Zygote **23** (August), pp. 485–493. © Cambridge University Press 2014
doi:10.1017/S0967199414000100 First Published Online 15 April 2014

Phosphorylated H2AX in parthenogenetically activated, *in vitro* fertilized and cloned bovine embryos

A.F. Pereira[2], *L.M. Melo*[2], *V.J.F. Freitas*[2] *and D.F. Salamone*[1,3]
School of Veterinary Medicine, Ceará State University, Fortaleza, Brazil; and School of Agronomy, Buenos Aires University, Buenos Aires, Argentina

Date submitted: 1.10.2013. Date revised: 24.1.2014. Date accepted: 25.1.2014

Summary

In vitro embryo production methods induce DNA damage in the embryos. In response to these injuries, histone H2AX is phosphorylated (γH2AX) and forms foci at the sites of DNA breaks to recruit repair proteins. In this work, we quantified the DNA damage in bovine embryos undergoing parthenogenetic activation (PA), *in vitro* fertilization (IVF) or somatic cell nuclear transfer (SCNT) by measuring γH2AX accumulation at different developmental stages: 1-cell, 2-cell and blastocyst. At the 1-cell stage, IVF embryos exhibited a greater number of γH2AX foci (606.1 ± 103.2) and greater area of γH2AX staining (12923.6 ± 3214.1) than did PA and SCNT embryos. No differences at the 2-cell stage were observed among embryo types. Although PA, IVF and SCNT were associated with different blastocyst formation rates (31.1%, 19.7% and 8.3%, $P < 0.05$), no differences in the number of γH2AX foci or area were detected among the treatments. γH2AX is detected in bovine preimplantation embryos produced by PA, IVF and SCNT; the amount of DNA damage was comparable among those embryos developing to the blastocyst stage among different methods for *in vitro* embryo production. While IVF resulted in increased damage at the 1-cell embryo stage, no difference was observed between PA and SCNT embryos at any developmental stage. The decrease in the number of double-stranded breaks at the blastocyst stage seems to indicate that DNA repair mechanisms are functional during embryo development.

Keywords: Chromatin, Cloning, DNA breakage, Histone phosphorylation, Preimplantation embryo

Introduction

Since the birth of the first *in vitro* produced cattle (Lu *et al.*, 1988), several studies have been carried out to improve the efficiency of *in vitro* embryo production systems (Brandão *et al.*, 2004; Gonçalves *et al.*, 2010; La Rosa *et al.*, 2011). *In vitro* production (IVP) of embryos by parthenogenetic activation (PA), *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT) are used for different purposes, including basic research in developmental biology, the production of transgenic animals and commercial applications (Gali *et al.*, 2003; Salamone *et al.*, 2006; Paffoni *et al.*, 2008; Pereira *et al.*, 2013).

Under the conditions of *in vitro* culture, pre-fusion gametes and embryos are exposed to different types of stress according to methods for IVP. Oxidative stress and reactive oxygen species damage cell membranes and cause DNA fragmentation and mitochondrial dysfunction (Aitken & Clarkson, 1987; Aitken *et al.*, 1989; Takahashi *et al.*, 1999), decreasing the efficiency of IVP systems. Also, epigenetic responses during preimplantation development are affected by *in vitro* conditions in some species, such as cattle, mouse and man (Barton *et al.*, 2001; Shi & Haaf, 2002; Santos *et al.*, 2003). In addition, environmental stresses such as ionizing radiation (Ismail & Hendzel, 2008) may also induce DNA damage such as double-stranded breaks (DSBs). In response to these stresses or other types of damage, a group of key proteins cell cycle arrested and allowed DNA repair. The activation of cell cycle

[1]All correspondence to: Daniel Felipe Salamone. Laboratory of Animal Biotechnology, School of Agronomy, Buenos Aires University, Av. San Martín 4453, C1417, Buenos Aires, Argentina. Tel: +54 11 4524 8000. Fax: +54 11 4514 8737. e-mail: salamone@agro.uba.ar
[2]Laboratory of Physiology and Control of Reproduction, School of Veterinary Medicine, Ceará State University, Fortaleza, Brazil.
[3]Laboratory of Animal Biotechnology, School of Agronomy, Buenos Aires University, Buenos Aires, Argentina.

https://doi.org/10.1017/S0967199414000100 Published online by Cambridge University Press

checkpoints is crucial to allow appropriate progression through the cell cycle, efficiently maintain the integrity of the genetic material and ensure genomic stability (Fernandes-Capetillo *et al.*, 2003; Stiff *et al.*, 2004; Gawecka *et al.*, 2013).

The histone H2AX is an H2A family variant with a conserved Ser–Gln–Glu motif at the carboxyl terminal of the protein; the serine of this motif (Ser139) is rapidly phosphorylated by ataxia telangiectasia mutated within minutes after DNA damage (Rogakou *et al.*, 1998; Redon *et al.*, 2002; Sedelnikova *et al.*, 2003). The phosphorylated form of H2AX is referred to as γH2AX, and the γH2AX foci that form at the site of DNA damage serve to recruit repair proteins (Fernandez-Capetillo *et al.*, 2004). In this way, γH2AX plays an important role in DNA repair and chromatin remodelling during the DNA damage response. It has been proposed that γH2AX leads to recombination and conformational changes in chromatin (Fernandez-Capetillo *et al.*, 2003; Reina-San-Martin *et al.*, 2003), preventing the early separation of broken ends.

Different factors could be involved in the induction of the DNA damage that occurs during the preimplantation period of *in vitro* manipulated embryos. The DNA damage induced by transgene injection in early embryonic stages is reflected by the formation of γH2AX foci (Bevacqua *et al.*, 2012). Moreover, H2AX phosphorylation can be used as a DNA DSB marker in spermatozoa before intracytoplasmic sperm injection (Li *et al.*, 2009). Therefore, the aim of this study was to evaluate the expression pattern of γH2AX, which is an indicator of DNA DSBs, in parthenogenetically activated, *in vitro* fertilized and cloned bovine embryos at different times of the preimplantation period to quantify the DNA damage and the dynamics of these lesions over time.

Materials and methods

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. M199 and the media used for the preparation of donor cells were obtained from Gibco (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Internegocios (Buenos Aires, Argentina). Bovine oocytes were collected from abattoir-derived ovaries in Buenos Aires Province, Argentina. All trials were conducted in accordance with the guidelines for animal care (ASAB, 2006).

Oocyte preparation

Cumulus–oocyte complexes (COCs) with more than three layers of cumulus cells and with a compact and evenly granulated cytoplasm were cultured in fresh, preincubated bicarbonate-buffered M199 medium that contained 10% FBS, 10 μg/ml follicle-stimulating hormone (NIH-FSH-P1, Follitropin, Bioniche, Caufield Junction Caufield North, Australia), 0.3 mM sodium pyruvate (P2256), 100 μM cysteamine (M9768) and 2% antibiotic-antimycotic (15 240–096; Gibco). The oocytes were incubated under mineral oil (M8410) in 100 μl droplets at 39°C in a humidified atmosphere that contained 6.5% CO_2 in air for approximately 21–24 h.

After maturation, the oocytes were isolated for PA and SCNT. Cumulus cells were completely removed by vortexing COCs in 0.1% hyaluronidase (H4272) in Dulbecco's phosphate-buffered saline (DPBS, 14287–072; Gibco) for 2 min and washed three times in HEPES-buffered Tyrode's albumin, lactate and pyruvate (HEPES-TALP) medium. Denuded mature oocytes, which were confirmed by visualization of the first polar body, were immediately used for PA and SCNT, whereas intact COCs were used for *in vitro* fertilization (IVF).

In vitro embryo production

In vitro matured oocytes were divided in three groups: PA, IVF and cloned embryos derived by SCNT.

PA embryos

In vitro matured oocytes were activated with 5 μM ionomycin (I24222; Gibco) in HEPES-TALP for 4 min at 37°C, followed by washing in HEPES-TALP. The oocytes were subsequently transferred to 1.9 mM 6-dimethylaminopurine (6-DMAP; D2629) in synthetic oviductal fluid (SOF) droplets for 3 h at 39°C. The oocytes were then washed three times in HEPES-TALP to remove the 6-DMAP, and culture was continued as described below.

IVF embryos

In vitro matured oocytes were fertilized in Brackett's fertilization medium, as described by Brackett & Oliphant (1975). Briefly, bovine semen from two bulls was thawed at 37°C for 30 s. The spermatozoa were washed twice by centrifugation at 490 *g* for 5 min in Brackett's defined medium. The sperm concentration was adjusted to 15×10^6/ml, and sperm were then co-incubated for 5 h with COCs in 100 μl droplets. The IVF was carried out in an atmosphere of 6.5% CO_2 in humidified air at 39°C. After IVF, the presumptive zygotes were washed three times in HEPES-TALP and cultured *in vitro* as described below.

SCNT embryos

The recipient cytoplast preparation and nuclear transfer procedures were performed as described previously (Oback *et al.*, 2003). Briefly, denuded

https://doi.org/10.1017/S0967199414000100 Published online by Cambridge University Press

mature oocytes were incubated in a drop of 1.5 mg/ml pronase (P8811) in HEPES-TALP to remove the zona pellucida. Zona-free oocytes were incubated with 1 μg/ml Hoechst 33342 (B2261) and 10 μg/ml cytochalasin B (C6762) for 10 min, after which they were enucleated with a glass micropipette by aspirating the metaphase II chromosomes along with a small volume of the surrounding cytoplasm. Following aspiration, approximately 10 individual fetal fibroblast cells were picked up with a mouth pipette and added to a drop of 10 mg/ml phytohemagglutinin (PHA-P; L8754) in HEPES-TALP that already contained 10 cytoplasts. Fetal fibroblasts were obtained through primary culture from tail biopsies of day 30 fetus. Cytoplasts and donor cells were pushed together with the mouth pipette and incubated for at least 5 min. Then, groups of 10 couplets were transferred into HEPES-TALP without PHA-P. After cell adhesion, the reconstructed embryos were electrofused by two 1.2 kV/cm DC pulses of 30 μs (Electroporator ECM830; BTX Instrument Division; Harvard Apparatus, Holliston, USA). All embryos were activated 2–3 h post-fusion using a combination of ionomycin and 6-DMAP, as described for the PA embryos.

In vitro culture conditions and evaluation of embryo development

Presumptive zygotes were cultured *in vitro* in 50 μl droplets of SOF supplemented with 2.5% FBS and 0.4% BSA (A6003) under mineral oil (Bevacqua *et al.*, 2012). Cloned embryos were placed individually into microwells using the well-of-the-well (WOW) system based on Vajta *et al.* (2000). The embryos were cultured in an incubator with 6.5% CO_2 in humidified air at 39°C. The PA and IVF zygotes were transferred to new droplets at days 2 and 5, while for SCNT, 50% of the medium was replaced with fresh medium supplemented with 5% FSB at day 5. For the detection of γH2AX, one-cell (1-cell) embryos were fixed at 16 h after either the beginning of oocyte activation or the spermatozoa-oocyte co-incubation. Two-cell (2-cell) embryos and blastocysts (Bl) were fixed at 48 h and 7 days after the beginning of *in vitro* culture, respectively. Embryo cleavage was evaluated on days 1–2, and the number of blastocysts was counted on days 7–8 post *in vitro* culture.

Indirect immunofluorescence analysis

Whole-mount immunofluorescence for quantitative γH2AX detection was performed on the bovine embryos obtained by the three methods (PA, IVF and SCNT) at each of three developmental stages (1-cell, 2-cell and blastocyst). Briefly, embryos were treated to remove the zona pellucida (PA and IVF zygotes), fixed in 4% paraformaldehyde (F1635) in PBS for 30 min and then permeabilized for 30 min at room temperature in PBS that contained 0.1% v/v Triton X-100 (T9284) and 3% FBS. The embryos were then washed three times in 0.1% Tween PBS (PBS-T; Promega, Madison, IL, USA) and blocked and incubated with antiphospho-histone H2AX antibody (1:100 dilution, 05–636, Upstate Biotechnology, Upstate, SC, USA) in 3% FBS 0.1% Tween in PBS for 2 h at 37°C. All embryos were then washed twice in PBS-T, blocked for 30 min and incubated for 45 min at 37°C with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (F6257, anti-rabbit IgG, 1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS-T in the dark. After additional washing, the embryos were incubated in PBS that contained propidium iodide (P4170; 1:100 dilution) in the dark for 10 min. Negative controls for γH2AX detection were treated with secondary antibody only. Stained embryos were mounted on slides in 70% glycerol.

Confocal laser scanning microscopy

All embryos were analysed on a Nikon Confocal C.1 scanning laser microscope. An excitation wavelength of 488 nm was selected from an argon-ion laser to excite the secondary antibody, and a wavelength of 544 nm was used to excite the propidium iodide. When necessary, images of serial optical sections were recorded every 1.5–2 μm vertical step along the Z-axis of each embryo. Three-dimensional images were constructed using EZ-C1 2.20 software (Nikon Corporation, Melville, USA).

Image analysis and γH2AX quantification

Images of embryos from each *in vitro* embryo production method (PA, IVF and SCNT) were obtained for γH2AX quantification. At least eight, 10 and four images derived from independent replicates were collected for 1-cell embryos, 2-cell embryos, and blastocysts, respectively. At the 1-cell stage, only IVF embryos with two pronuclei were used, and the pronuclei were classified as small and large. Both pronuclei were evaluated separately or combined in each structure. For the 2-cell stage, a mean of two nuclei was used. For the blastocyst stage, only 10 independently observed nuclei were analyzed.

The integrated fluorescence intensities of the nuclei were measured using ImageQuantiTL software (GE Healthcare, Amersham Biosciences Corporation, Piscataway New Jersey, USA). After subtracting the background, each nucleus was manually outlined and exposed to quantify the number of γH2AX foci and area of damaged chromatin. The number of γH2AX foci in each image was defined as the total number of spots measured by the software. The area of

https://doi.org/10.1017/S0967199414000100 Published online by Cambridge University Press

Table 1 *In vitro* preimplantation development in parthenogenetically activated (PA), *in vitro* fertilized (IVF) and cloned by somatic cell nuclear transfer (SCNT) bovine embryos

Embryo type	*n*	Cleavage (%)	Blastocyst (%)[A]
PA	350	264 (75.4)[a]	82 (31.1)[a]
IVF	184	142 (77.2)[a]	28 (19.7)[b]
SCNT	270	218 (80.7)[a]	18 (8.3)[c]

[A] The blastocyst rate was calculated with respect to the number of cleaved embryos.
[a,b,c] Within a column, means with a different superscript are significantly different ($P < 0.05$).

the nucleus was considered as the area of the spot displaying the number of pixel quantified in the image to produce the measurements. This area in each image was also used to adjust the count with respect to the average nucleus area because nucleus size varied considerably within the cell population.

Data and statistical analysis

All data were expressed as the means of individual values obtained from at least 10 replicates in an *in vitro* embryo production system. GraphPad software was used for all statistical analyses. The cleavage rate was calculated as the number of cleaved embryos at days 1 to 2 divided by the total number of embryos in culture. Blastocyst rate was calculated as the number of blastocysts at days 7 to 8 divided by the number of cleaved embryos. Cleavage and blastocyst rates were compared among groups by Fisher's exact test.

Differences in the numbers of γH2AX foci and damaged chromatin area per nucleus were analysed using one-way analysis of variance (ANOVA) of log10 transformed data, followed by Tukey's post-hoc test. Differences were considered significant at $P < 0.05$.

Results

After 10 replications, 804 structures were distributed in different groups for *in vitro* embryo production: PA ($n = 350$), *in vitro* fertilization, IVF ($n = 184$), and SCNT ($n = 270$). Cleavage rates were similar for all embryo types ($P > 0.05$). Totals of 82, 28 and 18 blastocysts were produced in the PA, IVF and SCNT groups, respectively, and the highest proportion of blastocysts was observed in PA embryos ($P < 0.05$, Table 1).

High levels of γH2AX were detected in bovine preimplantation embryos produced by PA (Fig. 1*A*–*C*), IVF (Fig. 1*A*′–*C*′) and SCNT (Fig. 1*A*″–*C*″) at all developmental stages. At the 1-cell stage, IVF embryos exhibited a higher γH2AX focus number (606.1 ± 103.2) and area (12923 ± 3214.1 pixels) than did PA- and SCNT-derived embryos (Table 2). Both pronuclei of IVF embryos exhibited similar γH2AX patterns in terms of γH2AX focus number (377.5 ± 67.7 vs 228.6 ± 42.5; large vs small pronuclei, respectively, $P > 0.05$) and damaged chromatin area (8698.9 ± 294.8 vs 4224.8 ± 1017.2; large vs small pronuclei, respectively, $P > 0.05$). In contrast, at the 2-cell stage, both PA and SCNT tended to show greater numbers of γH2AX foci than did IVF embryos (Table 2).

Although PA, IVF and SCNT yielded different blastocyst production rates, no differences in the number and area of γH2AX foci were detected among the groups (Table 2). Moreover, when evaluating the dynamics of γH2AX for the different IVP systems, similar numbers of γH2AX foci were observed in PA (1-cell: 163.3 ± 14.2; 2-cell: 367.9 ± 119.7; Bl: 133.8 ± 15.6) and SCNT embryos (1-cell: 200.8 ± 37.2; 2-cell: 682.9 ± 251.0; Bl: 134.5 ± 20.0). An increase in the number of γH2AX foci per nucleus tended to be detected by the 2-cell stage, followed by a reduction at the blastocyst stage. Interestingly, the γH2AX dynamics of IVF embryos were different (1-cell: 606.1 ± 103.2; 2-cell: 260.8 ± 88.0; Bl: 160.0 ± 22.2) from those of the PA and SCNT embryos.

Discussion

Although it has been employed successfully for nearly 3 decades, IVP of embryo by PA, IVF and SCNT still has several limitations that make it less efficient than *in vivo* production systems. The present study demonstrated that bovine embryos derived by PA, IVF and SCNT exhibited a high number of DNA DSBs by measuring γH2AX accumulation. In addition, the pattern of H2AX phosphorylation exhibited dynamic changes during preimplantation development (1-cell, 2-cell and blastocyst stages) within each treatment, and these dynamics varied among different IVP methods. While IVF embryos exhibited higher numbers of DSBs by the 1-cell stage, PA and SCNT embryos showed a tendency of greater numbers of breaks by day 2. In all embryo types, the numbers of DSBs decreased from the 2-cell stage to the blastocyst stage, indicating that DNA repair mechanisms are functional in blastocysts. In porcine embryos also DSBs occur in a high proportion in structures produced by either IVF or SCNT, proposing that the presence of H2AX foci can be a useful marker of embryo quality of this species (Bohrer *et al*., 2013).

Initially, it was observed that PA embryos showed greater developmental potential than IVF and SCNT IVP embryos. This effect could be due to the efficiency of this *in vitro* system, which depends only oocyte competence, while IVF and SCNT embryos are

https://doi.org/10.1017/S0967199414000100 Published online by Cambridge University Press

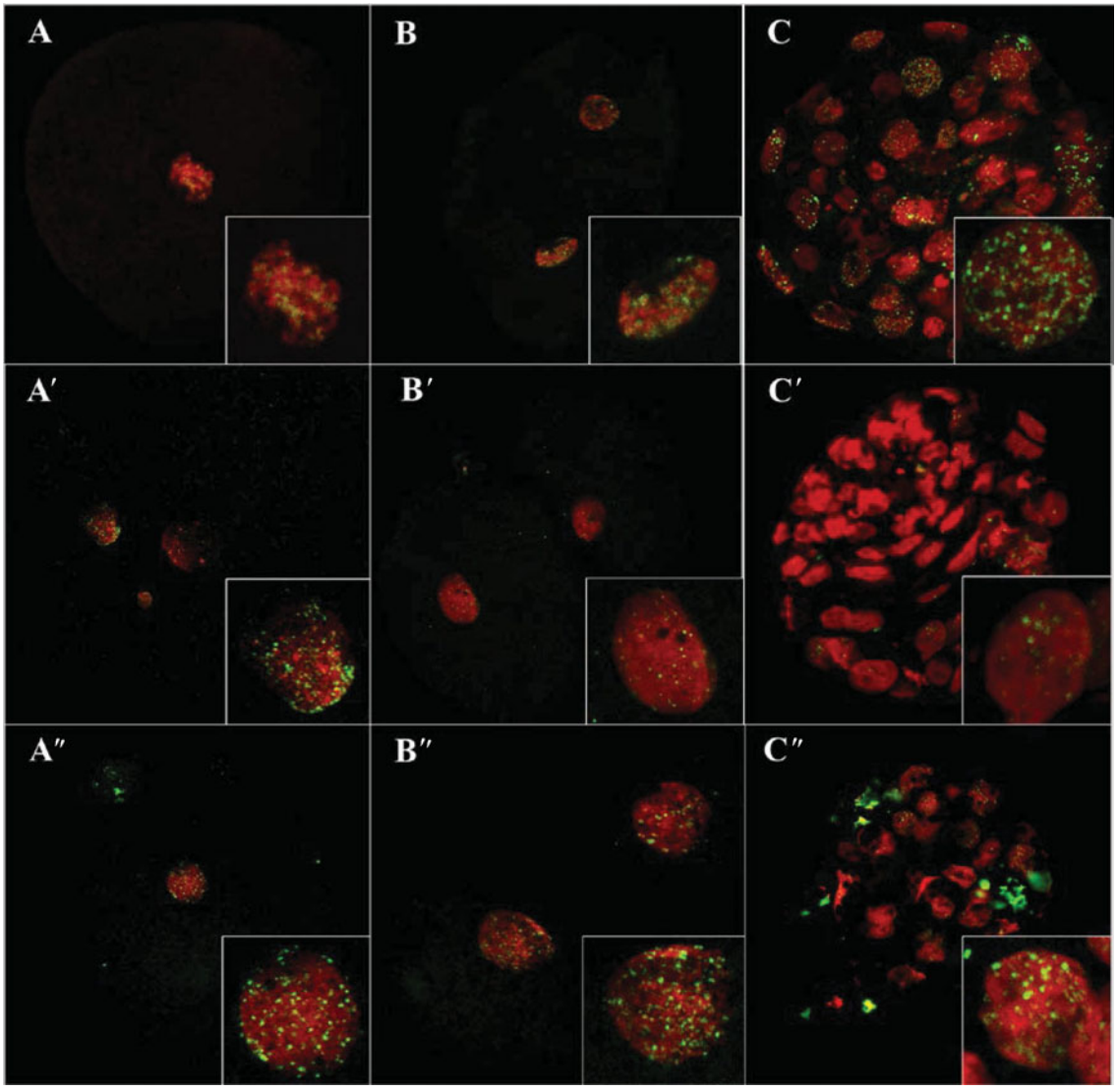


Figure 1 The phosphorylation of histone H2AX (γH2AX) in bovine preimplantation embryos. Data are presented for parthenogenetically activated (A–C), *in vitro* fertilized (A′–C′) and cloned by somatic cell nuclear transfer (A″–C″) embryos. The developmental stages analyzed include the 1-cell stage (A–A″), collected at 16 h after initial oocyte activation, spermatozoa–oocyte co-incubation or reconstructed zygote activation; the 2-cell stage (B–B″), recovered after 24 h of *in vitro* culture; and blastocysts (C–C″), recovered after 7 days of *in vitro* culture. Embryos were labelled by immunofluorescence using an antibody to γH2AX (green); DNA was counterstained with propidium iodide (red). All images are presented as a juxtaposition of the red and green channels (×400).

influenced also by spermatozoa quality and donor cell reprogramming, respectively.

In a subsequent step, this work aimed to evaluate the correlation between IVP system efficiency and the induction of DNA damage. Other methodologies may be used to assess DNA damage in cells and embryos (Hosseini *et al.*, 2011; Gawecka et al., 2013), however the use of γH2AX can explain how embryos respond to such damage. Phosphorylated H2AX was detected in 1-cell embryos, 2-cell embryos and blastocysts, suggesting that the mechanisms involved in the G2/M checkpoint and DNA repair are active in these IVP embryos. It has been demonstrated previously that approximately 2000 histone H2AX molecules near the site of the DSB are phosphorylated at Ser-139 (Pilch *et al.*, 2003), producing one focus that corresponds to a single DSB site in the nucleus (Kaneko *et al.*, 2005). Interesting, H2AX was highly phosphorylated throughout development in the absence of any induced DNA damage in mouse embryo produced by *in vivo* fertilization (Ziegler-Birling *et al.*, 2009), mammalian cell lines and primary cell cultures (McManus & Hendzel, 2005). Moreover, H2AX levels ranged significantly during the cell cycle, suggesting

https://doi.org/10.1017/S0967199414000100 Published online by Cambridge University Press

Table 2 Quantification of γH2AX foci and chromatin area with damage in bovine embryos produced by parthenogenetic activation (PA), *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT)

	Mean ± SEM foci			Mean ± SEM foci area (pixel)		
Embryo type	1-cell	2-cell	Bl	1-cell	2-cell	Bl
PA	$163.3 \pm 14.2^{A,a}$	$367.9 \pm 119.7^{A,a}$	$133.8 \pm 15.6^{A,a}$	$1204.3 \pm 256.2^{A,a}$	$5263.2 \pm 2163.9^{A,a}$	$2495.9 \pm 738.4^{A,a}$
IVF	$606.1 \pm 103.2^{A,b}$	$260.8 \pm 88.0^{B,a}$	$160.0 \pm 22.2^{B,a}$	$12923 \pm 3214.1^{A,b}$	$3463.2 \pm 1081.7^{B,a}$	$1729.7 \pm 378.1^{B,a}$
SCNT	$200.8 \pm 37.2^{A,a}$	$682.9 \pm 251.0^{A,a}$	$134.5 \pm 20.0^{A,a}$	$2152.0 \pm 507.6^{A,a}$	$10130.0 \pm 6021.6^{A,a}$	$1896.3 \pm 748.5^{A,a}$

$^{a\,b,c}$Within a column, means with a different superscript are significantly different ($P < 0.05$).
^{A,B} Within a row, means with a different superscript are significantly different ($P < 0.05$). For all embryos types, 10, eight and four structures were analyzed at the 1-cell, 2-cell and blastocyst stage, respectively, and the numbers of H2AX foci and focus areas were compared. SEM, standard error of the mean.

that the H2AX phosphorylation is an important event in the fast dividing cells of the early embryo in the absence of any induced DNA damage (Ziegler-Birling *et al.*, 2009), opening the possibility for a role in chromatin assembly and remodelling during these stages.

H2AX is known to be phosphorylated by the ataxia telangiectasia mutated kinase (Rogakou *et al.*, 1998; Kobayashi, 2004), and this phosphorylation is one of the first steps in the DNA damage response pathway, as phosphorylated H2AX triggers the assembly of response proteins (Adiga *et al.*, 2007). γH2AX focus formation is used as a marker of DNA DSBs in irradiated cells, and these foci are even visible at the break and exchange points of metaphase chromosomes (Rogakou *et al.*, 1998). Thus, the γH2AX foci may play an essential role in the efficient recruitment of proteins that are involved in DNA repair.

It was observed that IVF embryos exhibit a higher number of DSBs by the 1-cell stage than PA and SCNT embryos. One interesting observation in this experiment was that this increase was observed not only in the number of γH2AX foci per embryo but also in the number of γH2AX foci in the large IVF pronucleus only. The large IVF pronucleus tended to exhibit a greater number of DSBs than did the IVF female pronucleus (small). The observations from our work are supported by previous studies of male pronucleus formation. Prior to gamete fusion, during spermatogenesis, the postmeiotic spermatid nucleus undergoes a rigorous alteration of its chromatin, with the stepwise removal of most of the nucleosomes from the DNA and their replacement with protamines, which enable the high degree of compaction found in the sperm nucleus (Ward & Coffey, 1991). After entry into the oocyte, the reversal of this nucleosome-based chromatin state is necessary to enable the formation of a pronucleus that can enter S-phase in conjunction with the maternal pronucleus (Loppin *et al.*, 2005). The spermatid nucleus is devoid of DNA repair machinery upon the onset of protamine-induced compaction at stage 12 of spermatogenesis (Kofman-Alfaro & Chandley, 1971; Sega *et al.*, 1978), 2 weeks before ejaculation. Hence, any DNA lesions present in the paternal nucleus at this time must be repaired after entry into the oocyte, by oocyte-stored factors. Consequently, the large number of γH2AX foci observed in the paternal chromosome (large pronucleus) can be attributed to DNA damage induced during the compaction process, as the activity of phosphorylated H2AX has been demonstrated during chromatin restructuring of the male genetic complement in the zygote (Derijck *et al.*, 2006). DNA-damaged spermatozoa can fertilize oocytes and the capacity of early embryos to repair a damaged male genome could be inadequate, suggesting that this damage response may be useful in assessing embryo developmental competence (Grenier *et al.*, 2012).

In addition, during the first cell cycle after fertilization, extensive chromatin remodelling occurs in the zygote, and this appears to be the determining factor for the onset of gene expression (Renard, 1998). This remodelling is accompanied by alterations in DNA methylation, histone modification and the recruitment of various chromatin proteins into the pronucleus. The processes of histone modification and methylation affect male and female pronuclei differently (Adenot *et al.*, 1997; Mayer *et al.*, 2000), even though these structures reside in the same cytoplasm (Liu *et al.*, 2005). In mice, it was demonstrated that the male pronucleus has a larger volume, a higher concentration of transcription factors, exhibits a higher level of histone acetylation (Bouniol *et al.*, 1995; Adenot *et al.*, 1997) and undergoes more extensive DNA demethylation (Mayer *et al.*, 2000) than the female pronucleus. The bovine male pronucleus is morphologically distinct from the female version and can be identified based on two distinct features: (1) it is slightly larger than its female counterpart (Beaujean *et al.*, 2004) and distal from the polar body; and (2) it is more decondensed than the female pronucleus (Park *et al.*, 2007).

https://doi.org/10.1017/S0967199414000100 Published online by Cambridge University Press

We observed that the number of DSBs tended to increase by day 2 in both PA and SCNT embryos, in contrast with the behaviour observed in IVF embryos. While IVF embryos exhibited a higher γH2AX foci number per nucleus and greater chromatin area with damage at the 1-cell stage, these numbers were reduced at the 2-cell stage, showing that the greatest damage was present in early stages and that repair had occurred by day 2. The epigenetic modification of DNA (methylation of cytosine in the dinucleotide CpG) and/or associated proteins (e.g. histone phosphorylation, acetylation and methylation) regulates gene expression without changing the DNA sequence (Li, 2002). DNA methylation is recognized as a principal contributor to the stability of gene expression (Bird, 2002). The pattern of genomic methylation is stably inherited by differentiated somatic cells; however, genome-wide reprogramming occurs in germ cells and preimplantation embryos (Dean & Walter, 2001). The reprogramming process includes the erasure of existing epigenetic marks and the re-establishment of cell-specific marks to generate cells with nuclear totipotency and broad developmental potential (Dean & Walter, 2001; Meehan, 2003). However, this reprogramming process leads to DNA damage. Thus, we hypothesize that the greater number of γH2AX foci in 2-cell SCNT embryos is observed due to an increased need for nuclear reprogramming.

Finally, blastocysts showed a decreased level of DSBs with respect to 2-cell embryos in all IVP methods. These results indicate that the early embryo stages are more prone to DNA damage and that these lesions are repaired in embryos that reach the blastocyst stage. Poor γH2AX foci formation in early stages is likely to result in defective damage response, leading to improper DNA repair. Thus, poor H2AX phosphorylation in 1-cell and 2-cell stage embryos may serve as a mechanism to eliminate irreparably damaged embryos and prevent the development of defective offspring (Norimura *et al*., 1996), as division prior to DSB repair leads to cell death.

In summary, γH2AX is detected at the 1-cell, 2-cell and blastocyst stages in preimplantation bovine embryos produced by PA, *in vitro* fertilization and SCNT. While IVF embryos exhibited higher numbers of DSBs by the 1-cell stage, no difference was observed between PA and SCNT embryos at any developmental stage. The decrease in the number of DSBs at the blastocyst stage seems to indicate that DNA repair mechanisms are functional in blastocysts.

Acknowledgements

The authors thank the Amancay (Dr Silva) and Nueva Escocia (Dra Saenz) slaughterhouses for providing the biological material and Pablo Do Campo for support with confocal image analysis. V.J.F. Freitas is a CNPq senior investigator (Fortaleza, Brazil), and D.F. Salamone is a CONICET independent investigator (Buenos Aires, Argentina).

References

Adenot, P.G., Mercier, Y., Renard, J.P. & Thompson, E.M. (1997). Differential H4 acetylation of paternal and maternal chromatin precedes DNA replication and differential transcriptional activity in pronuclei of 1-cell mouse embryos. *Development* **124**, 4615–25.

Adiga, S.K., Toyoshima, M., Shimura, T., Takeda, J., Uematsu, N. & Niwa, O. (2007). Delayed and stage specific phosphorylation of H2AX during preimplantation development of g-irradiated mouse embryos. *Reproduction* **133**, 415–22.

Aitken, R.J. & Clarkson, J.S. (1987). Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *Reprod. Fertil.* **81**, 459–69.

Aitken, R.J., Clarkson, J.S. & Fishel, S. (1989). Generation of reactive oxygen species, lipid peroxidation and human sperm function. *Biol. Reprod.* **40**, 183–97.

ASAB (2006). Guidelines for the treatment of animals in behavioral research and teaching. *Anim. Behav.* **71**, 245–53.

Barton, S.C., Arney, K.L., Shi, W., Niveleau, A., Fundele, R., Surani, M.A. & Haaf, T. (2001). Genome-wide methylation patterns in normal and uniparental early mouse embryos. *Hum. Mol. Genet.* **10**, 2983–7.

Beaujean, N., Taylor, J.E., McGarry, M., Gardner, J.O., Wilmut, I., Loi, P., Ptak, G., Galli, C., Lazzari, G., Bird, A., Young, L.E. & Meehan, R.R. (2004). The effect of interspecific oocytes on demethylation of sperm DNA. *Dev. Biol.* **101**, 7636–40.

Bevacqua, R.J., Pereyra-Bonnet, F., Olivera, R., Hiriart, M.I., Sipowicz, P., Fernandez-Martín, R., Radrizzani, M. & Salamone, D.F. (2012). Production of IVF transgene-expressing bovine embryos using a novel strategy based on cell cycle inhibitors. *Theriogenology* **78**, 57–68.

Bohrer, R.C., Che, L., Gonçalves, P.B., Duggavathi, R. & Bordignon, V. (2013). Phosphorylated histone H2A.x in porcine embryos produced by IVF and somatic cell nuclear transfer. *Reproduction* **21**, 325–33.

Brackett, B. & Oliphant, G. (1975) Capacitation of rabbit spermatozoa *in vitro*. *Biol. Reprod.* **12**, 260–74.

Brandão, D.O., Maddox-Hyttel, P., Lovendahl, P., Rumpf, R., Stringfellow, D. & Callesen, H. (2004). Post hatching development: a novel system for extended *in vitro* culture of bovine embryos. *Biol. Reprod.* **71**, 2048–55.

Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Gene Dev.* **16**, 6–21.

Bouniol, C., Nguyen, E. & Debey, P. (1995). Endogenous transcription occurs at the 1-cell stage in the mouse embryo. *Exp. Cell. Res.* **218**, 57–62.

Dean, W. & Walter, J. (2001). Epigenetic reprogramming in mammalian development. *Science* **293**, 1089–93.

Derijck, A.A.H.A., Heijden, G.W.V.D., Giele, M., Philippens, M.E.P., Bavel, C.C.A.W.V. & Boer, P. (2006). γH2AX

https://doi.org/10.1017/S0967199414000100 Published online by Cambridge University Press

signalling during sperm chromatin remodeling in the mouse zygote. *DNA Repair* **5**, 959–71.

Fernandez-Capetillo, O., Chen, H.T., Celeste, A., Ward, I., Romanienko, P.J., Morales, J.C., Naka, K., Xia, Z., Camerini-Otero, R.D., Motoyama, N., Carpenter, P.B., Bonner, W.M., Chen, J. & Nussenzwig, A. (2003). DNA damage-induced G2-M checkpoint activation by histone H2A.X and 53 bp1. *Nat. Cell. Biol.* **4**, 993–7.

Fernandez-Capetillo, O., Lee, A., Nussenzweig, M. & Nussenzweig, A. (2004). H2AX: the histone guardian of the genome. *DNA Repair* **3**, 959–67.

Gali, C., Duchi, R., Crotti, G., Turini, P., Ponderato, N., Colleoni, S., Lagutina, I. & Lazzari, G. (2003). Bovine embryo technologies. *Theriogenology* **59**, 599–616.

Gawecka, J.E., Marh, J., Ortega, M., Yamauchi, Y., Ward, M.A. & Ward, W.S. (2013). Mouse zygotes respond to severe sperm DNA damage by delaying paternal DNA replication and embryonic development. *PLoS One* **8, 8**(2): e56385.

Gonçalves, F.S., Barretto, L.S.S., Arruda, R.P., Perri, S.H.V. & Mingoti, G.Z. (2010). Effect of antioxidants during bovine *in vitro* fertilization procedures on spermatozoa and embryo development. *Reprod. Dom. Anim.* **45**, 129–35.

Grenier, L., Robaire, B. & Hales, B.F. (2012). The activation of DNA damage detection and repair responses in cleavage-stage rat embryos by a damaged paternal genome. *Toxicol. Sci.* **127**, 555–66.

Hosseini, S.O., Aghaee, F., Hosseini, S.M., Hajian, M., Forouzanfar, M, Noorbakhshnia, M., Gourabi, H., Shahverdi, A.H., Dizaj, A.V.T. & Nasr-Esfahani, M.H. (2011). Effect of culture condition and cell-permeable superoxide dismutase on levels of reactive oxygen species (ROS) production in "in vitro" produced sheep embryos. *Small Rum. Res.* **97**, 88–93.

Ismail, H.I. & Hendzel, M.J. (2008). The γH2AX: is it just a surrogate marker of double-strand breaks or much more? *Environ. Mol. Mutagen.* **49**, 73–82.

Kaneko, H., Igarashi, K., Kataoka, K. & Miura, M. (2005). Heat shock induces phosphorylation of histone H2AX in mammalian cells. *Biochem. Biophys. Res.* **328**, 1101–6.

Kobayashi, J. (2004). Molecular mechanism of the recruitment of NBS1/hMRE11/hRAD50 complex to DNA double-strand breaks: NBS1 binds to gamma through FHA/BRCT domain. *J. Radiat. Res.* **45**, 473–8.

Kofman-Alfaro, S. & Chandley, A.C. (1971). Radiation-initiated DNA synthesis in spermatogenic cells of the mouse. *Exp. Cell. Res.* **69**, 33–44.

La Rosa, I., Camargo, L.S.A., Pereira, M.M., Fernandez-Martin, R., Paz, D.A. & Salamone, D.F. (2011). Effects of bone morphogenic protein 4 (BMP4) and its inhibitor, Noggin, on *in vitro* maturation and culture of bovine preimplantation embryos. *Reprod. Biol. Endocrinol.* **9**, 18–25.

Li, E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. *Nat. Rev. Genet.* **3**, 662–73.

Li, C., Mizutani, E., Ono, T. & Wakayama, T. (2009). Production of normal mice from spermatozoa denatured with high alkali treatment before ICSI. Reproduction **137**, 779–92.

Liu, H.L., Kentaro, T., Hara, T. & Aoki, F. (2005). Role of the first mitosis in the remodeling of the parental genomes in mouse embryos. *Cell. Res.* **15**, 127–32.

Loppin, B., Bonnefoy, E., Anselme, C., Laurencon, A., Karr, T.L. & Couble, P. (2005). The histone H3.3 chaperone HIRA is essential for chromatin assembly in the male pronucleus. *Nature* **437**, 1386–90.

Lu, K.H., Gordon, I., Chen, H.B., Gallagher, M. & Mcgovern, H. (1988). Birth of twins after of cattle embryos produced by *in vitro* techniques. *Vet. Rec.* **122**, 539–40.

Mayer, W., Niveleau, A., Walter, J., Fundele, R. & Haaf, T. (2000). Demethylation of the zygotic paternal genome. *Nature* **403**, 501–2.

McManus, K.J. & Hendzel, M.J. (2005). ATM-dependent DNA damage-independent mitotic phosphorylation of H2AX in normally growing mammalian cells. *Mol. Biol. Cell* **16**, 5013–25.

Meehan, R.R. (2003). DNA methylation in animal development. *Semin. Cell. Dev. Biol.* **14**, 53–65.

Norimura, T., Nomoto, S., Katsuki, M., Gondo, Y. & Kondo, S. (1996): p53-dependent apoptosis suppresses radiation-induced teratogenesis. *Nat. Med.* **2**, 577–80.

Oback, B., Wiersema, A.T. & Gaynor, P. (2003). Cloned cattle derived from a novel zona-free embryo reconstruction system. *Cloning Stem Cells* **5**, 3–12.

Paffoni, A., Brevini, T.A., Gandolfi, F. & Ragni, G. (2008). Parthenogenetic activation: biology and applications in the ART laboratory. *Placenta* **29**, 121–5.

Park, J.S., Jeong, Y.S., Shin, S.T., Lee, K.K. & Kang, Y.K. (2007). Dynamic DNA methylation reprogramming: active demethylation and immediate remethylation in the male pronucleus of bovine zygotes. *Dev. Dyn.* **236**, 2523–33.

Pereira, A.F., Feltrin, C., Almeida, K.C., Carneiro, I.S., Avelar, S.R.G., Alcântara Neto, A.S., Sousa, F.C., Melo, C.H.S., Moura, R.R., Teixeira, D.I.A, Freitas, V.J.F., Bertolini, L.R. & Bertolini, M. (2013). Analysis of factors contributing to the efficiency of the *in vitro* production of transgenic goat embryos (*Capra hircus*) by handmade cloning (HMC). *Small Rum. Res.* **109**, 163–72.

Pilch, D.R., Sedelnikova, O.A., Redon, C., Celeste, A., Nussenzweig, A. & Bonner, W.M. (2003). Characteristics of gamma-H2AX foci at DNA double-strand breaks sites. *Biochem. Cell. Biol.* **81**, 123–9.

Redon, C., Pilch, D., Rogakou, E., Sedelnikova, O., Newrock, K. & Bonner, W.M. (2002). Histone H2A variants H2AX and H2AZ. *Curr. Opin. Gen. Dev.* **12**, 162–9.

Reina-San-Martin, B., Difilippantonio, S., Hanitsch, L., Masilamani, R.F., Nussenzweig, A. & Nussenzweig, M.C. (2003). H2AX is required for recombination between immunoglobulin switch regions but not for intra-switch region recombination or somatic hypermutation. *J. Exp. Med.* **197**, 1767–78.

Renard, J.P. (1998). Chromatin remodeling and nuclear reprogramming at the onset of embryonic development in mammals. *Reprod. Fertil. Dev.* **10**, 573–80.

Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S. & Bonner, W.M. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J. Biol. Chem.* **273**, 5858–68.

Salamone, D., Barañao, L., Santos, C., Bussmann, L., Artuso, J., Werning, C., Prync, A., Carbonetto, C., Dabsys, S.,

https://doi.org/10.1017/S0967199414000100 Published online by Cambridge University Press

Munar, C., Salaberry, R., Berra, G., Berra, I., Fernández, N., Papouchado, M., Foti, M., Judewicz, N., Mujica, I., Muñoz, L., Alvarez, S.F., González, E., Zimmermann, J., Criscuolo, M. & Melo, C. (2006). High level expression of bioactive recombinant human growth hormone in the milk of a cloned transgenic cow. *J. Biotechnol.* **124**, 469–72.

Santos, F., Zakhartchenko, V., Stojkovic, M., Peters, A., Jenuwein, T., Wolf, E., Reik, W. & Dean, W. (2003). Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. *Curr. Biol.* **13**, 1116–21.

Sedelnikova, O., Pilch, D.R., Redon, C. & Bonner, W.M. (2003). Histone H2AX in DNA damage and repair. *Cancer Biol. Ther.* **2**, 233–5.

Sega, G.A., Sotomayor, R.E. & Owens, J.G. (1978). A study of unscheduled DNA synthesis induced by X-rays in the germ cells of male mice. *Mutat. Res.* **49**, 239–57.

Shi, W. & Haaf, T. (2002). Aberrant methylation patterns at the two-cell stage as an indicator of early developmental failure. *Mol. Reprod. Dev.* **63**, 329–34.

Stiff, T., O'Driscoll, M., Rief, N., Iwabuchi, K., Lobrich, M. & Jeggo, P.A. (2004). Atm and DNA-pk function redundantly to phosphorylate h2ax after exposure to ionizing radiation. *Cancer Res.* **64**, 2390–6.

Takahashi, M., Keicho, K., Takahasi, H., Ogawa, H., Schultz, R.M. & Okano, A. (1999). Effect of oxidative stress on development and DNA damage in *in-vitro* cultured bovine embryos by comet assay. *Theriogenology* **54**, 137–45.

Vajta, G., Peura, T.T., Holm, P., Paldi, A., Greve, T., Trounson, A.O. & Callesen, H. (2000). New method for culture of zona-included or zona-free embryos: the Well of the Well (WOW) system. *Mol. Reprod. Dev.* **55**, 256–64.

Ward, W.S. & Coffey, D.S. (1991). DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. *Biol. Reprod.* **44**, 569–74.

Ziegler-Birling, C., Helmrich, A., Tora, L. & Torres-Padilla, M. (2009). Distribution of p53 binding protein 1 (53BP1) and phosphorylated H2A.X during mouse preimplantation development in the absence of DNA damage. *Int. J. Dev. Biol.* **53**, 1003–1.

https://doi.org/10.1017/S0967199414000100 Published online by Cambridge University Press