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Trimethylation of histone 3 at lysine 4 in cryopreserved bovine embryos produced *in vivo* with sexed semen

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ABSTRACT

The production rates of viable embryos using sexed semen through the conventional methodologies of multiple ovulation and embryo transfer are generally not satisfactory. However, the cryopreservation of these embryos is considered efficient. Knowledge of epigenetics can provide new tools or allow for adapting new protocols that could enhance the efficiency of reproductive biotechnologies. The aim of this study was to characterize the pattern of trimethylation of histone 3 at lysine 4 (H3K4me3) in bovine embryos produced *in vivo* with sexed semen that were submitted to cryopreservation. *Bos taurus* × *Bos indicus* cows (n = 5) were superovulated and inseminated with sexed (two sessions) or conventional (two sessions) semen. A portion of the embryos collected on Day 7 was immediately stored in paraformaldehyde (3%) and another portion was stored in paraformaldehyde after cryopreservation/thawing. All embryos from the four groups (fresh, conventional semen; fresh, sexed semen; cryopreserved, conventional semen; and cryopreserved, sexed semen; 15 embryos per group) were evaluated by immunofluorescence under confocal microscopy to identify and quantify the H3K4me3 status. In total, 190 embryos were recovered, 100 of which were produced with conventional semen and 90 with sexed semen. The use of conventional semen after superovulation yielded 72% (72 of 100) viable embryos, which were mostly (81%; 59 of 72) in advanced stages of development (blastocysts and expanded blastocysts). Embryos produced with sexed semen had a lower viability rate (36.7%; 33 of 90), and most of them were collected at earlier stages of development (morulae and early blastocysts; $P < 0.05$). The H3K4me3 signal was similar among groups; however, there was a difference between morulae and blastocysts. A high intensity of H3K4me3 was observed in bovine embryos produced *in vivo*, and this pattern did not vary using sexed semen and the slow cryopreservation process. The lower viability of bovine embryos produced with sexed semen could be not explained by differences in H3K4me. Cryopreservation did not alter the pattern of H3K4me3; in this sense, we suggest that it is a process that exerts minimal damage to the embryos.

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1. Introduction

The production rates of viable embryos using sexed semen with the conventional methodologies of multiple ovulation and embryo transfer do not yield satisfactory results. Therefore, it is necessary to use a greater number of doses of semen per superovulated cow to obtain high embryo production [1], including a change in the artificial insemination (AI) schedule [2]. Semen sexing does not affect the sperm's ability to fertilize the oocytes, but it can affect the ability of embryos to develop normally after fertilization [3,4].

The progress of reproductive biotechnologies seems to have reached a limit, and its implementation relies on basic knowledge of the biology of gametes and embryos. These limiting factors provide an opportunity for integration of epigenetics knowledge. Gametes and embryos must pass through a broad epigenetic reprogramming process to be able to be fertilized and form a viable embryo [5,6]. However, epigenetic patterns may be susceptible to environmental changes such as temperature stress and/or physical and chemical processes [7].

Epigenetic modifications can occur through different mechanisms by which chromatin is modulated, including DNA methylation, that is, covalent modifications on histones and chromatin remodeling factors. The regulation of transcription can be accomplished through modifications in the amino terminal regions of histones, such as methylation, phosphorylation, acetylation, and ubiquitination [8]. DNA methylation involves the addition of a methyl group to the cytosine base in the DNA strand, which promotes transcriptional repression and is especially prevalent in mammals [9].

The high number of residues that can be modified on the tails of histones and the correlation of individual changes with various nuclear processes and specific combinations of histone modifications characterize the "histone code," which determines specific functions for each cell [10]. In this sense, the histone code has an important relationship with the chromatin structure [11].

The start and end points of the epigenetic reprogramming have not been well studied in many mammalian species. Certain modifications of histones are known to be essential for the establishment of meiotic maturation and development of the oocyte [12].

In general, methylation of histones is associated with transcriptional silencing, except in some cases such as methylation of histone 3 at lysine 4 (H3K4me), which is mainly associated with transcriptionally active chromatin, in forms mono-, di-, or trimethylated. The dynamic alteration of lysine methylation contributes to reversible and plastic regulation of gene expression in varieties of the cellular process, which contrasts with stable gene inactivation by DNA methylation [13]. The importance of H3K4me has been described for amphibian oogenesis [14], swine folliculogenesis [15], and mouse embryogenesis [16,17]. However, little is known about this epigenetic target with respect to bovine reproduction.

Although the general rate of transcription decreases during oocyte growth, histone acetylation and methylation of H3K4, which are associated with transcriptional activation, increase [12]. The need for increased transcription in this period is justified by the need for formation of an mRNA reserve for early embryo development [18]. However, the heterogeneous presence of histone modifications (H3K4me1, H3K9me2, H3K4me3, H3K79me2, and H3K36me3) was reported in human sperm, and a potential association of this pattern with sperm quality was proposed [19].

In the mouse male pronucleus, there is a rapid replacement of protamines by acetylated histones after fertilization, which subsequently become methylated at the H3K4 position [20,21]. H3K4 trimethylation (H3K4me3) is associated with critical events such as differentiation and activation of the embryonic genome, which has been demonstrated in swine [22], human [23], and bovine [24] embryos. Environmental factors and reproduction biotechnologies such as *in vitro* culture, nuclear transfer, and cryopreservation can modify the epigenetic profile of gametes and embryos [25]. According to this background, we hypothesized that H3K4me3 would be less frequent in cryopreserved bovine embryos produced *in vivo* with sexed semen, owing to stress, with a consequent negative influence on their quality.

The knowledge generated from studies on the epigenetic patterns of embryos can be used to support the development of new tools or to adapt new protocols that enhance the efficiency of reproductive biotechnologies. Thus, the aim of this study was to characterize the pattern of H3K4me3 of bovine embryos produced *in vivo* with sexed or conventional semen before and after cryopreservation.

2. Materials and methods

2.1. Experimental design

The production of *in vivo* embryos was carried out at the Dairy Cattle Department and the Laboratory for Biotechnology of Animal Reproduction of the State University of Mato Grosso do Sul—Aquidauana University Unit (UEMS/UUAq). All procedures were approved by the Committee of Ethic and Animal Use of the State University of Mato Grosso do Sul, Aquidauana–MS (protocol CEUA-UEMS 012–2013).

Nonpregnant, nonlactating, cyclic *Bos indicus* × *Bos taurus* cows (n = 5) were used. The mean age of the animals was 7 years with an average body condition score of 3 (scale of 1–5). The animals were maintained on a rotational grazing system with Mombasa (*Panicum maximum* cv. Mombasa) and Tanzania grass (*P maximum* cv. Tanzania), with mineral supplementation *ad libitum*.

The animals were held for four sessions of superovulation and insemination, including two sessions with sexed semen and two with conventional semen. All animals were subjected to AI with sexed (n = 2) and conventional (n = 2) semen to minimize individual effects. The mean interval between sessions was 60 days. Before this study, the animals were never submitted to superovulation procedures.

In each superovulation session, the collected embryos were divided into four groups: fresh/conventional semen, fresh/sexed semen, cryopreserved/conventional semen, and cryopreserved/sexed semen. The embryos were also

separated according to their stage of development. Morulae and early blastocysts were considered as earlier stages of development, and the blastocysts and expanded blastocysts were considered as advanced stages of development.

Epigenetic analyses were conducted with 15 embryos per group. Therefore, the number of thawed embryos was established to be sufficient to achieve 15 viable embryos after thawing.

2.2. Embryo production

The hormonal synchronization began at Day 0 (random day, in the morning) of the estrous cycle using the intravaginal device containig 1.9 g progesterone-P4 (Eazi Breed CIDR; Zoetis) and an intramuscular injection of 2 mg of estradiol benzoate (Estrogin; Biofarm). Starting 5 days later, the animals received a total amount of 200 IU porcine pituitary extract of FSH (Foltropin; Tecnopec) intramuscularly in eight decreasing doses every 12 hours (40, 40, 30, 30, 20, 20 10, and 10 IU); 48 hours after the first FSH injection (Day 6), animals received 25 mg of dinoprost tromethamine (Lutalyse; Zoetis) intramuscularly. On Day 7, two doses of 200 IU eCG (Novormon; Zoetis) were applied with a 12-hour interval. The progesterone devices were removed at the same period as the second injection of eCG on Day 7. Application of 12.5 mg LH (Lutropin; Tecnopec) took place 24 hours after withdrawal of the P4 device. Two AIs, at 12 and 24 hours after LH injection, were carried out with conventional or sexed semen from a single *Bos indicus* × *B taurus* bull of proved fertility. Semen of a single ejaculate was used in all procedures. Semen collection, sexing, and cryopreservation were conducted in a commercial AI Center (Semex Brazil). The experimental design and schedule are summarized in Figure 1.

The uterine flushing was performed 7 days after the first AI through nonsurgical transcervical uterine flushing. The collection medium used was Dulbecco's PBS (Cultilab) plus 5% fetal bovine serum (Sigma–Aldrich) previously heat treated at 37 °C. After flushing, each donor was treated with 25 mg of a prostaglandin F2α analogue (Lutalyse; Zoetis). The uterine content was transferred to sterile Petri dishes (100 × 20 mm), and the embryo handling was performed using a binocular stereomicroscope at fivefold magnification. The embryos found were classified as unfeasible (degenerate, not fertilized), morula, early blastocyst, blastocyst, and expanded blastocyst, according to the specifications of the International Embryo Technology Society [26]. Qualities 1 to 3 of Morulae and blastocysts were considered to be viable. The embryos were randomized among groups to reduce the effect of the individual animal on the results. An aliquot of embryos was stored at 4 °C in 3% paraformaldehyde (Sigma–Aldrich) in water containing 2% sucrose (Sigma–Aldrich) for further analysis. The other part was cryopreserved by a slow freezing method. In brief, the embryos were exposed to the cryoprotectant solution (1.5 M ethylene glycol; Vigro) for 10 minutes. During this period, the embryos were loaded into 0.25-mL straws, with a central column containing the embryo and adjacent columns containing Holding (Vigro). The straws were placed in a freezing machine (Beltron Instruments, EFT-3002 embryo freezer) at a cooling rate of 2 °C/min until reaching −6 °C, when seeding was performed. Thereafter, the cooling rate was maintained at 0.6 °C/min until reaching −30 °C. The embryos were then stored in liquid nitrogen at −196 °C. The thawing was performed in a water bath (35 °C) and then the embryos were reclassified [15] and stored in groups of 15 embryos in 3% paraformaldehyde plus 2% sucrose at 4 °C for further analysis.

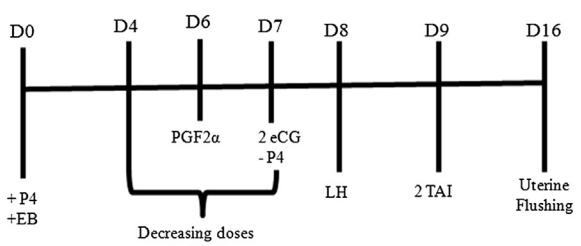

Fig. 1. Experimental design: superovulation protocol, TAI (twice on Day 9), and uterine flushing. EB, estradiol benzoate; P4, progesterone-P4 intravaginal device; PGF2α, prostaglandin F2α; TAI, time-fixed artificial insemination.

2.3. Immunofluorescence staining of H3K4me3

The evaluations of H3K4me3 status were performed at the Leibniz Institute for Farm Animal Biology (FBN, Dummerstorf, Germany); 15 embryos per group were used, including five for the negative control and 10 for investigation of H3K4me3.

After washing (100 mL PBS, 0.5 g bovine serum albumin, and 50 μL Triton X-100) at room temperature (RT), the embryos were permeabilized (20 mM HEPES, 300 mM saccharose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100, pH 7.4) at 0 °C for 15 minutes. After washing three times for 5 minutes each at RT (as mentioned earlier), the embryos were blocked (Roti ImmunoBlock 1:50 in H_2O; Carl Roth, Karlsruhe, Germany) for 2 hours at RT. Subsequently, they were exposed to a polyclonal antibody against methylated H3K4 (Cell Signaling; 1:1000 in Roti ImmunoBlock 1:100 in PBS + 0.02% Tween 20) overnight at 4 °C. After washing five times for 10 minutes each at RT (as mentioned earlier), the embryos were incubated with the secondary antibody (anti-rabbit-Alexa647; Invitrogen A21245, 1:200 in Roti ImmunoBlock (Carl Roth, Karlsruhe, Germany) 1:100 in PBS + 0.02% Tween 20) for 5 hours at RT. After washing five times for 10 minutes each at RT (as mentioned earlier), the nucleus was counterstained (SYBR Green; Thermo Fischer, Waltham, Massachusetts, USA; 1:500) and the embryos were incubated at 4 °C for 15 minutes. Finally, after washing four times for 10 minutes each at RT (as mentioned earlier), the embryos were individually mounted on coverslips.

2.4. Microscopic analysis of H3K4me3

For analysis of stained embryos, a confocal laser-scanning microscope (Pascal 5; Zeiss, Oberkochen, Germany) was used. Image stacks of individual embryos with a distance of 1 μm between images in the z-direction were recovered in dual tracks (40-fold oil lens): track 1:

Table 1
Viability of embryos produced *in vivo* with conventional or sexed semen before and after cryopreservation.

Semen	Viable (viable/total)	Cryopreserved embryos	Viability after thawing (viable/total of thawed embryos)[c]
Conventional	72%[a] (72/100)	57	88.2%[a] (15/17)
Sexed	37.70%[b] (34/90)	19	78.9%[a] (15/19)

Different lowercase letters indicate significant differences within the column ($P < 0.05$).
[c] The number of thawed embryos was sufficient to achieve 15 viable embryos after thawing for epigenetic analyses.

Laser: Argon 30 mW (458/488/514 nm) 488 nm/12%; HFT/NFT: 488/545 nm; filter: BP 505- to 530-nm channel 1 (channel 2 closed); track 2: Laser: HeNe 1 mW (543 nm) 543 nm/60%; HFT/NFT: 543/545 nm; filter: LP 560-nm channel 2 (channel 1 closed); pinhole: 1 AU, gain constant, averaging: 2; resolution: 1024 × 1024.

For localization and quantification of H3K4me3, the intensities of the fluorescence signals were analyzed by three-dimensional densitometry (ImageJ) evaluating at least 10 cells per embryo. Regions of interest (ROIs) were defined around the relevant nuclei in the stack. For background correction, an additional ROI was set outside the nuclei but inside the embryo. The measured intensities in these ROIs were subtracted from the intensities of the measurements in the ROIs around the nuclei. The fluorescence intensity depends on the location in the z-direction in the stack. Nevertheless, the intensity of nuclear staining (green channel, track 1) should be constant. Therefore, the incident intensity of nuclear staining (green channel) in the z-direction was used to calculate the fluorescence fading in the z-direction. The fading correction of the fluorophore (red channel) intensity in the z-direction was calculated using this result. The data were normalized to the controls (without the first antibody). For blastocysts, only cells from the inner cell mass (embryoblast, determined morphologically) were analyzed.

2.5. Statistical analyses

The experimental design was completely randomized and arranged in a factorial 2 × 2 structure (two treatments [semen and conservation] by two embryo development stages [morula and blastocyst]). First, the normality of the data was checked by the Shapiro–Wilks test. Then, ANOVA was performed, and when a significant difference was found, the post hoc Tukey test was carried out at a 5% probability (R Program).

3. Results

After all procedures (n = 4), 190 embryos (7.8 ± 6.6 embryos per cow per session) were obtained, comprising 100 embryos from groups produced with conventional semen and 90 produced with sexed semen. Thus, the production of viable embryos with conventional semen was superior to that of embryos produced with sexed semen (72% [72 of 100] vs. 37.7% [33 of 90], respectively; $P < 0.05$; Table 1). Fifty-seven viable embryos from the conventional semen group and 19 from the sexed semen group were cryopreserved. There were a sufficient number of thawed embryos to achieve 15 viable embryos per group for further analyses. The viability rate of embryos (qualities 1–3) after thawing was 78.9% for embryos produced with sexed semen and was 88.2% for embryos produced with conventional semen ($P > 0.05$; Table 1).

Considering that flushing was always conducted on Day 7 in the morning, the procedures performed with sexed semen generated more ($P < 0.05$) embryos at the early stages of development (21.21% morulae and 21.21% early blastocysts) compared with those produced with conventional semen (4.16% morulae and 13.88% early blastocysts; Table 2).

H3K4me3 was detected in both morulae (Fig. 2) and blastocysts (Fig. 3). A higher H3K4me3 signal intensity was observed in morulae compared with blastocysts for all groups, and there was no influence of cryopreservation or the use of sexed semen on H3K4me3 levels ($P > 0.05$). In addition, there was no interaction between treatments ($P > 0.05$; Table 3).

4. Discussion

For many years, the superiority of conventional semen for a higher embryonic viability rate has been observed, regardless of the animal breed or category [27–29]. However, in spite of the smaller number of embryos produced using sexed semen, the production of the desired sex of calves makes this a feasible biotechnology [30,31]. In this study, the number and viability of embryos were reduced when sexed semen was used (Table 1), and in general, the recovered embryos were at later stages of development (blastocysts and expanded blastocysts; Table 2). It has been reported that embryos of *B taurus* cattle obtained after superovulation and AI with conventional semen are generally in the early stages of development such as morulae, compact morulae, and early blastocysts [32,33]. In contrast, in zebu, it was observed that 80% of the collected embryos were in advanced stages of development [34]. This difference may be related to some endocrine

Table 2
Stages of development of embryos obtained after uterine flushing 7 days after artificial insemination (Day 7), with sexed or conventional semen.

Semen	D7			
	Mo	Bi	Bl	Bx
Conventional	4.16%[b] (3/72)	13.88%[b] (10/72)	29.16%[a] (21/72)	52.77%[a] (38/72)
Sexed	21.21%[a] (7/33)	21.21%[a] (7/33)	24.24%[a] (8/33)	33.33%[b] (11/33)

Different superscript letters indicate significant differences within the column ($P < 0.05$).
Abbreviations: Bi, early blastocyst; Bl, blastocyst; Bx, expanded blastocyst; Mo, morula.

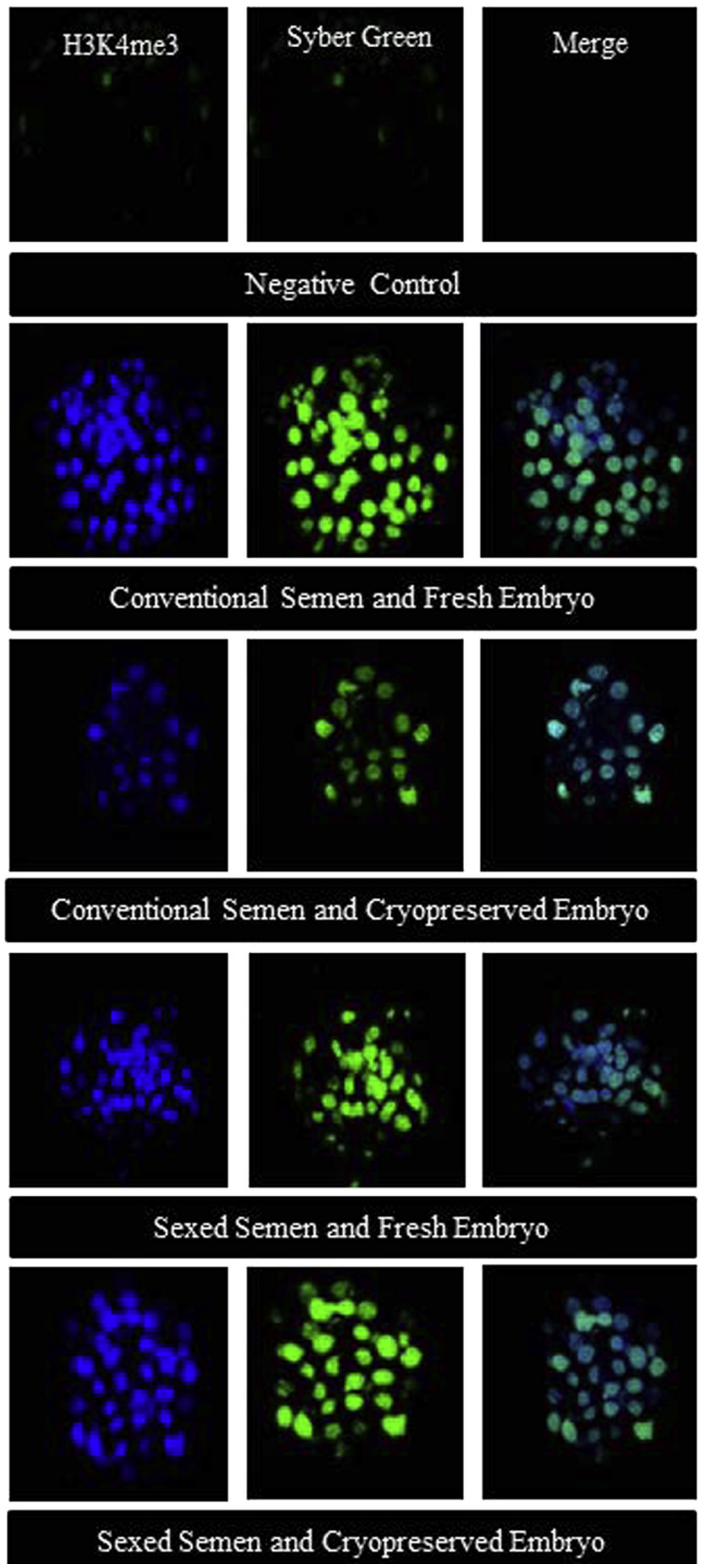

Fig. 2. Identification of H3K4me3 in morulae obtained after uterine flushing 7 days after artificial insemination with conventional or sexed semen. Immunofluorescence analysis by confocal microscopy. Blue: anti-H3K4me3 antibody, green: SYBR Green, merge: anti-H3K4me3 + SYBR Green. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

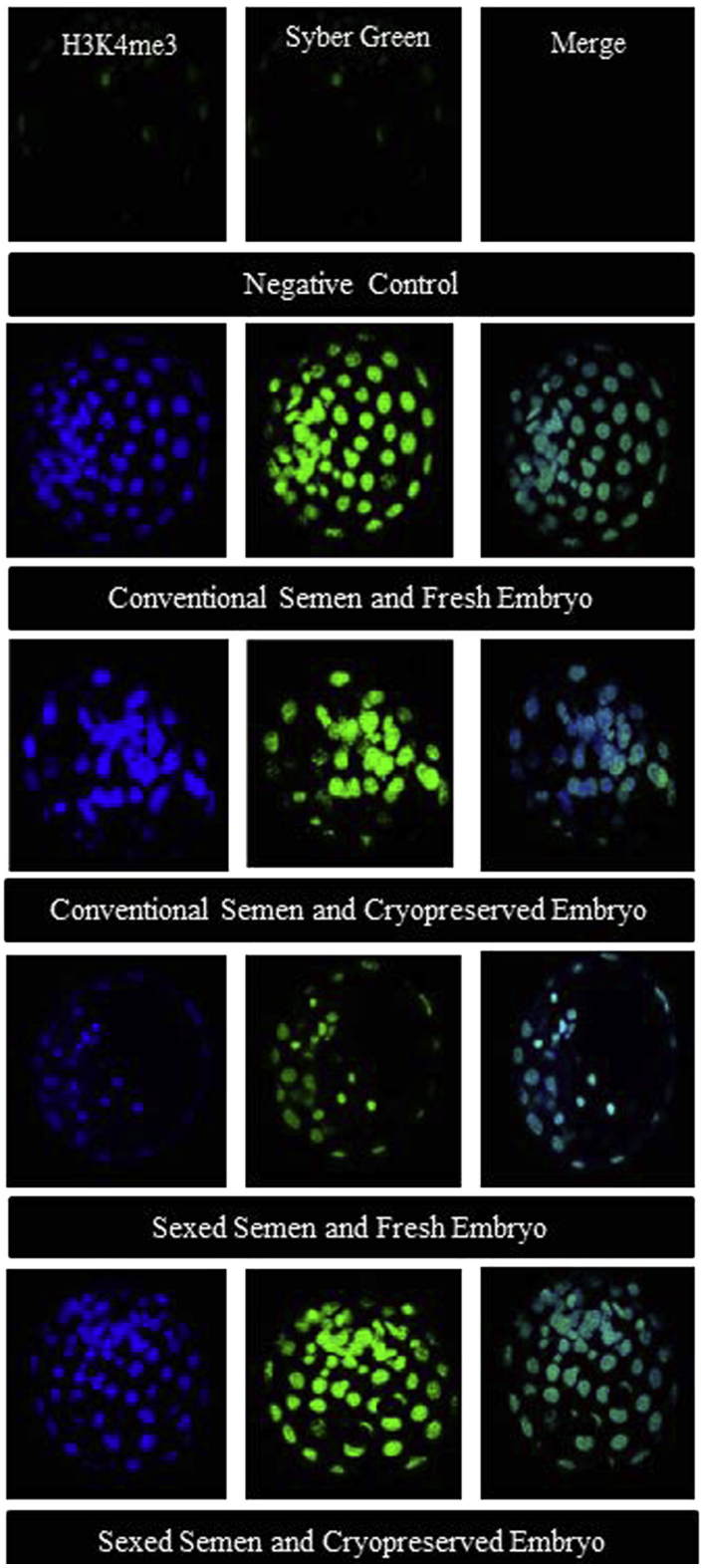

Fig. 3. Identification of H3K4me3 in blastocysts obtained after uterine flushing 7 days after artificial insemination with conventional or sexed semen. Immunofluorescence analysis by confocal microscopy. Blue: anti-H3K4me3 antibody, green: SYBR Green, merge: anti-H3K4me3 + SYBR Green. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Mean ± SD of the intensity of H3K4me3 signals in morulae and blastocysts (embryoblasts) produced *in vivo* using sexed and conventional semen before and after cryopreservation.

Semen	Signal intensity	P value
Sexed	178.51 ± 32.89	0.4321
Conventional	191.10 ± 25.26	
Conservation		
Fresh	180.67 ± 25.95	0.6049
Cryopreserved	188.94 ± 33.26	
Stage		
Morulae	209.26 ± 10.95	0.0041[a]
Blastocysts (embryoblasts)	160.35 ± 10.52	
Interaction		
Semen × conservation	—	0.7637
Semen × stage	—	0.7285
Conservation × stage	—	0.6370
Semen × conservation × stage	—	0.8522

[a] Significant at 5% probability.

differences because zebu have a pre-ovulatory LH surge closer to ovulation [35] than taurine females [36]. Consequently, fertilization occurs earlier, allowing more time and favorable conditions for embryos to develop [35].

In this study, the number of morulae and early blastocysts was significantly higher when sexed semen was used (Table 2). Higher pregnancy rates have been demonstrated in crossbred cows when embryos were transferred at more advanced stages of development (blastocysts and expanded blastocysts) [37]. The different steps required for the sperm sexing technique with flow cytometry may generate some morphologic damage, including exposure to the laser, the pressure during the passage through the cytometer, the fall at high speed within the collection tube, and holding for a few hours at RT before processing [38,39]. We speculate that changes in sperm chromatin because of the sexing procedures could reduce the ability of embryos to reach advanced stages of development or cause their degeneration. This would explain the observed lower pregnancy rates when sexed semen is used and the higher rate of nonviable embryos found in the present study and reported previously [27,40].

Some modifications of histones are essential for establishment of oocyte competence and development [12], spermatogenesis, and embryogenesis [5]. During spermatogenesis in mammals, histones are replaced in part or completely by specific nucleoproteins of spermatozoa known as protamines, which have a structural character [41]. Furthermore, after fertilization, protamines and almost all paternal histones are degraded and replaced by histones of the oocyte [42]. The presence of H3 was described in both human spermatozoa and in the male pronucleus of the zygote [43]. This pattern suggests the possible importance of paternal histone in the embryo. However, the presence of histone modification marks, including H3K4me3, was found to be higher in poor functional quality sperm, suggesting that defects in the process of spermatogenesis may alter the correct epigenetic programming in mature sperm [19]. Nevertheless, it is unknown whether this feature has a specific function during early embryonic development [19], and information in this regard is particularly lacking for cattle. Considering the small amount of histones identified in the spermatozoa of humans and mice [44,45], little influence of paternal histones in embryonic development is expected. However, this does not rule out the possibility that the small amount of histones present in the sperm might have a significant function.

The results of this study reported no different pattern of H3K4me3 among embryos produced with sexed and conventional semen (Table 3), suggesting that this epigenetic mark is not related to the stress experienced by the spermatozoa from the sexing procedure and its impact on embryonic development. However, it is possible that other epigenetic marks may be involved in the development of embryos produced with sexed semen.

Cryopreservation favors the storage of embryos for prolonged periods with little loss of the capacity of development, allowing for the genetic material of interest to be used as needed and at the most appropriate time for efficient embryo transfer.

The classic freezing method is used worldwide for the cryopreservation of embryos produced *in vivo*. This method has the advantage of using low concentrations of cryoprotectants; however, it also permits the formation of ice crystals to a greater or lesser extent, resulting in injury to the membranes and organelles [46]. Despite this potential for damage, the cryopreservation of embryos produced *in vivo* is a well-established strategy and can yield embryo survival, pregnancy, and birth rates similar to those achieved with fresh embryos [47,48]. However, basic research is important to determine the metabolic pathways, molecular mechanisms, and epigenetic events most affected by cryopreservation that could help to improve other reproduction biotechniques. In this study, a high signal of H3K4me3 was detected in morula and blastocysts obtained 7 days after AI. Our results reported no significant difference in the signal intensity of H3K4me3 from blastocysts and morulae, regardless of cryopreservation (Table 3, Figs. 1 and 2). It has been reported that there is no change in pattern of H3K4me3, H3K9ac, and H3K9me3 in mouse embryos, produced *in vitro* and *in vivo*, and cryopreserved or not [48]. These authors concluded that cryopreservation did not seem to have any additional effect on epigenetic changes (histone, H3K4me3) and that it could be considered as a process that exerts minimal damage to the embryos. The results of the present study corroborate these previous findings because the process of cryopreservation of the bovine embryos produced *in vivo* did not alter the pattern of H3K4me3.

In vitro bovine blastocysts subjected to slow freezing reported a reduction in the permissive mark of gene transcription, H3K4me3, and an increase in the repressive mark of gene transcription, H3K27me3, in comparison with fresh embryos [49]. However, in mouse, contradictory results were observed. After thawing and culturing to the blastocyst stage, there was an increase in the permissive mark H3K9ac and a decrease in the repressive mark, H3K9me2, associated with an increase in expression of H19 and MEST genes, important for embryo development, fetal growth, placental function, and postnatal behavior [48].

In this study, the signal intensity of H3K4me3 was higher in morulae than in the inner cell mass of blastocysts

(Table 3, Figs. 2 and 3). These results differ from those reported previously for mice, in which the H3K4me3 signal intensity was highest in zygotes at the pronuclear stage and two-cell embryos but significantly decreased at the eight-cell stage and gradually increased from the morula to blastocyst stages [50,51]. However, a higher signal intensity of H3K4me3 in the inner cell mass from thawed hatched blastocysts of mice in comparison with fresh hatched blastocysts was reported [47]. The differences between the present study and these mouse data may be because of species-specific differences, but further studies are required to elucidate the cause.

4.1. Conclusions

A high-intensity H3K4me3 signal was observed in bovine embryos produced *in vivo*, and this pattern did not vary depending on the use of sexed semen and the slow cryopreservation process.

The production of viable embryos with conventional semen was higher to that of embryos produced with sexed semen, which were mostly in early stages of development. This fact could be not explained by differences in H3K4me3.

Cryopreservation did not alter the pattern of H3K4me3, and in this sense, we suggest that it is a process that exerts minimal damage to the embryos.

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