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## Heat shock during *in vitro* maturation induces chromatin modifications in the bovine embryo

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**Abstract:** Heat stress compromises bovine oocyte developmental competence but the effects of high temperature during oocyte maturation on embryo chromatin organization is unknown. In this study bovine oocytes were exposed to heat shock (41°C) for 12h during *in vitro* maturation and then submitted to *in vitro* fertilization. The heat shock did not affect ( $P>0.05$ ) the cleavage but reduced ( $P<0.01$ ) the blastocyst rate on Day 7 and Day 8. No effect ( $P>0.05$ ) on total cell number was found but the heat shock increased ( $P<0.05$ ) the proportion of apoptotic cells in blastocysts at Day 8. Immunofluorescence analysis of H3K9me3 and HP1 was performed in embryos at 52 h post *in vitro* fertilization. An accumulation of H3K9me3 in the nuclei of embryos derived from heat-shocked oocytes at 4-cell and 8-cell stages was found. Also, a non-expected higher proportion ( $P<0.05$ ) of 4-cell stage embryos displaying nuclei with increased HP1 fluorescence was observed, suggesting an abnormal chromatin compaction in embryos from heat-shocked oocytes. Embryos at 8-cell stage derived from heat-shocked oocytes displayed lower ( $P<0.05$ ) relative amount of *HSP40* transcripts than control ones. In conclusion, heat shock before fertilization has an effect on embryo chromatin, influencing the accumulation of H3K9me3 and HP1 in early embryos as well as further development.

### Introduction

Heat stress is one of the main causes of bovine low reproductive performance in the tropics as well as in hot seasons in subtropical countries. Its effects on reproduction is supported by *in vivo* and *in vitro* studies which show that bovine oocytes are sensitive to heat stress at various stages of follicular development. It can disturb the physiology of the follicle-enclosed oocyte during the lengthy period of follicular development, leading to an oocyte with low competence for fertilization and subsequent embryo development (Roth 2015).

Exposure of cultured cumulus-oocyte complexes (COCs) to elevated temperatures (41°C) during the first 12 h of *in vitro* maturation decreases the cleavage rate (Roth & Hansen, 2004, Roth & Hansen 2005) and the proportion of oocytes that develop toward blastocyst stage (Edwards & Hansen 1997, Roth & Hansen 2004, Ju *et al.* 2005). The heat shock impairs both nuclear and cytoplasmic maturation events, such as translocation of cortical granules to the oolemma (Payton *et al.* 2004), cytoskeleton rearrangement [Roth & Hansen 2005], and spindle formation [Roth & Hansen 2005, Ju *et al.* 2005). Those alterations may potentially lead to incomplete nuclear maturation, fertilization failure, and/or abnormal zygote formation (Payton *et al.* 2004, Roth & Hansen 2005). Moreover, heat shock-induced apoptosis has been documented for bovine oocytes exposed to elevated temperatures during maturation (Roth & Hansen 2004).

One of the little-studied effects of heat stress on oocytes is epigenetic modification. It is clear that the epigenome can be modulated by nutritional (Murdoch *et al.* 2016, Longo *et al.* 2017) and environmental conditions (Horowitz 2016). Indeed, the epigenome provides an interface between genes and the environment and may be considered as a potential mechanism for environmental adaptation (Rissman & Adli 2014). For instance, heat acclimation can cause transcriptional changes in genes associated to chromatin remodeling, heat shock proteins and apoptosis in cardiac cells of rats, and those changes on chromatin structure may take part in an acclimation memory phenomenon (Tetievsky *et al.* 2008). Maternal heat stress increases embryo mortality in hens but supplementation of zinc in the maternal diets can reduce such effect, which was suggested to be due to increased antioxidant ability via epigenetic activation (Zhu *et al.* 2017). Heat shock can alter the expression of some target genes in bovine immature oocytes (Gendelman & Roth 2012) and *in vitro*-fertilized embryos (Camargo *et al.* 2016), suggesting an epigenetic modification during oocyte growth and embryo development caused by the elevated temperature.

Epigenetic modifications related to chromatin structure include changes on DNA methylation and post-translational modifications on histones. Highly condensed chromatin (heterochromatin) is typically transcriptionally repressive whereas non-condensed chromatin (euchromatin) is permissive to transcription (Cheung & Lau 2005). During early development the embryo undergoes genome activation that requires epigenetic modifications such as changes on histone methylation and chromatin organization (Sepulveda-Rincon *et al.* 2016). In bovine, the major embryonic genome activation (EGA) occurs between 8-16 cell stages (Frei *et al.* 1989, Graf *et al.* 2014) and is associated with chromatin remodeling (Pinchugin *et al.* 2010). Those modifications are essential to reprogram the parental genome to embryonic genome and then generate healthy embryos (Beaujean 2014). In cloned bovine embryos derived by nuclear transfer, abnormal reprogramming of heterochromatin from the somatic donor cells has been observed (Pinchugin *et al.* 2010) and is believed to affect embryonic development (Pinchugin *et al.* 2010, Le Bourhis *et al.* 2010). We postulate that one of consequences of heat shock during oocyte *in vitro* maturation is modifications on chromatin organization of early stages embryos. Those changes could disturb the EGA, impairing further development toward the blastocyst stage.

In order to test whether heat shock during oocyte maturation could affect chromatin remodeling in the early stages embryo we evaluated the distribution of histone H3 lysine 9 trimethylation (H3K9me3) and heterochromatin protein 1 (HP1) by immunostaining of embryos at 4-cell and at 8-cell stages obtained by *in vitro* fertilization of heat-shocked oocytes. Histone H3 lysine 9 (H3K9) methylation is a transgenerational epigenetic modification conserved from yeast to humans (Rissman & Adli 2014). H3K9 methylation has been shown to correlate with transcriptional repression and serve as a specific binding site for heterochromatin protein 1 (HP1), which recognizes H3K9-methylated chromatin, oligomerizes and forms a versatile platform that participates in diverse nuclear functions, ranging from gene silencing to chromosome segregation (Eissenberg & Elgin 2000, Lachner *et al.* 2001, Grewal & Jia 2007). During early development there is an asymmetry on H3K9 methylation between maternal and paternal chromatin in mice (Liu *et al.* 2004) and cattle (Pinchugin *et al.* 2010). In bovine *in vitro*-fertilized embryos, H3K9 methylation is reduced between the 2- and 4-cell stages and increases from the 8-cell stage onward (Santos *et al.* 2003, Wu *et al.* 2011). Despite the presence of methylated H3K9, there is no clear co-localization between HP1 and H3K9me3 in bovine embryos at the 2- and 4-cell stages. In fact, co-localization between H3K9me3 and HP1 is only evident at the 8-cell stage (Pinchugin *et al.* 2010), concurrently with EGA. Changes in HP1 and/or H3K9 methylation levels could potentially disturb the EGA and impair embryonic development.

Thus, this study aimed to evaluate the effect of heat shock during oocyte *in vitro* maturation on development, gene expression and chromatin organization of *in vitro*-fertilized embryo.

## Materials and Methods

Chemicals were purchased from the Sigma Chemical Co. (Saint Louis, MO, USA) unless otherwise indicated. All experimental procedures followed ethical guidelines for animal experimentation and were approved by local Committee (CEUA EGL 02/2015 protocol).

### *Experimental design*

Three experiments were performed in order to evaluate the developmental competence of heat-shocked oocyte after *in vitro* fertilization (n=569), the chromatin organization of 4- and 8-cell stage embryos derived from heat-shocked oocytes and the gene expression of 8-cell stage embryos derived from heat-shocked oocytes. Control groups were comprised of *in vitro*-fertilized embryos derived from oocytes not exposed to heat shock.

For the first experiment immature COCs were cultured in maturation medium at 38.5°C for 24 h (control) in 5.0% CO<sub>2</sub> and humidified air or at 41.5°C for the first 12 h of a 24 h culture period (heat shock) in 7.0% CO<sub>2</sub> and humidified air. After *in vitro* maturation (IVM), COCs were co-cultured with spermatozoa for 20 h for *in vitro* fertilization (IVF). Putative zygotes (n=470) were denuded of cumulus cells and then cultured in CR2aa at 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub> at 38.5°C in humidified air. Cleavage on day three (72 h post insemination) and blastocyst rates on day seven and day eight post-insemination was recorded. Hatching rates were recorded on day eight. Six replicates were performed. Total cell number and apoptosis index was determined in blastocysts at day eight. In the second experiment the heat shock, IVM, IVF and *in vitro* culture (IVC) were performed as described in the first experiment. Embryos at 4- and 8-cell stage at 52h post fertilization (n=58) were fixed in 4% (w/v) paraformaldehyde and chromatin staining was performed with primary and secondary fluorescent antibody to detect H3K9me3 and HP1. Embryos from four replicates were used in this experiment. For the third experiment, IVM, IVF and IVC were also performed as described in the first experiment. Embryos at 8-cell stage at 52h post fertilization were washed in PBS plus 0.1% polyvinyl alcohol and then rapidly frozen in liquid nitrogen and stored until total RNA extraction. Three pools of 10 embryos per group were used.

#### *Oocytes recovery and in vitro maturation (IVM)*

Oocytes recovery and IVM were performed as previously described by Camargo et al. (2016), with a few modifications. Ovaries from predominantly *Bos indicus* crossbred cows were obtained at a local commercial slaughterhouse (Fripai, Juiz de Fora, MG, Brazil) and transported to the laboratory in saline solution at 34–36°C. Follicles with 3–8 mm diameter were aspirated and COCs with homogeneous cytoplasm and compact layers of cumulus cells were selected for IVM and randomly allocated into control (38.5°C) or heat shock (41.5°C for 12h) groups. *In vitro* maturation was performed for 24 h in tissue culture medium (TCM-199; Gibco Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% estrus cow serum and 20 µg/mL follicle stimulating hormone (FSH; Pluset, Calier, Barcelona, Spain) in 5% CO<sub>2</sub> and humidified air. Heat shock was performed for 12h at 41.5°C under 7% CO<sub>2</sub> followed by 12 h at 38.5°C under the same conditions of control group.

#### *In vitro fertilization (IVF) of oocytes*

Frozen/thawed motile spermatozoa from a single Holstein bull were obtained after centrifugation at 3.600×g for 7 min in Percoll (Nutricell Nutrientes Celulares, Campinas, SP, Brazil) discontinuous density gradient (45 and 90%). The pellet was then centrifuged again at 520×g for 3 min in Fert-TALP medium. For *in vitro* fertilization, *in vitro*-matured oocytes were co-cultured with 2 x 10<sup>6</sup>/mL spermatozoa for 20 h in Fert-TALP in a humidified atmosphere of 5% CO<sub>2</sub> and 38.8°C in air.

#### *In vitro embryo culture (IVC)*

Presumptive zygotes were denuded in a PBS solution with 0.1% hyaluronidase and then cultured for eight days in modified CR2aa medium supplemented with 2.5% fetal calf serum (Nutricell Nutrientes Celulares, Campinas, SP, Brazil) in incubator at 38.5°C under 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N<sub>2</sub> and saturated humidity. Cleavage rate was evaluated on day three post-fertilization and blastocyst rate was evaluated on day seven and day eight as well as hatching rate.

#### *TUNEL Labeling*

Blastocysts on day eight from different replicates were submitted to terminal deoxynucleotidyltransferase-mediated dUTP-digoxigenin nick end-labelling (TUNEL) staining using a commercially available kit (Dead End Fluorimetric TUNEL System; Promega, Madison, WI, USA) according to manufacturer's instructions. Briefly, embryos were fixed in 4% paraformaldehyde at 4°C and then permeabilized with 0.2% Triton X-100 (Promega), both in PBS (Nutricell). Positive control embryos were previously treated with 8 units/mL DNase (Promega). After permeabilization, samples

were incubated in 100  $\mu$ L drops with reagent mix containing enzyme solution and 90% staining solution for 1 h at 37°C in a dark humid chamber. Negative control embryos were incubated only in the staining solution without enzyme solution. After that, embryos were stained with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) plus 406-diamidino-2-phenylindole (DAPI) and mounted on slides for evaluation by fluorescence microscopy. Total cell number and apoptotic cell number per embryo were counted, and apoptotic cell index was calculated as the proportion of apoptotic cell/ total cell number.

#### *Immunofluorescence, confocal microscopy and image analysis*

Immunofluorescence analysis of H3K9me3 and HP1 was performed in embryos with 4- and 8-cells at 52 h post *in vitro* fertilization. Embryos were fixed in 4% paraformaldehyde and permeabilized with 1% Triton X-100 in PBS. Afterwards all samples were blocked with 2% BSA in PBS and then incubated with primary antibody. H3K9me3 labeling was performed with rabbit anti-H3K9me3 primary antibody (Millipore/Merk; 1:500 in PBS-BSA) and Cy3 anti-rabbit secondary antibody (1:200 in PBS+BSA). HP1 labeling was performed with mouse anti-HP1beta primary antibody (Euromedex, 1:200 in PBS-BSA) and FITC anti-mouse secondary antibody (1:200 in PBS+BSA).

Embryos were mounted on slide in Vectashield (Vector Laboratories, Burlingame, CA, USA) under conditions of preservation of their three-dimensional (3D) nuclear structure. They were observed with an inverted Zeiss LSM 700 confocal microscope (MIMA2 platform, INRA). The Z-stacks were acquired using a frame size of 1024x1024 with a pixel depth of 8 bits and 0.370  $\mu$ m z-steps and with sequential multitrack scanning using the 405-, 488- and 555-nm wavelengths of the lasers. Images were processed using an in-house pipeline analysis software, developed with Python script language and ITK library ([www.itk.org](http://www.itk.org)).

Spatial 3D datasets were imported into the pipeline using the Bio-Formats library (<http://www.loci.wisc.edu>), and multi-channels images were split to individual DNA, HP1 and H3K9me3 Z-stacks. Segmentation of nuclei, H3K9me3 and HP1 spots was done using three different processes. To outline correctly the nucleus, the DNA channel and the HP1 protein were combined (AddImageFilter operation) to improve the detection of the nuclear shape, because in cattle embryos, DAPI staining alone did not allow a perfect detection of the nucleus. The binary mask of each nucleus was obtained by selecting (threshold filter) the 10% of brightest pixels in the combined image and suppressing the smallest binary areas (opening filter). Then binary structures with empty areas were filled (fill holes filter) and nuclei were discriminating from artifacts by fixing a minimal size of objects (725  $\mu$ m<sup>3</sup>). Finally, a color label to identify each nucleus separately was assigned. This nuclear mask was the region of interest (ROI) in the following image processing of proteins channels. Since H3K9me3 was accumulated in bright spots in the nuclei, these ones were segmented using a maximum entropy threshold filter in the ROI. The H3K9me3 were labeled individually. For the HP1 signal, accumulation of proteins resulted in spots of very different sizes and of different brightness according to the embryo developmental stages. A 2D maximum entropy thresholding was performed to detect spots, but a lot of punctuate structures were detected when the HP1 signal was more homogeneous. To remove unspecific spots, a mask, which detected approximately the regions enriched in HP1, was used. It was obtained by applying a Gaussian filter (sigma = 0.3  $\mu$ m) on the HP1 Z-stack, and a threshold filter keeping 5% of the brightest pixels. Then the HP1 spots were labeled individually. Measurements were done from the segmented nuclei, and spots image datasets.

In addition, detailed image analysis of H3K9me3 and HP1 fluorescent staining was performed in order to identify the proportion of embryos displaying different patterns of chromatin staining (Figure 1). Figure 1-A illustrates representative merged images of immunofluorescence in 4-cell and 8-cell stages embryos. Figure 1-B illustrates the patterns of chromatin compaction observed (as previously established in Pichugin *et al.* 2010): spread (uniform staining), fibrous (small spots displaying various levels of staining) or globular (evident and big spots displaying strong staining).

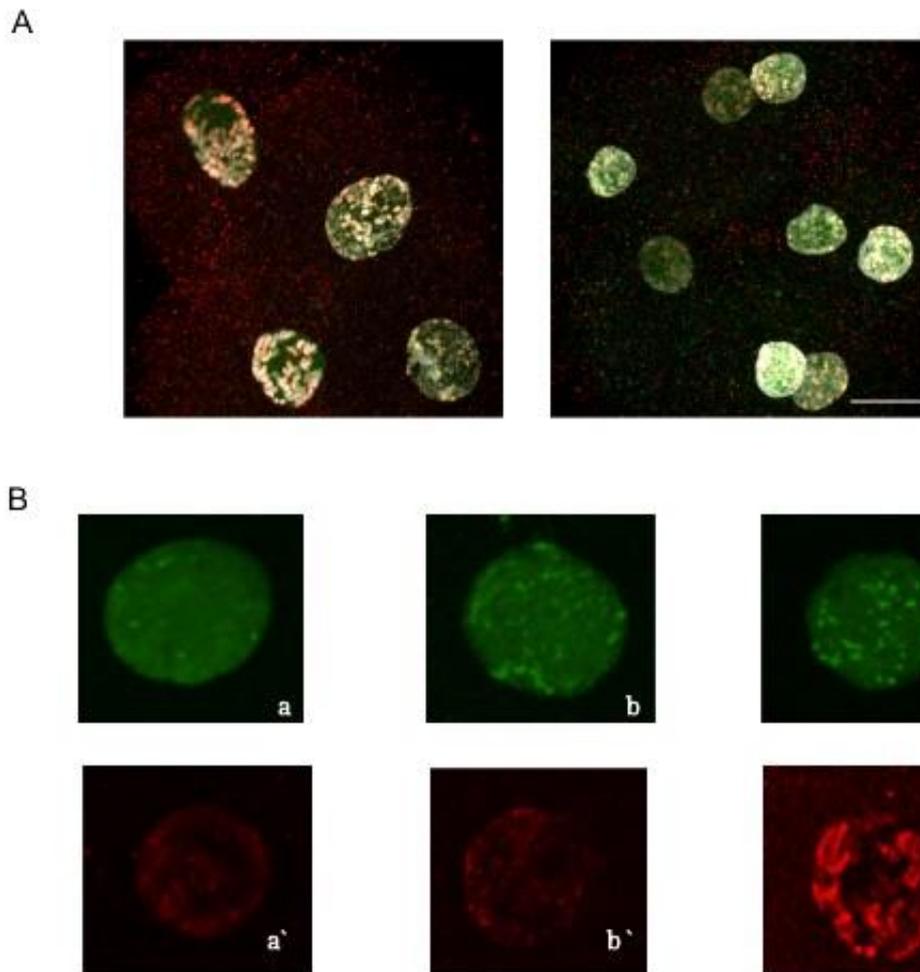


Figure 1. Representative images of the different chromatin compaction patterns established thanks to HP1 and H3K9me3 immunostaining in the nuclei of 4-cell (left) and 8-cell bovine embryos (right) showing that within each embryo the distribution pattern was similar within blastomeres (A, merged images). Scale bar: 15  $\mu$ m. Detailed images analysis with computing tools was performed on individual nuclei (B) in which three different patterns were distinguished: (a) Spread: uniform staining, (b) fibrous: small spots displaying various levels of staining, and (c) globular: evident and big spots displaying strong staining. Those analysis were performed in 3D on confocal z-scan that were merged on these representative examples for better visualization with HP1 in green (a, b and c) and H3K9me3 in red (a', b' and c').

*RNA extraction, reverse transcription and relative quantification by polymerase chain reaction (RT-PCR)*

Embryos at 8-cell stage were washed in PBS and then rapidly frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Three pools of ten embryos per group were used for total RNA extraction with RNeasy Micro Kit (Quiagen, Valencia, CA, USA) according to the manufacturer's instructions and treated with DNase to prevent DNA contamination. Elution was performed with  $14\mu\text{L}$  of RNAase-free water. RNA samples ( $6\mu\text{L}$ /reverse transcription reaction, equivalent to 4.3 embryos) were reverse transcribed using the SuperScript III First-Strand Synthesis Supermix (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions using oligo(dT)<sub>20</sub> primers in a final volume of  $20\mu\text{l}$  (equivalent to 0.21 embryos/ $\mu\text{L}$ ). Samples were first incubated at  $65^{\circ}\text{C}$  for 5 min and then for  $50^{\circ}\text{C}$  for 50 min. The reaction was terminated at  $85^{\circ}\text{C}$  for 5 min and then chilled on ice. After that, RNase H was added to the samples and incubated at  $37^{\circ}\text{C}$  for 20 min.

Relative quantification was performed in triplicate using real-time polymerase chain reaction (ABI Prism 7300 Sequence Detection Systems, Foster City, CA, USA). Reactions were prepared using a mixture of SYBR Green PCR Master Mix (Applied Biosystems),  $0.1\mu\text{M}$  primers, nuclease-free water and reverse transcribed cDNA. The volume of reverse transcribed cDNA for every primer was previously calculated and it is shown in microliter and as embryo equivalent. For *YWHAZ* (Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta) and *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) were added  $0.32\mu\text{L}$  of reverse transcribed cDNA per PCR reaction (equivalent to 0.07 embryo/reaction); for *ACTB* (beta actin), *HSP40* (Heat shock 40kDa protein) and *OCT4* (Octamer-binding transcription factor 4) were added  $0.64\mu\text{L}$ /PCR reaction (0.14 embryo/reaction); for *HSF1* (Heat Shock Factor 1) was added  $1.92\mu\text{L}$ /PCR reaction (0.42 embryo/reaction) and for *HSP90A1* (Heat Shock Protein 90 KDa class A member 1) was added  $0.96\mu\text{L}$ /PCR reaction (0.21 embryo/reaction). *YWHAZ*, *ACTB* and *GAPDH* genes were used as endogenous references. The cDNA template was denatured at  $95^{\circ}\text{C}$  for 15 min, followed by 45 cycles of  $94^{\circ}\text{C}$  for 15 sec, annealing at specific temperature for each primer for 15 sec (Table 1) and extension at  $60^{\circ}\text{C}$  for 30 sec. Negative controls, comprising the PCR reaction mixture without nucleic acids, were also run with each group of samples. After each PCR run, a melting curve analysis was performed to confirm that a single specific product was generated. Primer efficiency was calculated using LinRegPCR software (Ramakers *et al.* 2003) for each reaction. The primer efficiency average was 1.92; 1.89; 1.63; 1.80; 1.86; 1.92; 1.92 to *YWHAZ*, *GAPDH*, *ACTB*, *HSF1*, *HSP90*, *HSP40* and *OCT4*, respectively

**Table 1.** Primers used for real-time polymerase chain reaction

Gene	Primer sequence 5'-3'	Annealing temperature	Fragment size (bp)	Gene bank access number
<i>YWHAZ</i>	F GCAAAAGACGGAAGGTGCTG	59°C	197	NM_174814.2
	R CCTCAGCCAAGTAGCGGTAG			
<i>GAPDH</i>	F GCGTGAACCACGAGAAGTAT	53°C	119	NM_001034034
	R CCCTCCACGATGCCAAAGT			
<i>β-ACTINA</i>	F GAGGCTCTCTTCCAGCCTTC	53°C	184	NM_173979.3
	R GGCAGTGATCTCTTTCTGC			
<i>HSF1</i>	F ACTCCAACCTGGACAACCTG	59°C	209	XM_010811828.3
	R TGAGTCTGGGCTGCTTTTCT			
<i>HSP90A1</i>	F GAGGATCCCCAGACACATGC	62°C	143	NM_001012670.2
	R GACGTGTCGTCTCCTTC			
<i>HSP40</i>	F AGGACTGACCAGGGACCTTT	55°C	220	NM_001033763.1
	R AGACTGCATTGCCTTTGCTT			
<i>OCT4</i>	F GCTTGATCGTTTGCCCTTCT	55°C	105	NM_174580
	R CCCACCCTGCAGCAAATTA			

### Statistical Analysis

The rates of cleavage were evaluated on day three and blastocysts on day seven and eight after fertilization. Data of cleavage, blastocyst production and spots number and size were compared by analysis of variance. Apoptosis index was analyzed by logistic regression and total cell and apoptotic cell number by linear mixed model. Proportion of embryos displaying nuclei with different staining patterns was compared by Fischer Exact test. Relative transcript quantification was performed by Comparative Ct method and the data analyzed by mixed model using the Wise Fixed Reallocation Randomization TEST in REST software (Pfaffl *et al.* 2002). Values are shown as mean  $\pm$  standard error mean (S.E.M.) and the significance level was  $P < 0.05$ .

## Results

### *Embryonic development and blastocyst total cell number and apoptosis*

The heat shock during *in vitro* maturation did not affect the cleavage rate, ranging from 64.5% to 74.2% between treatments. However, a significant reduction in the proportion of putative zygotes that reached the blastocyst stage was observed in the heat shock group on day seven ( $P<0.01$ ) and day eight ( $P<0.05$ ) after fertilization (Table 2). In addition, heat shock reduced ( $P<0.05$ ) the proportion of hatched embryos at day 8 when compared to control, suggesting a reduction in the embryo quality (Table 2).

Blastocyst total cell number was not affected ( $P>0.05$ ) by the heat shock during oocyte *in vitro* maturation but the apoptotic cells index increased when compared to control (Table 3). Figure 2 shows representative images illustrating the total cell number and apoptotic nuclei in blastocysts from control (A and A') and heat shock (B and B') groups.

**Table 2.** Cleavage rate (D3) and blastocysts rate at Day 7 (BLD7) and Day 8 (BLD8) and hatched blastocysts at Day 8 (HBLD8) for groups Control and Heat Shock during *in vitro* maturation.

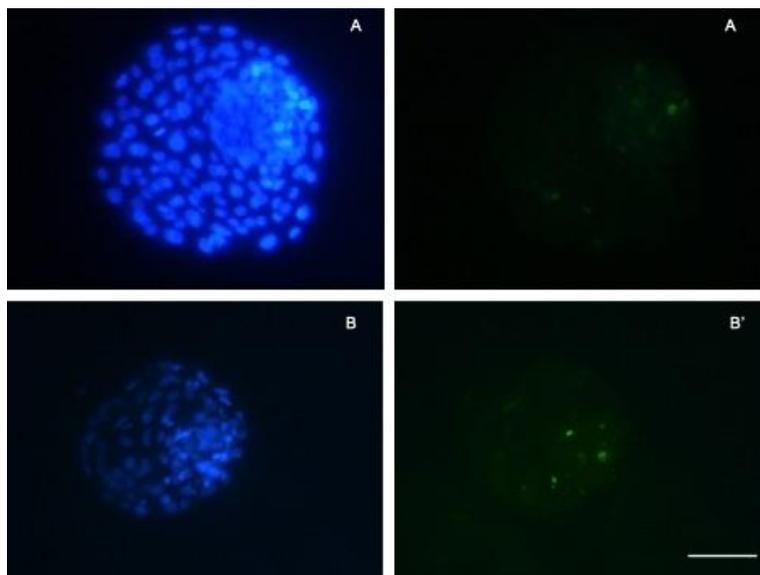
Treatments	n	Cleavage (%)	BLD7 (%)	BLD8 (%)	HBLD8 (%)
Control	240	74.2±4.1 <sup>a</sup>	31.8±2.5 <sup>a</sup>	34.1±2.9 <sup>a</sup>	35.8±3.9 <sup>a</sup>
Heat Shock	230	64.5±5.4 <sup>a</sup>	19.0±3.5 <sup>b</sup>	21.2±3.8 <sup>b</sup>	13.8±5.1 <sup>b</sup>

Values with different superscript letters in the same column differ ( $P<0.05$ ). Values are shown as mean ± s.e.m.

**Table 3.** Means of total cells number, apoptotic cells number and apoptotic index of blastocysts to Control and Heat Shocked oocytes groups from oocytes *in vitro* matured.

Treatments	n	Total Cells	Apoptotic Cells	Apoptotic Index
Control	20	113.15±6.62 <sup>a</sup>	10.05±0.99 <sup>a</sup>	8.75±0.69 <sup>b</sup>
Heat Shock	21	96.29±6.59 <sup>a</sup>	12.48±1.16 <sup>a</sup>	12.34±0.80 <sup>a</sup>

Values with different superscript letters in the same column differ ( $P<0.05$ ). Values are shown as mean ± s.e.m. N= embryos number.



*Figure 2. Representative images of DAPI and TUNEL-labeling embryos illustrating the total cell number and apoptotic nuclei (TUNEL positive) in bovine blastocysts derived from control oocytes or oocytes exposed to 12h of heat shock during *in vitro* maturation. Scale bar: 50  $\mu$ m. DAPI labeling: A and B, TUNEL labeling: A' and B'. Control group: A and A', heat shock group: B and B'.*

### Chromatin structure analysis of embryos at early stages

For the quantitative analysis, number and size of spots (representing accumulation of H3K9me3 or HP1) in each nucleus were counted and measured. The number of H3K9me3 spots in the nuclei of 4-cell stage embryos was greater ( $P<0.05$ ) in heat shock group than in control one, whereas the spots mean size was not different. At 8-cell stage the number of spots was significantly lower ( $P<0.01$ ) but the mean size was higher ( $P<0.02$ ) in the heat shock than in the control group (Figure 3). That increase of spots size with a decrease of spot number at 8-cell stage in the heat shock group is likely due to increase of H3K9 trimethylation that results in the fusion of several spots forming bigger but fewer spots than in the control group. The mean size of H3K9me3 spots increased between 4- and 8-cell stages for both control ( $385\pm65$  and  $509\pm44$  voxels, respectively) and heat shock ( $347\pm32$  and  $692\pm42$  voxels, respectively) groups.

The number and mean size of HP1 spots in the nuclei were not statistically different ( $P>0.05$ ) between heat shock and control group (Figure 3). However, in both cases, the total number of HP1 spots slightly decreased suggesting that the same mechanisms of fusion occur for HP1 and H3K9me3. It is also noteworthy that while HP1 spots increased in size between 4-cell and 8-cell stages (from  $250\pm39$  to  $398\pm47$  voxels, respectively) in the control group, it decreased in the heat shock group ( $404\pm88$  and  $280\pm27$  voxels for 4-cell and 8-cell stages, respectively).

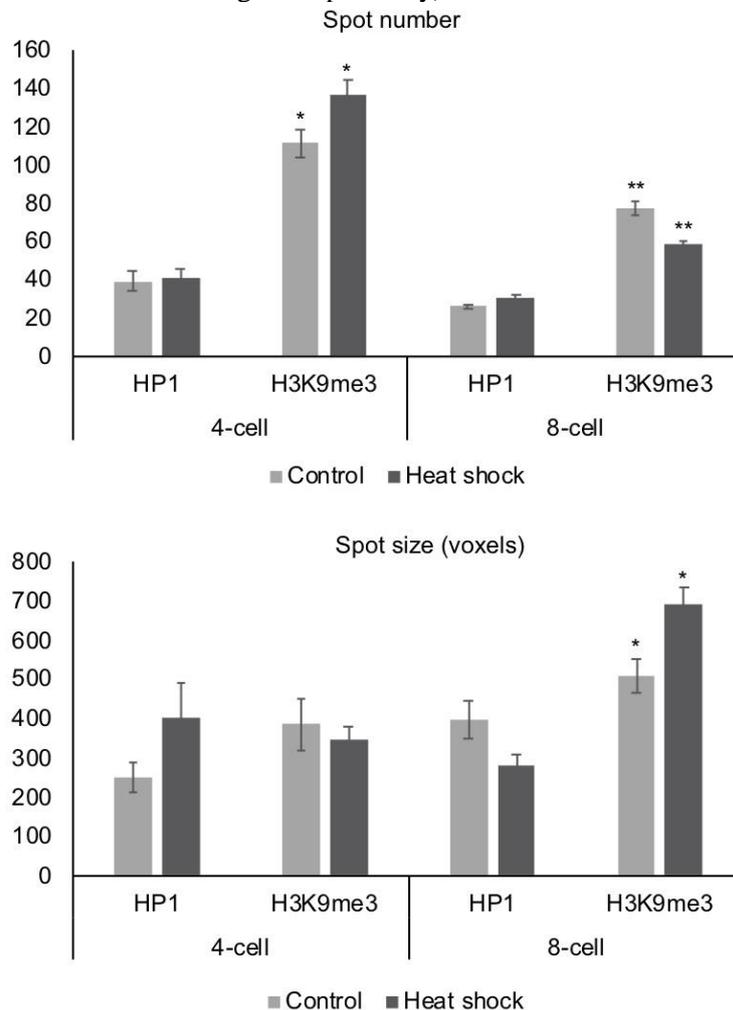


Figure 3: Analysis of trimethylation of histone3 lysine 9 (H3K9m3) and heterochromatin protein 1 (HP1) in nuclei of four-cell and eight-cell stage bovine embryos derived from control oocytes or oocytes exposed to 12h of heat shock during in vitro maturation. Accumulation of HP1 or H3K9m3 is given by spot size and number per nucleus. Asterisks indicate differences between control and heat shock groups within the same patterns of staining (\* $P<0.05$ ; \*\* $P<0.01$ ). Spots size (volume) = voxels. Data of spots number and size were compared by analysis of variance.

The analysis of fluorescence enabled to distinguish embryos displaying different patterns of chromatin staining between control and heat shock groups. For H3K9me3 immunofluorescence there was an increased ( $P<0.01$ ) proportion of embryos at 4-cell stage from heat shock group displaying nuclei with globular pattern (43.7%) when compared to control group (5.5%), whereas no difference ( $P>0.05$ ) was found for the fibrous and spread patterns (Figure 4-A). At 8-cell stage, distribution of H3K9me3 was similar ( $P>0.05$ ) between control and heat shock groups for globular and fibrous patterns (Figure 4-B) and no spread distribution was found in both groups. For HP1, the heat shock group presented higher ( $P<0.05$ ) proportion of 4-cell stage embryos with fibrous pattern and lower ( $P<0.05$ ) proportion with spread pattern than the control group, and no globular pattern was found (Figure 4-C). At 8-cell stage there was no statistical difference between the groups except that no spread pattern was found in nuclei of embryos in the heat shock group (Figure 4-D). Those results show that the heat shock during *in vitro* maturation increases the proportion of embryos displaying nuclei with an elevated accumulation of H3K9me3 (globular pattern) and HP1 (fibrous pattern) at the 4-cell stage.

Overall, the higher number of immunofluorescence spots indicates that an increased accumulation of H3K9me3 in the nuclei of embryos derived from heat-shocked oocytes can be found at 4-cell stage, one cell cycle earlier than nuclei of embryos derived from non-heat-shocked oocytes. This resulted in larger spots but in a small amount at 8-cell stage probably due to the union of the several spots found at 4-cell stage. That precocious accumulation can also be perceived by the high proportion of 4-cell embryos displaying nuclei with strong immunofluorescence staining for H3K9me3 when they are derived from heat-shocked oocytes. Similarly, the higher proportion of embryos displaying nuclei with HP1 fibrous patterns in the heat shock group at the 4-cell stage correlates with the slight increase observed in the size of HP1 spots between embryos from heat shock and control groups at that stage ( $250\pm 39$  vs.  $404\pm 88$  voxels, respectively).

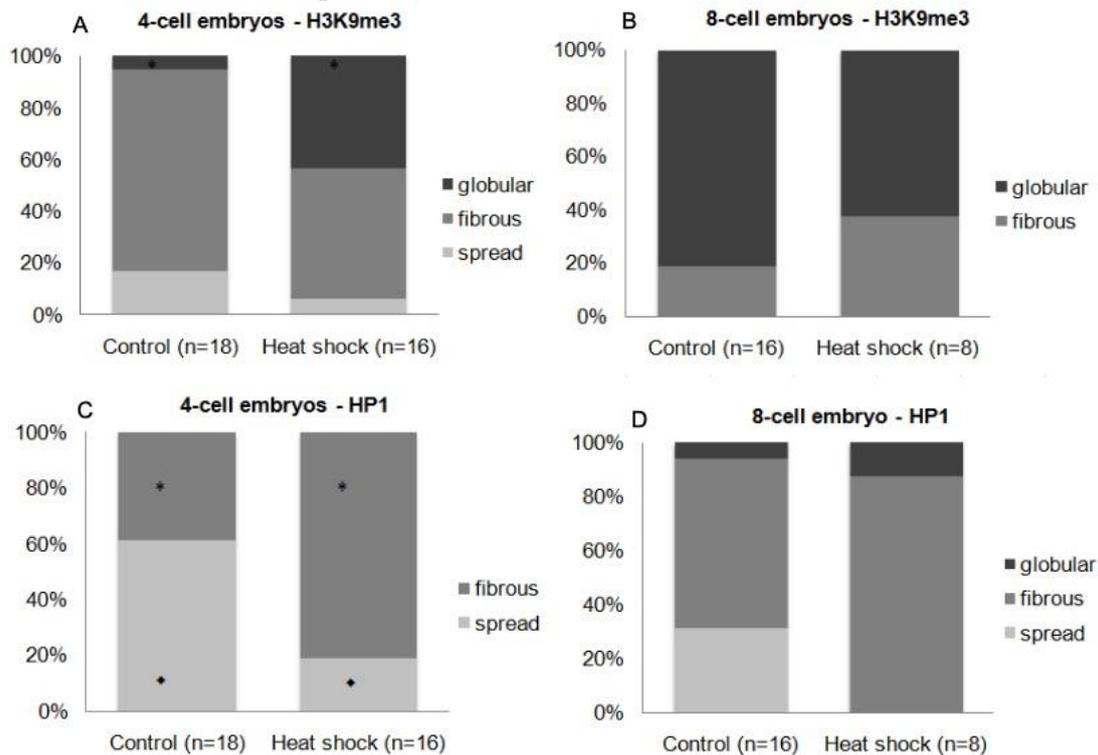


Figure 4: Percentage of four-cell and eight-cell stage bovine embryos displaying different patterns of histone 3 lysine 9 trimethylation (H3K9me3) and heterochromatin protein 1 (HP1) staining in the nucleus. Spread: uniform staining, fibrous: small spots displaying various levels of staining, and globular: evident and globular spots displaying strong staining. Control: embryos derived from non-heat-shocked oocytes; Heat shock: embryos derived from oocytes exposed to 12h of heat shock during *in vitro* maturation. N = number of embryos. Asterisks (\*) indicate differences between control and heat shock groups within the same patterns of staining ( $P<0.01$  for H3K9me3 and  $P<0.05$  for HP1). Data of embryos displaying nuclei with different staining patterns was compared by Fischer Exact test.

### Effect of heat shock on transcript levels in embryos

The relative expression of four target genes (*HSF1*, *HSP90A1*, *HSP40* and *OCT4*) was investigated in order to associate the heat shock during *in vitro* maturation with gene expression in embryos at 8-cell stage. The amount of *HSP40* transcripts was lower ( $P < 0.05$ ) in embryos derived from heat-shocked oocytes while no difference ( $P > 0.05$ ) between groups was found for *HSF1*, *HSP90* and *OCT4* genes (Figure 5).

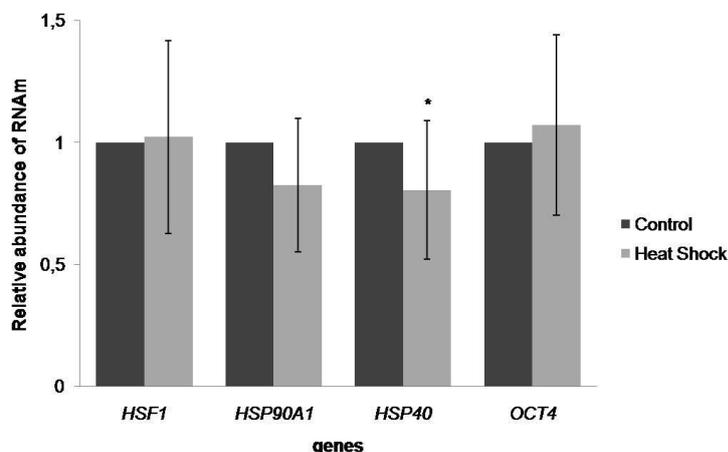


Figure 5: Relative abundance transcripts evaluation of *HSF1*, *HSP90A1*, *HSP40* and *OCT4* in 8-cell stage bovine embryos derived from oocytes exposed to 12h of heat shock during *in vitro* maturation. Data of Heat Shock were compared to Control group (calibrator). Asterisk (\*) indicates difference ( $P > 0.05$ ) between control and heat shock groups. Data from three replicates. Data of relative transcript quantification was performed by Comparative Ct method and analyzed by mixed model using the Wise Fixed Reallocation Randomization TEST in REST software (Pfaffl *et al.* 2002).

### Discussion

Heat stress has been a constant concern during hot seasons in both tropical and subtropical regions in cattle production systems because of its effects on reproductive and productive performance. The deleterious effect on reproduction is associated to oocytes with poor developmental competence. As previously reported (Edwards & Hansen 1997, Hansen *et al.* 2001, Roth & Hansen 2004, Roth & Hansen 2005,) we found that the heat shock during *in vitro* maturation decreases oocyte ability to develop toward blastocysts stage after *in vitro* fertilization and compromises embryo quality by increasing the apoptosis index. Such low developmental competence has been associated to several cytoplasmic, molecular and nuclear alterations (Payton *et al.* 2004, Roth & Hansen 2005, Roth 2015); however, little is known about the effect of heat shock during oocyte *in vitro* maturation on chromatin remodeling post-fertilization. In this study we showed that alterations on chromatin organization can be found in early embryos derived from oocytes exposed to heat shock during *in vitro* maturation. The immunofluorescence analysis revealed that the nuclei of 4- and 8-cell embryos had higher accumulation of H3K9me3. Also, a higher proportion of 4-cell embryos presented nuclei with strong fluorescence for H3K9me3 and HP1 when compared to embryos derived from control oocytes.

In bovine, H3K9 methylation is normally low in embryos at 4-cell stage and increases from 8-cell stage onwards [Santos *et al.* 2003, Wu *et al.* 2011). In our study we found that the size of H3K9me3 spots increased from 4-cell to 8-cell stage in embryos from control and heat shock groups, confirming those previous studies. Interestingly, the mean size of HP1 spots increased from 4-cell to 8-cell in the control group only. As H3K9me3 is a binding site for HP1 (Lachner *et al.* 2001), the increase of heterochromatinization at the 8-cell stage was expected as in the control group. However, it was not observed in the heat shock group. The size of HP1 spots reduced between the 4- and 8-cell stages in the heat shock group ( $404 \pm 88$  vs  $280 \pm 27$  voxels, respectively), despite the notable increase of H3K9me3 spots size. The large HP1 spots size as well as the proportion of embryos with the fibrous pattern nuclei suggest that heterochromatinization is stronger in 4-cell stage embryos from heat-shocked oocytes when compared to control group or even to the 8-cell stage. Those findings indicate that the heat shock during oocyte maturation may hasten H3K9 trimethylation and heterochromatin formation in 4-cell stage embryos.

Methylation of H3K9 associated with heterochromatinization has a repressive role on gene expression (Eisemberg & Elgin 2000, Lachner *et al.* 2001, Grewal & Jia 2007) and this silencing is critical to ensure the appropriated gene activation during the EGA (Nothias *et al.* 1995, Mason *et al.* 2012). Previous study showed that there is a co-localization between H3K9me3 and HP1 at 8-cell stage in bovine embryos fertilized *in vitro* (Pichugin *et al.* 2010), which coincides with major EGA. The unexpected accumulation of H3K9me3 and HP1 in the nuclei of 4-cell embryos and the abnormal formation of numerous H3K9me3 condensed spots in 8-cell embryos derived from heat-shocked oocytes indicate alterations of chromatin remodeling that can perturb the EGA and, consequently, gene expression.

It was shown previously that expression of some genes, as *POU5F1* and *GAPDH*, can be altered in 8-cell embryos derived from oocytes collected in the hot season (Gendelman & Roth 2012). In the present study we also found that the relative expression of specific gene can be altered in 8-cell embryos derived from oocytes exposed to heat shock during *in vitro* maturation. We analyzed the expression of genes that encode proteins associated to heat-shock (*HSF1*, *HSP90* and *HSP40*) and pluripotency (*OCT4*). Despite there was no difference on relative abundance of *HSF1*, *HSP90* and *OCT4* transcripts between treatments, the expression of *HSP40* was downregulated in those embryos derived from heat-shocked oocytes, suggesting that the heat shock during oocyte maturation may perturb the expression of some genes in embryos at 8-cell stage. *HSP40* is a co-chaperone with important role on cell protection under stress conditions (Gotoh *et al.* 2004, Lanneau *et al.* 2007) and alterations on its expression may impair further embryonic development under *in vitro* suboptimal environment. However, alterations on gene expression caused by the heat shock during *in vitro* maturation may be not gene specific presuming that such alterations are due to a random alteration on chromatin remodeling. This may be one of the reasons for some early stage embryos develop toward blastocyst stage whereas others do not.

How the heat shock during IVM can affect embryo chromatin is not clear but it may be related to disturb on redox balance. Accumulation of reactive oxygen species (ROS) is known to cause DNA damage and affects histones and DNA methylation (Niu *et al.* 2015, Mikhed *et al.* 2015). Recently it was shown that the paternal pronucleus retains the DNA methylation when the oocyte is fertilized with spermatozoa submitted to oxidative stress (Wyck *et al.* 2018). As the heat shock increases the ROS production in bovine oocytes matured *in vitro* (Ascari *et al.* 2017), the alterations found in our study could be in part associated to an increased level of ROS interfering in the chromatin organization in the first cell cycles of embryos derived from heat-shocked oocytes.

This is the first study showing that the heat shock during oocyte *in vitro* maturation can alter the chromatin organization in early *in vitro*-fertilized embryos. We suggest that alterations on accumulation of H3K9me3 and HP1 caused by heat shock during *in vitro* maturation can negatively influence the chromatin remodeling during the EGA, affecting the expression of some genes, and contribute to impair the embryo development and quality, represented by lower blastocysts rate and higher apoptotic index.

In conclusion, the heat shock during *in vitro* maturation can induce alterations of H3K9me3 and HP1 accumulation in early bovine embryos, affecting embryo development and quality. Our data indicates that heat shock before fertilization can have an epigenetic effect on embryos and highlights the need of studies to understand how such alterations can influence the minor and major genome activation and how they can be modulated, in an effort to contribute to mitigate the heat stress effects on reproduction.

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### **Disclosure statement**

No potential conflict of interest was reported by the authors.

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