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ORIGINAL

Effect of age and coasting period on oocytes quality and their *in vitro* development from prepubertal cattle

Efecto de la edad y la hora de la aspiración sobre la calidad oocitaria y el desarrollo *in vitro* en hembras bovinas prepúberes

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ABSTRACT

Objective. This study evaluated the effect of age and coasting period over oocyte quality and their posterior development under *in vitro* conditions from prepubertal *Bos indicus* crossbred donors. **Material and methods.** Donors females received a norgestomet implant and estradiol benzoate. Four days later a unique dosage of 150 IU of eCG was administered. Three coasting periods (24, 48, and 56 h) and four ages (3, 6, 10, and 12 months) were proved. All antral follicles were aspirated and an *in vitro* culture proceed was done. **Results.** 439 follicles were aspirated, of which 385 (87.7%) were 2-5 mm, 41 (9.33%) were 5-10 mm, and 13 (2.3%) were ›10 mm. After aspiration, a total of 373 oocytes (84.9%) were recovered, finding differences ($p<0.05$) between averages of 6 (9.3) and 10 months old animals (32.3). 285 (76.4 %) recovered oocytes were subjected to *in vitro* process. Cleavage values were significantly higher ($p<0.05$) in 10 (27.1%) and 12 months animals (26.8%). Although the number of transferable embryos was low, there were differences between ages ($p<0.05$) obtaining a higher percentage in age 3 (12.6%). **Conclusions.** A coasting period higher than 24 h has a negative effect on oocyte quality. Some oocytes from 3 months old calves were competent for *in vitro* embryo development; however, higher numbers of embryos were produced from 10 and 12 months of age prepuber females, indicating they have higher competency *in vitro.*

Key words: ECG, *in vitro* embryo production, oocytes donation, stimulation. (Sources: DeCS, AIMS).

RESUMEN

Objetivo. Este estudio evaluó el efecto de la edad y el período de aspiración folicular sobre la calidad del oocito y su posterior desarrollo *in vitro* con donadoras prepúberes *Bos indicus* mestizas. **Materiales y métodos.** Las hembras donantes recibieron un implante de norgestomet y benzoato de estradiol y cuatro días más tarde una dosis única de 150 UI de eCG. Las aspiración folicular se realizó en tres tiempos (24, 48, y h 56) y en cuatro edades (3, 6,10, y 12 meses). Todos los folículos antrales fueron aspirados y se realizaron procedimientos de maduración, fertilización y cultivo *in vitro*. **Resultados.** Se aspiraron 439 folículos en total, de los cuales 385 (87.7%) fueron de 2-5 mm, 41 (9.33%) fueron de 5-10 mm, y fueron 13 (2.3%) > 10 mm. Se recuperó un total de 373 oocitos (84.9%) y se encontraron diferencias significativas ($p<0.05$) entre los promedios de los animales de 6 (9.3) y 10 meses (32.3). 285 (76.4%) oocitos fueron sometidos a los procesos *in vitro*. La tasa de división fue significativamente mayor ($p<0.05$) en los animales de 10 (27.1%) y 12 meses (26.8%). Aunque el número de embriones transferibles fue bajo, existieron diferencias entre las edades ($p<0.05$), obteniendo el porcentaje más alto los animales de 10 meses (12.6%). **Conclusiones.** Un período de aspiración superior a 24 h tiene un efecto negativo sobre la calidad del oocito. Algunos oocitos procedentes de terneras de 3 meses fueron competentes en el desarrollo *in vitro*, sin embargo, un mayor número de embriones se produjeron con hembras de 10 y 12 meses de edad.

Palabras Clave: Donación de óvulo, eCG, estimulación, producción de embriones *in vitro*. (Fuentes: DeCS, AIMS).

INTRODUCTION

The potential of commercial application of *in vitro* embryo production (IVP) programs in fetal (1) and prepubertal females (2) offers accelerated genetic gain through a reduction in the generation interval since these animals represent a rich source of germ plasm (3-5). Furthermore, the use of IVP in prepubertal females is mainly useful in the genetic improvement of cattle breeds where puberty is achieved later, such as *Bos indicus* breeds (6). *Bos indicus* breeds and their crossbreeding with *Bos taurus* offers broad characteristics of tolerance and resistance to the extreme conditions in the tropics (7). These selective breeding effects (heterosis) on *in vitro* embryo production have been evaluated by Camargo et al (8) who evidenced that blastocyst development of Gyr (*Bos indicus*) and crossbred embryos is greater than with Holstein embryos.

Through IVP, it would be possible to produce embryos from crossbred prepubertal donors with greater genetic merit for dairy or beef crossbred herds (6). Nevertheless, previous studies have reported that developmental competence of oocytes from prepubertal donors is low in both *Bos taurus* and in *Bos indicus* (2, 6, 9-11). Low follicular activity needs to be stimulated in some donors, mostly by using Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), their combinations or equine chorionic gonadotropin (eCG) (12-14). Some studies have demonstrated that the administration of gonadotropin increases the number of viable oocytes and the follicular development per session. These have been administrated at different schemes, dosages, and products with different results (10, 15-20). Few studies have been conducted with prepubertal *Bos indicus* and *Bos indicus* crossbreeds as donors of oocytes. But none have evaluated the effect of the time interval between hormonal stimulation and oocyte recovery (coasting period) (21,22) and the donor age in prepubertal *Bos indicus* crossbred females. A correct costing period is required to have an excellent process of *in vivo* prematuration and *in vivo* final maturation (22). This study was developed

to evaluate the effect of age of the donor at the OPU's time and the coasting period after gonadotropin stimulation over oocyte quality and their posterior development under *in vitro* conditions from prepubertal *Bos indicus* crossbred donors.

MATERIALS AND METHODS

Gonadotropin stimulation. Twenty one *Bos indicus* crossbred (F1, Gyr x Holstein) prepubertal females, received a norgestomet sub cutaneous implant (Day 0; Crestar; Intervet International B.V.) and estradiol benzoate (1 mg; Syntex Laboratories). Four days later (day 4) a sole total dosage of 150 IU of Equine Chorionic Gonadotrophin (eCG; Folligon; Intervet International B.V.) was administered to each female. After the follicular aspiration, the implant was removed.

Chemical reagents. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Chemical Co.

Oocyte Recovery. After stimulation, three coasting periods were established. The first group of animals was aspirated 24 hours after the eCG administration, the second group of animals was aspirated 48 hours after the gonadotropin administration, and the last group was aspirated 56 hours after the eCG administration. Prior to follicular aspiration, the animals were anesthetized with a xilacine and ketamine combination. The ovaries were exposed by midventral laparotomy, and all antral follicles were aspirated using a 16-gauge needle attached to a 10 mL disposable syringe (15).

The oocytes were aspirated in a medium consisted of 0.9% saline sterile solution supplemented with 1% penicillin and streptomycin, 10% fetal bovine serum (FBS), and 5 IU/mL of sodium heparin. Immediately the content of the syringe was transferred into an Em-Con filter and washed 2-3 times. Cumulus-oocyte complexes (COCs) were collected, evaluated, and washed in this medium and then subjected to *in vitro* maturation. The oocytes were classified under the following parameters: Quality I (QI) - oocytes with homogeneous cytoplasm and at least four compact cumulus cell layers; Quality II (QII) - oocytes with some granulation in cytoplasm and less than four compact cumulus cell layers; Quality III (QIII) - oocytes with homogeneous or some granulation in cytoplasm and some cumulus cells expanded; Quality IV (QIV) - oocytes with expanded cumulus; Quality V (QV) - naked oocytes.

***In vitro* maturation (IVM).** Maturation was performed from 22 to 26 hours. The maturation medium was bicarbonate-buffered TCM-199, containing 10% FBS, 0.01 mL/IU of FSH, (0.1 IU/mL of LH, 0.055 mg/mL of pyruvate, 100 IU/mL of penicillin, and 100 µg/ mL of streptomycin) (23). Oocytes from individual donors were placed into cryotubes containing 700 µL of maturation medium covered with 300 µL of mineral oil. Each cryotube was gassed with a mixture of 5% CO_2 and 5% O_2 before being sealed. They were stored and transported at 38°C during approximately ten hours to the *in vitro* fertilization (IVF) laboratory. At the laboratory, each cryotube cap was unscrewed and the cryotubes were transferred to the incubator in a humidified atmosphere of 5% CO_2 in air at 38.5°C.

***In vitro* fertilization (IVF).** After maturation, matured COCs were transferred into 50 µL droplets under a mineral oil fertilization medium (TALP-FERT, 23) enriched with BSA, piruvate, and antibiotics. Motile spermatozoa were obtained by frozen-thawed spermatozoa on a Percoll discontinuous density gradient. Spermatozoa were diluted to obtain a final concentration of 1 x 10^6 cells/mL and coincubated with COCs for 18 h in a humidified atmosphere of 5% CO_2 in air at 38.5°C.

***In vitro* embryo culture (IVC).** Presumptive zygotes were denuded by agitation for a minute and cultured under mineral oil in 50 µL droplets of CR2aa medium (24) enriched with 10% fetal calf serum, and 1 mg/mL of BSA. Every 48 h, half of the medium was replaced. Seven days post-insemination embryo development was evaluated.

Statistical analysis. This study involved four age groups of animals: 3, 6, 10, and 12 months. Each group was conformed by three groups of different aspiration hours after stimulation 24, 48, 56 h. All groups were managed as a single experiment. Statistical computations were performed by using variance and student-t test analysis on all data (SAS 9.1.3 Version, SAS Institute Inc.)

RESULTS

The prepubertal status of the animals was confirmed by the absence of the corpus luteum or corpus albicans prior to the time of follicle aspiration (6). Before aspirations, the follicles were counted, measured, and classified according to their size: < 5 mm, 5-10 mm, and > 10 mm (25). From the total number of 439 aspirated follicles, 385 (87.7%) were 2-5 mm, 41 (9.33%) were 5-10 mm, and 13 (2.3%) were >10 mm. There were no differences among the coasting periods ($p<0.05$), but differences were found ($p<0.05$) between 6 and 10 months animals for the aspirated follicle number: 10.5 (8.7%) and 37.3 (34.67%), respectively. Differences were not found ($p>0.05$) for the 5-10 and > 10 mm sizes. After aspiration, a total of 373 oocytes (84.9%) were recovered, and differences were located ($p<0.05$) between the oocite recovery averages of 6 (9.3) and 10 months (32.3) (Table 1).

Table 1. Effect of age and three different coasting periods post-stimulation on follicular size from *Bos indicus* crossbred prepuberal females.

Age (Months)	No. of animals	Aspirated Follicles	Size of aspirated follicles			Recovered oocytes (%)
			2-5 mm (%)	5-10 mm (%)	>10 mm (%)	
3	6	26.1	23.3 (92.9)	2.6 (6.2)	0.1 (0.9)	20.5 (89)
6	6	10.5 ^{A}	8.1 (76.1) ^{B}	1.6 (17.4)	0.6 (6.4) ^{A}	9.3 (87)
10	3	37.3 ^{B}	34.6 (92.1) ^{A}	2.3 (6.7)	0.3 (0.8) ^{B}	32.3 (90.7)
12	6	17.8	15.3 (86.4)	1.3 (7)	1.1 (6.5)	16.1 (74.3)

(A,B) Mean values followed by different letters in the same column differ statistically between ages ($p<0.05$). There were no differences between hours of aspiration post stimulation for each variable ($p<0.05$).

The oocytes were classified according to their quality. Although the number of oocytes for the QI and QII were low, significantly different values ($p<0.05$) (Tables 2 and 3) were found among coasting periods; 24 h being the period with the highest averages. Furthermore, there were no differences among ages for these qualities.

Table 2. Effect of age on quality of oocytes recovered from *Bos indicus* crossbred prepuberal females.

Age (Months)	No. of animals	Recovered oocytes	Quality I (%)	Quality II (%)	Quality III (%)	Quality IV (%)	MIV (%)
3	6	21	0.5(1.3)	0.6(1.8)	17.1(78.3)	2.1(18.5) ^{A}	16.8(75.8) ^{A}
6	6	9.3 ^{B}	0(0)	0(0)	5.8(62.5) ^{B}	3.5(37.4)	6.1(65) ^{B}
10	3	32 ^{A}	0.3(0.8)	0.3(0.8)	29.3(90) ^{A}	2.3(8.3)	28.3(86.6) ^{A}
12	6	16	0(0)	0(0)	11.3(69.8)	4.8(30.1) ^{B}	10.3(64.1) ^{B}

(A,B) Mean values followed by different letters in the same column differ statistically between ages ($p<0.05$).

Quality III had the greatest number of oocytes and differences were found ($p<0.05$) (Tables 2 and 3) only between the ages 6 (62.5%) and 10 months (90%). For the QIV, there were differences between the ages 3 and 12 months with the latter having the highest value (30.15%). Moreover, 56 h was the coasting period with the highest averages for this quality (4.1,31.4%). From the total recovered oocytes, 285 (76.4%) were subjected to *in vitro* maturation/*in vitro* fertilization/*in vitro* embryo culture (IVM/IVF/IVC).

Table 3. Effect of three coasting periods post stimulation on quality of oocytes recovered from *Bos indicus* crossbred prepuberal females.

Coasting periods (h)	No. of animals	Quality I (%)	Quality II (%)	Quality III (%)	Quality IV (%)
24	7	0.571(1.5) ^{a}	0.7(1.9) ^{a}	19.5(80.2)	1.61(16.4) ^{a}
48	7	0(0) ^{b}	0(0) ^{b}	11.5(70.5)	4.3(29.5) ^{b}
56	7	0(0) ^{b}	0(0) ^{b}	10.8(68.5)	4.1(31.4) ^{b}

(a,b) Mean values followed by different letters in the same column differ statistically between hours of aspiration post-stimulation ($p<0.05$).

The cleavage values were significantly higher ($p<0.05$) in 10 (27.1%) and 12 months of ages animals (26.8%). Although the number of transferable embryos was low, there were differences between ages ($p<0.05$) and, 10 months of age animals obtained the highest percentage (12.6%).

Table 4. Effect of age and three different coasting periods post-stimulation on embryo development of *in vitro* fertilized oocytes from *Bos indicus* crossbred prepuberal females.

Age (Months)	No. of animals	CIV (%)	1 cell (%)	Cleavage (%)	Transferable embryos (%)
3	6	16.8(75.8) ^{A}	14.8(87.8)	2(12.2) ^{A}	0.5(1.5) ^{A}
6	6	6.1(65.1) ^{B}	5.1(84.7)	1(15.2) ^{A}	0(0) ^{A}
10	3	28.3(86.6) ^{A}	20.6(72.8)	7.6(27.1) ^{B}	4(12.6) ^{B}
12	6	10.3(64.1) ^{B}	7.3(73.1)	3 (26.8) ^{A}	0.3(2.8) ^{A}

(A,B) Means followed by different letters in the same column differ statistically between ages ($p<0.05$). There were no differences between hours of aspiration post stimulation for each variable ($p<0.05$).

There were no differences ($p<0.05$) among coasting periods for cleavage and transferable embryo values (Table 4).

DISCUSSION

This is the first study to investigate the effect of time interval between hormonal stimulation and oocyte recovery (coasting period, 21) and the donor age in prepubertal *Bos indicus* crossbred cattle.

When the ovarian response was evaluated, we found that follicular development was similar among the different coasting periods. In contrast, Blondin et al (21) evidenced that a longer coasting period resulted in a greater percentage of 5-10 mm follicles in stimulated cows. We only found differences for the total aspirated follicles and follicles of 2-5mm between 6 and 10 months of age, of which 10 months had greater values (92.1% for ‹5mm follicles). These results differ from Snel-Oliveira et al (26), who did not find differences in the total follicle number (≥3mm) among Nelore donors at 10, 11, and 12 months, or differences in stimulation treatments. We did not find differences with the 12 months of age animals in which the number of follicles was lower than 10 months of age ones (26).

Molina et al (27) showed that hormonal stimulation with gonadotropins increased oocyte quality and their in vitro development. On the contrary, Snel-Oliveira et al (26) reported that there were no differences in the total recovered oocytes or in the viable oocytes between ages and stimulation treatments. In the present study, qualities I and II of recovered oocytes were only found in the 24-h coasting period, the quantity of expanded follicles increased with the coasting period after stimulation, most oocytes were quality III, and 10 months of age animals had the highest quantity of QIII oocytes. Thus, indicating that oocyte quality declines with the increase of the coasting period. In contrast, Blondin et al (21) found no effect of 33 h and 48 h coasting periods on types of COCs from stimulated cows. Oropeza et al (28), reported 53, 61 and 60% of competent oocytes from un-stimulated *Bos taurus* (Holstein) calves at 6-7, 9-10, and 11-12 months of age, as well as blastocyst rates of 1, 9, and 10% for the same ages; therefore, showing that age affects cleavage rates.

In this study, the cleavage and transferable embryos were higher in older donors (9.5-10 and 11.5-12 months) and these results agree with previous reports. For example, the oocytes collected from non-stimulated calves at 1-4 months (9), between 5 and 9 months of age (16), and from 6-8 months *B. taurus* (11), lacked embryonic competence to produce viable transferable embryos. Additionally, Oropeza et al (28) evidenced that oocytes from *Bos taurus* calves between 6-7 months of age produced lower blastocyst development rate. Furthermore, Camargo et al (6), showed that non-stimulated *B. indicus* crossbred 4-7 month old calves are less competent than oocytes derived from cows, but oocytes from non-stimulated 9 to 14 month old heifers achieve similar developmentally competent as oocytes from cows. Camargo et al (6) showed that oocytes from non-stimulated *B. taurus* (Holstein) females reached competence at 11 months of age, suggesting that these oocytes have similar competence to those of cows. The reduced developmental competence of oocytes from prepubertal calves is attributed to a deficient expression of facilitative glucose transporters, insufficient protein translation (28), size, ultrastructure, metabolism, and cytoplasmic maturation (29).

Through this study, we observed broad individual variability among donors of

the same age. We found values among 0 and 64 recovered oocytes and embryo production rates from 0 to 7 in the same groups. These observations have been previously reported in prepubertal animals by other authors (15, 26, 30, 31). In conclusion, the results suggest that a coasting period higher than 24 h has a negative effect on oocyte quality and on their subsequent *in vitro* development. Additionally, some oocytes from 3 month old calves were competent for *in vitro* embryo development. However, a higher number of embryos were produced by 10 and 12 month prepuberal females, thus, indicating higher *in vitro* competence.

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