n=144) for 12h in LFF plus 12h in EMS. Fertilization was performed by incubation of sperma and oocytes in FERT-TALP medium for 18h. Zygotes were cultured in medium Synthetic Oviduct Fluid with amino acids, sodium citrate and myo-inositol (SOFaaci). The cleavage on Day 2 (D0 = fertilization day) and hatching rates on Day 9 were similar among groups (P>0.05). However, oocytes matured in medium with LFF showed higher (P<0.05) blastocyst yield on Day 7 compared to oocytes in vitro matured in medium with SFF. The addition of follicular fluid from small and large follicles to the maturation media supports *in vitro* maturation and embryonic development.

Key words: oocytes, maturation, follicular fluid, embryo.

O FLUÍDO FOLICULAR OBTIDO DE FOLÍCULOS DE DIFERENTES DIAMETROS INFLUENCIA O DESENVOLVIMENTO DE OÓCITOS BOVINOS?

RESUMO

O efeito do fluido folicular (FF) de pequenos (PFF = <8 mm) e grandes (GFF => 8mm) folículos no meio de maturação *in vitro* foi avaliado sobre a maturação, fecundação e

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desenvolvimento de oócitos bovinos. Os oocitos do grupo Soro de Égua em Estro (SEE) receberam 10% de soro de égua no meio. Nos demais quatro grupos acrescentaram-se 20% de fluído folicular proveniente de folículos de diversos diâmetros. No grupo SEE (n = 147) os oócitos foram mantidos por 24h em 10% SEE; no grupo PFF (n = 149) por 24h em PFF; no grupo GFF (n = 147) por 24h em GFF; no grupo PFF + GFF (n = 149) por 12h no PFF e 12h em GFF; no grupo GFF + SEE (n = 144) por 12h em GFF e 12h em SEE. A fecundação foi realizada pela incubação de espermatozóides e oócitos no meio FERT-TALP por 18h. Os zigotos foram cultivados em meio *Synthetic Oviduct Fluid* acrescido de aminoácidos, citrato de sódio e myo-inositol (SOFaaci). As taxas de clivagem no dia dois (D0=fecundação) e de blastocistos no dia nove foram similares entre os grupos (P> 0,05). No entanto, oócitos maturados no meio com GFF apresentaram maior (P <0,05) taxa de blastocistos no dia sete, quando comparados aos oócitos maturados no meio com PFF. A adição de fluido folicular de folículos pequenos e grandes aos meios de maturação possibilita a maturação e o desenvolvimento embrionário *in vitro*.
Palavras-chave: oócito, maturação, líquido folicular, embriões.

EL LIQUIDO FOLICULAR OBTENIDO DE FOLÍCULOS DE DIFERENTES TAMAÑOS INFLUENCIA EL DESARROLLO DE OVOCITOS BOVINOS?

RESUMEN

El efecto del líquido folicular (LF) de pequeño (PFF = <8mm) y de grande (GFF => 8mm) folículos en el medio de maduración *in vitro* fueron evaluados en la maduración, fecundación y desarrollo de los ovocitos de los bóvidos. Los ovocitos fueron distribuidos aleatoriamente en cinco grupos. En grupo que el SYC (Suero yegua en celo) el 10% del SYC fue utilizado y para los cuatro grupos restantes se utilizó el 20% de liquido folicular (LF) de los folículos de diversos tamaños. En el grupo el SYC (n = 147) los ovocitos fueron mantenidos por 24h en 10% SEE; en el grupo PFF (n = 149) por 24h en PFF; en el grupo GFF (n = 147) por 24h en GFF; en el grupo PFF + GFF (n = 149) por 12h en el PFF más 12h en GFF; en el grupo GFF + SEE (n = 144) por 12h en GFF más 12h en SEE. La fecundación fue llevada a través de la incubación de espermatozoides y de ovocitos en el medio FERT-TALP por 18h. Los zigotos habían sido cultivados en medio *Synthetic Oviduct Fluid* con aminoácido, citrato de sódio y myo-inositol (SOFaaci). Las tasas de escisión en el día dos (D0=fecundación) y de blastocistos en el día nueve fueron similares entre los grupos (P>0,05). Sin embargo, los madurados de los ovocitos con la adición de GFF presentaran mayor (P<0.05) tasa de blastocitos en el día siete, en comparación con los ovocitos madurados en el medio con PFF. La adición del LF de pequeños y grandes folículos al medio de maduración es posible para la maduración y el desarrollo embrionario *in vitro*.

Palabras clave: ovocitos, liquido folicular, maduración, embriones.

INTRODUCTION

The *in vitro* maturation (IVM) system is broadly applied for in vitro production (IVP) of embryos in domestic animals and humans. However, there is still a wide variation in blastocyst developmental rates of *in vitro* matured oocytes. This may be explained by inappropriate cytoplasmic maturation (1). It is known that the follicular fluid (FF) influences the ability of *in vitro*-matured bovine oocytes to acquire developmental competence (2). The FF contains, among other specific components, steroids and glycoproteins synthesized by the theca and granulosa cells and it is believed to be the source of nutritional and developmental

support of the oocytes (3). Follicular and oocyte maturation are parallel events that are functionally related (4). The concentration of FF, and the timing of addition of FF to the maturation medium could influence programming of the maturation process (5).

The aim of this study was to evaluate the effect of the supplementation of maturation media with FF aspirated from bovine follicles of different diameter, on developmental capacity of bovine oocytes fertilized *in vitro*.

MATERIALS AND METHODS

Ovaries were collected at an abattoir and transported within 2 hours after slaughter to the laboratory, at 30°C, in a 0.9% NaCl solution (Embryolab, UFSM, Santa Maria/RS Brazil) containing 100mg/mL Streptomycin and 50UI/mL Penicillin-G potassium salt.

Prior to aspiration of FF the follicles were dissected, the diameter measured by the use of a micrometer, and allocated into two groups according to the follicular diameter: small (<8 mm) and large (>8 mm) follicles. Aspiration of FF was performed using a 21-gauge needle connected to a vacuum pump (11 mL/min, Nevoni-Equip.Med.Hosp.Ltda, São Paulo, SP, Brazil). The FF of several follicles was mixed to build a pool of each follicular diameter. Collins and Wright (6) reported that some factors in FF that improve bovine oocyte maturation may include heat-labile proteins inactivated by heat treatment or factors removed by filtration, thus the follicular fluid was only centrifuged and frozen at –20ºC for later use in the maturation medium.

Oocyte collection was performed from follicles between 2 and 8mm in diameter. The follicles were aspirated using a 21-gauge needle in a 15 mL collection tube with suction provided by a vacuum pump. After collection, oocytes were held in FF for searching and selection under a stereomicroscope (Carl Zeiss Jena GmbH, Jena, Germany). Oocytes of quality I and II, with compact cumulus mass and uniform ooplasm were morphologically selected according to De Loos et al. (7). The oocytes were randomly allocated into one of five groups of 25 oocytes each. Oocytes were washed in 4 drops of TCM-HEPES [modified TCM-199; (GIBCO BRL, 31100-27, Grand Island, NY, USA) with 5.95mg/mL HEPES (SIGMA, H7006), 0.025mg/mL sodium pyruvate (SIGMA, P4562), 2.2 mg/mL $NaHCO_3$ (SIGMA, S-5761) and 10% Estrous Mare's Serum (EMS; Embryolab/UFSM, Santa Maria, RS, Brazil)] for the control group or 20% FF for each follicular diameter group. Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

In vitro maturation (IVM) was performed in 400μL drops of TCM-199 (GIBCO Cat. 31100-035), Hepes (SIGMA, H6147) and 0.01UI rFSH-h/mL (L1930300; Serono Pharma S.p.a, Bari, Italy), using four-well culture plates (Nunc Brand Prod., Roskilde, Denmark). Oocytes were randomly distributed into five groups of maturation. In the group EMS (n=147) oocytes were matured for 24h with 10% EMS. In groups Small follicular fluid (SFF) (n=149) and Large follicular fluid (LFF) (n=147) oocytes were matured for 24h in the presence of 20% of SFF and LFF, respectively. In the group SFF+LFF (n=149) maturation was performed for 12h in the medium with SFF followed by 12h with LFF. In the group LFF+EMS (n=144) oocytes were matured for 12h in the medium with LFF and for 12h in the presence of EMS. Oocytes were matured at 39°C, in a humidified 5% CO_2 in air atmosphere (W.C.Heraeus GmbH. Hanau, Germany) for 24 hours.

The *in vitro* fertilization and culture were performed according the methodology described by Rauber et al. (8). From the second day after fertilization onwards, the embryos were cultured in gasified plastic bags with 5%CO_2, 5%O_2 and 90% N_2 held in an incubator (W.C Heraeus GmbH, Hanau, Germany) at 39ºC. *In vitro* maturation, fertilization and culture were performed simultaneously in six replicates.

Cleavage, blastocyst and hatching rates were evaluated on Day 2, 7 and 9, respectively. Data were analyzed by GLM procedure (SAS 9.2). Percentages were submitted to the arcsine square root transformation before being analyzed through GLM procedure (SAS 9.2). Treatments were compared using the Tukey's test at a 5% probability level.

RESULTS

No treatment effect was observed on the maturation and fertilization. The cleavage and hatching rates on Day 2 and Day 9 were similar among treatments, respectively (Table 1). Blastocyst yield on Day 7 was lower for oocytes matured in medium supplemented with small follicular fluid compared to oocytes matured with EMS and to those matured in follicular fluid derived from large follicles ($P < 0.05$).

Table 1. Embryo development after *in vitro* fertilization of bovine oocytes matured in medium with estrous mare serum or bovine follicular fluid derived from follicles with different diameter.

Groups of matured CCO (n)	Cleavage % (n)	Blastocyst yield % (n)	Hatched blastocyst yield % (n)
10% EMS [1] (n=147)	89,8[a] (132)	38.1[a] (56)	30.6[a] (45)
20% SFF [2] (n=149)	82.5[a] (123)	20.8[c] (31)	22.8[a] (34)
20% LFF [3] (n=147)	92.5[a] (136)	34.0[ab] (50)	20[a] (29)
20% SFF + 20%LFF [4] (n=149)	91.3[a] (136)	39.5[a] (59)	25.5[a] (38)
20% LFF + 10% EMS [5](n=144)	88.8[a] (128)	29.9[abc] (43)	25.6[a] (37)

[a, b, c] Means within columns of groups according stage of embryo development without common superscripts differ (P<0.05).
[1]EMS: 24h in 10% estrous mare serum
[2]SFF: 24h in 20% follicular fluid of follicles ≤8mm in diameter
[3]LFF: 24h in 20% follicular fluid of follicles >8mm in diameter
[4]SFF+LFF: 12h in 20% SFF + 12h in 20% LFF
[5]LFF+EMS: 12h in 20% LFF + 12h in 10% EMS

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24h in 20% follicular fluid of follicles ≤8mm in diameter (n=149)	82.5[a] (123)	20.8[c] (31)	22.8[a] (34)
24h in 20% follicular fluid of follicles >8mm in diameter (n=147)	92.5[a] (136)	34.0[ab] (50)	20[a] (29)
12h in 20% SFF+12h in 20% LFF (n=149)	91.3[a] (136)	39.5[a] (59)	25.5[a] (38)
12h in 20% LFF+12h in 10% EMS (n=144)	88.8[a] (128)	29.9[abc] (43)	25.6[a] (37)

[a, b, c] Means within columns of groups according stage of embryo development without common superscripts differ (P<0.05).

DISCUSSION

It has been reported that bovine oocytes maturation with 30% and 60% follicular fluid has a detrimental effect on embryonic development (5, 9). In addition, undiluted follicular fluid (100%) has been shown to be unsuitable for maturation of bovine oocytes (4, 10). On the other hand, follicular fluid added to the maturation media at 10% or 20% improves the developmental capacity of bovine oocytes (2, 5, 11).

In the present study, blastocyst development was higher when the maturation occurred with LFF compared to SFF ($P < 0.05$). This may be explained by the possibility that FF from follicles larger than 8mm in diameter contains substances that simulates the resumption of meiosis and cytoplasmic maturation. During follicular growth, the endocrine and other biochemical profiles (2, 12, 13) of the follicular fluid undergo important changes for oocytes maturation and achievement of developmental ability after fertilization (12).

The group of oocytes treated with SFF resulted in lower cleavage ($P > 0.05$) and blastocyst yield ($P < 0.05$) than LFF and EMS groups. Ali et al. (14) observed that supplementation of completely defined maturation medium with 5% of bovine FF derived from competent follicles (>8mm) led to a higher proportion of blastocysts compared to FF derived from small follicles (2 to 5mm) or BSA-V, suggesting that FF from follicles >8mm produces and releases signals that are favourable to the acquisition of developmental competence of immature oocytes. The hatching blastocyst yield was lower ($P > 0.05$) for oocytes fertilized in group treated with LFF. Although embryo development to the blastocyst stage was supported by diluted follicular fluid from large follicles, its influence was not superior to diluted serum for *in vitro* maturation of bovine oocytes, contrasting with Elmileik et al. (5) who observed significantly higher blastocyst and hatching yield of oocytes matured in the 10% FF group compared to the control group with 10% estrous cow's serum.

CONCLUSION

The follicular diameter influenced the capacity of in vitro matured oocytes to acquire developmental competence. As the supplementation of maturation media with FF derived of small (<8 mm) and large follicles (>8mm) did not inhibit the developmental capacity of bovine oocytes, it could be used as an alternative to serum for in vitro production of embryos.

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