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Effect of the genetic group of cows of the Gyr and Holstein breeds on the *in vitro* production technique of bovine embryos

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Abstract

The aim of this study was to evaluate the genetic group effect of oocyte donors of the Gyr and Holstein breeds on the performance of the *in vitro* embryo production technique. Twelve multiparous cows were used, non-lactating, six of the Gyr breed (*Bos indicus*), and six of the Holstein (*Bos taurus*) breed. Prior to the implementation of the follicular aspiration technique, the animals were subjected to estrus synchronization through the administration of 3 mg estradiol benzoate (RIC-BE®, Tecnopec Ltda., Brazil) plus the insertion of an ear implant with norgestomet (Crestar®, Intervet, Brazil). Aspiration was performed seven days after the start of the synchronization protocol. The results showed that the cows of the Gyr breed had a higher number of visualized follicles and recovered oocytes, as well as a higher cleavage and blastocyst rate on day 7. These results allow considering that, the application of the *in vitro* embryo production technique in the Gyr breed offers a better performance in specific parameters in comparison to the Holstein breed.

Keywords: *Bos indicus*, *Bos taurus*, follicular aspiration, follicular wave, reproductive performance

Efecto del grupo genético de vacas de las razas Gyr y Holstein sobre la técnica de producción *in vitro* de embriones bovinos

Resumen

El objetivo del actual estudio fue evaluar el efecto del grupo genético de donadoras de ovocitos de las razas Gyr y Holstein sobre el desempeño en la técnica de producción *in vitro* de embriones. Se utilizaron 12 vacas multíparas, no lactantes, seis de la raza Gyr (*Bos indicus*) y seis de la raza Holstein (*Bos taurus*). Previo a la aplicación de la técnica de aspiración folicular, los animales fueron sometidos a sincronización del estro, mediante la administración de 3 mg de benzoato de estradiol (RIC-BE®, Tecnopec Ltda., Brasil), más la inserción de un implante auricular de norgestomet (Crestar®, Intervet, Brasil). La aspiración fue realizada siete días después del inicio del protocolo de sincronización. Los resultados determinaron que las vacas de la raza Gyr presentaron mayor número de folículos visualizados y de ovocitos recuperados, así como mayor tasa de clivaje y de blastocistos en el día siete. Estos resultados permiten considerar que la aplicación de la técnica de producción *in vitro* de embriones en la raza Gyr puede presentar mejor desempeño en ciertos parámetros en relación con la Holstein.

Palabras clave: aspiración folicular, *Bos indicus*, *Bos taurus*, desempeño reproductivo, onda folicular

Introduction

The use of the follicular aspiration technique associated with the *in vitro* production of embryos (ovum pick up-*in vitro* embryo production (OPU-PIV)), is a biotechnology that is highly widespread worldwide. This technology allows increasing the number of births per female and obtaining an estimated annual genetic gain of 2.5 % (Vishwanath, 2003).

According to the International Embryo Technology Society (IETS), in South America, 450,613 embryos were produced in 2017 through the *in vitro* production technique, consolidating itself as the largest embryo-producing region in the world (Viana, 2018).

Multiple factors affect the good results of the OPU-PIV programs, such as performing the technique in the random phase of the estrous cycle, obtaining oocytes with different maturity degrees (Hendriksen et al., 2000; Vassena et al., 2003), the variability of the response of the animals as well as of the *in vitro* culture systems used (Lonergan & Fair, 2008). The influence of these factors decreases the competence of oocytes for *in vitro* development, affecting the production and quality of embryos, causing losses of genetic material and economic losses in OPU-PIV programs (Ealy et al., 2019). Various works have been carried out to increase the efficiency of the follicular aspiration technique associated with the *in vitro* production of embryos, through the synchronization of the follicular growth wave together with the application of gonadotrophins. These procedures seek to improve the recovery rate, the quality of the collected oocytes and the number of embryos produced (Chaubal et al., 2007; Pfeifer et al., 2009; El-Sherry et al., 2010).

For this reason, the aim of the current study was to evaluate the effect of the genetic group of donors of the Gyr and Holstein breeds on their performance in the application of the *in vitro* embryo production technique.

Materials and methods

For the execution of the current research work, the National Statute for the Protection of Animals with Resolution 008430 of October 4, 1993, was adopted in compliance with the aspects mentioned in Article 87 of this Resolution. This study was carried out according to the following criteria: provision of adequate care for animals according to their ethology, and avoiding unnecessary pain, suffering, stress or prolonged injury; furthermore, avoiding unnecessary duplication or repetition of experiments and reducing the number of animals to the minimum necessary to guarantee the validity of the study (Garcés & Giraldo, 2012).

Selection of animals

Twelve cows (non-lactating and non-pregnant) were selected from a group of donors, including six females from the Gyr breed (*Bos indicus*) and six females from the Holstein breed (*Bos taurus*), with ages between 3 and 7 years, with a body condition of 3.5 to 4.0 on the scale of 0 to 5 points, and a healthy reproductive tract at a structural and functional level.

The study was carried out under tropical conditions; cows followed a grazing regime on pastures based on *Brachiaria decumbens* Stapf and *Brachiaria brizantha* (Hochst. Ex A. Rich.) Stapf (Poaceae), with mineral supplementation, water *ad libitum,* and without concentrate-based supplementation.

Ultrasound-guided follicular aspiration

The donors were located in two genetic groups: Gyr and Holstein. Follicular wave synchronization was carried out administering a dose of 3 mg of estradiol benzoate (RIC-BE®, Tecnopec Ltda., Brazil), plus the insertion of a norgestomet ear implant (Crestar ®, Intervet, Brazil). The OPU procedure was performed 7 days after the start of the synchronization protocol, hence, coinciding with the start of the emergence of the first follicular wave and before the formation of the dominant follicle.

The ear implants were removed 24 hours later. Follicular aspiration was performed in follicles with a diameter ≥ 3 mm to 5 mm and carried out with the aid of a disposable 20 G needle (WTA-Vet, Brazil) coupled to a 70 mm negative pressure Teflon line/Hg. Before carrying out the aspiration procedure, the follicles were counted in both ovaries, and an ultrasound machine (Mindray, DP 2200 VET, China) was used, equipped with a 7.5 MHz frequency microconvex transducer coupled to a transvaginal guide for follicular aspiration.

Immediately after the puncture was made, the follicular fluid was transferred to an EmCom filter (Agtech, USA), and 100 mL of D-PBS was added to remove the clots and cells. The structures were washed in HEPES buffered TCM 199 (TCM-199; Gibco BRL, Grand Island, NY), plus 10 % fetal bovine serum (Gibco BRL, Grand Island, NY), 16 µg/mL sodium pyruvate, and 83.4 µg/mL amikacin (Instituto Biochimico, Rio de Janeiro, Brazil). Cumulus-oocyte complexes (CCO) grade I, II, and III were considered viable for *in vitro* maturation, classified according to the number of layers of cumulus cells and the homogeneous appearance of the cytoplasm. The CCO classification was carried out as described by Sato et al. (1990).

***In vitro* production of embryos**

The CCOs from each donor were washed three times in 100 µL drops of washing medium (TCM 199 supplemented with HEPES (25 mM) and 10 % SFB) and transferred separately to 100 µL drops of the TCM 199 medium and supplemented with 10 % SFB, 1.0 µg/mL FSH (Folltropin™, Bioniche Animal Health, Belleville, Canada), 50 µg/mL hCG (Profasi™, Serono, São Paulo, Brazil), 1.0 µg/mL estradiol, 16 µg/mL sodium pyruvate, ITS (5 ug/mL insulin-transferrin-selenium), and 83.4 µg/mL amikacin coated with sterile mineral oil (Sigma-Aldrich Co, USA). Maturation was carried out for 24 hours in an incubator at 38.5 ° C, 5 % CO_2 in atmospheric air, and 95 % humidity.

Sexed Gyr and Holstein semen of the same batch was used in both breeds with known fertility. The straws were thawed at 35 ºC for 30 seconds, and the contents were carefully poured onto the Mini-Percoll 45/90 gradient. The corresponding inseminating dose for each drop was 1.0×10^6 sperm/mL. Gyr breed semen was used to fertilize oocytes from Holstein cows, and Holstein semen was used to fertilize oocytes from Gyr cows. This fertilization protocol was carried out to obtain F1 embryos.

After maturation, the CCOs were washed three times in 100 µL drops of FERT-TALP medium and transferred together with the sperm into 50 µL drops of FERT-TALP medium supplemented with 0.6 % BSA, 10 µg/mL heparin, 18 µM penicillamine, 10 µM hypotaurine, 1.8 µM epinephrine, and covered with sterile mineral oil. The CCO remained under culture conditions together with the sperm for a period of 18 to 22 hours under the same conditions used for maturation.

After the fertilization period, the zygotes that were presumed to be formed were transferred separately per donor to 100 µL drops of SOF medium supplemented with 2.5 % SFB and 5 mg/mL BSA coated with sterile mineral oil for 7 days. After 72 hours post-fertilization, 50 % of the volume of each culture drop was removed and replaced using the same culture medium mentioned above. At this time, the cleavage rate was evaluated with the formation of two cells of the embryos in culture.

Seven days after fertilization, the blastocyst rate was quantified, and the quality of the embryos was evaluated according to the classification of the International Embryo Technology Society (Stringfellow & Givens, 2010).

Statistical analysis

The data were analyzed using the Software Analysis and Experimentation Group (SAEG, version 9.1), through the System for Statistical and Genetic Analysis application. The means and standard error were obtained and analyzed through an ANOVA, considering the effects of the genetic group and, in cases where a significant effect was obtained ($p < 0.05$), means were compared by Tukey's test.

Results and discussion

As observed in table 1, differences ($p < 0.05$) in the variables number of follicles displayed, number of oocytes recovered, cleavage rate, and number of blastocysts produced on day 7 were verified.

Table 1. Effect of the genetic group, according to the application of the follicular aspiration and *in vitro* embryo production techniques in cows of the Gyr and Holstein breeds

Variables	Gyr cows (n = 6)	Holstein cows (n = 6)	*p*-value
No. replicas	6	6	-
No. of follicles displayed	$21.1^{a} \pm 0.7$	$15.7^{b} \pm 0.6$	0.0002
No. recovered oocytes	$12.7^{a} \pm 1.1$	$8.2^{b} \pm 0.7$	0.0013
Recovery rate (%)	60.1 ± 5.0	52.2 ± 4.4	0.28
Grade I oocytes (%)	20.3 ± 0.4	22.6 ± 0.3	0.45
Grade II oocytes (%)	27.2 ± 0.3	23.0 ± 0.3	0.19
Grade III oocytes (%)	24.6 ± 0.4	19.9 ± 0.3	0.13
Viable oocyte rate (%)	70.8 ± 3.6	67.0 ± 3.7	0.86
Cleavage rate (%)	$81.8^{a} \pm 3.5$	$68.7^{b} \pm 4.5$	0.02
No. blastocysts on day 7 (D7)	$3.6^{a} \pm 0.5$	$2.1^{b} \pm 0.4$	0.03
Blastocyst rate (%)	40.2 ± 4.7	32.4 ± 5.5	0.28

Note. Data are described as mean ± SEM; a, b: different lowercase letters on the same line differ from each other ($p < 0.05$).

Source: elaborated by the authors

According to these results, the number of visualized follicles was higher in the Gyr cows ($p = 0.0002$) compared to the Holstein cows (21.1 ± 0.7 vs. 15.7 ± 0.6, respectively). The mechanisms associated with this trait are related to higher circulating concentrations of IGF-1 and insulin in zebuine breeds (Alvarez et al., 2000). Sales (2011) reported that *B. indicus* cows showed higher concentrations of IGF-1 in the follicular fluid, being this trait related to higher oocyte quality and better embryonic development in the initial phases of the technique. More recently, Mossa et al. (2017) and Tavares et al. (2018) correlated that those animals with a high follicular population show high levels of the anti-Müllerian hormone; these authors suggested that this hormone is a predictor of high follicular population and fertility. Other literature reports indicate that the lower follicular population found in the *B. taurus* breeds is associated with high follicular atresia rates compared to *B. indicus* females (Silva-Santos et al., 2011).

In a study carried out in Gyr cows subjected to one or two aspirations per week, the number of follicles visualized in each aspiration session was maintained in ranges from 14.0 ± 0.3 to 14.5 ± 0.3 (Viana et al., 2004). Carvalho et al. (2008) showed that *B. indicus* heifers showed a higher number of recruited follicles (33.4 ± 3.2) in relation to *B. indicus* × *B. taurus* and *B. taurus* heifers (29.6 ± 2.5 and 25.4 ± 2.5, respectively).

By carrying out the analysis, the number of oocytes recovered in the Gyr cows was shown to be higher than in the Holstein cows. These results are similar to those reported in the literature by Galli et al. (2001), Tamassia et al. (2003), Torres-Júnior et al. (2008), and Viana et al. (2004, 2010).

In a study carried out on Holstein cows, an average of 4.6 ± 0.2 oocytes was recovered per OPU session (Tamassia et al., 2003). However, in a subsequent work carried out on Holstein heifers (Gimenes, 2010), divergent results were obtained from those observed in the current study, in which 13.8 ± 1.8, 16.2 ± 2.2, and 16.2 ± 2.3 structures on days 1, 3, and 5, respectively, were recovered after follicular wave emergence. Similarly, Pontes et al. (2010) recovered 11.4 ± 3.9 oocytes on average in animals of the same genetic group. Contrary to all the results mentioned above, Dayan (2001) obtained a higher number of oocytes retrieved in Holstein cows concerning Gyr cows (8.0 ± 1.6 vs. 4.8 ± 3.5, respectively). The author reports that the follicular aspiration technique was performed on a random day of the estrous cycle, with cyclic and acyclic cows being aspirated.

Even though the results obtained on oocyte quality (grades I, II, and III) were similar in both genetic groups, it is important to highlight that in the current study, quality grades II and III were considered for the *in vitro* embryo production procedures. Recent studies have confirmed that these structures seem to have the same development capacity as those considered morphologically normal (grade I) (Urrego et al., 2015; Velez et al., 2017).

Regarding the number of viable oocytes, the Gyr breed showed higher values compared to the Holstein cows, a difference that is probably an inherent trait of the *Bos indicus* breeds, given that, when a higher number of follicles is visualized, a higher number of good quality oocytes are recovered. These observations from the current study coincide with what was previously described by Gimenes (2010) and Pontes et al. (2010).

Regarding cleavage rates, better results were obtained in Gyr cows compared to Holstein cows. These observations may be related to the oocyte quality and the ability of these structures to show adequate nuclear and cytoplasmic maturation, in addition to the quality of the semen used. However, Tamassia et al. (2003) reported that cleavage rates show a positive correlation ($r = 0.39$; $p < 0.01$) with respect to blastocyst rates. These reports are divergent from those observed in the current study, where the high cleavage rates found did not influence the blastocyst rates obtained.

Already in the number of blastocysts produced seven days after fertilization, the females of the Gyr breed showed a difference with respect to the Holstein cows (3.6 ± 0.5 vs. 2.1 ± 0.4, respectively). Other studies have observed that embryos from *B. taurus* breeds show higher heat stress in the *in vitro* culture phase, leading to higher apoptosis rates compared to the *B. indicus* breeds (Satrapa, 2011). Although in the current study, embryos from Holstein cows were inseminated with Gyr bulls, oocytes are considered to be more important than sperm in the development of thermo-tolerance (Nabhan et al., 2010). Gimenes (2010) verified the better performance of the *B. indicus* breeds in the *in vitro* production of embryos, compared to taurine breeds.

Conclusions

From the results obtained in the current study, it is possible to conclude that cows of the Gyr breed show a better response regarding the number of follicles visualized, the number of oocytes recovered, and the production of blastocysts seven days after fertilization when compared with cows of the Holstein breed.

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Disclaimers

All authors made significant contributions to the document, agree with its publication, and declare that there are no conflicts of interest in this study.

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