Received: 12 September 2023 | Accepted: 8 January 2024

DOI: 10.1111/ahe.13015

Anatomia Histologia Embryologia

WILEY

ORIGINAL ARTICLE

Morphology of 16-cell embryo in bovine: Inside cells, compaction, fragmentation and effects of X-sorted semen

Clara Slade Oliveira[1] | Naiara Zoccal Saraiva[1] | Leticia Zoccolaro Oliveira[2]

[1]Embrapa Dairy Cattle, Juiz de Fora, Minas Gerais, Brazil

[2]Department of Veterinary Clinics and Surgery, Veterinary School, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil

Correspondence
Clara Slade Oliveira, Embrapa Dairy Cattle, Juiz de Fora, MG, Brazil.
Email: clara.oliveira@embrapa.br

Funding information
Conselho Nacional de Desenvolvimento Científico e Tecnológico

Abstract

In mouse embryos, inside cells are allocated in 16-cell embryos through a well-orchestrated sequence of events involving compaction and polarization. The emergence of inside cells is of great importance as itl later gives rise to the inner cell mass and epiblast. In this study, we report the sequence of critical events in embryology (compaction, inside cells allocation and fragmentation) in bovine 72 h.p.i. 9–16 cell embryos, while also investigating the effects of X-sorted semen on these events. We found a wide distribution of total cell numbers among embryos, attributed to an asynchronous cleavage pattern and blastomere death. Additionally, 13% of embryos displayed irregular shapes. The establishment of the inside cell compartment increased ($p < 0.01$) in embryos with more cells. However, only 53.8% of 16-cell embryos presented inside cells. Compaction was present in 32.4% embryos and was positively correlated ($p = 0.03$, OR 3.02) with the establishment of inside cells, occurring independently of cell number. Fragmentation was present in 36% embryos, being more frequent ($p = 0.01$) in embryos with lower cell numbers. A possible association between irregular shape and fragmentation was considered ($p = 0.06$). The use of X-sorted semen had no effect on most evaluated parameters. However, it did have a marked effect on cleavage rate ($p < 0.01$) and the arrest of 2- and 4- cell embryos. In conclusion, bovine embryos exhibit an asynchronous cleavage pattern, high levels of fragmentation, and demonstrate compaction and inside cell allocation later in development compared to mouse embryos. Semen X-sorting has major effects on cleavage and embryo arrest. Further studies are needed to elucidate the association between irregularly shaped embryos and fragmentation, as well as the effects of sex on inside cell allocation.

KEYWORDS
bovine embryo, compaction, fragmentation, inner cell mass, inside cells, IVF

1 | INTRODUCTION

Early mammalian embryogenesis varies among species. While mouse embryos are currently considered the best model for studying human embryonic development, reports highlighting differences between human and mouse embryonic cells (Chen et al., 2009; Hassani et al., 2014; Zhang et al., 2013) underline the importance of multispecies studies (Rossant, 2011). Interestingly, features such as an extended duration of preimplantation development, low developmental rates and delayed zygotic genome activation were shown to

be similar between human and bovine embryos (Adjaye et al., 2007). This has led to an increased interest in bovine embryology and the necessity to characterize and understand basic biological mechanisms in this model organism.

Apart from its significance as a model for embryology, understanding bovine embryology and the emergence of the inside cells compartment could provide insights into the field of embryonic stem (ES) cell derivation. The ability to derive bovine ES cells holds major implications, especially for nuclear transfer techniques (Oback, 2008) and genetic engineering (Gjørret & Maddox-Hyttel, 2005; Keefer et al., 2007). However, despite extensive efforts (Cibelli et al., 1998; Iwasaki et al., 2000; Mitalipova et al., 2001; Saito et al., 2003; Wang et al., 2005), the successful establishment of bovine ES cell (bES) lines, including germline transmission and maintenance of pluripotency with further passaging, has not been achieved in bovine as it has for the mouse ES cells. The establishment of stable primed pluripotent embryonic stem cells from bovine blastocysts using a WNT signalling inhibition system has been reported (Bogliotti et al., 2018; Xiao et al., 2021), suggesting this mechanism, which affects pluripotency properties, is associated with the distinct properties observed in the bovine inner cell mass. Additionally, the expression pattern of pluripotency genes in bovine embryos differs from that in mouse embryos (Khan et al., 2012). This data reveals significant differences between mouse and bovine inner cell mass properties.

In mouse, polarization precedes the formation of inside cells, occuring at the 8-cell stage and transforming homogenous spherical cells into highly polarized radial blastomeres (Johnson & Ziomek, 1981). This involves the asymmetric distribution of polarity factors into apical and basolateral domains, along with mutually antagonistic interactions between them (Nance, 2014). Once established, polarity domains are differentially inherited during asymmetric divisions, contributing to the establishment of inside and outside cell identities in the mouse embryo (Bischoff et al., 2008; Surani & Handyside, 1983). Inside cells will develop into the inner cell mass (ICM), a group of cells that will mainly build the body of an organism, while outer cells will give rise to the trophectoderm (TE), the epithelium tissue that contributes to the embryonic part of the placenta (Rossant & Cross, 2001). However, in bovine embryos, little is known about the emergence of the inside cell compartment in 16-cell embryos and whether it precedes compaction and polarization.

Another important aspect often neglected in bovine embryology is the sex of embryos. It is well-known that male and female cells can behave differently (Pollitzer, 2013). Female embryo characteristics can be valuable for human assisted reproductive medicine, especially when the risk of X-linked disorders is detected. The sex of the bovine embryo can be controlled with over 90% accuracy using cytometer cell-sorted semen, based on small differences in chromosomal DNA content between X and Y sperms (Garner, 2006). For in vitro fertilization (IVF) routines using sexed semen in the animal reproduction field, where female embryos are in demand for herd replacement and milk production, understanding female-specific requirements is extremely relevant to improve in vitro production (IVP) systems, and the sex of the embryo should always be considered. It is known that dosage compensation of X-chromosome transcripts only occurs in bovine embryos after blastocyst formation (Bermejo-Álvarez et al., 2010, Bermejo-Alvarez et al., 2011), so most female embryos have both X chromosomes active during early development and display-specific gene expression patterns (Bermejo-Álvarez et al., 2010; Denicol et al., 2015).

In previous studies conducted by our group, female embryos exhibited a unique epigenetic profile (Oliveira et al., 2010, 2013). Furthermore, a noteworthy correlation between female embryos and compromised developmental outcomes was observed, as evidenced by diminished embryo quality characterized by elevated levels of fragmentation and apoptosis, as well as reduced cell numbers (Oliveira et al., 2016a, 2016b). Additionally, an increased incidence of developmental arrest at the early cleavage stages among female embryos was described (Oliveira et al., 2016a, 2016b).

Sex-related differences in the growth kinetics of human and bovine embryos produced in vitro have also been reported (Bronet et al., 2015; Larson et al., 2001; Oliveira et al., 2013; Oliveira, Saraiva, de Lima, Oliveira, Serapião, Borges, et al., 2016; Oliveira, Saraiva, de Lima, Oliveira, Serapião, Garcia, et al., 2016; Xu et al., 1992). Besides sex differences, there is also an effect of altered developmental kinetics and viability resulting from the sex-sorting processing of bovine semen (Magata et al., 2021).

The sex of embryos also affects ES cell derivation in mouse. XX-derived ES cells resembles a less differentiated state with epigenetic differences and exit pluripotency less efficiently than male ES cell lines (Choi et al., 2017; Schulz et al., 2014; Yagi et al., 2017). This outcome is particularly attributed to the presence of two active X chromosomes and its effect of increased inhibition of the Fgf–Erk signalling pathway, inhibition of Gsk3β (Wnt signalling activation) and Akt signalling stimulation (Hassani et al., 2019).

Given its importance in lineage specification and inner cell mass formation, the aim of this study was to characterize inside cell establishment in bovine 16-cell embryos produced with conventional or X-sorted semen, considering its relation to candidate predictors that can be easily assessed morphologically (compaction, fragmentation and irregular shape). We compared this pattern to the mouse embryo with the aim of bringing new insights into mammalian embryology and bovine ES cell and embryology field.

2 | MATERIALS AND METHODS

2.1 | Supplements

Reagents and culture media were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

2.2 | Preparation and selection of oocytes

Bovine ovaries were collected at a local slaughterhouse and processed within 2 h after slaughter. The ovaries were washed in

saline (37°C) and follicles measuring 3 to 8 mm in diameter were aspirated with an 18 gauge needle coupled to a 20 mL syringe. Cumulus-oocyte complexes (COCs) presenting at least three layers of cumulus cells and homogenous cytoplasm were selected under a stereomicroscope. The COCs were washed in HEPES-buffered TCM-199 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Cripion, Andradina, Brazil), 16 μg/mL sodium pyruvate and 83.4 μg/mL amikacin (Instituto Biochimico, Rio de Janeiro, Brazil).

2.3 | In vitro maturation (IVM)

Groups of 20 COCs were transferred to 100 μL drops of medium containing sodium bicarbonate-buffered TCM-199 supplemented with 10% FBS, 1.0 μg/mL FSH (Folltropin™, Bioniche Animal Health, Belleville, Canada), 50 μg/mL hCG (Profasi™, Serono, Sao Paulo, Brazil), 1.0 μg/mL estradiol, 16 μg/mL sodium pyruvate and 83.4 μg/mL amikacin, covered with sterile mineral oil (Dow Corning Co., Midland, MI) and incubated for 24 h at 38.5°C in an atmosphere of 5% CO_2 in air under saturated humidity.

2.4 | In vitro fertilization (IVF)

After IVM the cumulus cells were partially removed from the oocytes by pipetting. Groups of 25 oocytes were washed twice and transferred to 60 μL drops of TALP-IVF medium supplemented with 0.6% BSA, 10 μg/mL heparin, 18 μM penicillamine, 10 μM hypotaurine and 1.8 μM epinephrine, and covered with sterile mineral oil. Frozen straws of X-sorted and conventional semen from the same bull (CRV Lagoa, Sertaozinho, Brazil) were used at each replicate. Each straw containing approximately 2 million spermatozoa was centrifuged separately on a discontinuous 45/90 Percoll gradient for 7 min at 3600 G. The pellet was resuspended in 700 μL TALP-IVF medium and again centrifuged for 5 min at 520 G. After centrifugation, 30 μL of the medium containing the pellet was collected from the bottom of the tube and homogenized in a conic tube. Motility and concentration of sperm was calculated and adjusted to a final suspension of 10^4 spermatozoa for each oocyte. The plates were incubated at 38.5°C for 20 h in an atmosphere of 5% CO_2 in air under saturated humidity.

2.5 | In vitro culture

After IVF, presumptive zygotes were denuded of cumulus cells by vigorous pipetting and cultured in SOF medium supplemented with 1.5% FBS and 5 mg/mL BSA at 38.5°C in an atmosphere of 5% CO_2 in air under saturated humidity. Groups of 15–20 presumptive zygotes were cultured in 100 μL drops. Embryos were evaluated and fixed at 96 h.p.i. Since asymmetric cleavage divisions are observed in bovine embryos, 5–8-cell embryos were grouped as 8-cell and 9–16 cell embryos were grouped as 16-cell. Average number of each embryonic stage was assessed in order to characterize the embryos used in this study.

2.6 | Phalloidin and HOECHST staining

Embryos were fixed in 4% paraformaldehyde for 30 min at 37°C and stored at 4°C in PBS supplemented with 3% BSA and 0.2% tween 20 for up to 1 week. Phalloidin (Atto-568 conjugated, 0.5 μM) was used to stain actin filaments, for 50 min. The nuclei were stained with 10 μL/mL Hoechst 33342 solution for 50 min. Embryos were washed three times for 10 min in PBS and examined under a fluorescence microscope.

2.7 | Analysis of compaction, presence of inside cells, fragmentation and embryo shape

Embryos were imaged on an EVOS 5000 inverted microscope using Z-stack function. Z-sections were captured from bottom to top of each embryo, with intervals from 5 to 8 μm and a total of 12–15 sections per embryo. Images were analysed using ImageJ (NIH) Software.

Embryos were examined for the presence of radial cells and stronger cell membrane contacts. The initiation of compaction was evaluated in this assay, so partially compacted embryos (still presenting non-compacted cells) were classified as compacted. For inside cells, multiple optical sections from bottom to top of each embryo were examined. Cells fully surrounded by others, with no external contact, were categorized as inside.

Focus plane images from each embryo were captured and analysed for fragmentation and embryo shape. Embryos displaying enucleated cytoplasmic fragment totaling approximately 20% of the embryo's size were categorized as fragmented. Embryos with varying sizes in the Zona pellucida meridional and equatorial planes were classified as elongated.

2.8 | Statistical analysis

Binary logistic regression considering variables (i) presence of inside cells; (ii) compaction and (iii) fragmentation and predictors: presence of inside cells, compaction, fragmentation, irregular shape and X-sorted semen was used to estimate effects of each predictor on main variables. GLM was estimated to compare groups of embryos with and without: inside cells, compaction, fragmentation and irregular shape. Cleavage rate was compared between X-sorted semen and conventional semen groups using Fisher Exact Test. All analysis were performed at 5% significance level, at Minitab Software.

3 | RESULTS

We assessed 9–16 cell bovine embryos at 96 h.p.i. ($n = 105$), examining the presence of inside cells, compaction, fragmentation, their shapes (elongated/rounded) and the effects of X-sorted semen use on these features.

The first notable observation is the asynchronous cleavage pattern observed for this stage in the bovine species. Embryos displayed varying cell numbers: 9 ($n = 8$), 10 ($n = 10$), 11 ($n = 10$), 12 ($n = 16$), 13 ($n = 19$), 14 ($n = 18$), 15 (n = 11) and 16 ($n = 13$) cells. Moreover, some embryos presented arrested cells from previous cleavage cycle, indicating that they would never reach homogeneously the 16-cell stage—an observation typical in bovine development. The appearance of irregularly shaped (elongated) embryos was detected (Figure 1a) and accounted for 13% (14 out of 105 embryos). No difference between elongated and rounded embryos in terms of cell numbers was detected (Figure 1b).

3.1 | Establishment of the inside cell compartment in bovine embryos

In Figure 2a,b, embryos with and without inside cells are shown in three Z-sections. As expected, we found that embryos with inside cells had higher cell numbers than embryos without inside cells (12 vs. 14, $p < 0.01$) (Figure 2c). In Figure 2d, the percentage of embryos with inside cells is shown regarding the total cell number. Only one out of 44 embryos with fewer than 12 cells exhibited inside cells (2.2%), while 20 out of 61 embryos with 13–16 cells exhibited inside cells (32.8%). Still, only half (7/13) of 16-cell embryos have inside cells in bovine.

Next, we associated inside cell establishment with other phenomena of early embryo development. In Figure 2e, odds ratio and confidence interval are shown. Compacted embryos had a 3.02 times higher chance ($p = 0.03$) of exhibiting inside cells than non-compacted embryos.

Fragmented and irregularly shaped (elongated) embryos do not have a higher chance of having inside cells. Embryos produced with X-sorted semen exhibited a trend to display a higher percentage of embryos with inside cells ($p = 0.08$) (OR 2.69).

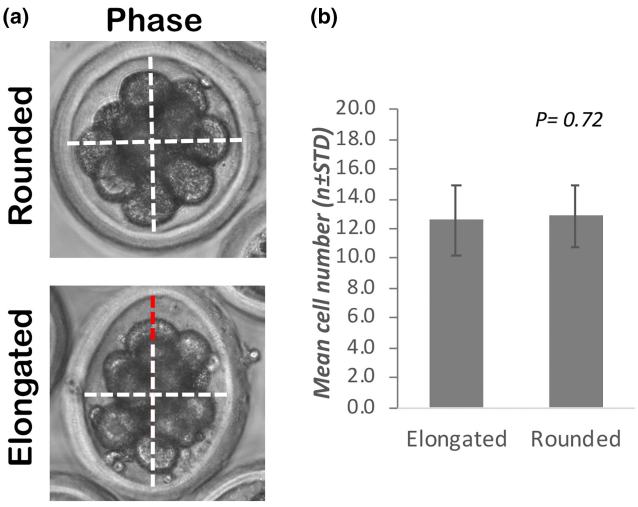

FIGURE 1 Elongated bovine 9–16 cell embryos. (a) Phase contrast images of embryos with rounded and elongated shape. White dotted lines with similar length are delimiting equatorial and meridional planes in each embryo. Red line represents the difference between meridional and equatorial planes in the elongated embryo. (b) Mean cell number of embryos with distinct shapes.

3.2 | Compaction in bovine embryos at 96 h.p.i.

A compacted embryo is shown in Figure 3a. Compaction occurrence was independent of embryo cell number, as no difference was found between compacted or non-compacted embryos' cell number (Figure 3b), and the percentage of compacted embryos was balanced among embryos from 9 to 16-cell stage (Figure 3c). Inside cell embryos presented a 2.94 higher chance of being compacted ($p = 0.04$), but no effect was detected for fragmented, irregular shape or X-sorted semen embryos regarding compaction establishment (Figure 3d). Overall, 32.4% (34 out of 105) 9–16 embryos were compacted at 96 h.p.i.

3.3 | Fragmentation in bovine embryos at 96 h.p.i.

Fragmented embryos (Figure 4a) were associated with lower ($p = 0.01$) cell numbers (Figure 4b,c). In 9–11 cell embryos, 68% (19 out of 28) presented fragmentation, while embryos with 12–16 cells presented a prevalence of 25% (19 out of 77) of fragmentation. Overall, 36% (38 out of 105) 9–16 embryos had fragmentation at 96 h.p.i.

Fragmentation in bovine embryos had apparently no relation to inside cell establishment, compaction, or X-sorted semen, but a trend ($p = 0.06$) to a 3.17 times higher chance of fragmentation was found in irregularly shaped embryos (Figure 4d).

3.4 | X-sorted semen effects

Comparing x-sorted semen and conventional semen embryos, the cleavage rate was lower ($p < 0.01$) for X-sorted semen (Figure 5a). Embryo evaluation at 96 h.p.i. revealed that cleaved embryos were also delayed in the X-sorted semen group, as presented in Figure 5b. In the X-sorted semen group, 16-cell embryos represented 28% (41 out of 147) of cleaved embryos, while in the Conventional semen group, it accounted for 58% (126 out of 217). This delay is apparently due to an arrest to a greater extent at the 2-cell and 4-cell stage observed in the X-sorted group, corresponding to 46% (68 out of 147) in X-sorted semen and only 17% (37 out of 217) in the conventional

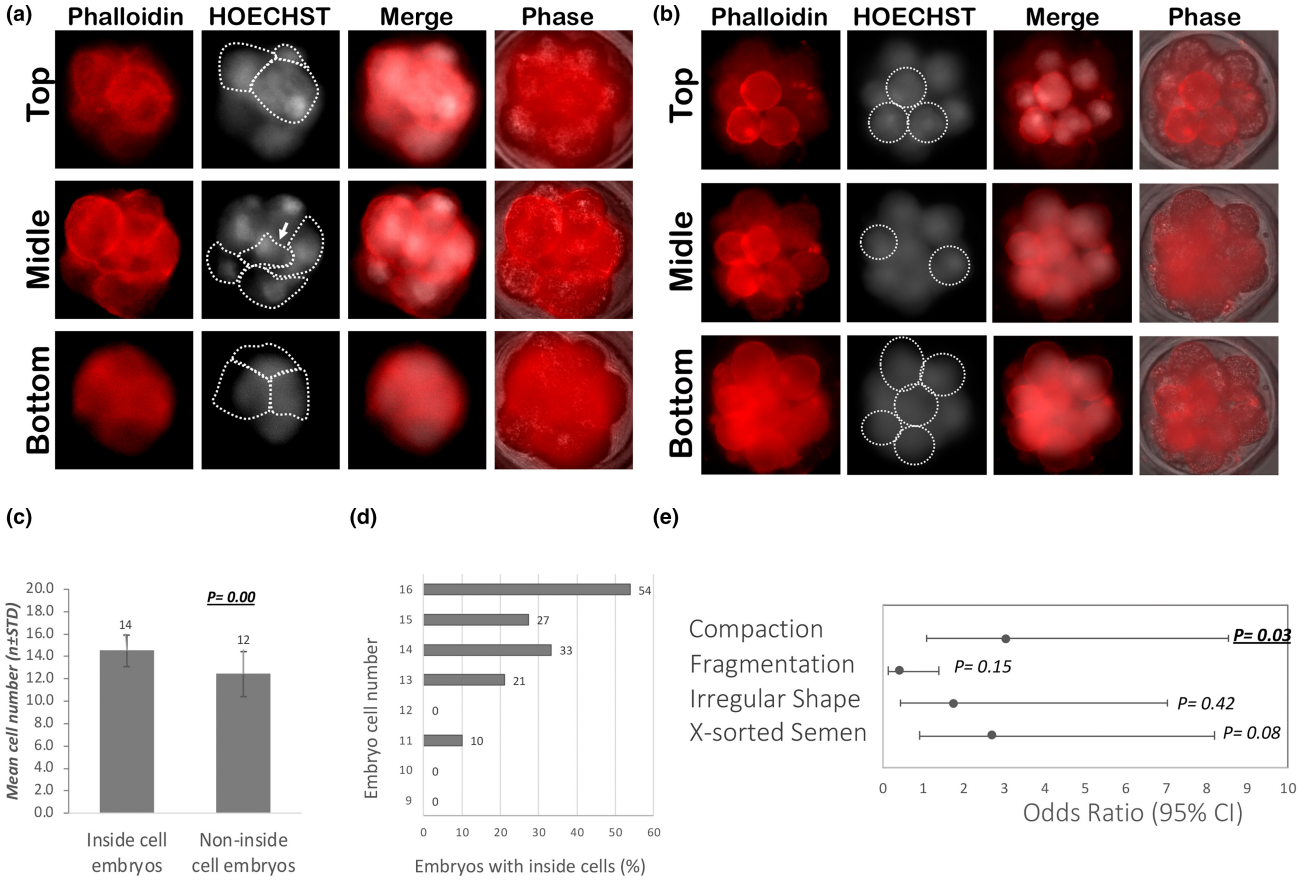

FIGURE 2 Establishment of inside cell compartment in bovine 9–16 cell embryos. (a,b) Images of phalloidin/ HOECHST-stained embryos exhibiting inside cells (a) or not (b). Images of a single embryo in top, middle and bottom Z-sections are shown. Membrane drawings of new cells at each plane are represented in HOECHST images. Arrow indicates an inside cell (a). (c) Mean cell number of inside cell embryos compared to non-inside cell conterparts. Embryos were at 9–16 cell stage (fixed at 96 h.p.i.). (c) Mean cell number of embryos exhibiting inside cells or not. (d) Percentage of inside cell embryos at each cell stage (9 to 16). (e) Logistic regression odds ratio and CI for inside cells presence considering binary predictors (Y/N) compaction, fragmentation, irregular shape and X-sorted semen.

semen group. However, X-sorted semen did not affect the mean cell number in stained 9–16 cell embryos (Figure 5c).

Associations between X-sorted semen and evaluated parameters of 16-cell embryos are presented in previous topics of this study. Briefly, no significant statistical association with inside cell establishment, compaction, fragmentation or irregular shape was detected at a 5% significance level. A trend to display a higher percentage of embryos with inside cells ($p=0.08$) (OR 2.69) was present in X-sorted semen embryos. Inside cell embryos corresponded to 29% (8 out of 28) X-sorted semen embryos and 17% (13 out of 77) conventional semen embryos ($p=0.09$).

4 | DISCUSSION

Embryo development in bovine is quite unique. In bovine, similar to human, an oocyte has a limited chance for blastocyst formation, and several events can influence and help predict its fate (Luciano & Sirard, 2018). Longer and asynchronous cell cycles, late zygotic genome activation and late lineage specification are some aspects that highlight the differences between bovine and mouse embryology. Here, we reaffirm this observation in compaction and inside cell establishment, events that were initiated later in bovine development.

Our study focused on the formation of the inside cell compartment at 9–16 cell embryos, collected at 96 h.p.i., and its relation to compaction, sex, fragmentation and embryo shape. Interestingly, we show here that the inside cell compartment was not formed in most 9–16 cell stage embryos, and even in embryos with 16 cells only approximately half of them had inside cells. In mouse, inside cells are allocated at 16-cells, after compaction, and blastomeres gradually commit to distinct developmental fates (Johnson, 2009). Inside and outside cells inherit different cytoskeletal regions through asymmetric divisions; outside cells assume polarized domains, and inside cells remains non-polarized. Polarized cells develop into trophectoderm (TE), whereas non-polarized inside cells form the inner cell mass (Bischoff et al., 2008). The later inside cell establishment could have impacts on polarity and inner cell mass formation, affecting bovine epiblast properties.

Inside cell establishment was positively associated to compaction onset. Compaction phenomena occurred regardless cell

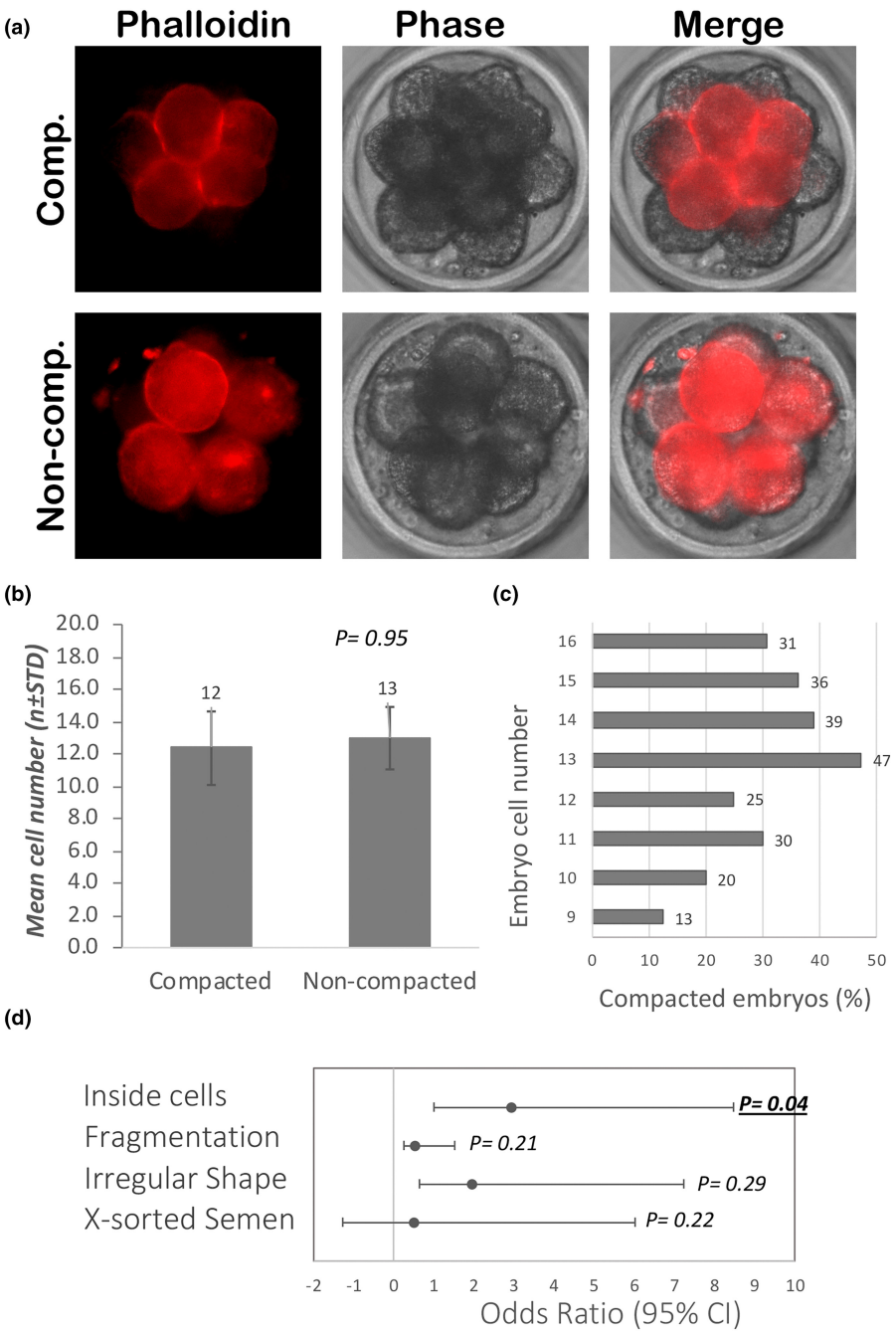

FIGURE 3 Compaction in bovine 9–16 cell embryos. (a) Images of phalloidin stained embryos exhibiting compacted and non-compacted morphology are shown. (b) Mean cell number of compacted embryos compared to non-compacted conterparts. Embryos were at 9–16 cell stage (fixed at 96 h.p.i.). (c) Percentage of compacted embryos at each cell stage (9 to 16). (d) Logistic regression odds ratio and CI for compaction considering binary predictors (Y/N) inside cells, fragmentation, irregular shape and X-sorted semen.

number, suggesting it has more relationship with the embryonic day than with embryonic stage. It is important to notice that before compaction, bovine embryos displayed big, rounded cells that are normally arranged in a configuration that lacks inside cells, as evidenced in our study. Previous studies on bovine embryos reported compaction to be initiated around the 16- to 32-cell stage (Betteridge & Fléchon, 1988; van Soom et al., 1997). Consistently, here we found less than half of embryos exhibiting radially oriented cells at the 16-cell stage (96 h.p.i.).

Fragmentation occurs when anucleated structures of blastomeric origin appear in the embryo (Keltz et al., 2006). Extensive fragmentation negatively correlates with embryo implantation and pregnancy establishment in human (Prados et al., 2012). In bovine embryos, we found that fragmentation was not associated with inside cells onset or compaction. However, fragmentation was associated with lower cell numbers in embryos, and a trend was detected for the association between fragmentation and elongated shape occurrence in bovine embryos. In this respect, Kamran et al. (2012)

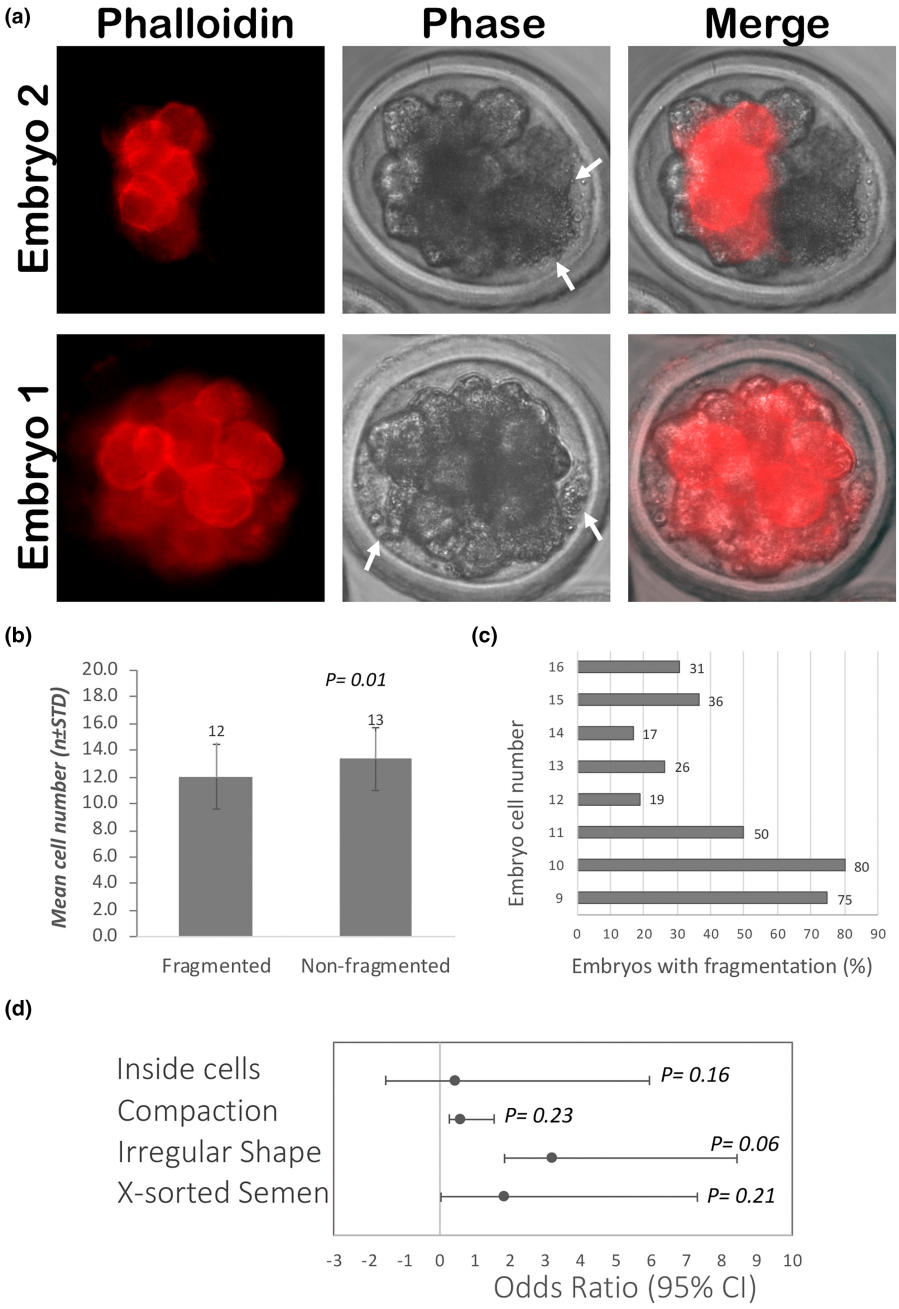

FIGURE 4 Fragmentation in bovine 9–16 cell embryos. (a) Images of phalloidin stained embryo exhibiting fragmentation (arrows). (b) Mean cell number of embryos with fragmentation compared to counterparts. Embryos were at 9–16 cell stage (fixed at 96 h.p.i.). (c) Percentage of embryos with fragmentation at each cell stage (9 to 16). (d) Logistic regression odds ratio and CI for fragmentation considering binary predictors (Y/N) inside cells, compaction, irregular shape, and X-sorted semen.

has shown a weak association of day 3 embryo shape with implantation potential in human embryos, suggesting indeed a lower developmental potential for elongated embryos. This result suggests fragmented and elongated embryos could represent a lower developmental competence class of embryos in bovine, which should be further investigated.

In this study, we analysed the contribution of sexed semen to the evaluated phenomena. Apparently, no effect of X-sorted semen was detected for inside cell establishment, compaction or fragmentation, but we detected a trend to an increased inside cell establishment percentage when X-sorted semen was used that deserves better investigation. In our study design, we dissected sperm sorting effects on the studied phenomena. It is possible, though, that no effect was detected for sperm sorting, but sex could have an influence on inside cell establishment. This is because in the X-sorted group we expect mostly female embryos, but in the conventional group, a mix of male and female embryos could mask a sex effect that would be better evidenced comparing X-sorted with Y-sorted groups.

Differences in embryo production efficiency were present comparing X-sorted and conventional groups. In previous studies,

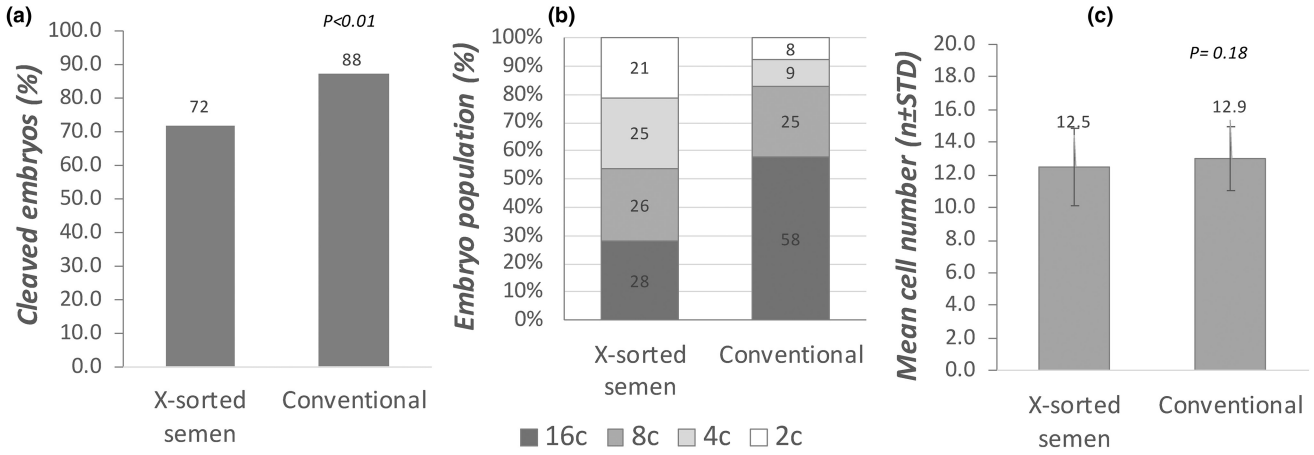

FIGURE 5 Effects of X-sorted semen in developmental kinetics. (a) Percentage of cleaved embryos in X-sorted and conventional semen groups. (b) Embryos at each cleavage stage at 96 h.p.i., in X-sorted and conventional semen groups (16c, 9–16 cells; 8c, 5–8 cells; 4c, 3–4 cells, 2c, 2 cells). (c) Mean cell number of phalloidin/ HOECHST stained 9–16 cell embryos (fixed at 96 h.p.i.) produced with X-sorted and conventional semen.

our research group demonstrated no differences in fertilization or cleavage rates between female and male embryos, suggesting X and Y spermatozoa has similar fertility. Still, some particularities were presented since delayed embryo development was increased in the female group (Oliveira et al., 2016a, 2016b). Other reports also demonstrated that male IVP embryos develop faster than female embryos, and that a higher percentage of male embryos develop to advanced stages beyond blastocysts compared to female embryos until 8 days post fertilization (Larson et al., 2001; Xu et al., 1992). Thus, it is expected that the conventional semen group has increased percentage of embryos at advanced developmental stage. However, the lower cleavage percentage and the higher percentage of embryo arrest observed in X-sorted semen group seems to overcome the expected male effect, since a major decrease in embryo viability was detected (X-sorted 46% vs. conventional 17% 2-4-cell arrest). For these reasons, the variation in the kinetics and viability of embryos derived from X-sorted and unsorted semen could be mostly due to the sex-sorting process of semen.

Bermejo-Álvarez et al. (2010) previously demonstrated that the use of sex-sorted sperm, rather than unsorted sperm, significantly delays the onset of first cleavage of in vitro-produced (IVP) bovine embryos. A recent study also revealed delayed development in bovine embryos produced with X-sorted compared to unsorted semen, providing further understanding of some mechanisms involved in the reduced fertility of sex-sorted sperm (Magata et al., 2021). Continuous monitoring of IVP embryos by time-lapse cinematography revealed that blastomere fusion during the first cleavage division occurred frequently in the sexed semen-derived embryos. The authors also demonstrated that abnormality during the first cleavage division could be a potential factor associated with the alterations in the development of bovine embryos produced with X-sorted semen.

In fact, the timing of the first cleavage seems to be related to implantation potential, in both human and bovine embryos (Meseguer et al., 2011; Rubio et al., 2012; Sugimura et al., 2012). Furthermore, reduced blastocyst hatchability, chromosomal aberrations and impaired cryotolerance were previously observed in embryos produced with X-sorted semen, suggesting that the compromised viability of those embryos is associated with first cleavage alterations (Kola et al., 1987; Lagalla et al., 2017; Magata et al., 2019, 2021). Regarding sex-sorted semen use for IVF, it is known that in vivo (DeJarnette et al., 2010; Diniz et al., 2021; Sá Filho et al., 2012; Sales et al., 2011) and in vitro (Magata et al., 2021; Schenk et al., 2009; Seidel, 2014) rates are usually lower than non-sorted sperm mainly due to potential cellular damages (DeJarnette et al., 2008, 2011; Mocé et al., 2006) and/or alterations in structural characteristics of sperm cells after the sex-sorting process (Carvalho et al., 2018). Sex-sorted sperm commonly exhibits capacitation-like phenomenon after cryopreservation, which are related to destabilization of sperm plasma membranes (Carvalho et al., 2013), sperm motility or acrosome integrity and has a direct impact on sperm fertilization capacity, whereas alterations in DNA integrity mainly affects gamete syngamy and embryo development (Blondin et al., 2009; Gosálvez et al., 2011).

Sperm DNA fragmentation can be related to compromised embryo development and quality (Ahmadi & Ng, 1999; Avendaño et al., 2010), and decreased embryo hatchability indicates reduced oxygen consumption and aberrant mitochondrial function (Sugimura et al., 2012). Bovine blastocysts derived from sex-sorted semen present increased proportion of immature mitochondria (Palma et al., 2008) indicating that sperm sex sorting process might modify the embryo metabolic function which results in reduced hatchability. Thus, regarding postfertilization embryo development, the reduced cleavage rates and the embryo development arrest here observed for X-sorted sperm might be related to damages in chromatin stability (Boe-Hansen et al., 2005; Gosálvez et al., 2011) and/or mitochondrial modifications (Rath et al., 2013).

In conclusion, our findings provide further support for the interspecies diversity during preimplantation embryo development.

Here, we report later inside cell establishment and compaction in bovine embryos, suggesting that in this species, polarity can be established after inside cells formation in a high proportion of embryos, and this could affect inner cell properties.

AUTHOR CONTRIBUTIONS

CSO performed the experiments, analysed data and wrote the original draft. NZS and LZO involved in conceptualization, reviewed and edited the manuscript.

ACKNOWLEDGEMENTS

This work was supported by CNPq [grant number 408435/2016-0].

CONFLICT OF INTEREST STATEMENT

The authors have no potential conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, CSO, upon reasonable request.

REFERENCES

Adjaye, J., Herwig, R., Brink, T. C., Herrmann, D., Greber, B., Sudheer, S., Groth, D., Carnwath, J. W., Lehrach, H., & Niemann, H. (2007). Conserved molecular portraits of bovine and human blastocysts as a consequence of the transition from maternal to embryonic control of gene expression. *Physiological Genomics*, *31*(2), 315–327. https://doi.org/10.1152/physiolgenomics.00041.2007

Ahmadi, A., & Ng, S. C. (1999). Fertilizing ability of DNA-damaged spermatozoa. *Journal of Experimental Zoology*, *284*(6), 696–704. https://doi.org/10.1002/(SICI)1097-010X(19991101)284:6<696::AID-JEZ11>3.0.CO;2-E

Avendaño, C., Franchi, A., Duran, H., & Oehninger, S. (2010). DNA fragmentation of normal spermatozoa negatively impacts embryo quality and intracytoplasmic sperm injection outcome. *Fertility and Sterility*, *94*(2), 549–557. https://doi.org/10.1016/j.fertnstert.2009.02.050

Bermejo-Alvarez, P., Rizos, D., Lonergan, P., & Gutierrez-Adan, A. (2011). Transcriptional sexual dimorphism in elongating bovine embryos: Implications for XCI and sex determination genes. *Reproduction*, *141*(6). https://doi.org/10.1530/REP-11-0006

Bermejo-Álvarez, P., Lonergan, P., Rath, D., Gutiírrez-Adan, A., & Rizos, D. (2010). Developmental kinetics and gene expression in male and female bovine embryos produced in vitro with sex-sorted spermatozoa. *Reproduction, Fertility and Development*, *22*(2), 426–436. https://doi.org/10.1071/RD09142

Betteridge, K. J., & Fléchon, J. E. (1988). The anatomy and physiology of pre-attachment bovine embryos. *Theriogenology*, *29*(1), 155–187. https://doi.org/10.1016/0093-691X(88)90038-6

Bischoff, M., Parfitt, D. E., & Zernicka-Goetz, M. (2008). Formation of the embryonic-abembryonic axis of the mouse blastocyst: Relationships between orientation of early cleavage divisions and pattern of symmetric/asymmetric divisions. *Development*, *135*(5), 953–962. https://doi.org/10.1242/dev.014316

Blondin, P., Beaulieu, M., Fournier, V., Morin, N., Crawford, L., Madan, P., & King, W. A. (2009). Analysis of bovine sexed sperm for IVF from sorting to the embryo. *Theriogenology*, *71*(1), 30–38. https://doi.org/10.1016/j.theriogenology.2008.09.017

Boe-Hansen, G. B., Morris, I. D., Ersbøll, A. K., Greve, T., & Christensen, P. (2005). DNA integrity in sexed bull sperm assessed by neutral comet assay and sperm chromatin structure assay. *Theriogenology*, *63*(6), 1789–1802. https://doi.org/10.1016/j.theriogenology.2004.08.004

Bogliotti, Y. S., Wu, J., Vilarino, M., Okamura, D., Soto, D. A., Zhong, C., Sakurai, M., Sampaio, R. V., Suzuki, K., Izpisua Belmonte, J. C., & Ross, P. J. (2018). Efficient derivation of stable primed pluripotent embryonic stem cells from bovine blastocysts. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(9), 2090–2095. https://doi.org/10.1073/pnas.1716161115

Bronet, F., Nogales, M. C., Martínez, E., Ariza, M., Rubio, C., García-Velasco, J. A., & Meseguer, M. (2015). Is there a relationship between time-lapse parameters and embryo sex? *Fertility and Sterility*, *103*(2), 396–401.e2. https://doi.org/10.1016/j.fertnstert.2014.10.050

Carvalho, J., Sartori, R., Rodello, L., Mourão, G. B., Bicudo, S. D., & Margot, M. A. (2018). Flow cytometry sex sorting affects bull sperm longevity and compromises their capacity to bind to oviductal cells. *Livestock Science*, *207*, 30–37. https://doi.org/10.1016/j.livsci.2017.11.005

Carvalho, J. O., Silva, L. P., Sartori, R., & Dode, M. A. N. (2013). Nanoscale differences in the shape and size of X and Y chromosome-bearing bovine sperm heads assessed by atomic force microscopy. *PLoS One*, *8*(3), e59387. https://doi.org/10.1371/journal.pone.0059387

Chen, A. E., Egli, D., Niakan, K., Deng, J., Akutsu, H., Yamaki, M., Cowan, C., Fitz-Gerald, C., Zhang, K., Melton, D. A., & Eggan, K. (2009). Optimal timing of inner cell mass isolation increases the efficiency of human embryonic stem cell derivation and allows generation of sibling cell lines. *Cell Stem Cell*, *4*(2), 103–106. https://doi.org/10.1016/j.stem.2008.12.001

Choi, J., Huebner, A. J., Clement, K., Walsh, R. M., Savol, A., Lin, K., Gu, H., di Stefano, B., Brumbaugh, J., Kim, S. Y., Sharif, J., Rose, C. M., Mohammad, A., Odajima, J., Charron, J., Shioda, T., Gnirke, A., Gygi, S., Koseki, H., … Hochedlinger, K. (2017). Prolonged Mek1/2 suppression impairs the developmental potential of embryonic stem cells. *Nature*, *548*(7666), 219–223. https://doi.org/10.1038/nature23274

Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce De León, F. A., & Robl, J. M. (1998). Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells. *Nature Biotechnology*, *16*(7), 642–646. https://doi.org/10.1038/nbt0798-642

DeJarnette, J. M., Leach, M. A., Nebel, R. L., Marshall, C. E., McCleary, C. R., & Moreno, J. F. (2011). Effects of sex-sorting and sperm dosage on conception rates of Holstein heifers: Is comparable fertility of sex-sorted and conventional semen plausible? *Journal of Dairy Science*, *94*(7), 3477–3483. https://doi.org/10.3168/jds.2011-4214

DeJarnette, J. M., McCleary, C. R., Leach, M. A., Moreno, J. F., Nebel, R. L., & Marshall, C. E. (2010). Effects of 2.1 and 3.5×106 sex-sorted sperm dosages on conception rates of Holstein cows and heifers. *Journal of Dairy Science*, *93*(9), 4079–4085. https://doi.org/10.3168/jds.2010-3181

DeJarnette, J. M., Nebel, R. L., Marshall, C. E., Moreno, J. F., McCleary, C. R., & Lenz, R. W. (2008). Effect of sex-sorted sperm dosage on conception rates in holstein heifers and lactating cows. *Journal of Dairy Science*, *91*(5), 1778–1785. https://doi.org/10.3168/jds.2007-0964

Denicol, A. C., Leão, B. C. S., Dobbs, K. B., Mingoti, G. Z., & Hansen, P. J. (2015). Influence of sex on basal and dickkopf-1 regulated gene expression in the bovine morula. *PLoS ONE*, *10*(7). https://doi.org/10.1371/journal.pone.0133587

Diniz, J. H. W., Peres, R. F. G., Teixeira, A. C. B., Riveros, J. A. N., Noronha, I. M., Martins, C. F. G., Oliveira, C. S., Pohler, K. G., Pugliesi, G., & Oliveira, L. Z. (2021). Administration of PGF2α at the moment of timed-AI using sex-sorted or conventional semen in suckled nelore cows with different intensity of estrus behavior. *Theriogenology*, *174*, 169–175. https://doi.org/10.1016/j.theriogenology.2021.08.023

Garner, D. L. (2006). Flow cytometric sexing of mammalian sperm. *Theriogenology*, *65*(5), 943–957. https://doi.org/10.1016/j.theriogenology.2005.09.009

Gjørret, J. O., & Maddox-Hyttel, P. (2005). Attempts towards derivation and establishment of bovine embryonic stem cell-like cultures. *Reproduction, Fertility and Development*, *17*(1–2), 113–124. https://doi.org/10.1071/rd04117

Gosálvez, J., Ramirez, M. A., López-Fernández, C., Crespo, F., Evans, K. M., Kjelland, M. E., & Moreno, J. F. (2011). Sex-sorted bovine spermatozoa and DNA damage: II. Dynamic features. *Theriogenology*, *75*(2), 206–211. https://doi.org/10.1016/j.theriogenology.2010.09.011

Hassani, S. N., Moradi, S., Taleahmad, S., Braun, T., & Baharvand, H. (2019). Transition of inner cell mass to embryonic stem cells: Mechanisms, facts, and hypotheses. *Cellular and Molecular Life Sciences*, *76*(5), 873–892. https://doi.org/10.1007/s00018-018-2965-y

Hassani, S. N., Totonchi, M., Gourabi, H., Schöler, H. R., & Baharvand, H. (2014). Signaling roadmap modulating naive and primed pluripotency. *Stem Cells and Development*, *23*(3), 193–208. https://doi.org/10.1089/scd.2013.0368

Iwasaki, S., Campbell, K. H. S., Galli, C., Akiyama, K., & Iwasaki, S. (2000). Production of live calves derived from embryonic stem-like cells aggregated with tetraploid embryos. *Biology of Reproduction*, *62*(2), 470–475. https://doi.org/10.1095/biolreprod62.2.470

Johnson, M. H. (2009). From mouse egg to mouse embryo: Polarities, axes, and tissues. *Annual Review of Cell and Developmental Biology*, *25*, 483–512. https://doi.org/10.1146/annurev.cellbio.042308.113348

Johnson, M. H., & Ziomek, C. A. (1981). The foundation of two distinct cell lineages within the mouse morula. *Cell*, *24*(1), 71–80. https://doi.org/10.1016/0092-8674(81)90502-X

Kamran, S. C., Reichman, D. E., Missmer, S. A., Correia, K. F., Karaca, N., Romano, A., & Racowsky, C. (2012). Day 3 embryo shape as a morphologic selection parameter in in vitro fertilization. *Journal of Assisted Reproduction and Genetics*, *29*(10), 1135–1139. https://doi.org/10.1007/s10815-012-9842-7

Keefer, C. L., Pant, D., Blomberg, L., & Talbot, N. C. (2007). Challenges and prospects for the establishment of embryonic stem cell lines of domesticated ungulates. *Animal Reproduction Science*, *98*(1–2), 147–168. https://doi.org/10.1016/j.anireprosci.2006.10.009

Keltz, M. D., Skorupski, J. C., Bradley, K., & Stein, D. (2006). Predictors of embryo fragmentation and outcome after fragment removal in in vitro fertilization. *Fertility and Sterility*, *86*(2), 321–324. https://doi.org/10.1016/j.fertnstert.2006.01.048

Khan, D. R., Dubé, D., Gall, L., Peynot, N., Ruffini, S., Laffont, L., le Bourhis, D., Degrelle, S., Jouneau, A., & Duranthon, V. (2012). Expression of pluripotency master regulators during two key developmental transitions: EGA and early lineage specification in the bovine embryo. *PLoS One*, *7*(3), e34110. https://doi.org/10.1371/journal.pone.0034110

Kola, I., Trounson, A., Dawson, G., & Rogers, P. (1987). Tripronuclear human oocytes: Altered cleavage patterns and subsequent karyotypic analysis of embryos. *Biology of Reproduction*, *37*(2), 395–401. https://doi.org/10.1095/biolreprod37.2.395

Lagalla, C., Tarozzi, N., Sciajno, R., Wells, D., di Santo, M., Nadalini, M., Distratis, V., & Borini, A. (2017). Embryos with morphokinetic abnormalities may develop into euploid blastocysts. *Reproductive Biomedicine Online*, *34*(2), 137–146. https://doi.org/10.1016/j.rbmo.2016.11.008

Larson, M. A., Kimura, K., Kubisch, H. M., & Roberts, R. M. (2001). Sexual dimorphism among bovine embryos in their ability to make the transition to expanded blastocyst and in the expression of the signaling molecule IFN-τ. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(17), 9677–9682. https://doi.org/10.1073/pnas.171305398

Luciano, A. M., & Sirard, M. A. (2018). Successful in vitro maturation of oocytes: A matter of follicular differentiation. *Biology of Reproduction*, *98*(2), 162–169. https://doi.org/10.1093/biolre/iox149

Magata, F., Ideta, A., Okubo, H., Matsuda, F., Urakawa, M., & Oono, Y. (2019). Growth potential of bovine embryos presenting abnormal cleavage observed through time lapse cinematography. *Theriogenology*, *133*, 119–124. https://doi.org/10.1016/j.theriogenology.2019.04.031

Magata, F., Urakawa, M., Matsuda, F., & Oono, Y. (2021). Developmental kinetics and viability of bovine embryos produced in vitro with sex-sorted semen. *Theriogenology*, *161*, 243–251. https://doi.org/10.1016/j.theriogenology.2020.12.001

Meseguer, M., Herrero, J., Tejera, A., Hilligsøe, K. M., Ramsing, N. B., & Remoh, J. (2011). The use of morphokinetics as a predictor of embryo implantation. *Human Reproduction*, *26*(10), 2658–2671. https://doi.org/10.1093/humrep/der256

Mitalipova, M., Beyhan, Z., & First, N. L. (2001). Pluripotency of bovine embryonic cell line derived from precompacting embryos. *Cloning*, *3*(2), 59–67. https://doi.org/10.1089/15204550152475563

Mocé, E., Graham, J. K., & Schenk, J. L. (2006). Effect of sex-sorting on the ability of fresh and cryopreserved bull sperm to undergo an acrosome reaction. *Theriogenology*, *66*(4), 929–936. https://doi.org/10.1016/j.theriogenology.2006.01.063

Nance, J. (2014). Getting to know your neighbor: Cell polarization in early embryos. In. *Journal of Cell Biology*, *206*(7), 823–832. https://doi.org/10.1083/jcb.201407064

Oback, B. (2008). Climbing mount efficiency—small steps, not Giant leaps towards higher cloning success in farm animals. *Reproduction in Domestic Animals*, *43*(SUPPL.2), 407–416. https://doi.org/10.1111/j.1439-0531.2008.01192.x

Oliveira, C. S., Saraiva, N. Z., Cruz, M. H. C., Mazeti, B., Oliveira, L. Z., Lopes, F. L., & Garcia, J. M. (2013). HDAC inhibition decreases XIST expression on female IVP bovine blastocysts. *Reproduction*, *145*, 9–17. https://doi.org/10.1530/REP-11-0343

Oliveira, C. S., Saraiva, N. Z., de Lima, M. R., Oliveira, L. Z., Serapião, R. V., Borges, C. A. V., Garcia, J. M., & Camargo, L. S. A. (2016). Kinetics data from bovine sex-specific embryo development from three different bulls. *Data in Brief*, *7*, 1211–1216. https://doi.org/10.1016/j.dib.2016.03.066

Oliveira, C. S., Saraiva, N. Z., de Lima, M. R., Oliveira, L. Z., Serapião, R. V., Garcia, J. M., Borges, C. A. V., & Camargo, L. S. A. (2016). Cell death is involved in sexual dimorphism during preimplantation development. *Mechanisms of Development*, *139*, 42–50. https://doi.org/10.1016/j.mod.2015.12.001

Oliveira, C. S., Saraiva, N. Z., de Souza, M. M., Tetzner, T. A. D., de Lima, M. R., & Garcia, J. M. (2010). Effects of histone hyperacetylation on the preimplantation development of male and female bovine embryos. *Reproduction, Fertility and Development*, *22*(6), 1041–1048. https://doi.org/10.1071/RD09238

Palma, G. A., Olivier, N. S., Neumüller, C., & Sinowatz, F. (2008). Effects of sex-sorted spermatozoa on the efficiency of in vitro fertilization and ultrastructure of in vitro produced bovine blastocysts. *Journal of Veterinary Medicine Series C: Anatomia Histologia Embryologia*, *37*(1), 67–73. https://doi.org/10.1111/j.1439-0264.2007.00795.x

Pollitzer, E. (2013). Cell sex matters. *Nature*, *500*(7460), 23–24. https://doi.org/10.1038/500023a

Prados, F. J., Debrock, S., Lemmen, J. G., & Agerholm, I. (2012). The cleavage stage embryo. *Human Reproduction (Oxford, England)*, *27 Suppl 1*, i50–i71. https://doi.org/10.1093/humrep/des224

Rath, D., Barcikowski, S., de Graaf, S., Garrels, W., Grossfeld, R., Klein, S., Knabe, W., Knorr, C., Kues, W., Meyer, H., Michl, J., Moench-Tegeder, G., Rehbock, C., Taylor, U., & Washausen, S. (2013). Sex selection of sperm in farm animals: Status report and developmental prospects. *Reproduction*, *145*(1), R15–R30. https://doi.org/10.1530/REP-12-0151

Rossant, J. (2011). Developmental biology: A mouse is not a cow. *Nature*, *471*(7339), 457–458. https://doi.org/10.1038/471457a

Rossant, J., & Cross, J. C. (2001). Placental development: Lessons from mouse mutants. *Nature Reviews Genetics*, *2*(7), 538–548. https://doi.org/10.1038/35080570

Rubio, I., Kuhlmann, R., Agerholm, I., Kirk, J., Herrero, J., Escribá, M. J., Bellver, J., & Meseguer, M. (2012). Limited implantation success of direct-cleaved human zygotes: A time-lapse study. *Fertility and Sterility*, *98*(6), 1458–1463. https://doi.org/10.1016/j.fertnstert.2012.07.1135

Sá Filho, M. F., Girotto, R., Abe, E. K., Penteado, L., Campos Filho, E. P., Moreno, J. F., Sala, R. V., Nichi, M., & Baruselli, P. S. (2012). Optimizing the use of sex-sorted sperm in timed artificial insemination programs for suckled beef cows. *Journal of Animal Science*, *90*(6), 1816–1823. https://doi.org/10.2527/jas.2011-4523

Saito, S., Sawai, K., Ugai, H., Moriyasu, S., Minamihashi, A., Yamamoto, Y., Hirayama, H., Kageyama, S., Pan, J., Murata, T., Kobayashi, Y., Obata, Y., & Yokoyama, K. K. (2003). Generation of cloned calves and transgenic chimeric embryos from bovine embryonic stem-like cells. *Biochemical and Biophysical Research Communications*, *309*(1), 104–113. https://doi.org/10.1016/S0006-291X(03)01536-5

Sales, J. N. S., Neves, K. A. L., Souza, A. H., Crepaldi, G. A., Sala, R. V., Fosado, M., Filho, E. P. C., de Faria, M., Filho, M. F. S., & Baruselli, P. S. (2011). Timing of insemination and fertility in dairy and beef cattle receiving timed artificial insemination using sex-sorted sperm. *Theriogenology*, *76*(3), 427–435. https://doi.org/10.1016/j.theriogenology.2011.02.019

Schenk, J. L., Cran, D. G., Everett, R. W., & Seidel, G. E. (2009). Pregnancy rates in heifers and cows with cryopreserved sexed sperm: Effects of sperm numbers per inseminate, sorting pressure and sperm storage before sorting. *Theriogenology*, *71*(5), 717–728. https://doi.org/10.1016/j.theriogenology.2008.08.016

Schulz, E. G., Meisig, J., Nakamura, T., Okamoto, I., Sieber, A., Picard, C., Borensztein, M., Saitou, M., Blüthgen, N., & Heard, E. (2014). The two active X chromosomes in female ESCs block exit from the pluripotent state by modulating the ESC signaling network. *Cell Stem Cell*, *14*(2), 203–216. https://doi.org/10.1016/j.stem.2013.11.022

Seidel, G. E. (2014). Update on sexed semen technology in cattle. *Animal*, *8*(SUPPL. 1), 160–164. https://doi.org/10.1017/S1751731114000202

Sugimura, S., Akai, T., Hashiyada, Y., Somfai, T., Inaba, Y., Hirayama, M., Yamanouchi, T., Matsuda, H., Kobayashi, S., Aikawa, Y., Ohtake, M., Kobayashi, E., Konishi, K., & Imai, K. (2012). Promising system for selecting healthy in vitro-fertilized embryos in cattle. *PLoS One*, *7*(5), e36627. https://doi.org/10.1371/journal.pone.0036627

Surani, M. A. H., & Handyside, A. H. (1983). Reassortment of cells according to position in mouse morulae. *Journal of Experimental Zoology*, *225*(3), 505–511. https://doi.org/10.1002/jez.1402250320

van Soom, A., Boerjan, M. L., Bols, P. E. J., Vanroose, G., Lein, A., Coryn, M., & de Kruif, A. (1997). Timing of compaction and inner cell allocation in bovine embryos produced in vivo after superovulation. *Biology of Reproduction*, *57*(5), 1041–1049. https://doi.org/10.1095/biolreprod57.5.1041

Wang, L., Duan, E., Sung, L. Y., Jeong, B. S., Yang, X., & Tian, X. C. (2005). Generation and characterization of pluripotent stem cells from cloned bovine embryos. *Biology of Reproduction*, *73*(1), 149–155. https://doi.org/10.1095/biolreprod.104.037150

Xiao, Y., Amaral, T. F., Ross, P. J., Soto, D. A., Diffenderfer, K. E., Pankonin, A. R., Jeensuk, S., Tríbulo, P., & Hansen, P. J. (2021). Importance of WNT-dependent signaling for derivation and maintenance of primed pluripotent bovine embryonic stem cells. *Biology of Reproduction*, *105*(1), 52–63. https://doi.org/10.1093/biolre/ioab075

Xu, K. P., Yadav, B. R., King, W. A., & Betteridge, K. J. (1992). Sex-related differences in developmental rates of bovine embryos produced and cultured in vitro. *Molecular Reproduction and Development*, *31*(4), 249–252. https://doi.org/10.1002/mrd.1080310404

Yagi, M., Kishigami, S., Tanaka, A., Semi, K., Mizutani, E., Wakayama, S., Wakayama, T., Yamamoto, T., & Yamada, Y. (2017). Derivation of ground-state female ES cells maintaining gamete-derived DNA methylation. *Nature*, *548*(7666), 224–227. https://doi.org/10.1038/nature23286

Zhang, B., Krawetz, R., & Rancourt, D. E. (2013). Would the real human embryonic stem cell please stand up? *BioEssays*, *35*(7), 632–638. https://doi.org/10.1002/bies.201200162

How to cite this article: Oliveira, C. S., Saraiva, N. Z., & Oliveira, L. Z. (2024). Morphology of 16-cell embryo in bovine: Inside cells, compaction, fragmentation and effects of X-sorted semen. *Anatomia, Histologia, Embryologia*, *53*, e13015. https://doi.org/10.1111/ahe.13015