Reproductive Biology 20 (2020) 288–292

Contents lists available at ScienceDirect

Reproductive Biology

journal homepage: www.elsevier.com/locate/repbio

Original article

Crossbreeding effect of double-muscled cattle on *in vitro* embryo development and quality

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ARTICLE INFO

Keywords:
Belgian Blue
fertility
semen
oocyte

ABSTRACT

Nowadays, several developing countries have started to breed double-muscled cattle to their autochthonous cattle to improve meat production. However, the developmental competence of the resultant crossbreeding embryos is unknown. The objective of this study was to evaluate the effect of crossbreeding double-muscled (*Belgian Blue*; BB) semen with beef (*Limousin*; LIM) and dairy (*Holstein-Friesian*; HF) derived oocytes on embryo development and quality, using purebred BB as a control (BB oocytes fertilized by BB sperm). A single ejaculate of a BB bull was evaluated by Computer Assisted Sperm Analysis before using for *in vitro* fertilization. Ovaries from each breed were collected at the local slaughterhouse (n = 1,720 oocytes). All statistical analyses were performed using R-core ($P < 0.05$). Embryo quality was evaluated via differential-apoptotic staining of day 8 blastocysts. Cleavage (48 h post insemination) and day 8 blastocyst rates were greater ($P < 0.05$) for LIM (82.9 ± 6 and 27 ± 4.3%, respectively) than for BB (69.8 ± 8.5 and 19.6 ± 3.1%, respectively) and HF (45.1 ± 10 and 12.3 ± 2.2%, respectively). *Holstein-Friesian* presented lower cleavage and day 8 blastocyst rates than BB ($P < 0.05$). *Limousin* blastocysts presented a higher number ($P < 0.05$) of inner cell mass cells (ICM; 68 ± 7.8) than HF (40.4 ± 8.2). In conclusion, crossbreeding double-muscled cattle by *in vitro* fertilization with LIM oocytes yielded better embryo compared with the purebred combination, while the combination with HF oocytes produced the lowest rate of blastocysts.

1. Introduction

Double-muscled cattle might be a valid alternative for crossbreeding autochthonous cattle in developing countries to improve meat production. Double-muscling in cattle is a genetic condition that occurs in several breeds such as *Belgian Blue* (BB), *Piedmontese*, and *Marchigiana* [1]. It has been reported that double muscling in BB cattle significantly increases carcass yield and meat tenderness [[2]], characteristics that are desirable in the meat industry. Some research groups uncovered that double-muscling is caused by mutations in the *myostatin gene* [3]. Among other functions, *myostatin* appears to play a role in the regulation of protein synthesis and glucose metabolism [4]. Although this condition results in improved meat quality and quantity, it also comes along with problems associated with reduced reproductive performance [5]. The sub-development of the hip bone (relative to the size of the animal) [6], the extended gestation period [7], and the higher birth weight of the offspring is associated higher risk of dystocia and perinatal calf mortality in double-muscled cows than non-double-muscled cows [8].

Fertility is a multifactorial trait that could be affected by many extrinsic factors such as estrus detection, timing of artificial insemination (AI) relative to ovulation, AI technique, reproductive pathologies, among others [9]. The use of an in vitro approach could help us to eliminate these extrinsic confounders to focus on the inputs of the oocyte and sperm on early embryonic development. A previous study reported more competent oocytes in double-muscled BB than in *Holstein-Friesian* (HF) dairy cows [10]. In contrast, semen characteristics are known to be markedly lower in BB than HF or crossbreed bulls [11]. The latter might be associated with smaller reproductive organ sizes and a not well defined scrotal neck in BB bulls in comparison to non-double muscled cattle breeds [3]. However, no study has been performed to associate the potential contribution of BB semen crossbred

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https://doi.org/10.1016/j.repbio.2020.07.007
Received 23 May 2020; Received in revised form 20 July 2020; Accepted 22 July 2020
Available online 30 July 2020

with oocytes derived from other breeds on their subsequent embryo development and quality.

We hypothesized that crossbreeding double-muscled semen with oocytes derived from distinct beef and dairy breeds, will affect their subsequent *in vitro* embryo assessments. Thus, the objective of this study was to evaluate the effects of *in vitro* crossbreeding of double-muscled (BB) sperm with beef (*Limousin*; LIM), dairy (HF) as well as with double-muscled purebred (BB) derived oocytes on blastocyst development and quality. The evaluation of blastocyst development included cleavage rate (CR), and day 7 and 8 embryonic rates. For blastocyst quality, we evaluated the total cell number (TCN), trophectoderm cells (TE), inner cell mass (ICM), ICM/TCN ratio, total apoptotic cells (AC), and AC/TCN ratio via differential/apoptotic staining of day 8 blastocysts. This could be considered as a first step to study the potential contribution of BB bulls in crossbreeding systems to maintain the benefit of meat production without an undesirable toll in fertility.

2. Materials and methods

2.1. Sperm collection, cryopreservation, and post-thawing quality assessment

A single ejaculate from a BB bull located in the breeding center of AWE in Ciney (Belgium; 50°29 N, 5°11 E) was used for all the experiments. The semen of the BB bull used for this experiment is commercially available. Thus, its fertility performance is continuously monitored via multiple reproductive parameters (e.g., non-return rate after AI) at the field level. The ejaculate was collected in spring 2016 using an artificial vagina after which it was cryopreserved via the standard procedure. Briefly, the semen sample was extended via OptydilTM (Minitüb GmbH, Germany) and cooled (4 °C for 4 h). Then, it was loaded into 0.25 mL straws (IMV Technologies) at 4 °C. The freezing was carried out via a programmable freezing system (IMV, Technologies-Digitcool, L'Aigle, France). After that, the straws were cooled to -10 °C at −5 °C/min, from -10 °C to −140 °C at −40 °C/min and were subsequently plunged into liquid nitrogen.

A frozen sperm sample was thawed at 37 °C for 30 seconds. After thawing, the sample was evaluated for motion kinetics using a computer assisted sperm analyzer (CASA; ISAS® v1 CASA system, Proiser, Spain) with three replicates. Briefly, a droplet (3 μL) of the sample was placed in a pre-warmed Leja chamber (37 °C; Leja counting chambers, depth 20 mm; Microptics®, Barcelona, Spain) and automatically assessed for motion kinetics using a 10x negative phase contrast microscope objective (connected to the CASA system). The settings of the CASA software are presented in Supplemental Table S1 and motion kinetic parameters of the frozen-thawed semen sample are presented in Supplemental Figure S2.

2.2. Oocyte collection and embryo production

Ovaries from each breed (BB as for double-muscled, LIM as for beef, and HF as for dairy) were collected at the local slaughterhouse and transferred to the laboratory facilities allocated in independent and well identified bags. *Belgian Blue* derived oocytes fertilized with BB sperm (purebred) was used as control for embryo development and quality. Experiments were run in three replicates (three different days using different batches of media), and for each replicate we included the following combinations: BB semen and BB oocytes vs. BB semen and HF oocytes vs. BB semen and LIM oocytes. In all the cases, frozen-thawed semen samples from the same BB bull were used (as described above). Basic Eagle's Medium amino acids, minimal essential medium non-essential amino acids (100 x), TCM-199-medium, kanamycin, and gentamycin were purchased from Life Technologies Europe (Ghent, Belgium). All other components were obtained from Sigma (Schnelldorf, Germany) unless otherwise stated. All the media were filter-sterilized using a 0.22 mm filter (Pall Corporation, Ann Arbor, MI, USA) before use.

The production of *in vitro* bovine blastocysts was done as described by Wydooghe et al. (2014) [12]. Briefly, using an 18-gauge needle attached to a 10 ml syringe, the cumulus-oocytes complexes (COCs) were recovered from follicles with a diameter of 2–8 mm. Oocytes with homogeneous dark cytoplasm and compact cumulus cells were selected and matured in groups of 60 in 500 μl of bicarbonate-buffered TCM199 medium supplemented with 50 mg/ml gentamycin and 20 ng/ml of epidermal growth factor. Oocytes were matured for 22 h at 38.5 °C in 5% CO_2 in humidified air. Frozen-thawed BB sperm (same bull and batch) was used for fertilization. The sperm was passed through a discontinuous Percoll® gradient (45:90% (v:v)) and adjusted to a final concentration of 1×10^6 cells/ml in IVF TALP medium.

The matured COCs were washed in 500 ml IVF-TALP and co-incubated with sperm for 21 h at 38.5 °C in 5% CO_2 in humidified air. After fertilization, presumptive zygotes were first vortexed to remove the excess of the sperm and cumulus cells, and then cultured in groups of 25 in 50 μl droplets of SOF medium at 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2. Embryo development was evaluated at 48 h post-insemination (cleavage), and at day 7 and day 8.

2.3. Differential staining of blastocysts

In vitro-produced day-8 blastocysts were fixed in 4% PFA for 20 min at RT and stored at 4 °C until use. Differential staining was performed as described by Wydooghe et al. (2011) [13]. Blastocysts were permeabilized with 0.5% Triton X-100 + 0.05% Tween for 1 h at RT. Then, DNA of the blastomeres was denatured by exposure to 2 N HCl (1.09063.1000, HC98019763, Tritipur®, Germany) for 20 min followed by 100 mM Tris-HCl (pH 8.5) for 10 min at RT. Blocking solution consisting of 10% goat serum and 0.5% BSA prepared in phosphate buffer saline (PBS; 0000333482 Gibco®, UK) was added and the embryos were incubated at 4 °C overnight. The embryos were washed and incubated in the ready-to-use primary CDX2 antibody (Biogenex, San Ramon, USA) at 4 °C overnight. Embryos were washed and incubated overnight at 4 °C in rabbit active caspase-3 antibody (0.768 μg/ml in blocking solution, Cell Signaling Technology, Leiden, the Netherlands). After another wash step, test embryos and negative controls (not exposed to CDX2 and Caspase-3 antibodies) were transferred to goat anti-mouse Texas Red secondary antibody (20 μg/ml in blocking solution, Molecular Probes, Merelbeke, Belgium) for 1 h at RT and to goat anti-rabbit FITC antibody (10 μg/ml in blocking solution, Molecular Probes) for 1 h at RT. Next, blastocysts were rinsed three times in PBS, counterstained with Hoechst 33,342 (50 μg/ml in PBS/BSA, Molecular Probes) for 20 min at RT, and mounted with DABCO mounting medium. This differential staining allowed the assessment of the number of TE cells, inner cell mass number (ICM), total cells number (TCN; TE + ICM), ICM/TCN ratio and total number of apoptotic cells (AC) as well as apoptotic cells ratio (ACR; AC/ TCN). These assessments were done by fluorescence microscopy (Leica DM 5500 B) using a triple bandpass filter, evaluated by an experienced observer.

2.4. Statistical analyses

All statistical analyses were performed using R-core (version 3.6.1; R Core Team, Vienna, Austria). The zygote/embryo was considered as the unit of interest. Generalized mixed effects models were used to test the effect of breed (BB vs. LIM vs. HF) on developmental (cleavage and blastocyst rates) and differential staining parameters (TCN, TE, ICM, ICM/TCN, AC, and AC/TCN). Differential staining parameters were analyzed by Shapiro-Wilk's test and when not normally distributed ($P < 0.05$), were $\log_{10}$ transformed. For all the models the replicate was set as a random effect. Results are expressed as least squares means and standard errors. The differences between treatment groups were assessed using Tukey's post hoc test. The significance and tendency levels

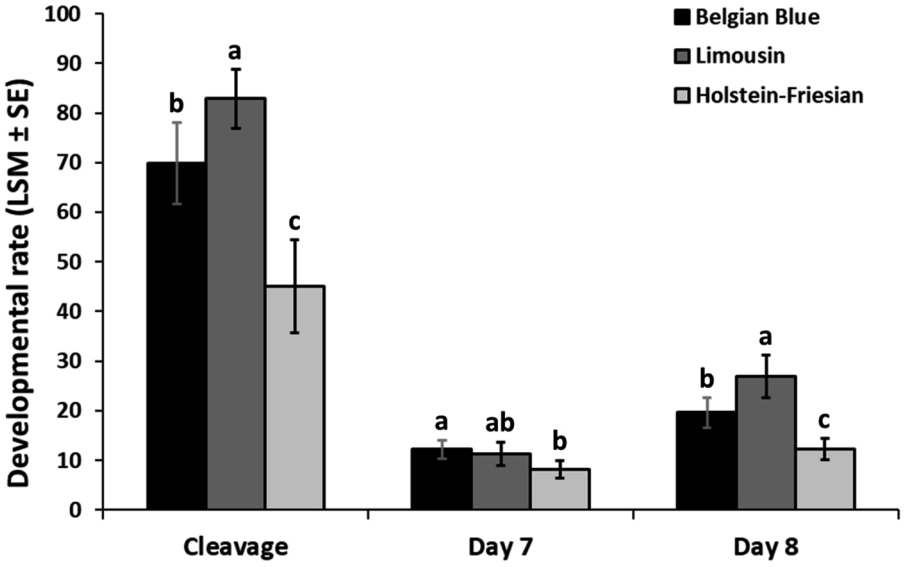

Fig. 1. Cleavage, day 7, and day 8 blastocyst rates expressed as percentage from presumed zygotes. Cumulus oocyte complexes (n = 1,720) of double-muscled (*Belgian Blue*; BB), beef (*Limousin*; LIM), and dairy breed (*Holstein-Friesian*; HF) were fertilized with semen from a single double-muscled (BB) bull. *In vitro* embryos were standardly produced in groups and cultured in serum-free medium in three replicates. Different superscripts (a, b, and c) represents statistical differences ($P < 0.05$) among groups. Results are expressed as least square means ± standard error (LSM ± SE).

were set at $P < 0.05$ and $P < 0.1$, respectively.

3. Results

A total of 1,720 oocytes (644 for BB, 274 for LIM, and 802 for HF) in three replicates were used for this study. Cleavage and day 8 blastocyst rates were greater ($P < 0.05$) for LIM (82.9 ± 6 and 27 ± 4.3%, respectively) than for BB (69.8 ± 8.5 and 19.6 ± 3.1%, respectively) and HF (45.1 ± 10 and 12.3 ± 2.2%, respectively) derived oocytes (Fig. 1). *Holstein-Friesian* presented lower cleavage and day 8 blastocyst rates than BB derived oocytes ($P < 0.05$; Fig. 1). *Belgian Blue* presented a greater ($P < 0.05$) day 7 blastocyst rate (12.2 ± 1.8%) than HF (8.2 ± 1.8%) but the day 7 blastocyst rate in LIM (11.3 ± 2.3%) was not different than for BB or HF oocytes ($P < 0.05$; Fig. 1). Blastocysts of LIM derived oocytes possessed higher cell numbers in ($P < 0.05$) ICM and ICM/TCN (68 ± 7.8 and 65.2 ± 4.3, respectively) than HF (40.4 ± 8.2 and 45.9 ± 4.7, respectively; Fig. 2). No other difference in differential nor apoptotic staining parameter was found among breeds ($P < 0.05$; Figs. 2 and 3).

4. Discussion

We investigated the effects of crossbreeding double-muscled (BB) semen with LIM (beef), and HF (dairy) derived oocytes on further *in vitro* embryo development and quality, using the development and quality of purebred derived embryos (semen and oocytes from BB cattle) as a control. Our results show greater developmental capacity in LIM derived oocytes compared to BB and HF. Furthermore, development was significantly higher in BB than in HF. *Limousin* blastocysts possessed more cells in the ICM than HF. Crossbreeding double-muscled cattle by *in vitro* fertilization with LIM oocytes yields better embryo development compared with the purebred combination, while the combination with HF oocytes produced the lowest rate of blastocysts. This finding suggests that cross-breeding BB semen with oocytes from other beef breeds may have positive effects on fertility. The cross-breeding effect of BB semen with dairy cows remains inconclusive since culled HF cows are often culled for infertility or health reasons.

This experiment consisted of a preliminary study to mimic what might be happen concerning aspects of potential fertility in a BB crossbreeding program in Indonesia. We are aware of the multiple limitations of the model. However, results suggest that at least for *in vitro* settings, crossbreeding BB bulls with other breeds (especially beef)

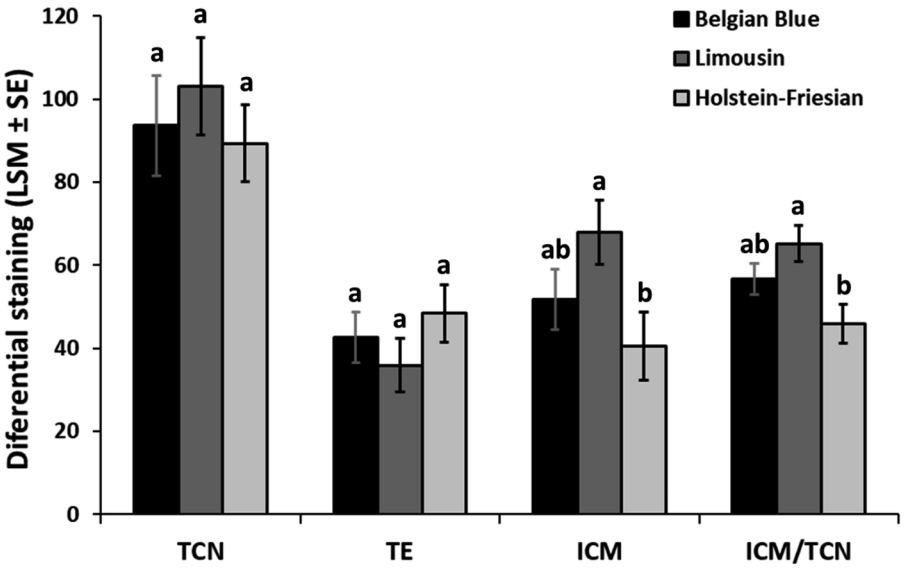

Fig. 2. Total cell number (TCN), trophectoderm cells (TE), inner cell mass (ICM) and ICM/TCN of day 8 blastocyst differentially stained (n = 63). Cumulus oocyte of double-muscled (BB), beef (LIM), and dairy (HF) were fertilized with semen from a single double-muscled (BB) bull. *In vitro* embryos were standardly produced in groups and cultured in serum-free medium in three replicates. Different superscripts (a, b, and c) represents statistical differences ($P < 0.05$) among groups. Results are expressed as least square means ± standard error (LSM ± SE).

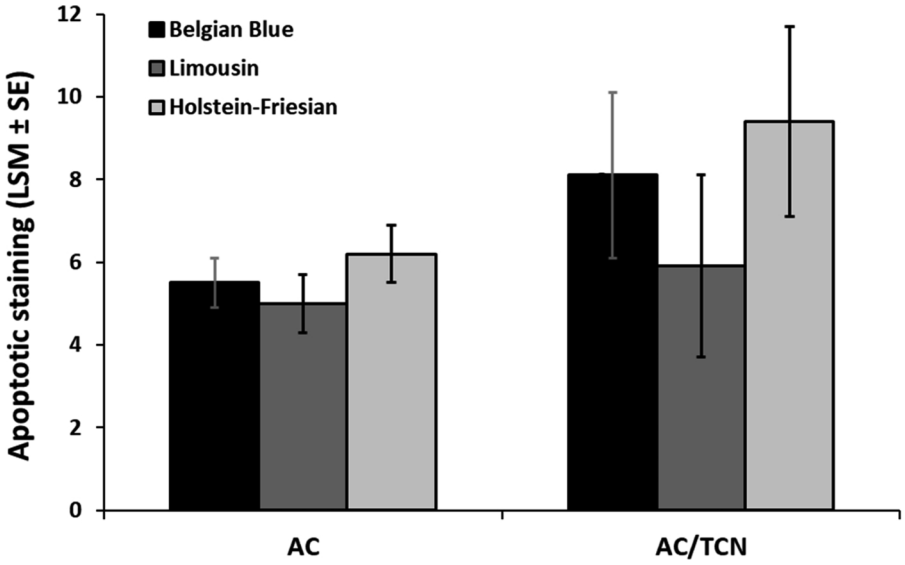

Fig. 3. Total apoptotic cells (AC) and AC/Total cell number (TCN) of day 8 blastocysts subjected to an apoptotic staining (n = 63). *In vitro* matured oocytes of double-muscled (BB), beef (LIM), and dairy (HF) were fertilized with semen from a single double-muscled (BB) bull. *In vitro* embryos were standardly produced in groups and cultured in serum-free medium in three replicates. No significant differences ($P < 0.05$) were found among groups. Results are expressed as least square means ± standard error (LSM ± SE).

will not result in reduced embryonic development. In aspects of *in vitro* production and fertility, this is one of the first studies reporting the crossbreeding effect of BB with other beef cattle.

Heterosis advantages associated with beef quantity and quality is expected while crossbreeding gametes of double-muscled cattle with other beef cattle. However, this might be accompanied by undesirable, negative effects associated with fertility. Heterosis or hybrid vigour is defined as increased vigor of growth, survival, and fertility of hybrids between two breeds, especially in the first generation. Heterosis is well established in beef cattle, and systems of production that exploit both maternal and individual heterosis may increase production levels by 30% [14]. Here, we found that the crossbreeding of BB with LIM gametes presented higher blastocyst development than the BB purebred embryos. This indicates that a crossbreeding program of BB with other beef (non-double muscled) breeds may not result in lower conception rates. On the other hand, it could be an excellent solution to improve beef quality and fertility simultaneously. Interestingly, LIM oocytes yield embryos with greater ICM numbers compared with the HF crossbreed. In this regard, a recent study showed that ICM number is the best indicator of the blastocysts' ability to implant [15]. Still, results should be interpreted with caution since there is still a large gap between embryo implantation and the delivery of a healthy calf.

In most European countries, the culling of dairy cows represents 10 to 15% of the total number of cattle slaughtered to fulfill the meat demand [16]. The most common reasons for culling dairy cows are associated with reproductive failure (primary reason), mastitis, udder problems, low milk production, and old age [17]. Oppositely, BB cows are mainly culled to fulfill the meat demand and they are mostly young at culling. In addition, beef cows are not bred for high milk production. Hence, since beef cows lack most of the husbandry and milk production related stressors, they should have better fertility. Milk production in dairy cows is associated with an altered metabolic profile that is characterized by a reduced concentration of circulating insulin growth factor-1, hypoglycemia, and increased concentrations of non-esterified fatty acids and β-hydroxybutyrate, and haptoglobin. This altered metabolic profile influences the follicular environment, which reverberates on the growing and maturing of the oocyte [18]. Additionally, the altered metabolic profile in dairy cows affects the oocyte developmental capacity and granulosa cell function *in vitro* [19]. These conditions might then yield oocytes of lower quality impacting on their inferior blastocyst quality as we found in this study.

It is important to mention that we used ovaries derived from the slaughterhouse. Therefore, we do not know the background of the oocytes at the individual cow level. As a consequence, results of this study should be carefully interpreted. Still, all oocytes were processed in the same way for the *in vitro* production process, and in each replicate, we used oocytes derived from BB, LIM, and HF ovaries. These results should be considered as a first step to study the cross-breeding effect of BB semen with oocytes from other breeds. More studies should be done at the molecular and genetic levels to establish definitive conclusions.

5. Conclusion

We used BB semen to fertilize LIM and HF oocytes to study the potential crossbreeding effect on *in vitro* embryo development and quality. Our results show that blastocyst development is greater in LIM than HF crossbreed or even the BB purebred. Interestingly, LIM presented a higher ICM number than the HF crossbred. This finding might be due to the fact that HF are often culled due to health or infertility reasons. This experiment consisted of a preliminary study to mimic what may happen in aspects of potential fertility in a BB breeding program. Hence, more studies need to be conducted to draw definitive conclusions.

Declaration of Competing Interest

None.

Acknowledgments

This research was funded by LPDP (Indonesia Endowment Fund for Education) with number 201707220411226. Thanks to for Euro Meat Group, Belgium, who provided the oocyte samples from different breeds, and Association Wallonne De L'Élevage, Belgium, who provided the sperm samples. Osvaldo Bogado Pascottini was granted by Fonds voor Wetenschappelijk Onderzoek–Vlaanderen (FWO, Research Foundation, Flanders) under project number 12Y5220N.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.repbio.2020.07.007.

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