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Research Article

Effect of Follicle Size of Boran Cows on Glucose 6 Phosphate Dehydrogenase Activity and Developmental Competence

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ABSTRACT

In vitro embryo production (IVEP) has been used to improve of cattle genotypes in many parts of the world. However IVEP is not without some challenges. Oocyte developmental competence is a key area affecting IVEP and it is associated with the glucose 6 phosphate dehydrogenase (G6PDH) activity and follicle size. Ovarian follicular dynamics differ between *B. indicus* and *B. taurus*, which may influence oocyte competence. This study sought to evaluate the effect of follicle size on G6PDH activity and oocyte developmental competence in the Boran (African Zebu breed) cattle using brilliant cresyl blue (BCB) staining. Oocytes were collected from Boran cows at slaughter, their follicles were measured and classified into 3 groups; 1-3mm, >3-6mm and >6mm in diameter. Cumulus oocytes complexes (COC) were exposed to BCB stain for 1 hour and observed for cytoplasmic coloration. Those that retained the blue coloration (BCB positive) were deemed competent (low activity of G6PDH) while those that did not have any cytoplasmic coloration (BCB negative) were deemed incompetent (high activity of G6PDH). Higher proportion ($P<0.01$) of BCB positive oocytes was found in >6mm follicles (81.1%) than in 1-3mm (73.1%) and >3-6 mm (76.5%) follicles. BCB positive oocytes from 1-3 mm follicles had higher ($P<0.05$) blastocyst rate (18.94%) than BCB negative oocytes (9.7%), however no significant difference was found in their cleavage rates ($P > 0.05$). No difference in cleavage and blastocyst rates was found between BCB positive and negative oocytes from follicles with >3-6 mm in diameter. The cleavage and blastocysts rates with BCB positive oocytes increased as follicle diameter increased. In conclusion, in Boran cows as follicle size increases the activity of G6PDH decreases in the whereas COC developmental competence increases and follicles as small as 1-3 mm in diameter are able of producing developmentaly competent oocytes.

Key words: *In vitro* embryo production, Boran cow, Brilliant Cresyl Blue, Follicle size

INTRODUCTION

Reproductive technologies have been largely responsible for the development of the livestock industry the world over. These have resulted in the improvement of cattle genotypes and enhanced their utilization. In vitro embryo production is one such technology that has gained popularity over the years for its ability to improve the genetic quality of a breed. This technology has been predominantly used in the improvement of *Bos taurus* herds although in the recent past countries such as Brazil have utilized the technology to improve their *Bos indicus* breeds (Viana *et al*., 2010a).

In Africa there exist vast genetic pools of cattle breeds that are well adapted to the unfavorable tropical climates but are yet to be fully utilized. In East Africa the Boran cattle, a *Bos indicus* breed has been identified as a suitable candidate for improvement for increased utilization (Rege *et al*., 2001).

Despite its success, IVEP is still faced with some challenges and thus is unable to mimic the success rates observed in *in vivo* production. At present blastocyst development from the IVEP system has only achieved a 30- 40% success rate (Camargo *et al*., 2006)

Oocyte competence prior to in vitro maturation has been identified as one of the key areas affecting this process. Developmental competence is the ability of the oocyte to produce normal, viable and fertile offspring after fertilization (Duranthon and Renard, 2001). The developmental competence of the oocyte is acquired

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within the ovary during the stages that precede ovulation or in case of IVEP, during the stages preceding isolation of the oocyte from its follicle. It has been shown that immature oocytes are heterogeneous in terms of quality and developmental competence (Gordon and Lu 1990).

The ability of the oocyte to complete meiosis is known as meiotic competence, which is acquired gradually during follicular growth and correlated with oocyte and follicle size (Miyano, 2003). However, to achieve developmental competence oocytes also need to undergo cytoplasmic maturation which involves organelle reorganization and RNA and protein accumulation (Mermillod *et al*., 1999, Ferreira *et al*., 2009). It is known that oocyte developmental competence for IVEP increases with follicle size (Hazeleger *et al*., 1995, Mermillod *et al*., 1999, Hendriksen *et al*., 2000). For instance, Blondin and Sirard (1995) reported that oocytes from follicles with diameters of 3 mm or smaller did not develop after 16-cell stage, in contrast to oocytes from larger follicles. However, differences in ovarian follicular dynamic between *B. indicus* and *B. taurus* cattle should be taken in account when evaluating the oocyte competence. For *B. indicus* the maximum diameter of dominant follicle is around 10-12 mm in diameter whereas for *B. taurus* is around 14-20 mm (Bo *et al*., 2003). In contrast, number of follicles is higher in Bos indicus, but mainly number of follicles with less than 5 mm in diameter (Segerson *et al*., 1984, Alvarez *et al*., 2000).

Several methods have been utilized to evaluate the competence of oocytes prior to maturation. One such method is evaluating the activity of glucose 6 phosphate dehydrogenase activity. Immature oocytes are known to synthesize a variety of proteins (Wassarman, 1988), such as glucose-6-phosphate dehydrogenase (G6PDH) enzyme. This enzyme is active in growing oocytes (Mangia and Epstein, 1975) and its activity is decreased in oocytes that have finished their growth phase (Wassarman, 1988) and, therefore, achieved developmental competence. Evaluation of glucose 6-phosphate dehydrogenase (G6PDH) activity in oocytes can be performed by brilliant cresyl blue (BCB) staining (Alm *et al*., 2005). G6PDH can convert BCB from its blue colour into a colorless state (Ericsson *et al*., 1993). A BCB colour change represents high G6PDH activity which is a sign of incompetence (immaturity) in oocytes tested (Ferrandi *et al*., 2002). BCB stain can be used to select oocytes with a higher developmental competence in cows, with the BCB negative oocytes (colorless cytoplasm, high G6PDH) being of lower developmental competence than BCB positive oocyte (blue cytoplasm, lower G6PDH activity) (Ericsson *et al*., 1993). Despite the effects of follicle size on oocyte competence of *B. taurus* cattle and the association between oocyte competence and G6PDH activity, no study has reported associations between follicle size and oocyte G6PDH activity.

Considering the importance of IVEP as a tool for improvement of Boran cattle and the differences in ovarian dynamics between *B. taurus* and *B. indicus*, this study aimed to evaluate the effect of follicle size of Boran cows on glucose-6-phosphate dehydrogenase (G6PDH) activity and oocyte developmental competence.

MATERIALS AND METHODS

Experimental design

Ovaries were collected from mature Boran cows at slaughter. Their follicle diameter was measured and grouped based on their size: - group 1: 1-3 mm, group 2; >3-6 mm and group 3; > 6 mm in diameter. COC's were aspirated from these groups separately, selected and the morphologically viable ones (grade A and B) were exposed to 26 µM of BCB stain for 60 minutes in order to evaluate the G6PDH activity. After that, oocytes from each follicle size group were separated according to the cytoplasm color (BCB positive and BCB negative) and taken to the in vitro maturation and fertilization in order to evaluate their potential to develop until blastocyst stage.

Ovary collection and oocytes recovery

Ovaries from Boran cows were collected from a local slaughter house. The time between collection and transportation to the laboratory was maintained below 4 hours. Each ovary was examined for visible follicles and the ovaries without visible follicles were discarded. The size of visible follicles on the ovary was measured and number of follicles was counted.

COC's morphological analysis was performed according to De Loos *et al*. (1989) with modification from Tamassia *et al*. (2003). Briefly, grade A COC's corresponds to intact immature COCs with three or more layers of dense cumulus cells and homogeneous cytoplasm while Grade B COCs have fewer layers (2-3) of compact cumulus investment or is partially denuded oocyte with homogeneous cytoplasm. Only compact grade A and B COC were utilized for the study.

G6PDH activity analysis by BCB staining

Staining with BCB was performed for grade A and B COC's obtained from follicle of group 1, 2 and 3. The COC's were washed twice in TALP-Hepes media and once in modified Dulbecco's PBS and then transferred to an Eppendorf tube containing 500µL of modified Dulbecco's PBS supplemented with 26 µM of BCB stain. The COC's were exposed to BCB stain for 60 minutes at 38.5 ºC in a water bath (Mota *et al*., 2010). After BCB exposure period, the COC's were transferred to modified Dulbecco's PBS and washed twice before being examined under a stereo microscope. During this examination, COC's were divided into two groups according their cytoplasm coloration and counted. BCB positive oocytes were the ones with any degree of blue cytoplasmic coloration while BCB negative oocytes were those without blue coloration in the cytoplasm. The COC's were then transferred to TALP hepes media (Gordon, 1994) and washed twice before being placed into maturation media for *in vitro* maturation procedure.

In vitro maturation (IVM)

Before IVM the COC's were washed twice with TALP hepes media and once in Tissue culture medium (199 medium; Invitrogen, Grand Island, USA) supplemented with 10% FCS, and 10mg/ml of FSH, 10mg/ml LH and 1mg/ml of 17βEstradiol. The COC's were then placed in a sterile four-well plate (Nunc, Roskilde, Denmark) containing 400ul of a pre-prepared

TCM 199 medium that was previously incubated at 38.8°C and 5% CO2. IVM was performed at 38.8°C and 5% CO_2 in air for 22-24h.

In Vitro Fertilization (IVF)

Semen from one Jersey bull was used for this study. This semen had been pre tested for suitability in vitro embryo production. After maturation, COC's were examined and judged on the basis of their cumulus expansion. COC's with full expansion of cumulus cells were considered mature. The matured oocytes were then washed twice with TALP-hepes media and once with Fert -TALP medium (Gordon, 1994). Matured oocytes (n=20-40) in 20 ul volume were then transferred into a prior prepared IVF droplet of Fert-Talp medium supplemented with 20 µg/µL heparin.

Motile sperm were obtained by Percoll discontinuous (45-90%) density gradient (Nutricell, Campinas, Brazil) and added to the IVF droplet. The semen concentration was adjusted to contain 1 X 10^6 sperm per ml and the determined volume of semen picked up and added to this droplet containing the matured oocytes. The droplet volume was then topped up to 100ul using Fert-TALP medium. The droplets containing matured oocytes and capacitated sperms were then cultured in an incubator at 38.8°C at 5% CO_2 and maximum humidity for a period of 18- to 22 h.

***In Vitro* culture, cleavage and blastocyst rate assessment**

After 18-22hr incubation, the fertilized zygotes were transferred into a Petri-dish containing TALP-hepes medium. The zygotes were then washed twice in TALP-hepes medium and once in CR2aa culture medium (Wilkinson *et al*. 1996). The zygotes were then co-cultured with their cumulus in 100ul droplets of CR2aa culture medium covered with a film of oil for 168 h in an incubator at an atmosphere of 5% CO_2 at 38.8°C and maximum humidity. Cleavage and blastocyst rates were evaluated at 72 and 168 h post-insemination, respectively.

Statistical analyses

Proportional data were analyzed by chi square, except cleavage and blastocyst rates that were analyzed by ANOVA in SAS ® 9.1(2008) statistical package. All materials were sourced from Sigma Chemical Company, unless otherwise stated.

RESULTS

The proportion of follicles with 1-3 mm diameter (58.7%; n=1132) found in Boran ovaries was higher (P<0.001) than the proportion of follicles with >3-6 mm (29.8%; n=575) or >6 mm (11.5%; n=222) diameter. As consequence, more COC's (P<0.001) were isolated from follicles between 1-3mm diameter (57.6%, n=938) as compared to those of 3-6mm (31.8%, n=519) and those larger than 6mm (10.5%; n=171) in diameter. The overall cleavage and blastocyst rates after 10 replicates were 56.7% (696/1226) and 24.0% (168/698) respectively.

The G6PDH activity analysis, based on BCB staining, showed that greater (P<0.01) proportion of oocytes obtained from >6 mm diameter follicles (88.1%) has low activity for this enzyme than oocytes from other groups (Table 1), whereas no difference (P>0.05) was found between proportion of oocytes with low activity but obtained from 1-3 mm and >3-6 mm diameter follicles.

The oocyte ability to cleave and generate blastocysts is shown in the Table 2. Oocytes with low G6PDH activity and from follicle with 1-3 mm diameter produced higher (P<0.05) blastocyst rate than oocytes with high G6PDH activity whereas oocytes with low G6PDH activity but obtained from follicles with >3-6 mm diameter cleaved and generated blastocyst at similar rate (P>0.05) to those ones with high G6PDH activity. Due to the low number of oocytes with high G6PDH from follicles larger than 6 mm diameter no statistical analysis was performed between high and low G6PDH activity in this follicle group and, therefore, data are not shown in the table 2. When considering only oocytes with low G6PDH activity, the cleavage and blastocysts rates increased as follicle diameter increased, with oocytes from follicles larger than 6 mm diameter producing the highest rate of blastocyst (29.03%).

Table 1: Influence of follicle size on G6PDH activity based on brilliant cresyl blue (BCB) staining

Follicle size	BCB positive n (%)	BCB Negative n (%)
1-3 mm	445 (73.1)[a]	163 (26.8) [a]
>3 -6 mm	235 (76.5) [a]	72 (23.4) [a]
>6 mm	97 (88.1) [b]	13 (11.8) [b]

[a, b] Different letters in the same column differ statistically by Chi-square, P<0.01; BCB Negative: oocytes showing no cytoplasm coloration representing high G6PDH activity; BCB Positive: oocytes showing blue cytoplasm coloration representing low G6PDH activity

Table 2: Cleavage and blastocyst rates of immature oocytes from different follicle sizes and with different G6PDH activity, based on brilliant cresyl blue (BCB) staining

Follicle size group	BCB	N	Cleavage rate % (n)	Blastocyst rate % (n)
1-3 mm	Negative	134	67.91% (91) [a]	9.7% (13) [a]
1-3 mm	Positive	417	72.18% (301) [ab]	18.94% (79) [b]
>3 -6 mm	Negative	51	66.66% (34) [ab*]	13.72% (7) [ab]
>3 -6 mm	Positive	221	67.87% (150)[a]	19.45% (43) [bc]
>6 mm	Positive	93	80.64% (75) [b*]	29.03% (27) [c]

[a, b, c] Different letters in the same column differ statistically by Chi-square, P<0.05; * Tend to differ, P=0.62; BCB Negative: oocytes showing no cytoplasm coloration representing high G6PDH activity; BCB Positive: oocytes showing blue cytoplasm coloration representing low G6PDH activity.

DISCUSSION

The current study aimed to evaluate what effect the follicle size would have on in vitro embryo production and oocyte G6PDH activity, which is associated to oocyte competence in Boran cows. Boran cow is an African breed that is well adapted to tropical conditions.

This study observed higher proportion of 1-3 mm diameter sized follicles in the Boran ovaries and lower G6PDH activity oocytes from large-sized (over 3 mm) follicles (indicating higher oocyte competence in oocytes from such follicles). Thus, chances to generate blastocyst after in vitro fertilization increases as the follicle diameter size increases because of increased oocyte competence. This information establishes that during ovum pick from

live Boran donors, oocytes should be collected from follicles with diameter sizes of over 3 mm in order to increase the success rate of IVEP. It can also be recommended that the population of follicles with 1-3 mm diameter size can be stimulated using hormonal protocols to stimulate their growth to over 3mm size prior to harvesting to boost oocyte competence for improved embryo production during in vitro fertilization

The Boran is predominantly *B. indicus*, but also contains *B. taurus* background (Rege *et al*., 2001). Thus, physiological differences are expected whenever the Boran is compared to purebred *B taurus* and *B indicus* breeds. Therefore, in the Boran cow, there is an agreement with the several studies that showed an effect of follicle size influencing oocyte competence of *B. taurus* breeds (Mermillod *et al*., 1999, Hendriksen *et al*., 2000).

Oocytes from follicles larger than 6 mm diameter showed the lowest G6PDH activity. Follicles in their growth phase have high G6PDH activity (Tsutsumi *et al*., 1992, Pujol *et al*., 2004). For the Boran cow, follicles larger than 6 mm in diameter have finished their growth phase and therefore have low G6PDH. Consequently, such follicles have oocytes that have attained competence to develop into blastocysts. It is also known that oocyte competence is achieved at dominance phase of folliculogenesis (Hyttel *et al*., 1997) and that for *B. indicus* breeds like the Boran, follicle dominance is reached at 6 mm diameter (Gimenes *et al*., 2008; Viana *et al*., 2010). However, for European breeds, dominance is reached at larger follicle diameter of over 6mm (Savio *et al*., 1988, Ginther *et al*., 1997).

An increase in blastocyst rate was observed as the follicle diameter size increased, with the highest rate seen for oocytes from follicles larger than 6 mm diameter (Table 2). This data confirms the developmental competence indicated by the low G6PDH activity in oocytes from larger follicles and is in agreement with other studies (Lonergan *et al*., 1994, Blondin and Sirard, 1995, Lequarre *et al*., 2005). Evaluating Brazilian *B indicus*, Caixeta *et al*. (2009) reported high blastocyst rate when oocytes were aspirated from follicles larger than 6 mm in diameter size. This was also the case with the Boran cow.

Some oocytes obtained less than 3 mm follicles showed low activity of G6PDH (Table 1), suggesting that they were fully grown and competent. The same oocytes also produced blastocysts (Table 2). This data contrast the work of Blondin and Sirard (1995) that reported no development after 16-cell stage for oocytes from follicles with 3 mm or smaller. A reason for this difference may be that some of the oocytes were close to 3mm diameter size, which could be the critical size for competence. Although a follicle with 3 mm diameter has an ooycte with an approximate diameter of 110 µm (Hyttel *et al*., 1997, Fair, 2003), it was noted that Boran follicles, like the rest of Bos indicus cows, had oocytes of 124 µm diameter size arising from follicles of 1-3 mm diameter sizes (Caixeta *et al*., 2009). Considering that the oocyte acquires full developmental competence at 120 µm diameter (Otoi *et al*. 1997), then it can be speculated that Bos indicus oocytes from smaller diameter follicles may be competent. Another possible explanation for the ability of oocytes from 1-3 mm diameter follicle in producing embryos is based on follicular development. There is a possibility that some oocytes of atretic smaller follicles had reduced G6PDH activity as suggested by Hendriksen *et al*. (2004). As only oocytes with compact cumulus cells were selected for the present study (grade A and B), it is also possible that oocytes from follicles in the beginning of atresia were used, explaining also the likely proportion of oocyte competence within follicles of 1-3 mm diameter size.

Conclusion

It can therefore be deduced that the G6PDH activity (oocyte competence) of Boran oocytes is influenced by oocyte and the follicle size. Oocyte competence increases as G6PDH decreases and the oocyte and follicle diameter size increases. Although some follicles as small as 1-3 mm in diameter are able to produce oocytes with low G6PDH activity and are competent to develop to blastocyst stage, the surest size of follicle diameter size to yeild competent oocytes in the Boran cow is 3mm and above.

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