



EFFECT OF LACTATION ON OOCYTE QUALITY AND *IN VITRO* EMBRYO PRODUCTION OF *BOS INDICUS* COWS

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Summary

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The objective of the current study was to evaluate the effect of lactation on oocyte quality and *in vitro* embryo production in Gyr (*Bos indicus*) cows. A total of 187 multiparous cows (92 lactating and 95 non-lactating) were subjected to a single session of follicular aspiration. Follicle puncture was performed on a random day of the estrous cycle, without prior application of hormonal drugs. The recovered oocytes were subjected to maturation, fertilisation, and *in vitro* culture. The results indicated that in lactating Gyr cows had the following values of studied variables: number of follicles visualised (22.1 ± 11.38), number of recovered oocytes (17.4 ± 10.21), number of grade III oocytes (7.0 ± 5.86), rate of viable oocytes ($72.1 \pm 13.11\%$), number of blastocysts D7 (2.5 ± 2.26) and blastocyst rate ($18.3 \pm 15.61\%$), in relation to non-lactating cows (27.7 ± 11.53 ; 21.1 ± 10.06 ; 9.8 ± 6.08 , $77.4 \pm 10.08\%$; 4.9 ± 3.34 ; $30.2 \pm 15.41\%$, respectively). In conclusion, the lactation period in Gyr cows affects the performance of *in vitro* embryo production programmes.

Key words: bovine, *in vitro* maturation, milk production oocyte competence

INTRODUCTION

The maximisation of meat and milk production is closely related to the reproductive efficiency of herds and the appropriate use of assisted reproduction biotechnologies for the rapid multiplication of animals of high genetic value, becoming the fundamental basis of the global livestock industry to face part of the challenges that will arise in the coming decades (Moore & Hasler, 2017). Despite the evolution of reproductive techniques, some biological and environmental vari-

ables are still important barriers to the reproductive performance of female cattle. Particularly in dairy cattle, low herd fertility is related to environmental heat stress (Ferreira *et al.*, 2016), negative energy balance (NEB) (Maillo *et al.*, 2012) and high levels of milk production (Lucy, 2001). However, with respect to *in vitro* embryo production (IVP) programmes there are other biological variables that significantly affect the results such as breed or genetic group (Baruselli *et al.*,

2017), age (Merton *et al.*, 2003), reproductive status (Bayeux *et al.*, 2016), the phase of the estrous cycle (Machatková *et al.*, 1996), metabolic status (Mingoti, 2018), oocyte recovery at different degrees of development (Hendriksen *et al.*, 2000; Vassena *et al.*, 2003) and the *in vitro* culture systems employed (Lonergan & Fair, 2008). The influence of these factors can decrease oocyte competence for *in vitro* development and affect embryo production and quality, resulting in economic and genetic material losses (Ealy *et al.*, 2019).

Few studies have been developed in zebu breeds indicating the effect of lactation on embryo development *in vitro*. Snijders *et al.* (2000) observed a negative effect in high milk production cows on blastocyst production rates. A later study reported no difference on *in vitro* embryo production between lactating cows and heifers (Rizos *et al.*, 2005). Matoba *et al.* (2012) demonstrated a negative effect of metabolic status of postpartum cows on the ability of oocytes to be fertilised and develop to the blastocyst stage.

For this reason, the objective of the current study was to evaluate the effect of lactation on oocyte quality and *in vitro* embryo development in *Bos indicus* (Gyr) cows.

MATERIALS AND METHODS

The present study was conducted under the criteria of providing adequate care to the animals according to their ethology, avoiding unnecessary pain, suffering, stress, or prolonged injuries, reducing the duplication or unnecessary repetition of experiments, and using the minimum number of animals necessary to guarantee the validity of the present research (Garcés & Giraldo, 2012).

Animals and facilities

A total of 187 multiparous Gyr cows were used as oocyte donors, of which 92 were lactating females with calves at foot and an average milk production per day of 8.2 kg and 50 to 150 days in lactation, and 95 non-lactating animals. The age of the cows used ranged from 5 to 10 years, with an average weight for lactating cows of 473 ± 43 kg and 509 ± 51 kg for non-lactating cows. Body condition of lactating and non-lactating cows was 2.5 to 3 and 3.5 to 4, respectively. All animals were kept under similar pasture conditions (*Brachiaria decumbens* and *Brachiaria brizantha*), with mineral supplementation and water *ad libitum*. The lactating cows were fed corn silage and commercial supplementation based on 23% crude protein, to provide the necessary nutritional requirements. The cows in this category were milked twice a day at 12-hour intervals.

The donors of both productive stages were submitted to a single session of follicular aspiration; the technique was performed randomly and without previous application of hormonal drugs for the synchronisation of the follicular wave. The study was conducted under tropical conditions.

Ultrasound-guided follicular aspiration

The study animals were not subjected to follicular wave synchronisation; therefore, aspirations were performed on a random day of the estrous cycle. Prior to the application of the technique, epidural anaesthesia was performed between the last sacral vertebra and the first coccygeal vertebra by administering 0.2 mg/kg lidocaine (Roncaina[®], Ropsohnlab, Colombia) and subsequently the follicle count of both ovaries was advanced with the help of an ultrasound scanner (Mindray, DP 2200

VET, China), equipped with a 7.5 MHz micro convex transducer, coupled to a transvaginal guide for follicular aspiration. Aspirations were performed with an 18 G $\times$ 75 mm disposable needle, coupled to a Teflon line with negative pressure between 10 to 12 mL of water/minute (70 mm/Hg) produced by a vacuum pump with 50 mL tube heater (WTA, Cravinhos, SP, Brazil) and 1% foetal bovine serum (Gibco BRL, Grand Island, NY), maintained at 37 °C. Immediately after aspiration, the follicular fluid was transferred to a gamete wash filter (Agtech, USA) and 100 mL of D-PBS was added to remove clots and cells. Prior to oocyte selection, oocytes were washed in TCM 199 buffered with HEPES (25 mM) (TCM-199; Gibco BRL, Grand Island, NY) plus 10% fetal bovine serum (Gibco BRL, Grand Island, NY), 16 μ g/mL of sodium pyruvate and 83.4 μ g/mL of amikacin (Biochemical Institute, Rio de Janeiro, Brazil). Oocytes considered viable for *in vitro* maturation were grade I, II and III, classified according to the number of cumulus cell layers and homogeneous appearance of the cytoplasm. The classification of the COCs was carried out according to Wright (1998).

In vitro embryo production

CCOs classified as viable were conditioned in 1.5 mL cryotubes under the presence of TCM 199 supplemented with HEPES (25 mM), 10% SFB, 1.0 μ g/mL FSH (FolltropinTM, Bioniche Animal Health, Belleville, Canada), 50 μ g/mL of hCG (ProfasiTM, Serono, São Paulo, Brazil), 1.0 μ g/mL estradiol, 16 μ g/mL sodium pyruvate, ITS (5 μ g/mL insulin - transferrin - selenium) and 83.4 μ g/mL of amikacin. The cryotubes were introduced into an oocyte carrier at a temperature of 35°C and controlled gaseous atmosphere

(6% CO₂, 5% O₂ and 89% nitrogen), the time elapsed until arrival at the laboratory was considered as the maturation period. Upon arrival at the laboratory, the cryotubes with the CCOs were transferred to an incubator until the end of 24 hours of *in vitro* maturation.

For fertilisation, Gyr semen with known *in vitro* fertility was used. The straws were thawed at 35 °C for 30 seconds and the contents were carefully poured over the Percoll gradient 45/90 to obtain the motile spermatozoa and remove the diluent and seminal plasma. The corresponding inseminating dose for each drop was 1.0×10^6 spermatozoa/mL.

Gametes remained incubated on 50 μ L microdroplet plates for a period of 20 hours in FERT-TALP medium supplemented with 0.6% BSA, 10 μ g/mL heparin, 18 μ M penicillamine, 10 μ M hypotaurine, 1.8 μ M epinephrine and covered with sterile mineral oil. The atmospheric conditions used were the same as those used for *in vitro* maturation.

After the fertilisation period, the probable zygotes were transferred by donor in drops of 100 μ L of SOF medium supplemented with 2.5% SFB, 6 mg/mL BSA, 16 μ g/mL sodium pyruvate, 83.4 μ g/mL amikacin, 2.8 mM myoinositol, 340 μ M trisodium citrate dihydrate. and coated with sterile mineral oil for a period of 7 days. Every 48 hours the medium was removed and 50% of the volume of each drop was replaced. At 72 to 96 hours post-fertilisation, the cleavage rate was evaluated with the formation of two cells. Embryos were graded according to the International Embryo Technology Society (Stringfellow & Givens, 2010).

Statistical analysis

For each of the variables, an exploratory analysis was performed to analyse the

distribution of the variables according to lactation and to detect the presence of outliers. For each of the variables of interest, hypothesis tests were performed for the difference of means of two independent populations, verifying compliance with the assumptions of normality and homoscedasticity. When the assumptions of normality and homoscedasticity were not met, the Wilcoxon group difference test was used for the following variables: oocytes grade I, oocytes grade II, oocytes grade III and blastocyst rate. The significance level was 5%. The data were analysed with the help of the statistical software R 4.0.2.

RESULTS

The performance of the cows that conformed both productive stages during the *in vitro* embryo production technique is represented in Table 1. Statistically significant differences were found out for the following variables: number of follicles visualised, number of oocytes recovered,

number of grade III oocytes, rate of viable oocytes, number of D7 blastocysts and blastocyst rate.

DISCUSSION

Lactating cows often enter a state of NEB, due to energy requirements and milk production exceeding dietary energy intake (Maillo *et al.*, 2012). NEB is associated with a decrease in circulating concentrations of insulin, glucose and insulin-like growth factor type 1 (IGF-1) and at high concentrations of the non-esterified fatty acids and β -hydroxybutyrate (Sales *et al.*, 2015). A consequence of this is the deterioration in fertility and decreased efficiency of *in vitro* embryo production programmes. In cattle the follicular population is a characteristic of high repeatability (0.84–0.95) and with a number varying from 8 to 56 structures (Burns *et al.*, 2005). Studies evidenced that the follicular population is directly correlated to the genetic group, with *Bos indicus* females having more antral folli-

Table 1. Effect of lactation on the performance of the *in vitro* embryo production technique in Gyr cows. Data are presented as mean $\pm$ SD.

Variables	Lactating Gyr cows (n=92)	Non-lactating Gyr cows (n=95)	P-value
No. follicles visualized	22.1 $\pm$ 11.38	27.7 $\pm$ 11.53	0.0016
No. oocytes retrieved	17.4 $\pm$ 10.21	21.1 $\pm$ 10.06	0.0162
Recovery rate (%)	76.4 $\pm$ 8.30	75.2 $\pm$ 8.02	0.3262
Oocytes grade I	0.9 $\pm$ 1.42	0.7 $\pm$ 1.21	0.4927
Oocytes grade II	3.3 $\pm$ 3.65	2.8 $\pm$ 2.38	0.2184
Oocytes grade III	7.0 $\pm$ 5.86	9.8 $\pm$ 6.08	0.0003
Viable oocyte rate (%)	72.1 $\pm$ 13.11	77.4 $\pm$ 10.08	0.0048
Cleavage rate (%)	52.5 $\pm$ 28.68	56.1 $\pm$ 21.82	0.3276
No. blastocysts day 7 (D7)	2.5 $\pm$ 2.26	4.9 $\pm$ 3.34	6.604e ⁻⁰⁷
Blastocyst rate (%)	18.3 $\pm$ 15.61	30.2 $\pm$ 15.41	2.458e ⁻⁰⁶

cles than *Bos taurus* (Pontes *et al.*, 2010; Gimenes *et al.*, 2015). Other works have classified the bovine follicular population into 3 categories: low (LFC) (≤ 15), intermediate (IFC) (16 to 24) and high follicular count (HFC) (≥ 25) (Burns *et al.*, 2001; Ireland *et al.*, 2008; Jimenez-Krassel *et al.*, 2009; Mossa *et al.*, 2012). According to these findings, lactating cows in the current study can be categorised as intermediate follicle count, while non-lactating females: as high follicle count. It is possible to indicate that lactation in Gyr cows exerts a transient effect on follicular population.

Batista *et al.* (2014) demonstrated high correlation between follicular population and anti-Müllerian hormone concentration in *Bos indicus* heifers. Alward & Bohlen (2020), on the other hand, determined low concentrations of this hormone in animals with a low number of antral follicles. A previous study established that HFC and IFC animals had higher plasma concentrations of anti-Müllerian hormone relative to LFC females; this hormone is considered a possible marker of ovarian reserve size and the number of growing follicles (Alward & Bohlen, 2020). Another mechanism that has been related to high follicular population is the effect of insulin and IGF-1: higher plasma concentrations of these hormones are observed in *Bos indicus* females than in *Bos taurus* (Alvarez *et al.*, 2000).

The effect of lactation on oocyte quality is a highly complex mechanism, influenced mainly by metabolic status and environment (Maillo *et al.*, 2012), lactating cows being the most susceptible to the effects of heat stress (Tao *et al.*, 2020). However, studies have shown that *Bos indicus* females also show a deleterious effect of heat stress on oocyte quality

(Torres-Júnior *et al.*, 2008). In the current study it was possible to observe that non-lactating cows presented a higher rate of viable oocytes than lactating cows. These results indicate that the oocyte viability of Gyr cows may be affected during the lactation period. According to Sales *et al.* (2015), *Bos indicus* cows present greater deleterious effects on reproductive performance due to energy-rich diets, which causes an excess of insulin in the blood, altering the expression of genes associated with glucose metabolism and oxidative stress, significantly affecting oocyte quality (Wrenzycki *et al.*, 2001).

As for embryos produced by non-lactating cows, these were higher than those obtained by lactating cows. These results are similar to those reported by Maillo *et al.* (2012) and Vieira *et al.* (2014) in Holstein cows as oocyte donors, although there are no reports in the literature evaluating the effect of lactation in *Bos indicus* cows on performance in the IVP technique. Lactating Holstein cows have lower progesterone and estradiol concentrations (Wiltbank *et al.*, 2006) and higher concentrations of non-esterified fatty acids and β -hydroxybutyrate (Leroy *et al.*, 2005); this metabolism is associated with a sub-optimal follicular microenvironment that compromises oocyte quality and subsequently affects IVP programmes (Sartori *et al.*, 2004; Wiltbank *et al.*, 2006; Walsh *et al.*, 2011).

CONCLUSION

From the results obtained in the current study, it may be concluded that the lactation period in Gyr cows had a negative effect on the number of follicles visualised, the number of oocytes recovered, the number of grade III oocytes, the rate of

viable oocytes, the number of D7 blastocysts and the blastocyst rate, affecting performance in IVP programmes.

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