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Comet assay on thawed embryos: An optimized technique to evaluate DNA damage in mouse embryos

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ABSTRACT

Our objective was to optimize the CA technique on mammal embryos.

Materials and methods: 1000 frozen 2-cell embryos from B6CBA mice were used. Based on a literature review, and after checking post-thaw embryo viability, the main outcome measures included: 1) comparison of the embryo recovery rate between 2 CA protocols (2 agarose layers and 3 agarose layers); 2) comparison of DNA damage by the CA on embryos with (ZP +) and without (ZP −) zona pellucida; and 3) comparison of DNA damage in embryos exposed to 2 genotoxic agents (H_2O_2 and simulated sunlight irradiation (SSI)). DNA damage was quantified by the % tail DNA.

Results: 1) The recovery rate was 3,3% (n = 5/150) with the 2 agarose layers protocol and 71,3% (n = 266/371) with the 3 agarose layers protocol. 2) DNA damage did not differ statistically significantly between ZP − and ZP + embryos (12.60 ± 2.53% Tail DNA vs 11.04 ± 1.50 (p = 0.583) for the control group and 49.23 ± 4.16 vs 41.13 ± 4.31 (p = 0.182) for the H_2O_2 group); 3) H_2O_2 and SSI induced a statistically significant increase in DNA damage compared with the control group (41.13 ± 4.31% Tail DNA, 36.33 ± 3.02 and 11.04 ± 1.50 (p < 0.0001)).

The CA on mammal embryos was optimized by using thawed embryos, by avoiding ZP removal and by the adjunction of a third agarose layer.

1. Introduction

The exposure of parents to environmental toxicants, such as polycyclic aromatic hydrocarbons (Einaudi et al., 2014; Perrin et al., 2011), Bisphenol A (Goldstone et al., 2015), solvents (Kolstad et al., 1999), metals (Thompson and Bannigan, 2008; Zhou et al., 2016) or various therapies (Bujan et al., 2014; Esquerré-Lamare et al., 2015; Pecou et al., 2009; Roti Roti et al., 2012) may induce DNA damage in male and female germ cells. DNA damage in parental germ cells can lead to reproductive issues, such as reduced fertilization, impaired early embryonic development, a decreased pregnancy rate and increased miscarriage rate (Simon et al., 2014; Zhao et al., 2014). The transmission of paternal germ cell DNA damage to preimplantation embryos has been demonstrated in humans (Zenzes et al., 1999), although DNA repair occurs in the zygote (Ménézo et al., 2010). This observation raises the question of evaluating DNA damage in preimplantation embryos because, currently, no genotoxicity test on the embryo has been validated.

The comet assay is a simple and rapid test for evaluating DNA damage in eukaryotic cells (Speit et al., 2009). It allows for the visualization of denatured DNA fragments after ex-nucleus migration by electrophoresis. After staining, the obtained shape mimics a “comet” with the head containing intact DNA and the remaining parts of the chromosome and the tail containing relaxed DNA loops or broken DNA fragments (Horváthová et al., 2004). This test is validated by toxicological regulatory agencies for the assessment of DNA damage in somatic cells (OECD, 2014). The comet assay is also used by researchers

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for the assessment of DNA damage in sperm and male germ cells (Baumgartner et al., 2009; Perrin et al., 2007; Preaubert et al., 2016) and in oocytes (Berthelot-Ricou et al., 2013, 2011a, 2011b; Courbiere et al., 2013; Einaudi et al., 2014). Studies using the comet assay on animal embryos are scarce and use heterogeneous protocols and species (Blerkom et al., 2001; Fabian et al., 2003; Harrouk et al., 2000; Hwang et al., 2013; Ju et al., 2010; Kitagawa et al., 2004; Müller et al., 1996; Natarajan et al., 2010; Rajesh et al., 2010; Sturmey et al., 2009; Takahashi et al., 1999, 2000; Thiyagarajan and Valivittan, 2009a, 2009b; Tranguch et al., 2003; Tsuda et al., 1998; Webster et al., 2000). As comet assay is a very sensitive test, the analysis of fresh embryos by comet assay requires the proximity between animal facilities and laboratory. Moreover, the analysis of fresh embryos does not allow control or additional analyses if required, notably after in vivo exposure or for an hypothetical use for regulatory issues.

The aim of our study was to optimize the comet assay for application on frozen mouse embryos, in order to simplify the handling and allow subsequent analysis.

2. Material and methods

All of the chemicals were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) unless otherwise stated.

2.1. Institutional Review Board

Institutional Review Board approval (C2EA-14) was obtained after submission to the National Ethics Committee on Animal Experimentation. All of the experimental protocols and animal handling procedures were approved by The National Ethics Committee on Animal Experimentation.

2.2. Source of embryos

In total, 1000 frozen 2-cell embryos were purchased from the ImmunoPHEnomique Center (CIPHE, Luminy, Marseille, France). Briefly, the embryos were obtained by natural mating on superovulated B6CBA females aged 5 weeks. 48 h after mating, the oviduct was flushed and the two-cells embryo obtained were cryopreserved. We used a slow freezing protocol with a controlled rate freezing machine and 1.5 M propanediol as a cryoprotectant, according to the procedure described by the European Mouse Mutant Archive (EMMA) (EMMA—The European mouse mutant archive, 2013a; Hagn et al., 2007).

2.3. Embryo thawing

The embryos were thawed according to the procedure described by the European Mouse Mutant Archive (EMMA) (EMMA—The European mouse mutant archive, 2013b; Hagn et al., 2007). Briefly, the straw was thawed at room temperature (RT), and then the embryos were rinsed in 4 successive M2 medium drops and placed in KSOM medium (EmbryoMax®, Merck Millipore, Darmstadt, Germany).

The number of thawed embryos used for each experiment performed in this study is presented in Table 1.

2.4. Embryo viability and culture conditions

To validate the use of frozen embryos instead of fresh embryos for comet assay, we assessed viability and blastulation rates in thawed embryos. After 1 h of recovery in KSOM, the 2-cells embryos were examined under a microscope and were classified as lysed or intact. The survival rate was defined as the ratio of the intact embryos to the total thawed embryos.

The embryos were then cultured for 24 h in a humidified chamber at 37 °C (95% air/5% CO_2) to determine viability. The viability rate was defined by the number of 2-cell embryos moving to the 4–8 cell stage to the total intact embryos. The embryos were cultured for 24 h more to determine the evolution rate to the blastocyst stage, assessing the number of blastocysts compared to the total intact embryos. For each separate experiment, 20 embryos were used (5 experiments = 100 embryos) (Table 1).

Literature review of the comet assay protocols used for mammalian embryos.

A literature review using PubMed with the key words “comet assay” and “embryo” was conducted. We excluded studies using non-mammalian species, post-implantation embryos, studies with incomplete data about the comet assay technique and studies applying the comet assay on other cells than embryos. We collected the following information from the protocols: species, embryo production, zona pellucida (ZP), embryo mixing technique in agarose, number of layers, lysis solution/duration and temperature used, incubation/electrophoresis duration, number and stage of embryos deposited per slide and technique used for the quantification of DNA damage.

The main technical details of the published protocols are presented in Table 2. We selected 14 studies using the comet assay on animal embryos matching our criteria, out of 118 studies retrieved. In these studies, fresh embryos were all obtained by natural mating (Fabian et al., 2003; Harrouk et al., 2000; Müller et al., 1996; Takahashi et al., 1999; Tranguch et al., 2003; Webster et al., 2000) or IVF (Hwang et al., 2013; Ju et al., 2010; Kitagawa et al., 2004; Natarajan et al., 2010; Rajesh et al., 2010; Sturmey et al., 2009; Takahashi et al., 2000; Thiyagarajan and Valivittan, 2009a, 2009b). This review of the literature shows that there is no standardization of the protocol: Seven different animal species were used; The ZP was removed in 4 studies; The protocols for the transfer of the embryos in the LMP agarose layer were heterogeneous: a minimum of half a blastocyst and a maximum of 20 cleavage embryos were transferred, and the volume of LMP varied from 4 μL to 50 μL; The lysis and electrophoresis protocols were also heterogeneous. No publication described the recovery rate of the embryos in the agarose layer after lysis and electrophoresis.

The findings of this literature review were used in the following paragraphs and tests to optimize comet assay.

Table 1
Experiments performed in the study and number of thawed embryos used for each one.

Experiments performed	Embryo stage used	Protocol used after embryo thawing	Nb of separate experiments	Total nb of 2-cells embryos used
Assessment of embryo viability and blastulation rates	2-Cell	2-Cells embryo in vitro culture to blastocyst stage	5	20 × 5 = 100
Impact of the nb of agarose layers on embryo recovery rate	2-Cell	CA Protocol 1	5	30 × 5 = 150
	2-Cell	CA Protocol 2	5	30 × 5 = 150
Impact of ZP on CA results	2-Cell	CA Protocol 2	5	60 × 5 = 300
Impact of genotoxic agents exposure on DNA damage	2-Cell	CA Protocol 2	5	60 × 5 = 300
Total				1000

CA: comet assay (Protocol 1 uses 2 agarose layers; Protocol 2 uses 3 agarose layers), ZP: zona pellucida.