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Cell death is involved in sexual dimorphism during preimplantation development

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ABSTRACT

In bovine preimplantation development, female embryos progress at lower rates and originate smaller blastocysts than male counterparts. Although sex-specific gene expression patterns are reported, when and how sex dimorphism is established is not clear. Differences among female and male early development can be useful for human assisted reproductive medicine, when X-linked disorders risk is detected, and for genetic breeding programs, especially in dairy cattle, which requires female animals for milk production. The aim of this study was to characterize the development of female and male embryos, attempting to identify sex effects during preimplantation development and the role of cell death in this process. Using sex-sorted semen from three different bulls for fertilization, we compared kinetics of bovine sex-specific embryos in six time points, and cell death was assessed in viable embryos. For kinetics analysis, we detected an increased population of female embryos arrested at 48 and 120 h.p.i., suggesting this time points as delicate stages of development for female embryos that should be considered for testing improvement strategies for assisted reproductive technologies. Assessing viable embryos quality, we found 144 h.p.i. is the first time point when viable embryos are phenotypically distinct: cell number is decreased, and apoptosis and cell fragmentation are increased in female embryos at this stage. These new results lead us to propose that sex dimorphism in viable embryos is established during morula-blastocyst transition, and cell death is involved in this process.

1. Introduction

Sex of preimplantation embryos is usually not considered in reproductive biology studies. However, recent evidence has suggested far from being irrelevant the sex of cell lines, and highlights how X-chromosome inactivation (XCI) and other singularities can be interfering with results in many cell types (Shah et al., 2014). In addition, NIH has recently proposed an initiative aiming the balance in sex of animals in preclinical studies (McCullough et al., 2014), emphasizing the importance of sex at biological responses.

In vitro fertilization (IVF) and embryo culture are extensively used biotechnologies, and are considered valuable tools for human assisted reproduction, developmental science, and for livestock breeding. In addition to notably importance on behalf of livestock, bovine embryonic development also consists in an important model for embryology. Among their similarities to human development are: the closer time of embryonic genome activation, the duration of preimplantation development and the low developmental rates (Niakan and Eggan, 2013). Critical differences between mouse and cattle early embryo development were pointed out in ES cell derivation (Keefer et al., 2007) and trophectoderm differentiation experiments (Berg et al., 2011), highlighting the importance of multi-species studies, and suggesting that mouse might not be the best model for studying embryonic development (Rossant, 2011). In this respect, growing interest in bovine embryology is observed, and knowledge of several basic mechanisms is urged.

The knowledge of embryo sex and its singularities can be useful for human assisted reproductive medicine, specially in cases when X-linked disorders risk is detected. For animal reproduction field, female embryos are especially important, since assisted reproductive technologies are often applied to increase the number of female calves aiming further herd replacement and milk production. Therefore, most commercial bovine systems nowadays apply frozen–thawed sex sorted sperm for oocyte fertilization, and the understanding of female specific requirements is extremely relevant to improve IVP systems. Also, onset of sexual dimorphism characterization could contribute to current knowledge of several phenotypic differences between females and males.

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An intriguing aspect of bovine species is the different behavior between female and male embryos during preimplantation development. Dosage compensation of X-chromosome transcripts occurs in bovine embryos after blastocyst formation (Bermejo-Alvarez et al., 2010, 2011), and sex-specific gene expression patterns are reported for day 6 (Denicol et al., 2015) and day 7 (Bermejo-Alvarez et al., 2010) embryos. However, when and how sex-specific embryos begin to diverge morphologically, and how can we direct culture conditions to mitigate detrimental effects, is not clear.

The aim of this study was to characterize female and male embryos through development, attempting to identify singularities and reveal sex effects in preimplantation embryos. Morphological differences were reported regarding dissemblance in speed of blastocyst formation (Avery et al., 1992) and blastocyst cell numbers (Oliveira et al., 2010; Xu et al., 1992) for day 7 embryos. Also, sensitivity during in vitro culture is reported for female embryos (Edwards et al., 2001). Together, those evidences could suggest female embryos present slower development than male counterparts do, although our personal observations did not support this theory — rates of compaction and cavitation in our system were apparently similar for both genders. Therefore, our hypothesis was that sexual dimorphism is present before day 7 of development, and the main mechanism leading to sex-specific differences reported at blastocyst stage would be cell death rather than delayed development.

2. Material and methods

2.1. Experimental design

Matured oocytes were randomly distributed among six groups for fertilization. Semen from three bulls sexed for X- or Y-chromosome spermatozoa were used. In assay 1, kinetics was assessed during development. For that, cleavage of blastomeres was monitored daily for each group, from 24 h.p.i. till 144 h.p.i., and results were compared between bulls and between sexes. In assay 2, quality parameters were assessed in viable embryos. For this analysis, embryos destined for collection and fixation were cultivated in parallel, and embryos with more than 4 cells at 72, 96 and 120 h.p.i.; or more than 8 cells at 144 h.p.i., were selected. Embryos were submitted to caspase 3 immunofluorescence for apoptosis evaluation, and total cell number was assessed by Hoechst 33342 counterstaining. Cell fragmentation, compaction and cavitation were also evaluated in bright field images.

2.2. Supplements

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

2.3. Oocyte recovery

Bovine ovaries were collected at a local slaughterhouse and processed within 3 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).

2.4. 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.

2.5. In vitro fertilization (IVF)

Groups of 20 matured COCs were washed twice and transferred to 30-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–thawed straws from three different bulls, containing X-chromosome (female embryo groups) or Y-chromosome (male embryo groups) bearing spermatozoa, sorted by flow cytometry (CRV Lagoa/Sexing Technologies, Sertaozinho, Brazil) were used. For each bull, X- and Y-spermatozoa straws were obtained from the same batch of semen. Flow cytometric sperm sorting based on differences in their DNA content is the best method for separation of X- and Y-chromosome bearing spermatozoa, and its accuracy is about 90% (Seidel, 1999; Hamano, 2007). 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, 80 μL of the medium containing the pellet was collected from the bottom of the tube and homogenized in a conic tube. The final suspension was divided among five TALP-IVF drops, in a final concentration of approximately 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.

2.6. In vitro culture (IVC)

After IVF, presumptive zygotes were partially denuded of cumulus cells by vigorous pipetting and cultured in SOF medium supplemented with 2.5% FBS and 6 mg/mL BSA at 38.5 °C in an atmosphere of 5% CO_2 in air under saturated humidity. Remaining cumulus cells were attached to plastic surface and formed a monolayer of granulosa cells. Groups of 20 presumptive zygotes were cultured in 100-μL drops, and medium was half replaced every 48 h.

2.7. Cleavage assessment

Embryos cultured side by side, female and male, obtained from fertilization with three different bulls were assessed daily (24, 48, 72, 96, 120 and 144 h.p.i.) for developmental progression rates. Presumptive zygotes cleavage was assessed at 48 h.p.i.

2.8. Compaction, cavitation and cell fragmentation analysis

Embryos destined for collection and fixation were cultivated in parallel, in groups of 20 structures per drop, and drops were collected at 72, 96, 120 and 144 h.p.i. Since our goal was to describe viable embryos and detect particularities of female and male development, embryos with less than 5 cells at 72, 96 and 120 h.p.i.; and embryos with less than 9 cells at 144 h.p.i. were considered non-viable and discarded from analysis.

Embryos were fixed in 4% paraformaldehyde for 30 min at 37 °C, and phase contrast images were used to quantify compaction (percentage of embryos exhibiting radial compacted cells), cavitation (percentage of embryos exhibiting fluid filled cavity) and cell fragmentation (estimated number of enucleated cytoplasm fragments inside zona pellucida). The onset of compaction was assessed in this assay, so partially compacted embryos (still presenting non-compacted cells) were considered positive.

2.9. Caspase 3 immunofluorescence and total cell number analysis

Viable embryos were stained for HOECHST (nuclei) and active caspase 3, an apoptosis downstream effector enzyme. Embryos were collected at 72, 96, 120 and 144 h.p.i., fixed in 4% paraformaldehyde for 30 min at 37 °C and stored at 4 °C in PBS supplemented with 3% BSA. Embryos were incubated in 0.5% Triton X-100 solution for 30 min at RT, and washed three times in PBS supplemented with 0.2% Tween-20 (PBS-T) for 10 min. After, structures were incubated with blocking solution (PBS-T supplemented with 3% BSA) for 4 h at 4 °C. Next, embryos were incubated with the primary antibody (mouse anti-active Caspase 3, 1:750) for 12 h at 4 °C. The structures were then washed three times in PBS-T for 10 min and incubated with the secondary antibody (AlexaFluor 555-conjugated donkey anti-mouse, 1:400) for 1 h. The nuclei were stained with 10 μL/mL Hoechst 33342 solution for 20 min, and embryos were washed three times for 10 min in PBS-T and examined under a fluorescence microscope. Reactions in which the primary antibody was omitted served as negative control. Images of each structure were captured with an AxioCam camera and stored using the AxioVision 4.7.1 software (Carl Zeiss, Jena, Germany). Apoptosis analysis was carried out for: 1) apoptotic embryos (percentage of embryos that displayed apoptotic cells — cas3 positive embryos); 2) apoptosis rate in cas3 positive embryos; 3) apoptotic cell number (mean number of apoptotic cells in cas3 positive embryos). Embryos were classified as "grade I" based on cell number (more than 8 cells at 72 h.p.i. and 96 h.p.i.; more than 16 cells at 120 h.p.i.; more than 32 cells at 144 h.p.i.).

2.10. Statistical analysis

All statistical analyses were performed at a 5% level of significance, using SAS 9.2, Graphpad Instat and R softwares. In assay 1 (kinetics assessment during development), logistic regression models were used to analyze the effects of bull, sex and their interaction on embryonic development, separately for each evaluated time point. Problems of quasi-complete separation of data points occurred in two cases (9–16 cell at 48 h, and 2-cell at 72 h), and models were readjusted using Firth's penalized maximum likelihood estimation method. Only well fitted models (in terms of global likelihood-ratio Chi-Square test) were considered. Results were reported as odds ratios (ORs) with 95% confidence intervals (CIs). SAS 9.2 software was used for this analysis.

Male and female results regarding percentage of cleaved embryos; percentage of compacted and cavitated embryos; and percentage of grade I embryos were compared using Fisher's Exact test. Percentage of apoptotic embryos was compared using Chi-Square Test. Graphpad Instat software was used for this analysis.

Analysis of cell number and apoptosis rate in Cas3 positive embryos at each time point; and cell death at 144 h.p.i. (mean rate of cell damage and mean number of damaged cells by apoptosis and fragmentation) was carried out using general linear model, testing interaction among bulls and sex. Least square means were compared by ANOVA and Tukey's post hoc test.

3. Results

3.1. Fertilization and cleavage

Quality of sorted spermatozoa and possible differences in fertilization capability of bulls and sex were inferred by analysis of presumptive zygotes cleavage, at 48 h.p.i. (n = 1070 presumptive zygotes, 222–459 per bull). No difference was detected between female and male embryos, suggesting X and Y spermatozoa has similar fertility and sex has minor influence during first cleavage (p values: Bull 1 — 0.38, Bull 2 — 0.38, Bull 3 — 0.65). Moreover, all selected bulls presented cleavage rates higher than 80%, and were considered appropriate for IVF. Mean first cleavage rate was 87% for X (454 / 523) and 85% (463 / 547) for Y spermatozoa groups.

3.2. Subsequent cleavage divisions

Rates of representative embryonic stages at 24, 48, 72, 96, 120 and 144 h.p.i. revealed similar distribution among embryos from the three bulls, although some particularities were present (see Fig. 1 in Oliveira et al., 2015).

Summary of statistical analysis for grouped cleavage division's data can be seen in Table 1, in Oliveira et al. (2015). In this experiment, two main factors were considered: sex and bull. Interaction between bull and sex was significant in two cases. The first case was for 2-cell embryos at 72 h.p.i. ($p = 0.042$); but its analysis revealed no difference among bulls for female embryos. The only significant difference was found for male embryos, where bull 2 (OR 24) or bull 3 (OR 38) embryos had more chance of arresting at the 2-cell stage than bull 1.

The other case was 9–16-cell embryos at 96 h.p.i. ($p = 0.0029$). In this situation, percentage of 9–16 cell embryos in female group was increased for bull 1 (OR 4.3); decreased for bull 2 (OR 3.6), and similar between sexes for bull 3.

The main effect of bull was significant in five cases: 2-cells at 24 h.p.i. ($p = 0.0005$); 2-cell ($p = 0.0006$) and 4-cell ($p < 0.0001$) at 48 h.p.i.; 4-cell at 96 h.p.i. ($p = 0.0011$) and blastocysts at 144 h.p.i. ($p < 0.0001$) (see Fig. 2 in Oliveira et al., 2015).

Fig. 1 illustrates the main significant effects of sex. At 48 h.p.i., percentage of 2-cell embryos was increased ($p = 0.0072$) in female group — female embryos had two times more chance ($OR_{F\ vs\ M} = 2.1$, $IC_{95\%} = [1.2, 3.5]$) of arresting at the 2-cell stage than male embryos. At 120 h.p.i., percentage of 4-cell embryos was increased ($p = 0.032$) in female group — female embryos had 70% greater chance of arresting at 4-cell stage than male embryos ($OR_{F\ vs\ M} = 1.67$, $IC_{95\%} = [1.05, 2.66]$). Both embryonic stages, considering the evaluated time point, correspond to a delayed class of embryos, which is increased in female group.

3.3. Compaction and cavitation

Embryos (n = 375) collected at 96, 120 and 144 h.p.i. were analyzed for the presence of radial cells and stronger cell membrane contacts (Fig. 2.1). No statistical difference was detected at any evaluated time point (Fig. 2.2) (p values: 96 h.p.i. — 0.24, 120 h.p.i. — 0.81, 144 h.p.i. — 0.64). Analysis revealed 82% female and 84% male embryos compacted at 120 h.p.i., and at the end of experiment (144 h.p.i.) 95% female and 97% male embryos were compacted.

Regarding blastocoel formation, a small fluid filled cavity was first detected at 120 h.p.i., at 3% female and 9% male embryos (Fig. 2.3). At 144 h.p.i., 33% female and 47% male evaluated embryos presented blastocoel already. No statistical difference was detected between groups (p values: 120 h.p.i. — 0.27, 144 h.p.i. — 0.10).

3.4. Apoptosis

First, the percentage of embryos that exhibited apoptotic cells (apoptotic embryos) was evaluated, to address if differences in population of damaged embryos existed between female and male embryos (n = 797 embryos). In Fig. 3.2, data is presented for each bull (1, 2, 3). Comparing time points (72, 96, 120 and 144 h.p.i.), 72 h.p.i. presented the lowest percentage of apoptotic embryos for all bulls, in both female and male groups ($p < 0.05$). Differences in percentage of apoptotic embryos between female and male embryos were only detected for bull 1 at 72 h.p.i. ($p = 0.025$) and 144 h.p.i. ($p = 0.016$) and bull 2 at 96 h.p.i. ($p = 0.017$).

Then, assessment of apoptosis rate in caspase 3 positive embryos was performed, to address if the extent of apoptosis differed between female and male embryos (n = 307 Cas3 positive embryos). Comparison of female and male groups at each time point (Fig. 3.3, 1) revealed increased apoptosis rates at 72 h.p.i. ($p = 0.03$) and 144 h.p.i. ($p = 0.04$) in female embryos. No interaction between bull and sex factors was detected at 72 h.p.i. ($p = 0.75$), 96 h.p.i. ($p = 0.80$), 120 h.p.i.

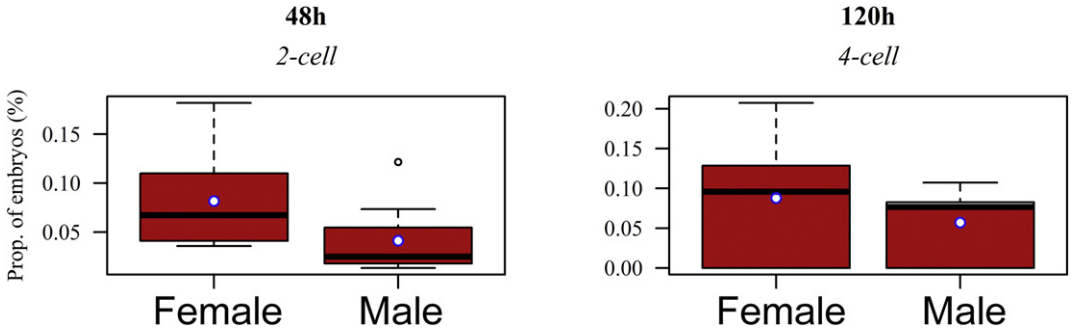

Fig. 1. Differences between female and male embryos during early development. Boxplot distribution of *p* between groups in cases where "sex" main effect was significant, where *p* is the proportion of embryos at a specific stage of development relative to the total number of embryos.

($p = 0.51$) or 144 h.p.i. ($p = 0.84$); and no effect of bull was present at 72 h.p.i. ($p = 0.49$), 96 h.p.i. ($p = 1.00$) or 144 h.p.i. ($p = 0.17$). At 120 h.p.i., bull effect was significant ($p = 0.04$).

Comparison among time points revealed increased apoptosis rates were present at 96 h.p.i. ($p = 0.00$).

3.5. Cell death at 144 h.p.i.

The degree of cytoplasm fragmentation is an important parameter of embryo quality. Fig. 4 presents a summary of apoptotic and fragmented cells at 144 h.p.i. At this stage, an increase ($p = 0.01$) in fragmentation rate in female embryos was detected (Fig. 4.2). No interaction between bull and sex factors was present ($p = 0.64$), and cellular fragmentation was similar ($p = 0.93$) among bulls. This result shows that the fragmentation increase detected for female embryos was consistent among bulls, suggesting that no genetic influence appears to be involved in this result.

While this increase would be expected, since fewer cells are present in female embryos at this time point, we also noticed an increase ($p = 0.01$) in mean number of fragmented cells in female embryos at this stage (Fig. 4.3). No difference among bulls or interaction between bull and sex factors was detected for this analysis as well. Therefore, both rate and number of fragmented cells were increased in female embryos at 144 h.p.i.

Even though apoptosis rate was also increased in female embryos at this time point (as presented in previous section and in Fig. 4.2), no difference in number of apoptotic cells was detected among bulls ($p = 0.60$) or sex ($p = 0.14$), and no interaction was present for bull and sex factors ($p = 0.65$). Therefore, the increase in apoptosis detected for female embryos was also not influenced by genetic background, being consistent among different bulls.

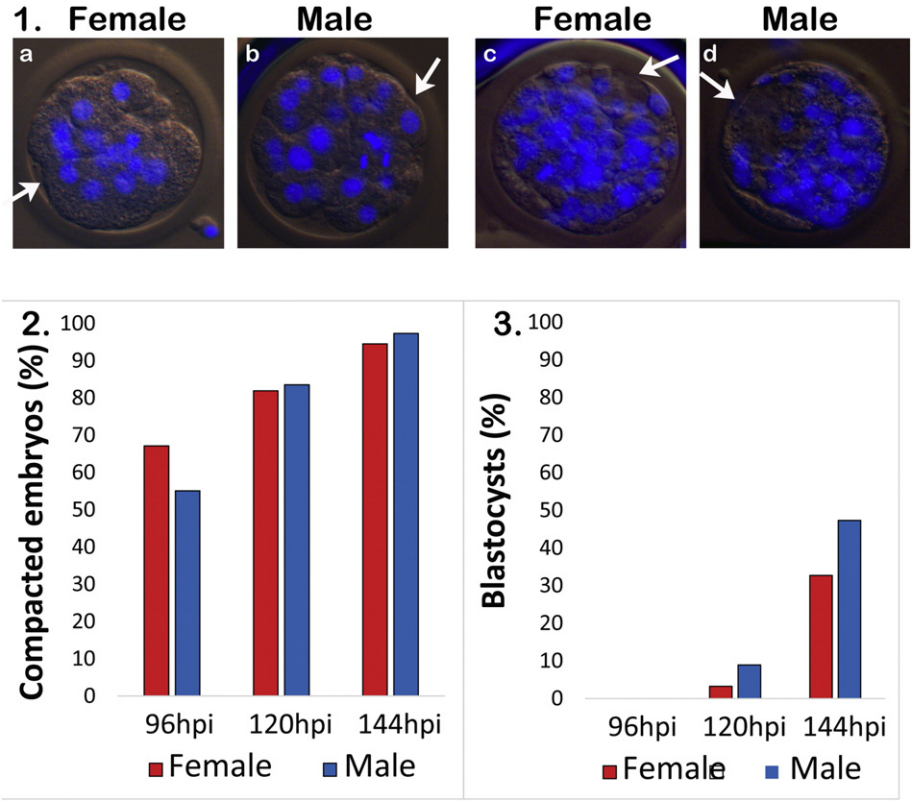

Fig. 2. Compaction and cavitation of female and male embryos (96, 120 and 144 h.p.i.). 1. Phase contrast images merged with HOECHST are shown. Embryos at 96 h.p.i. (a, female; b, male) showing compacted cells (arrows) and embryos at 144 h.p.i. (c, female; d, male) showing fluid filled cavity (arrows) are presented. 2–3. Graphs show percentage of embryos exhibiting signs of compaction (2) and blastocoel (3). No statistical difference was detected between female and male groups at each time point.

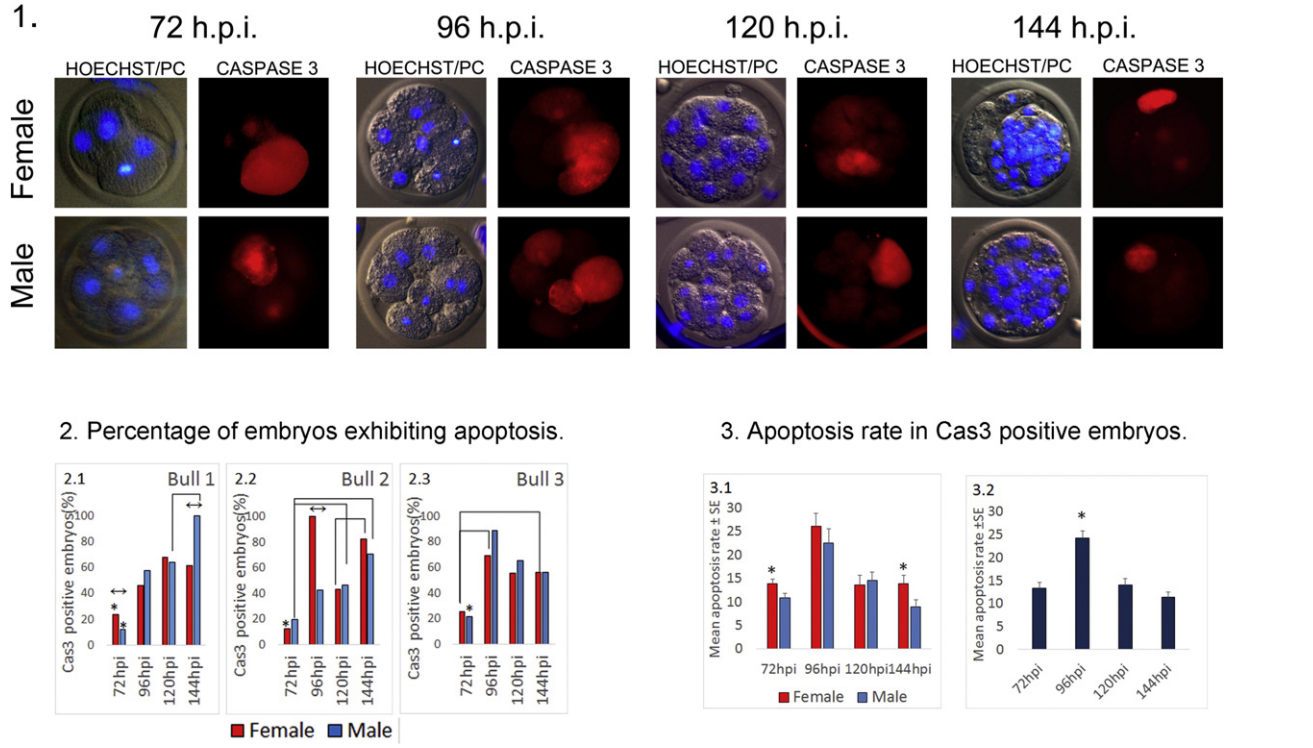

Fig. 3. Apoptosis during female and male embryonic development (72, 96, 120 and 144 h). 1. Images of caspase 3 (Alexa 555) and phase contrast merged with HOECHST of female and male embryos collected at 72, 96, 120 and 144 h.p.i. are shown. 2. Graphs show percentage of embryos exhibiting apoptosis in female and male embryos for bulls 1 (2.1), 2 (2.2) and 3 (2.3). Asterisks and connectors indicate statistical difference within each sex (female or male), and arrows indicate difference between sexes. 3. Graphs show mean apoptosis rate of embryos exhibiting apoptotic cells, comparing female and male groups at each timepoint (3.1), and comparing all embryos (female and males) among timepoints (3.2). Asterisks indicate statistical difference.

3.6. Cell number

Mean cell number of viable embryos was assessed within each group ($n = 797$ embryos). Mean cell numbers of female embryos at 72, 96, 120 and 144 h.p.i. were 8.6, 11.9, 15.4, and 28.1, respectively; and of male embryos were 8.5, 11.33, 16.6, 40.2, respectively. Even though no difference was detected between female and male embryos at 72, 96 and 120 h.p.i., total cell number was increased ($p < 0.001$) in male embryos at 144 h.p.i. (Fig. 5.1). This increase was independent of genetic background (bulls), since no interaction was detected between sex and bull factors. Number of cells in bull 2 embryos was increased ($p < 0.001$) comparing to bulls 1 and 3.

In order to address if mean cell number was representative of the majority of embryos, instead of masked by few embryos displaying an increased cell number, the percentage of grade I embryos in each time point was evaluated (Fig. 5.2). An increased ($p < 0.001$) percentage of grade I embryos in male group at 144 h.p.i. was detected. Therefore, not only male embryos presented a higher mean cell number at 144 h.p.i., but also a higher percentage of embryos with more than 17 cells (grade I) was present at this moment.

A scheme summarizing our main findings is presented in Fig. 6.

4. Discussion

The characterization of sex patterns in early embryo development is a valuable approach for understanding the effects of molecular mechanisms differentially orchestrated among sexes and for identification of critical stages of development. Differential behavior among female and male embryos can guide new strategies aiming the improvement of assisted reproductive technologies throughout preimplantation development, including reducing X-linked genetic disorder risks in human embryos, and increasing quantity and quality of female livestock embryos.

In the present study, development of female and male embryos from zygotes to early blastocysts was analyzed, searching for patterns that could guide innovation of sex-specific systems. This study was based on the hypothesis that sexual dimorphism was present before day 7 of development, and the main mechanism leading to sex-specific differences reported at blastocyst stage would be cell death rather than delayed development; which was confirmed by our findings. Based on presented data, we suggest 144 h is the first time point in development when female and male bovine embryos present evidence of morphological difference.

The main findings of this work were (i) a population of female delayed embryos was present first at 48 h.p.i., and then at 120 h.p.i.; (ii) apoptosis rates were increased in female embryos at 72 h.p.i. and at 144 h.p.i.; and (iii) embryo quality was decreased in female embryos at 144 h.p.i. Apart from sex, (iv) we also found a decreased percentage of apoptotic embryos at 72 h.p.i., and (v) an increased apoptosis rates in embryos at 96 h.p.i.

The use of sexed spermatozoa is an important tool for the presented experiments, since it allows the study of live embryos of known sex. Flow cytometric sperm sorting is based on differences in DNA content of X- and Y-chromosomes, and its accuracy is about 90% (Seidel, 1999; Hamano, 2007), with similar rates for resulting in vitro fertilized embryos (Bermejo-Alvarez et al., 2008). Production of sex-specific embryos using sexed spermatozoa was standardized in our system previously, and accuracy rates up to 98% were reported (Oliveira et al., 2013).

In the present study, no effect of sex was detected regarding first cleavage. Studies evaluating presumptive zygotes cleavage revealed differences in kinetics of development between female and male embryos (Beyhan et al., 1999; Gutiérrez-Adán et al., 1999; Kochhar et al., 2003). However, oocyte maturation state (Gutiérrez-Adán et al., 1999) and

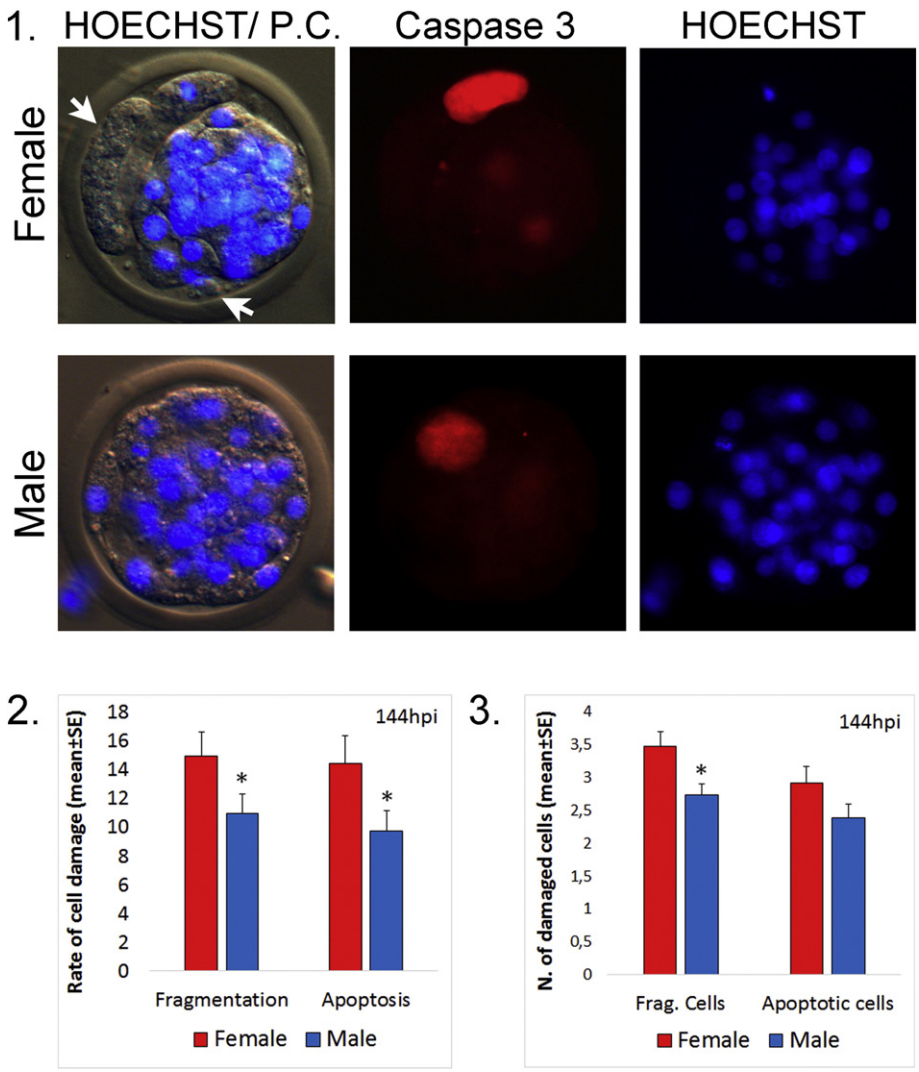

Fig. 4. Cell death in female and male 144 h.p.i. embryos. 1. Images of phase contrast merged with HOECHST, caspase 3 (Alexa 555) and HOECHST of female and male embryos are shown. Arrows indicate cell fragmentation. 2. Mean rate of cell damage per embryo. 3. Mean number of damaged cells. Asterisks denote statistical difference between female and male groups.

sperm incubation time at IVF apparently were involved with those differences — increased fertilization rates for Y-sperm were detected when low incubation time was applied (Kochhar et al., 2003). In our study, standard sperm incubation time was performed, and high rates of fertilization were achieved with all tested bulls, similarly among X and Y groups. In this sense, it is interesting to notice that our results seems to confirm the improvement in sex-sorted semen fertility which has been observed since the development of sperm sorting technique (Sharpe and Evans, 2009).

Further development assessment revealed, from all stages of embryos distributed among time points (20 evaluations in total), only two cases of interaction between sex and bull factors. In the 18 remaining cases, sex behavior was not affected by paternal genetic background influence, and consistent differences regarding embryonic sex could be

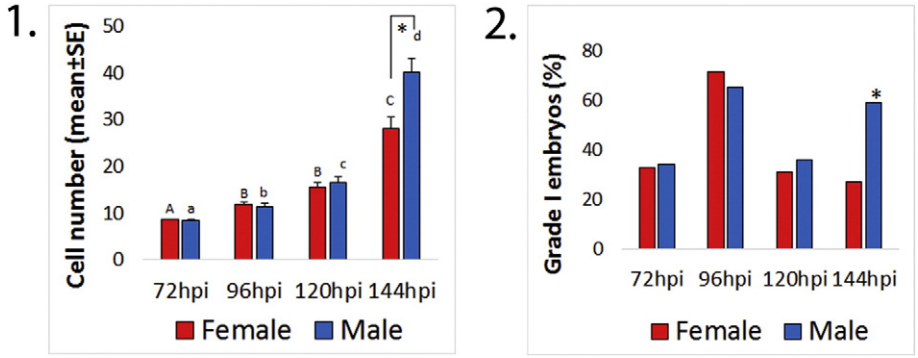

Fig. 5. Cell number and percentage of grade I embryos in female and male groups. 1. Mean cell number of female and male embryos at each timepoint. Connectors indicate difference among female and male groups at the same timepoint. Female (A, B, C) and male (a, b, c, d) columns marked with different letters are statistically different. 2. Percentage of grade I female and male embryos at each timepoint. Asterisks indicate statistical difference between female and male groups.

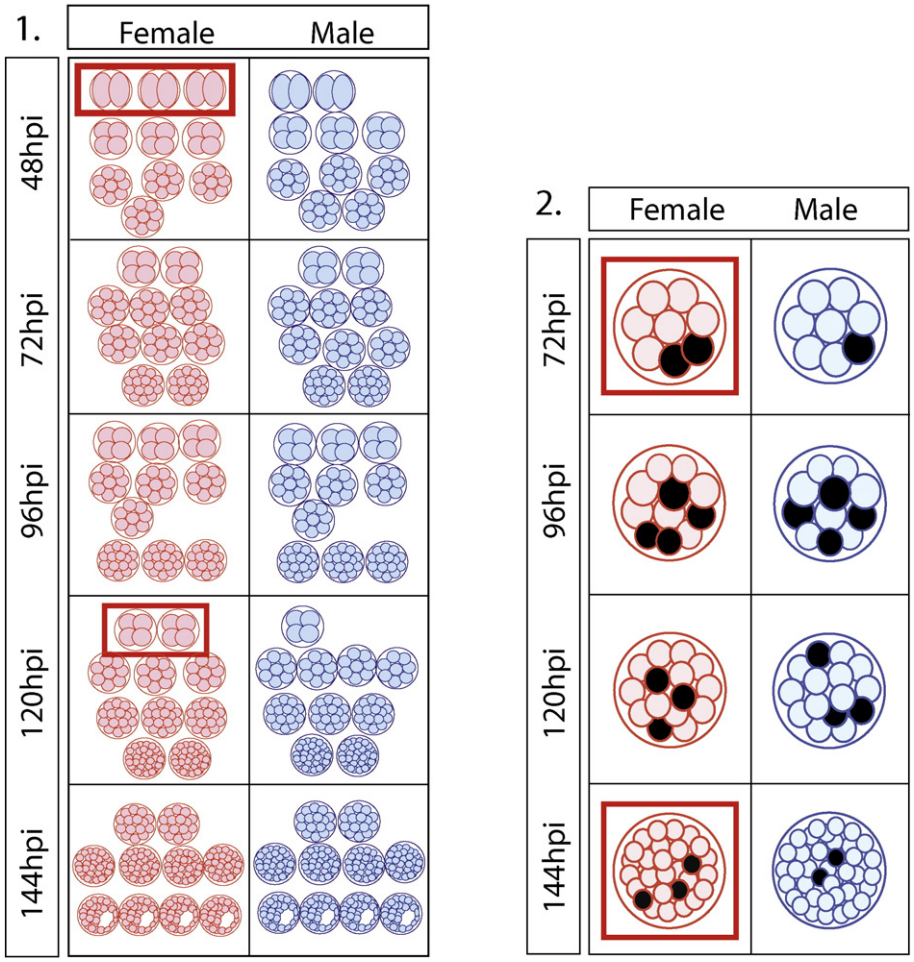

Fig. 6. Graphical abstract — cell death is involved in sexual dimorphism during preimplantation development. 1. Summary of main findings for kinetics assessment of female and male embryos development. Approximate percentages of 2, 4, 5–8, 9–16 morula and blastocysts are shown for 48, 72, 96, 120 and 144 h.p.i. evaluations. Red boxes indicate statistical difference between female and male groups. 2. Summary of main findings (mean cell number and apoptosis rate) in embryonic quality assessment. Black cells represent apoptotic blastomeres. Red boxes indicate statistical difference between female and male groups.

dissected. However, in five cases bull main effect was present. For this study, commercial frozen straws from three Nellore bulls were selected without considering any performance parameter, but still differences could be detected. Bull effect has been described earlier in bovine development (Ward et al., 2001). Recent studies using mouse and human embryos identified novel stage-specific monoallelic expression patterns for a significant proportion of polymorphic gene transcripts (Xue et al., 2013), suggesting paternal genetics influence during early embryo development that could also be present in bovine embryos and affect embryonic rates.

Regarding sex main effect, an increased population of 2-cell at 48 h.p.i., and 4-cell embryos at 120 h.p.i., was detected in female embryos comparing to male counterparts. In both situations, affected embryos consist in a group of delayed or arrested embryos with minor relevance, considering they should not be able to achieve blastocyst stage until 168 h.p.i. However, this difference highlights female increased sensitivity at 48 and 120 h.p.i. In bovine, major EGA only occurs at 8–16 cell transition, but it is known that transcriptional activity is present from 2-cell onwards (Barnes and First, 1991; Memili and First, 1998; Natale et al., 2000; Viuff et al., 1998; Peippo et al., 2002). In mouse embryos, transcription from zygote to 4-cell embryos regards to basic cellular machinery (Hamatani et al., 2004). In bovine 2- and 4-cell embryos, transcription related to cell cycle progression, chromatin remodeling and splicing or translational initiation is reported (Kanka et al., 2009). In bovine blastocysts, sex determines the expression level of one third of the actively expressed genes, and male embryos present increased expression of genes related to metabolic processes, protein transport and cell cycle, among other processes (Bermejo-Alvarez et al., 2010). Therefore, it is possible that at 2- and 4-cell stage embryos, those differences are also present and sex-related gene expression could trigger arrest of female embryos, in response to challenging in vitro culture environment.

Cleavage of female 4-cell embryos is delayed when glucose is added to culture media (Peippo et al., 2001). This outcome could be caused by the activation of pentose phosphate detoxification pathway, related to X-chromosome genes, as suggested by the authors. Female embryos might present twice the amount of X-chromosome related transcripts compared to male counterparts before blastocyst stage, since dosage compensation initiates in bovine after blastocyst formation (Bermejo-Alvarez et al., 2011). However, in addition to pentose phosphate pathway activation, glucose supplementation and the resulting environment could represent a stressful condition, to which female embryos could react worse than male counterparts.

Beside delayed embryos, if environmental conditions are specially challenging to all female embryos at 48 and 120 h.p.i., consequences could be present for all stages of embryos (4 and 8-cell at 48 h.p.i., and 8-, 16 and morula embryos at 120 h.p.i.). Interestingly, one time point after increase in delayed embryos percentage was detected, apoptosis rates were increased in female groups (72 and 144 h.p.i.). This finding suggests that the presence of delayed embryos could be indeed indicating a stressful condition time point, which would influence cell death rates in the next 24 h.

Even though female embryos were delayed at 120 h.p.i., no effect was detected in compaction rates. In mouse embryos, studies suggest that all proteins required for compaction are made earlier (maternally and/or zygotically) than its onset, and are post-translationally activated later on development (Johnson, 2009). Therefore, even though stressful conditions were detected at 120 h.p.i., no effect would be expected in compaction rates.

At 144 h.p.i., in addition to increased apoptosis, other evidences of lower quality were present in female embryos, such as increased fragmentation, decreased cell number and decreased percentage of grade I embryos. It has been shown that a high degree of fragmentation correlates negatively with blastocyst formation, implantation and pregnancy rates (Prados et al., 2012). Those findings converge to suggest that at 144 h.p.i. female embryos have distinct phenotype, characterized by four parameters. Since at 168 h.p.i. sexual dimorphism has already been well characterized (Oliveira et al., 2010; Kochhar et al., 2003; Xu et al., 1992), we identified 144 h.p.i. as the first dimorphic time point in bovine embryonic development, and suggest cell death is behind female and male differences observed at early embryos. This stage correlates with morula-blastocyst transition, a crucial period of development involving cell fate specification and transcriptional shifts (Sozen et al., 2014). Recently, it was reported that cell survival-related genes are up-regulated in female bovine morulas (Denicol et al., 2015). This finding could also suggest that female embryos are struggling for survival at this stage of development, given the increased cell death and inferior phenotype described here.

At 96 h.p.i., an increased apoptosis rate was detected for both female and male embryos, suggesting a delicate time point for bovine development. This time point also coincided with the highest rate of 8–16 cell embryos — overpassing major EGA-. Apoptosis is a physiological process in embryo development and may prevent a damaged cell from contributing to the formation of an individual, thus functioning as a survival mechanism under stressful conditions (Paula-Lopes and Hansen, 2002). However, high apoptosis rates are associated with reduced viability (Jousan et al., 2008) and embryonic death (Antunes et al., 2010). This stage of development is known to be crucial, since maternal–embryonic transition must occur (De Sousa et al., 1998), and this requires the overcome of repressive chromatin state and zygotic genome activation (Betts and King, 2001). Embryos that fail to achieve active transcription usually block at 8-cell stage (Meirelles et al., 2004). Since no differences between female and male embryos were detected at this time point, our results suggest that EGA and 8-cell block phenomena affect sex-specific embryos in a similar way.

In conclusion, our findings provide further support for sexual dimorphism during preimplantation embryo development. Here, we report 144 h.p.i. is the first time point in development when clear signs of decrease in female embryo quality are detectable, and cell death is involved in this process. Also, 48 h.p.i. and 120 h.p.i. were detected as delicate stages of development for female embryos.

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