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In vitro performance of Zebu (*Bos indicus*) and Taurus (*Bos taurus*) donor cow embryos

Desempenho de vacas doadoras zebuínas (Bos indicus) e taurinas (Bos taurus) na produção de embrião in vitro

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ABSTRACT

In this study, the *in vitro* production of bovine embryos from zebu and taurine donors was compared. Cumulus-oocyte complexes (COCs) were obtained from 167 *Bos taurus* and 161 *Bos indicus* donors by ovum pick-up. COCs were classified based on their morphological quality, matured in incubators for 22 to 24 h in maturation medium, and then fertilized for 18 to 22 h. The zygotes were transferred to the culture medium for seven days. The embryos were classified as morula (OM), initial blastocyst (BI), blastocyst (BL), and expanded blastocyst (BX), before being transferred to synchronized recipient cows. Pregnancy was diagnosed 30–45 days post-transfer. The *Bos indicus* donors had a higher oocyte yield (n = 2556) than *Bos taurus* donors (n = 1903) (P = 0.008). The COCs from zebu donors had a better morphological quality than those from taurine donors (n = 689 *vs*. 444 for grade 1 COC, $P < 0.0001$; n = 681 *vs*. 509 for grade 2 COC, $P = 0.010$, for zebu and taurine donors, respectively). There were differences in embryo production percentages obtained from OM (0.44% from zebu and 6.42% from taurine, $P = 0.017$), BL (14.18% from zebu and 3.74% from taurine, $P < 0.0001$), and BX (81.43% from zebu and 75.13% from taurine, $P < 0.0001$). No significant difference was observed for embryo production from BI and pregnancy rate ($P > 0.05$). The *Bos indicus* cows showed greater oocyte recovery, number of viable oocytes, and production of viable embryos than the *Bos taurus* cows.

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Keywords: blastocyst, *in vitro* cultivation, oocytes, reproductive biotechniques

RESUMO

O objetivo foi avaliar a produção *in vitro* de embriões bovinos a partir de doadoras zebuína e taurina. Os complexos cumulus oócitos (COC's) foram obtidos de 167 doadoras *Bos taurus* e 161 *Bos indicus*, por meio de *Ovum pick-up*. Os COC's foram classificados quanto à qualidade morfológica, maturados em incubadora por 22 a 24h em meio de maturação, e encaminhados à fertilização entre 18 e 22h. Os zigotos foram transferidos para meio de cultivo, onde permaneceram durante sete dias. Os embriões foram classificados em mórula (MO), blastocisto inicial (BI), blastocisto (BL) e blastocisto expandido (BX), envasados em palhetas contendo meio de cultivo, e inovulados em receptoras sincronizadas. Após 30 e 45 dias da inovulação, realizou-se o diagnóstico de gestação. Os animais *Bos indicus* apresentaram maior produção oocitária (n = 2556) em comparação aos *Bos taurus* (n = 1903) (P = 0,008). Os COCs das doadoras zebu apresentaram melhor qualidade morfológica do que das doadoras taurina (n = 689 *vs*. 444 para COCs grau I, P < 0,0001; n = 681 *vs*. 509 para COCs grau II, P = 0,010, para doadoras zebu e taurina, respectivamente). Quanto à produção de embriões, houve diferença nas porcentagens obtidas de MO (0,44% zebuínas e 6,42% taurinas, P = 0,017), BL (14,18% zebuínas e 3,74% taurinas, P < 0,0001) e BX (81,43% zebuínas e 75,13% taurinas, P < 0,0001). Não foi observado diferença para BI e taxa de prenhez (P > 0,05). Os animais *Bos indicus* apresentaram maior recuperação oocitária e maior número de oócitos viáveis em comparação às vacas *Bos taurus*, como também, maior produção de embriões viáveis.

Palavras-chave: biotécnicas reprodutivas, cultivo *in vitro*, oócitos, blastocisto

INTRODUCTION

In the search for the means to improve and increase livestock productivity, the development and use of animal reproductive biotechniques have been considered indispensable (BARUSELLI et al., 2019a). Such biotechniques, including artificial insemination (AI), embryo transfer (ET), and the *in vitro* production of embryos (IVPE), have been successfully used on these animals (BARUSELLI et al., 2019a; BARUSELLI et al., 2019b).

According to Silva et al. (2015), IVPE has been used extensively in Brazil, and has been contributing to improving the quality and quantity of the final product of livestock production decisively. The prime goal of IVPE is to produce a greater number of descendants per female donor, since with this technique, it is possible to obtain up to one calf per week per donor, which makes it possible to greatly increase the length of the useful life of breeding cows (MELLO et al., 2016).

However, this biotechnique still continues to present some limitations due to the wide diversity of factors influencing the final results of IVPE, among which factors related to donors and recipients may be highlighted (MELLO et al., 2016).

Within this context, it is important that knowledge of the idiosyncrasies of the reproductive physiology of zebu and taurine cows is applied for the optimal

implementation of such biotechniques (BATISTA et al., 2020). Studies that show, for example, the differences between ovarian follicular populations, production of growth factors, and Anti-Müllerian hormone (BATISTA et al., 2014) support this theme. The understanding of these differences has been crucial in developing reproductive strategies specific for each genetic group (BARUSELLI et al., 2017).

The differences in the reproductive physiology of *Bos indicus* and *Bos taurus* cattle influence their potential oocyte production, and consequentially, the success of IVPE. Given the above findings, the goal of the present study was to evaluate and compare the *in vitro* production of bovine embryos from zebu and taurine donors.

MATERIALS AND METHODS

The Animal Ethics Committee (CEUA) of the Federal University do Reconcavo da Bahia (UFRB) approved this study under the registration number 23007.032366/2017-30. This experiment was performed at the Fazenda Casa Branca Agropastoril, located in the city of Silvianópolis-MG, Brazil (latitude 22°01'44" South and longitude 45°50'06" West).

Three hundred and twenty-eight cows with a body condition score of between 3 and 4.5 were selected as donors for use in this study, with these being distributed between two experimental groups: Group 1 (n = 161), comprising Brahman cows (*Bos indicus*), and Group 2 (n = 167), comprising Angus and Simmental cows (*Bos taurus*). The cows were fed a balanced diet based on corn silage and a mineral lick and were provided with water *ad libitum*.

The donors were submitted to follicular aspiration through the ovum pick-up (OPU) technique using a 7.5 MHz intravaginal microconvex sector transducer coupled to ultrasound equipment (Mindray, model DP 2200 VET, China). A 17G disposable needle was inserted into the transducer, which was connected to a vacuum pump system, with the vacuum/flow pressure set at 18 mL/min.

To perform the procedure, each of the donors was held in a crush (chute) and perineal cleansing was performed. To avoid donor discomfort and facilitate ovary manipulation, the cows were treated with an epidural anesthetic containing 3 mL of a solution of 2% lidocaine hydrochloride and 0.1% xilazine hydrochloride. After anesthesia, the transducer was inserted into the vaginal base, and after transrectal manipulation, the tip of the transducer was positioned at the ovaries for the aspiration of all visible antral follicles. The aspirated contents and oocytes were stored in phosphate buffered saline (PBS) plus heparin at 36 °C.

Flushed oocytes were packed in 0.25-mL mini-straws containing maturation medium and were sent in carriers held at 38 °C to the IVPE laboratory, during which the average transportation time did not exceed 40 min. The aspirated oocytes were then washed with PBS, and classified as Grade I (GI), II (GII), III (GIII), or naked, based on their morphological quality, which was assessed by counting the number of layers of the cumulus-oocyte complex (COC) present (VIANA et al., 2004).

In the laboratory, the oocytes were transferred to 60-mm Corning (Corning Incorporated, United States of America-USA) slides, subjected to two to three washes with the maturation medium, and

then transferred to 35-mm Cornin slides that contained the maturation medium (containing 9.0 mL of Earle's Salts tissue culture medium (TCM) 199 (Sigma Aldrich, USA), 1.0 mL of fetal bovine serum (FBS) (Sigma Aldrich, USA), 20 µL of pyruvate, 10 µL of follicle-stimulating hormone (FSH), 100 µL of luteinizing hormone (LH), 10 µL of estradiol, 50 µL of amikacin, and 4 mL of sterile mineral oil (Oil for Embryo Culture, Irvine Scientific, USA). They were then allowed to mature *in vitro* (MIV) in an incubator (at 38.7 °C with 99% humidity and 5% CO_2 conditions) for between 22 and 24 h.

After MIV, the oocytes were evaluated to confirm the occurrence of maturation (cell expansion of the cumulus with the maintenance of cytoplasmic integrity), and were then subjected to *in vitro* fertilization (IVF) for between 18 and 22 h in the following medium: 10 mL of FERT TALP (Life Technologies, Brazil Ltd., Brazil), 0.06 g of bovine serum albumin – fatty acid free (BSA-FAF, Sigma Aldrich, USA), 20 µL of pyruvate, 440 µL of penicillin, hypotaurine, and epinephrine (PHE), 110 µL of heparin, 50 µL of amikacin, and 4 mL of sterile mineral oil (Oil for Embryo Culture, Irvine Scientific, USA).

For IVF, cryopreserved semen was used, which was thawed at 35°C for 40 s before use. Live and dead spermatozoa and spermatic diluents were separated using a Percoll Gradient (with 45% and 90% Percoll, Sigma Aldrich, USA). The semen used was from bulls of the Brahman (*Bos indicus*), and Angus and Simental (*Bos taurus*) breeds, according to the donor breed.

After IVF, all spermatozoa and other cells around each zygote were removed, leaving only the two to three layers of cumulus cells, if present. The zygotes were transferred to slides containing growth medium (9.3 mL of CR-2, 0.05 g of BSA-FAF (Sigma Aldrich, USA), 500 µL of fetal bovine serum (Sigma Aldrich, USA), 100 µL of alanine, 100 µL of glycine, 40 µL of amikacin, and 4 mL of sterile mineral oil (Oil for Embryo Culture, Irvine Scientific, USA)), where they remained for seven days, with the medium exchanged once (feeding) 48 h after the beginning of *in vitro* cultivation (CIV).

When the CIV had concluded, the embryos were evaluated and classified into different developmental stages, including the morula (OM), initial blastocyst (BI), blastocyst (BL), and expanded blastocyst (BX) stages, according to the International Embryo Technology Society - IETS (2014) criteria. The embryos were then packaged in straws containing culture medium for embryo maintenance (Holding Plus, Biodux, IMV, Brazil), and then transported in carriers at 36°C to be transferred into synchronized recipients.

The recipient cows were crossbred and reared in an extensive management system, during which they were fed a diet containing mineral lick and provided water *ad libitum*. They were subjected to the vaccination and deworming protocol appropriate to the region. The synchronizing protocol of the recipients was the same. On day 0, they received a progesterone (P_4) vaginal implant (CIDR, Zoetis, Brazil) and 2 mL of intramuscular (IM) estradiol benzoate (Estrogin, Biofarm, Brazil); on day 9, the P_4 implant was withdrawn, and they received 1 mL of estrogen (Sincrodiol, Ouro Fino, Brazil), 1.5 mL of equine chorionic gonadotropin (eCG, Novormon, Zoetis, Brazil), and 2 mL of

cloprostenol (Ciosin, MSD, Brazil) via the intramuscular route; on 11, 12, and 13, estrus detection occurred.

On day 18, the recipients were classified as being suitable or not for embryo transfer, based on the day of the estrus cycle and the size of the corpus luteum (CL): CL1: large; CL2: medium; CL3: small. The size of the CL was evaluated by ultrasonography (Mindray, model DP 2200 VET, China). Only the recipients that presented estrus on day 6 or day 7 after fertilization and with CL2 or CL3 were classified as suitable. Embryos were randomly transferred into the recipients in the uterine horn ipsilateral to the CL.

At 30 and 45 days post ET, pregnancy diagnosis (PD) was performed through rectal palpation and ultrasonography (Mindray, model DP 2200 VET, China), using a linear probe. At 30 days post transfer, the presence of the CL in the ovary and allantoic vesicles in the uterine body was determined. Confirmation of pregnancy was performed at 45 days post-transfer by ultrasonography and visualization of the embryonic vesicles, and embryonic losses were assessed.

The data were tested for normality using the Shapiro-Wilk test. Because they did not meet the assumption of normality, they were subjected to the nonparametric Mann-Whitney test, which is used to compare two independent samples. A significance level of 5% was used for all evaluations. Statistical analyses were performed using the Software Statistical Package for the Social Sciences (SPSS, 2015), version 23.0.

RESULTS AND DISCUSSION

A total of 328 follicular aspirations were performed: 161 in zebu and 167 in taurine cows. Of these aspirations, 4,440 viable oocytes were obtained (13.54% of the total number of oocytes extracted), which produced 1,284 viable embryos (29%), of which 886 were transferred to recipients (69%), resulting in 427 gestations (49%).

Of the recovered oocytes, a higher number of viable oocytes (15.9 oocytes/donor) was obtained from zebu cows (n = 2556) than that (11.4 oocytes/donor) obtained from taurine cows (n = 1903) (P = 0.008). Higher numbers of grade 1 oocytes were obtained from zebu cows (4.28 oocytes/donor) than from taurine cows (2.66 oocytes/donor), (P < 0.0001); further, more grade 3 oocytes were obtained from zebu cows than from taurine cows (4.23 oocytes/donor *vs.* 3.0 oocytes/donor, in zebu and taurine cows, respectively, P = 0.010). There was no difference in the numbers of grade 2 oocytes (P = 0.110) and naked oocytes obtained (Table 1; P = 0.311) between the breeds.

Table 1. Morphological quality of cumulus oocyte complexes obtained from zebu and taurine donors

Donors	Grade I n (%)	Grade II n (%)	Grade III n (%)	Naked n (%)	Viable oocytes n (%)
Zebu	689 (26.96)[a]	1101 (43.08)	681 (26.64)[a]	85 (3.33)	2.556 (100)[a]
Taurine	444 (23.33)[b]	889 (46.72)	509 (26.75)[b]	61 (3.21)	1.903 (100)[b]
P value	0.000	0.110	0.010	0.311	0.008

Different superscripts within the same column indicate significant differences, as calculated by the Mann-Whitney test (P < 0.05).

Recent studies have been carried out with the intention of explaining the mechanisms involved in the differences between the breeds of cattle (SARTORI et al., 2016), whether with regard to the follicular population (ALVAREZ et al., 2000), the amount of insulin produced, the production of a growth factor similar to insulin type I (IGF-1) (GIMENES et al., 2015), or the difference in the concentration of the Anti-Müllerian hormone (AMH) (BATISTA et al., 2014). In a recent study, Batista et al. (2020) emphasized again that there are important differences in the follicular dynamics, circulating P_4 levels, and LH pulse concentrations for zebu and taurine breeds.

Bos indicus cows have greater quantities of growing follicles in their ovaries, and consequently, higher numbers of COCs can be recovered from them by OPU compared to those that can be obtained from taurine cows (PONTES et al., 2009). Batista et al. (2014) also suggested that *Bos indicus* cows have higher numbers of viable oocytes due to them having larger populations of antral follicles.

The difference in the quantity of follicles in the ovary recruited per follicular growth wave appears to be related to the insulin-like growth factor (IGF) system (ALVAREZ et al., 2000). Studies performed with zebu cows of the Brahman breed suggested that these animals have higher IGF-1 concentrations in the plasma and lower follicle-stimulating hormone (FSH) concentrations than those in taurine cows of the Angus breed (ALVAREZ et al., 2000).

Even in the presence of low FSH levels, multiple studies have also raised the hypothesis that the higher numbers of follicles present in zebu cow ovaries could be due to their high IGF-1 concentrations (ALVAREZ et al., 2000; MONTEIRO et al., 2010).

Another aspect involved is the amount of AMH produced by different breeds. Batista et al. (2014) concluded that the antral follicular population is positively correlated with the plasma AMH concentration in both *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers. Furthermore, *Bos indicus* (Nelore) heifers presented both greater plasma AMH concentrations and populations of antral follicles than *Bos taurus* (Holstein) heifers. These results concur with the study of Baldrighi et al. (2014), who compared the Gir and Holstein breeds. Batista et al. (2016) have stated that the AMH level is a promising parameter for selecting oocyte donor cows to maximize the number of viable embryos during *in vitro* embryo production.

However, it is known that follicular recruitment can also be influenced by other factors besides genetics. The main factors are nutritional and metabolic factors (SARTORI et al., 2016) and environmental factors, mainly induced by thermal stress (BARUSELLI et al., 2017).

The percentage of viable embryos obtained was also influenced by the breed. Higher quantities of viable embryos were obtained from zebu (n = 910, 5.7 embryos/cow) than taurine (n = 374, 2.2 embryos/cow) donor cows (P = 0.0001). Zebu cows also produced lower quantities of morulae (zebu: 0.02/donor *vs.* taurine: 0.14/donor; P = 0.017) and higher quantities of blastocysts (zebu: 0.8/donor *vs.* taurine: 0.08/donor; $P <$ 0.001) and expanded blastocysts (zebu: 4.6/donor *vs.* taurine 1.68/donor; $P <$

0.001). There was no difference in the quantity of initial blastocysts obtained (Table 2; $P = 0.149$) between the breeds.

Table 2. Quality of embryos produced *in vitro* from zebu and taurine donors

Donors	Morula	Blastocyst Initial	Blastocyst	Blastocyst Expanded	Viable embryos
Zebu	4 (0.44%)[a]	36 (3.96%)	129 (14.18%)[a]	741 (81.43%)[a]	910 (100%)[a]
Taurine	24 (6.42%)[b]	55 (14.71%)	14 (3.74%)[b]	281 (75.13%)[b]	374 (100%)[b]
P *value*	0.017	0.149	0.000	0.000	0.000

Different superscripts within the same column indicate significant differences calculated by the Mann-Whitney test ($P < 0.05$).

In the present study, 29% of the viable oocytes obtained produced transferable embryos. In the same way that larger quantities of viable oocytes were obtained by follicular aspiration from zebu cows, a larger quantity of embryos was also produced. This concurs with the findings of the study Gimenes et al. (2015), who stated that the differences in embryo production between breeds are positively related to differences in the quantity and quality of oocytes recovered from them. These authors also reported that *Bos indicus* cows, due to higher numbers of viable recovered COCs, showed higher embryo production.

Camargo et al. (2007) demonstrated that due to high adaptability environmental stresses, there was better fetal development in *Bos indicus* cows than in *Bos taurus* cows, when they were reared in a tropical environment. The authors reported that Gir donors were less agitated during the summer, suggesting that they are less stressed than Holstein cows. In addition, they observed that oocytes from the cows of the Gir race were of the correct shape during *in vitro* development.

The nutritional and metabolic state of the cows may also participate in follicular growth alterations, hormonal secretion, and oocyte quality, and consequentially compromise the results of IVPE. Oocyte competence may be compromised due to endocrinal alterations (e.g., peripheral insulin resistance, hyperinsulinemia, and increased glucose and IGF-1 levels), which may lead to impaired glucose transport in embryonic cells (BATISTA et al., 2013; SALES et al., 2015).

It is important to highlight the fact that in the present study, both the breeds of donor cows, zebu and taurine, were reared in the same, climate-controlled environment. Further, the animals were managed in small groups, with an *ad libitum* water supply, and food was provided at the coolest times of the day. Therefore, thermal stress effects, even though they have been described in the literature as having strong influences on the quality of both oocytes and embryos, were not considered to be likely and were not analyzed in the present study; however, this does not mean that there was absolutely no influence from such factors.

The pregnancy rate did not differ between the groups (Figure 1; $P > 0.05$). Despite the differences in the oocyte quality and embryo production rates obtained in this study, the pregnancy rate

was not affected by the donor breed; it was similar to that described in previous literature for bovine recipients of embryos produced by IVPE, and was reported to vary between 30 and 51% (ANDRADE et al., 2012).

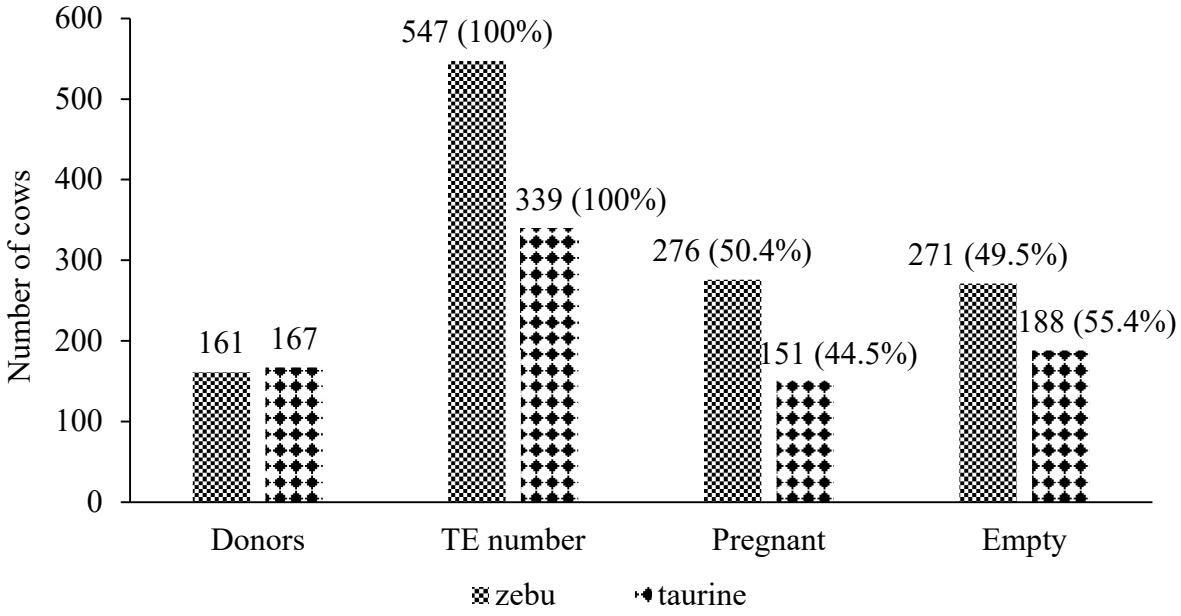

Figure 1. Pregnancy rate in recipients of bovine embryos produced by *in vitro* production of embryos from *Bos indicus* and *Bos taurus* donor cows

Pregnancy rates of the recipients of embryos produced by IVPE are inferior to those of embryos produced *in vivo* (HASLER et al., 2003). This could be due to the maternal recognition mechanisms of pregnancy, with IVPE embryos being slower to recognize (FARIN et al., 2006).

The embryos of ruminants show similar *in vitro* and *in vivo* development until the eight-cell stage, and subsequently, IVPE-derived embryos develop slower. The *in vitro* culture phase may also have an effect on fetal metabolism and alter the embryo quality (RIZOS et al., 2008). Hasler (2003) confirmed that metabolic and biochemical differences may also affect pregnancy rates after implantation. Moreover, after implantation, the embryo survival rate may suffer as a result of multiple factors, including chromosomal abnormalities, the age and quality of the implanted embryo, the location and method of transfer, heat stress, synchronization between donors and recipients, and the nutritional status and serum progesterone concentration in the recipients (HANSEN & EALY, 1991).

In vitro embryo production is influenced by the type of bovine breed; zebu cows yielded better results when compared with taurine cows, showing a greater recovery of viable oocytes and greater production of blastocysts, but with the same gestation rate.

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