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Association of recombinant bovine somatotropin (rBST) with equine chorionic gonadotropin (eCG) on antral follicle count and oocyte production in Holstein and Tabapuã heifers

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ABSTRACT - The objective of this study was to investigate whether the use of rbST and eCG prior to ultrasound-guided follicular aspiration (OPU) improves oocyte yield and quality in Tabapuã and Holstein heifers. The study was conducted in two phases, 20 days apart, in a change-over design. The dominant follicle was ablated two days (D-2) before two treatments: stimulation (6 Holstein and 8 Tabapuã), 500 mg of rbST (Boostin®) on D0 and 500 IU of eCG (Folligon) on D2; and control (6 Holstein and 8 Tabapuã), in which heifers received injections of the excipient. Heifers were aspirated on D4. Oocytes were subjected to a well established commercial *in vitro* embryo production protocol (Vitrogen®) and embryos were evaluated seven days after fertilization. There was an effect from the interaction of treatment and breed, so that hormonal stimulation increased antral follicle count (2-8 mm) in Tabapuã (29.9±2.6 to 41.4±2.6), but not in Holstein heifers (14.4±2.6 to 15.5±2.6). Tabapuã heifers had higher mean antral follicle count than Holsteins (35.6±1.8 vs. 15.0±2.1). The number of viable oocytes was not increased by stimulation in Tabapuã (from 4.7±1.0 to 5.2±1.1 in control and simulation, respectively) or in Holstein heifers (1.3±1.9 to 2.0±1.6 in control and simulation, respectively). There was no difference in the percentage of heifers with more than five viable oocytes in the group treated (33 vs 27%). The number of blastocysts was not affected by treatment (1.75 vs. 1.00 in hormonal stimulation and control, respectively). The increase in antral follicle count in the stimulated Tabapuã heifers did not reflect upon oocyte yield. The differential breed response to the hormonal treatment underscores the need for additional tests, especially for the Holstein breed, in order to enhance OPU efficiency.

Key Words: embryo, hormonal stimulation, ovary

Introduction

Oocyte yield obtained after bovine ovum pick up (OPU) and OPU-derived embryo yield after *in vitro* culture (IVP) are low, especially in relation to the antral follicle population available at the time of ultrasound-guided follicle aspiration (Blondin et al., 2002; Chaubal et al., 2007).

Equine chorionic gonadotrophin, with its FSH-like action and growth hormone, with its effect on IGF-I (Moreira et al., 2002), are important regulators of antral follicle development and their actions are related to oocyte viability (Cushman et al., 2001; Murugavel et al., 2009). In previous research (Sendag et al., 2008; Vasconcelos et al., 2009; Cushman et al., 1999), the individual potential of rbST and eCG on improving the number and quality of follicles and oocytes was demonstrated in cattle.

According to Vasconcelos et al. (2009), the use of FSH did not alter the number of follicles or oocytes, but oocyte quality, expressed as their proportion attaining the blastocyst stage, was greater. Sendag et al. (2008) concluded that FSH was superior to eCG in terms of OPU oocyte yield, but they did not use rbST in any combination with either hormone. Furthermore, in order to obtain the best results with FSH, repeated injections, extra labor and higher investment are necessary, compared with eCG (Chaubal et al., 2007).

However, it is important to notice that the studies of Blondin et al. (2002) and Rossa et al. (2009) showed that increases in oocyte yield after follicle growth stimulation protocols may adversely affect *in vitro* oocyte developmental competence. As for Zebu, there are limited reports, but it is clear that noticeable differences exist in constitutive and stimulated follicular dynamics and oocyte yields (Rossi, 2008; Carvalho et al., 2008; Rossa et al., 2009).

In the literature, there were no reports investigating the possible synergistic effects between eCG and rbST and much less considering the distinct antral follicle populations

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(Blondin et al., 2002) before treatment allocation. Finally, Holsteins usually produce less OPU-derived oocytes in relation to *Bos indicus* cows. This is also true for embryo production, either after superovulation (Sales et al., 2008) or OPU-derived oocyte culture (Merton et al., 2009).

The objective of this study was to investigate the effects of the combination of eCG and rbST prior to OPU on follicle growth and oocyte yield in Tabapuã and Holstein heifers.

Material and Methods

The trial took place at the dairy and beef research units of the animal science department at UFLA, Lavras, Minas Gerais state - Brazil, between December 2009 and January 2010.

Twenty-eight nulliparous Holstein (24.6±0.7 months old and 376.5±5.5 kg, n=12) and Tabapuã (a polled composite Zebu breed, 30.6±0.8 months old and 478.0±21.9 kg, n=16) heifers were used in this study. The Holstein heifers were kept on Tifton pastures and had free access to water and mineral mix. They also received corn silage and 1.0 kg of concentrate. The Tabapuã heifers were kept on a common Brachiaria pasture, with free access to water and mineral mix.

Two days before (D-2) the initiation of treatments, dominant follicles were aspirated by guided-ultrasonography using an Aloka SSD 500 unit, with a 5.0 MHz linear probe adapted to an ovum pick up vaginal rod (WTA®, Cravinhos, SP), under 45 mm Hg of vacuum pressure.

Follicle counts were performed by ultrasonography with the same equipment used for ablation of the dominant follicle. The number of subordinate follicles (2-8 mm in diameter) and the diameters of the dominant and co-dominant follicles were recorded on D-2, D0, D2 and D4. Images were digitally recorded and the image software Pro-Plus 4.5® (Media Cybernetics, MD- USA) was used for all measurements. Antral follicle counts from D4 were used for all statistical comparisons.

Treatments were applied on a change-over scheme, over two periods, and animals were randomly allocated to one of two treatments: simulation (6 Holstein and 8 Tabapuã) - 500 mg of rbST (Boostin®, Intervet, SP) on the first day (D0) and 500 IU of eCG (Folligon®, Intervet, SP) on D2; and control (6 Holstein and 8 Tabapuã) - heifers received excipient I.M. only. All follicle aspirations were performed on D4. This protocol was repeated 20 days after the first OPU, changing the treatment which each heifer had received previously. In period 2, one Tabapuã heifer allocated to the control group was injured and removed from the trial.

The OPU sessions were carried out with the methodology described by De Roover et al. (2005). Oocytes were classified under stereomicroscopy, according to Oropeza et al. (2004).

Oocytes were cultured, according to a commercial methodology (Vitrogen®, Sertãozinho, SP). The semen used was from a single bull of proven *in vitro* fertility.

All data were analyzed using Statistical Analysis System (version 9.1). Data relative to the effects of treatment, period, breed and interactions were analyzed via the GENMOD procedure. Means were compared by orthogonal contrasts and expressed as least square means and standart error of the mean. Significance was considered at $P<0.05$.

Results and Discussion

There was no interaction effect between experimental period and any other dependent variable, so the data illustrated here do not include period for clarification.

The combined rbST and eCG hormonal stimulus, prior to follicular aspiration, increased ($P<0.04$) the number of antral follicles (2-8 mm) in the Tabapuã breed, but not in the Holstein breed (Table 1). This result characterizes, then, an interaction effect between breed and treatment upon the average antral follicle count on the day of OPU or D4.

Table 1 - Effect of breed and recombinant bovine somatotropin/equine chorionic gonadotropin combined treatment on ovarian antral follicle count of Holstein and Tabapuã heifers

Treatment*	Breed				Mean treatment		Probability (P)		
	Tabapuã	n	Holstein	n		n	Treatment	Breed	T*B
rbST/eCG	41.4±2.6a	16	15.5±2.6c	12	28.4±1.9	28	0.001	0.000	0.040
Control	29.9±2.6b	15	14.4±2.6c	12	22.1±2.0	27			
Mean	35.6±1.8	31	15.0±2.1	24		55			

T*B - interaction between treatment and breed.
Values are least square means ± standart error of the mean.
Different letters within columns indicate statistical differences ($P<0.05$).
*Treatments were 500 mg of rbST (D0), followed by 500 IU of eCG on D2 and OPU on D4.

This interaction indicates that Holstein heifers might already have higher endogenous growth hormone concentrations and, therefore, would not respond equally to the given exogenous rbST, as the Tabapuã heifers did. Genetic selection for milk production, indeed, has increased circulating rbST in high milk producing dairy cattle lineages (Lefcourt et al., 1995). Perhaps, the rbST dosage should be increased for Holsteins to respond in the same pattern as the Tabapuã heifers did. This is an interesting finding, because, typically, Holstein cows yield much fewer oocytes than any of the Zebu breeds subjected to OPU. Any increase in viable oocytes in Holsteins should be very useful to improve OPU results in this breed.

The total number of antral follicles was higher (P<0.0001) in Tabapuã compared with Holstein heifers (Table 1). These numbers were comparable to those observed in the literature in Tabapuã (Rossi, 2008) and Holstein heifers (Chaubal, 2007). Similar findings have been reported when Gir and Holstein were compared in previous studies (Carvalho et al., 2008; Sales, 2010). The substantial increase in antral follicle number observed (35.6 ±1.8 vs 15.0±2.1 for Tabapuãs and Holteins, respectively) is in accordance with the much higher number of oocytes usually retrieved in *Bos indicus* cattle subjected to OPU (Carvalho et al., 2008; Sales, 2010). Whether this is a reflex of an inherent larger follicular population in Zebu ovaries or of a greater follicle turnover from the original ovarian pool is beyond the scope of the present study, but this basic difference certainly raises some interesting questions, reinforced by the differential breed responses to the hormones used in the present study.

The mean oocyte number was not influenced (P>0.5) by treatment (Table 2). The number of viable oocytes was greater (P<0.0001) for the *Bos indicus* heifers, which was also consistent with the breed effect observed on the antral follicle count and in the recent literature (Carvalho et al., 2008; Sales, 2010). Higher oocyte production was similarly found by Pontes et al. (2010) in *Bos indicus,* compared with Holstein cows. However, there was no interaction effect between breed and treatment (P = 0.52), so that, in neither breed, the hormonal treatment was able to increase the number of viable oocytes. It is possible that the combination of higher follicular fluid IGF-I concentrations (Sales, 2010) combined with less circulating FSH (Bastos et al., 2010) in *Bos indicus*, compared with those of *Bos taurus* cattle, may have prevented a similar effect of rbST on oocyte yield amongst the two breeds.

In the present study, the eCG treatment aimed to provide enough physiological support for follicle and oocyte development and to avoid superovulation and its deleterious effects on fertility (Nogueira et al., 2004). The relatively low eCG dosage also aimed to limit possible anti-eCG antibody production as well as to provide a cheaper and less labor-intensive option compared with regular FSH-protocols.

The average number of embryos reaching the blastocyst stage was not influenced by treatment (Table 3) and was comparable to those reported in the literature (Dode et al., 2002; Chaubal et al., 2007; Sales, 2010). Since the number of oocytes derived from the Holstein heifers was low, it was not possible to evaluate the main effect of breed on the final *in vitro* embryo development. Most embryos reaching the blastocyst stage derived from the *Bos indicus* heifers were reported as having higher follicular fluid IGF-I concentrations, which may have impaired any response to the rbST/eCG treatment. Regarding *in vitro* maturation capacity, IGF-I mediates expression of important genes encoding proteins responsible for the uptake of glucose by the oocyte, such as, GLUT 1, to ensure appropriate maturation and development, either *in vivo* or *in vitro*. Both IGF-I receptor and GLUT 1 transcripts have been found to be expressed in relative higher amounts in the follicular fluid of *Bos indicus* compared with that of *Bos taurus* (Sales, 2010) and, in this respect, help to explain the lack of a rbST/eCG treatment effect on the final *in vitro* embryo development, observed in the present study.

The mean number of oocytes recovered per OPU session was highly variable (0-17) between individuals and reflected an equally variable range in antral follicle counts. This observation underscores the need to evaluate donors before

Table 2 - Effect of breed and recombinant bovine somatotropin/equine chorionic gonadotrophin combined treatment on the mean number of viable oocytes after OPU in Holstein and Tabapuã heifers

Breed	Treatment*					Breed mean	Probability		
	n	Simulation	n	Control	n		Treatment	Breed	T*B
Tabapuã	16	4.7±1.0	15	5.2±1.1	31	4.9±0.7a	0.050	0.000	0.520
Holstein	12	1.3±1.9	12	2.0±1.6	24	1.6±1.2b			
Total	28	3.0±1.1	27	3.6±0.9	55	4.1±3.8			

T*B - interaction between treatment and breed; OPU - ovum pick up.
Different letters within the column indicate statistical differences (P<0.05).
Values are least square means ± mean standard error.
*Treatments were 500 mg of rbST (D0), followed by 500 IU of eCG on D2 and OPU on D4.

Table 3 - Mean number of *in vitro* developed oocytes derived from rbST and eCG-treated or untreated heifers

Treatment	n	Blastocyst	SEM	Probability (P)
				Treatment
Simulation	4	1.75	0.96	0.432
Control	3	1.00	0.00	

n - number of heifers producing oocytes that reached the final day in culture on each treatment. SEM - standart error of the mean.

their utilization in commercial OPU and embryo programs, as well as in research. The reasons for this variability, which resemble those found in reported superovulatory responses (Sales et al., 2008) are not clear, but should, in part, be explained by genetics. It has been shown that high antral follicle counts may be inherited and are highly repeatable within certain individuals (Merton et al., 2009).

It is possible that the OPU sessions in this trial were done too shortly after the rbST administration, and to the same effect, the eCG injection could have been delayed one or two days, so that extra time would be allowed for rbST to act. Especially for the Holstein heifers, higher numbers of follicles and oocytes could have been achieved with slight modifications in the protocol. For instance, higher rbST dosages could be applied in *Bos taurus* heifers.

However, another reason for the adoption of the present interval of only two days would be to avoid excessive follicle growth or maturation (Peres et al., 2009). Although larger follicles should be easier to visualize and aspirate than smaller ones, they do not necessarily produce more competent oocytes. In the present trial, the percentage recovery of viable oocytes relative to the number of follicles aspirated was approximately 11 and 14%, which is comparable to (Chaubal et al., 2007), higher (Fihri et al., 2005) or lower (Viana et al., 2010) than what has been reported.

Larger follicles, such as those produced under gonadotropin stimulation, may be mature and contain granulosa cell agglomerates and a more viscous follicular fluid (Goodhand et al., 1999). Consequently, a vacuum pressure of 45 mm Hg may not have been sufficient to aspirate the more dense follicular fluid of the larger follicles. In addition, a collapsed follicular wall around the needle may have impeded the flux or incarcerated the oocyte (Goodhand et al., 1999). The use of a needle of greater diameter could also have improved the results, although in combination with higher pressure it could damage oocytes and ovaries (Manik et al., 2003). Accumulation of luteal tissue may also be a consequence of advanced follicular development caused by eCG, which can interfere with the follicle: oocyte ratio in OPU (Stubbings & Walton, 1995).

In the present experiment, in spite of the dominant follicle ablation, variations in the follicular wave may have occurred, affecting oocyte yield directly. According to Adams et al. (1992), Fortune et al. (1985), Gibbons et al. (1999) and Ginther et al. (1999), at around 24 to 36 hours post ovulation, the growth of a follicular cohort occurs is characterized as a wave within the estrous cycle. In the present protocol, there was no rigid control of the follicular wave emergence. However, there are no records in the literature similar to the present stimulus in terms of follicular wave emergence. These data could be rescued from the recorded images for further analyses.

There are reports showing that gonatropin therapy decreases oocyte recovery (Goodhand et al., 1996; 1999; Sirard et al., 1999). An inverse relationship between follicle diameter and oocyte recovery rates (Seneda et al., 2001) was confirmed in the present study, by the observation of positive correlations between the number of antral follicles and total viable oocytes ($r^2 = 0.32$; $P<0.0002$) and the total number of oocytes retrieved ($r^2 = 0.36$; $P<0.0001$). Additionally, the number of structures retrieved increased linearly with the number of viable structures ($r^2=0.96$, $P<0.0002$), indicating that there was no loss of quality as a function of the amount of oocytes retrieved. This finding opens good possibilities to increase oocyte yield gains by stimulating ovarian follicular development in cattle.

Thus, the use of eCG and rbST may have improved oocyte morphological quality by decreasing the population of atretic follicles (Blondin et al., 1996; Cushman et al., 1999) and increasing antral follicle rescue from pre-established ovarian follicular pools (Chaubal et al., 2007). Some authors suggested that FSH-like effects synchronize the follicular development by accelerating growth and *in vivo* oocyte maturation, which could benefit later embryo development *in vitro* (Gibbons et al., 1994). Furthermore, the use of eCG may have altered the follicular environment around the oocytes, influencing their quality undetectably under the present experimental scope (Roth et al., 2002; Nogueira et al., 2004). It is well described that follicle stimulating hormone, as well as exogenous rbST, increase IGF-I receptor density (Spicer et al., 1994; Cushman et al., 2001) and decrease the amount of IGF 2-binding proteins in bovine follicles (Echternkamp et al., 1994).

Thus, the greater follicular development and the increase in the bioavailability and activity of IGF-I induced by the eCG FSH-like effect could be associated with the maintenance of oocyte quality observed in this experiment, which followed increased oocyte numbers. Although the blastocyst stage does not guarantee posterior *in vitro* development, it is a strong indicator of early development potential (McEvoy et al., 2000).

The mean number of embryos reaching the blastocyst stage was not influenced by treatment (Table 3) and was comparable to those reported in the literature (Chaubal et al., 2007). Multiple injections and OPU sessions once a week have been more efficient than single injection and shorter OPU session interval schemes (Goodhand et al., 1996), which differs from the results of the present study. Moreover, low cleavage rates may be the result of faulty pre-fertilization oocyte selection which have been reported in *in vitro* culture systems (Looney et al., 1994; Ushijima et al., 2008).

Similarly to the eCG used in this experiment, the FSH-based treatment improved pregnancy rates, according to Faber et al. (2003). In this trial, it was not possible to evaluate pregnancy rates, since embryos were not transferred to recipients and only a few embryos reached the blastocyst stage, limiting further conclusions.

Conclusions

Increases in antral follicle count by the combination of rbST and eCG should be recommended to *Bos indicus* only, according to the present experimental conditions. Since the number of viable oocytes is not affected by the hormonal treatment, further studies are necessary, especially in relation to change in the dosages for Holstein heifers.

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