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In vitro equine embryo production using air-dried spermatozoa, with different activation protocols and culture systems

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Summary

The aim of this work was to evaluate the use of air-dried spermatozoa for *in vitro* production of equine embryos and verify if sperm extract activation and *in vivo* culture improve *in vitro* embryo production. Cooled spermatozoa (control) and air-dried spermatozoa stored for 2, 14 or 28 days were used for ICSI sperm extract, or ionomycin was used for oocyte activation, and embryos were *in vitro* or *in vivo* (in mare′s oviduct) cultured for 7 days. With *in vitro* culture, cleavage rate was higher when activating with sperm extract ($P < 0.05$). No differences in embryo development were seen between the two activation treatments nor between storage periods ($P > 0.05$). Blastocysts were obtained with cooled spermatozoa, and morulae were achieved using *in vivo* culture with 28-day storage spermatozoa and ionomycin-activated oocytes. When *in vivo* culture was performed, sperm DNA fragmentation was assessed using the sperm chromatin dispersion test and did not show statistical correlation with cleavage nor embryo recovery rates. In conclusion, equine embryos can be produced using air-dried spermatozoa stored for several weeks. Sperm extract activation increased cleavage rates but did not improve embryo development. *In vivo* culture allowed intrauterine stage embryos to be achieved.

Introduction

In recent years, intracytoplasmic sperm injection (ICSI) has allowed embryo production using immotile spermatozoa in several species (Rahman, 2011). Sperm samples have been heat-dried (Lee & Niwa, 2006), lyophilised (Martins *et al.*, 2007a and b; Kwon *et al.*, 2004) or dehydrated by convective drying (Bhowmick *et al.*, 2003). Recently, a foal was produced using lyophilised spermatozoa in co-injection with sperm extract (Choi *et al.*, 2011). These techniques require difficult protocols and expensive equipment. Sperm preservation by nonconventional methods appears as an opportunity of preserving genetic material when cryopreservation is not possible, and it would be important to have a method that allows long-term storage. Mice have been born after ICSI with lyophilised spermatozoa stored for 1 year (Kaneko & Nakagata, 2006), and bovine blastocysts have been produced with lyophilised spermatozoa stored for 3 months (Martins *et al.*, 2007a). Bovine blastocyst production with heat-dried spermatozoa was lower when storage exceeded 3 months (Lee & Niwa, 2006). Air-dried human spermatozoa stored for 3 days achieved 8.3% blastocysts (Imoedemhe *et al.*, 2003), and our laboratory has produced equine embryos using dehydrated spermatozoa stored for 2–3 days (Alonso *et al.*, 2007). Air-drying allows transporting sperm samples at refrigeration temperature; therefore, it is more readily accessible than deep freezing methods.

It could also be used to avoid transmission of venereal viral diseases such as equine viral arteritis, which seriously affects international trading of frozen semen and stallions. This virus is sensitive and rapidly inactivated by low humidity and light (Timoney, 2002). The possibility of processing sperm samples in a way that eliminates viral

contamination would allow obtaining offspring from carrier individuals without the risk of disseminating diseases.

Despite the various advantages of preserving a sperm sample by air-drying, it is still unknown whether the genetic material suffers any alterations over storage time. DNA is known to have a very stable structure (Jacobs & Schär, 2012); however, in bovine and mouse, chromatin alterations have been reported when spermatozoa are preserved by nonconventional method requiring dehydration (Martins *et al.*, 2007b; Kusakabe *et al.*, 2008). Samples can be evaluated with the sperm chromatin dispersion test (SCD), which shows the percentage of spermatozoa with fragmented DNA after acid denaturation and removal of nuclear proteins. Thus, spermatozoa with fragmented chromatin fail to produce the characteristic halo of dispersed DNA loops that is observed in nonfragmented spermatozoa (Fernández *et al.*, 2003).

Spermatozoa with damaged DNA had similar fertilisation rates to normal spermatozoa, but the blastocyst rate was lower (Fatehi *et al.*, 2006). However, even if dehydrated spermatozoa keep DNA intact, some sperm factors necessary for embryo development might be affected. Therefore, it would be necessary to provide a sperm protein source that could supply this lack. Sperm extract has been used for oocyte activation (Hinrichs *et al.*, 2007; Choi *et al.*, 2009), and among its proteins, PLCz has been identified and described as the sperm borne oocyte activation factor (Swann *et al.*, 2004).

An efficient system of embryo culture is a fundamental aspect of assisted reproduction. Although *in vivo* culture requires maintenance of recipient mares and surgical transfer, it allows the limitations of *in vitro* culture systems to be overcome. Equine embryos produced by ICSI have been cultured in oviducts of recipient ewes (Galli *et al.*, 2002) and mares (Choi *et al.*, 2004), yielding better results than their respective control groups that included *in vitro* culture.

The aim of this study was to evaluate the use of a nonconventional technique for sperm preservation, such as air-drying, for *in vitro* production of equine embryos by ICSI and verify if sperm extract activation and *in vivo* culture of *in vitro* produced embryos improve their development Fig. 2.

Materials and methods

Unless otherwise stated, all reagents and media were purchased from Sigma (St. Louis, MO, USA).

All procedures were evaluated by the Institutional Committee for the Care and Use of Experimental Animals (Comité Institucional de Cuidado y Uso de Animales de Experimentación, CICUAL, protocol No. 2008/38 and 2010/23).

Oocyte collection and maturation

Mare ovaries were collected post-mortem at a slaughterhouse and were transported to the laboratory and processed within 4 h. Follicles between 7 and 30 mm were opened with a scalpel blade, and the inner follicle surface was scraped with a bone curette (Volkmann bone curette, oblong cup, 7 mm). The contents of the curette were washed into Petri dishes with Ringer lactate solution (Baxter®; Baxter Argentina S.A., Olivos, Bs As, Argentina) and examined under a stereomicroscope. Oocyte–cumulus complexes were washed in 15 mM Hepes-buffered DMEM/F12 (H-DMEM), with 15% foetal bovine serum (FBS) (Internegocios®; Internegocios S.A., Mercedes, Bs As, Argentina) and 50 IU heparin ml^{-1}. Oocyte maturation and transport to the ICSI laboratory (approx 22 h) were carried out in a portable incubator at 38 °C, in the same medium without heparin and supplemented with 5 μg/ml FSH (Folltropin®; Bioniche Animal Health, Belleville, ON, Canada), 100 ng ml^{-1} IGF-1 and 100 ng ml^{-1} EGF. Maturation continued in the laboratory using 5% CO_2 in air, in maturation medium without Hepes until completion of a maturation period of 26–30 h. For cumulus cell removal, oocytes were incubated in H-DMEM with 15% FBS and 0.1% hyaluronidase for 10 min, followed by vigorous pipetting through a fine-bore glass pipette. Oocytes having a polar body and intact oolemma were selected for ICSI.

Sperm preparation

Semen was collected from two stallions of proven fertility, using a Missouri artificial vagina. Visual evaluation of sperm motility was performed under light microscopy on a warm stage.

Cooled semen

Semen was diluted in Kenney's extender and stored in an Equitainer® (Hamilton Research Inc., Ipswich, MA, USA) for 24–28 h. At the time of ICSI, 1 ml was warmed for 5 min at 37 °C and placed in the bottom of a tube containing 2 ml of Ham's F-10 (Gibco®, Invitrogen Argentina S.A., CABA, Buenos Aires, Argentina) with antibiotic and 3 mg/ml bovine serum albumin (BSA). After 20 min of incubation at 38 °C, 600 μl was collected from the top of the tube and centrifuged at 327 x g for 3 min. The sperm pellet was resuspended in the same medium and used to provide spermatozoa for ICSI.

Air-dried spermatozoa

Semen was diluted 1:1 in Kenney's extender, centrifuged and resuspended in Hepes-TALP with 6 mg ml^{-1} BSA. Smears were made on sterile glass slides under laminar

flow and left to dry for 15–20 min at room temperature. Samples were kept at 5 °C for either 2 days, 2 weeks or 4 weeks, after which they were resuspended in Hepes-TALP for use in ICSI.

Sperm extract preparation

Sperm extract was prepared according to Choi *et al.* (2002) and was maintained in liquid nitrogen until use. Ejaculated stallion spermatozoa, from a stallion of proven fertility, were centrifuged at 900 x *g* for 10 min to remove seminal plasma. The pellet was then suspended in Sperm-TALP (Sp-TALP) containing 6 mg ml^{-1} BSA and centrifuged at 900 x *g* for 10 min. The resulting pellet was resuspended to a final concentration of 5 x 10^8 spermatozoa ml^{-1} in nuclear isolation medium (NIM: 125 mM KCl, 2.6 mM NaCl, 7.8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 3.0 mM EDTA disodium salt; pH 7.45) and centrifuged to remove Sp-TALP. The pellet was then resuspended to the same volume with NIM containing 1 mM dithiothreitol, 100 μM leupeptin, 100 μM antipain and 100 μg ml^{-1} soybean trypsin inhibitor. The suspension was subjected to four cycles of freezing (5 min per cycle in liquid N_2) and thawing (5 min per cycle at 15 °C), and then spermatozoa were pelleted at 20000 x *g* for 50 min at 2 °C. The resultant supernatant was carefully removed and stored.

Intracytoplasmic sperm injection

Microinjection was conducted in a microdroplet of H-DMEM under mineral oil, on a heated stage, using an inverted microscope Leica DMIL® (Leica Microsystems, Wetzlar, Germany) with Narishige® (Narishige International USA Inc., East Meadow, NY, USA) micromanipulators. For the ICSI procedure with cooled spermatozoa, a motile spermatozoon was selected, placed in a 20% polyvinylpyrrolidone (PVP; Sigma) droplet and immobilised by disruption of the plasma membrane with the injection pipette. Air-dried spermatozoa were placed in a droplet of Ham′s F-10 (when activating with ionomycin) or Ham′s F10 mixed 1 : 1 with sperm extract (when activating with sperm extract). Each oocyte was immobilised by the holding pipette, placing the polar body at the 6 or 12-h position, and was injected with a single spermatozoon.

Parthenogenetic control

As a control for parthenogenetic development, sham injections were performed in 36 oocytes, which were then incubated in ionomycin (Sham injection group). A further 36 oocytes were injected only with sperm extract (SE group).

Experimental design

Experiment I: Equine embryo production with air-dried spermatozoa and in vitro *culture*

Oocytes selected for ICSI were randomly assigned to the following groups: control group: injection with cooled spermatozoa and ionomycin activation, $n = 116$; Group 1: injection with air-dried spermatozoa stored for 2 days and activation with either ionomycin (Group 1a, $n = 11$) or with sperm extract (Group 1b, $n = 18$); Group 2: injection with air-dried spermatozoa stored for 2 weeks and activation with either ionomycin (Group 2a, $n = 36$) or with sperm extract (Group 2b, $n = 21$); Group 3: injection with air-dried spermatozoa stored for 4 weeks and activation with either ionomycin (Group 3a, $n = 31$) or with sperm extract (Group 3b, $n = 19$). Two to three replicates per group were performed.

After injection, oocytes from groups: sham injection, control, 1a, 2a, and 3a, were activated by incubation for 5 min with ionomycin (5 μM in DMEM/F12 with 10% FBS).

After a 3-h interval in DMEM/F12 with 10% FBS, all injected oocytes were incubated in 6-DMAP (2 mM in the same medium) for 3 more hours. *In vitro* embryo culture was performed at 38 °C under 5% CO_2, 5% O_2, 90% N_2, in DMEM/F12 with 10% FBS during 7 days. Cleavage and embryo development rates were recorded at 48, 120 and 168 h of culture.

Experiment II: In vivo *culture of equine embryos produced with air-dried spermatozoa and evaluation of sperm DNA fragmentation*

Oocytes selected for ICSI were randomly assigned to the same groups as in experiment I (control group: cooled spermatozoa, $n = 90$; treatment groups: 1a, $n = 35$; 1b, $n = 31$; 2a, $n = 33$; 2b, $n = 32$; 3a, $n = 29$; 3b, $n = 33$). Two to three replicates per group were performed.

Injected oocytes were incubated in 6-DMAP as in experiment I and were *in vitro* cultured for 24 to 48 h in the same conditions.

Intrafallopian transfer to recipient mares. Cleaved embryos were surgically transferred to the oviducts of recipient mares between 24 and 48 h after ICSI. Three days before surgery, mares having follicles of <22 mm were given 10 mg estradiol cypionate IM (ECP®; König Argentina, Avellaneda, Bs As, Argentina), and 24 h before surgery, they received 1500 mg long-acting progesterone IM (LAP_4Biorelease®, BetPharm, Lexington, KY, USA). The technique employed was a standing flank laparotomy. Sedation was induced by IV administration of 0.4 mg kg^{-1} xylazine (Rompun®; Bayer Argentina Div. Veterinaria, Munro, Bs As, Argentina) and

2.2 μg kg^{-1} detomidine (Dormosedan®; Pfizer Animal Health, New York, NY, USA) and maintained with 0.5 mg/kg xylazine and 0.025 mg kg^{-1} butorphanol IV (Torbutrol Plus®; Fort Dodge, La Plata, Bs As, Argentina). Local anaesthesia of the surgical area was carried out using 2% lidocaine (Equisystems, Buenos Aires, Argentina). After the peritoneum was punctured, the ovary was located and gently exteriorised through the incision. Embryos were loaded into a Sherwood® catheter (Agtech Inc., Manhattan, KS, USA) with a small volume of culture medium and gently deposited within the oviduct through the infundibular *os*.

Mares were given antibiotics (penicillin–streptomycin, 5 x 10^6 IU) and flunixin meglumine (500 mg), daily during 6 days.

Uterine flushing of recipient mares. Seven days after surgery, a uterine flushing was performed on each recipient mare using 2 l Ringer lactate solution. The number of embryos recovered was recorded and evaluated according to McCue *et al.* (2009).

Evaluation of sperm DNA integrity by sperm chromatin dispersion (SCD) test. This evaluation was performed on fresh spermatozoa, cooled spermatozoa and air-dried sperm samples, according to Carretero *et al.* (2010). Briefly, air-dried spermatozoa were resuspended in H-TALP and taken to a concentration of 5–10 x 10^6 spermatozoa/ml. Fresh and cooled sperm samples were taken to the same concentration using the same medium. Samples were then mixed with 1% agarose, placed on pre-treated slides and cooled for 10 min. at 4 °C before treatment with a denaturation acid solution. Following this, an incubation in lysing solution 1 (10 min.) followed by incubation in lysing solution 2 (5 min.) was carried out. Slides were rinsed with distilled water, dehydrated in increasing concentrations of ethanol (70%, 85% and 96%) for 2 min each and then air-dried. They were stained with Giemsa (20 min.), and a minimum of 400 spermatozoa per sample were observed at 1000X. Spermatozoa with large or medium halos were considered to have intact DNA, while those presenting small or no halo were considered to have fragmented DNA.

Statistical analysis

In experiment I, cleavage, embryo development and blastocyst production rates were analysed using ANOVA, considering 2 factors: 1) sperm conservation method (levels: 'cooled', '2 days air-dried', '2 weeks air-dried', '4 weeks air-dried', 'sham injection', 'sperm extract injection') and 2) activation method (with levels 'ionomycin' and 'sperm extract'). In experiment II, cleavage and embryo recovery rates were analysed using ANOVA, considering 2 factors: 1) sperm conservation method (levels: 'cooled', '2 days air-dried', '2 weeks air-dried', '4 weeks air-dried') and 2) activation method (with levels 'ionomycin' and 'sperm extract'). For sperm DNA evaluation, ANCOVA was made for cleavage and embryo recovery rates, considering the percentage of fragmented DNA spermatozoa as covariate and sperm conservation method as a factor (with levels: 'cooled', '2 days air-dried', '2 weeks air-dried', '4 weeks air-dried').

Results

Experiment I

A total of 116 oocytes were injected with cooled spermatozoa, and 136 oocytes were injected with air-dried spermatozoa. Results are shown in Table 1. Cleavage rate was significantly higher in the control group than in any other group ($P < 0.05$) and was significantly higher in treatment

Table 1 Oocytes injected with air-dried spermatozoa stored for different periods of time and cultured *in vitro* for 7 days

Group	Sperm conservation	Activation	Injected oocytes (n)	Cleavage (%)	Embryo development (8–16 cells) (%)	Blastocysts (%)
Control	Cooled (24 to 28 h)	Ionomycin	116	43.9^{a} (51/116)	12^{a} (14/116)	1.7^{a} (2/116)
1a	Air-dried (2 days storage)	Ionomycin	11	27.2^{b} (3/11)	0^{a}	0^{b}
1b		SE	18	38.8^{b} (7/18)	16.7^{a} (3/18)	0^{b}
2a	Air-dried (2 weeks storage)	Ionomycin	36	22.2^{bd} (8/36)	5.5^{a} (2/36)	0^{b}
2b		SE	21	33.3^{bd}(7/21)	9.5^{a} (2/21)	0^{b}
3a	Air-dried (4 weeks storage)	Ionomycin	31	29^{b} (9/31)	16.1^{a} (5/31)	0^{b}
3b		SE	19	36.8^{b} (7/19)	15.8^{a} (3/19)	0^{b}
Sham	_	Ionomycin	36	0^{c} (0/36)	0^{a}	0^{b}
SE injection	_	SE	36	22^{cd} (8/36)	0^{a}	0^{b}

Values with a different superscript within columns are significantly different ($P < 0.05$).
SE, sperm extract.

groups activated with SE compared to ionomycin activation ($P < 0.05$). No significant differences were detected in embryo development between groups, either for the different storage times evaluated or for the two different activation methods assayed ($P > 0.05$). Blastocyst production was only achieved in the control group and was significantly higher in the control group than in treatment groups ($P < 0.05$). Fertilised oocytes from the treatment groups only reached the 8- to 16-cell stage (Fig. 1).

Experiment II

In total, 90 oocytes were injected with cooled spermatozoa, and 193 oocytes were injected with air-dried spermatozoa. Results are shown in Table 2. Eight surgeries were carried out: 21 embryos (produced with cooled spermatozoa) were transferred to two recipient mares; 51 embryos (produced with air-dried spermatozoa) were transferred to the remaining six recipients (one surgery for each treatment group). In the control group, the recovery rate was 33.3% (7/21) obtaining grade 3 morulae and early blastocysts (Fig. 2*)*. No embryos were obtained in Groups 1a (air-dried spermatozoa stored for 2 days and ionomycin-activated oocytes), 1b (air-dried spermatozoa stored for 2 days and sperm extract-activated oocytes), 2a (air-dried spermatozoa stored for 2 weeks and ionomycin-activated oocytes), 2b (air-dried spermatozoa stored for 2 weeks and sperm extract-activated oocytes), and 3b (air-dried spermatozoa stored for 4 weeks and sperm extract-activated oocytes). In Group 3a (air-dried spermatozoa stored for 4 weeks and ionomycin-activated oocytes), the recovery rate was 28.5% (2/7) obtaining two grade three morulae. No significant differences were observed in cleavage ($P > 0.05$) and embryo recovery rates ($P > 0.05$) among all groups.

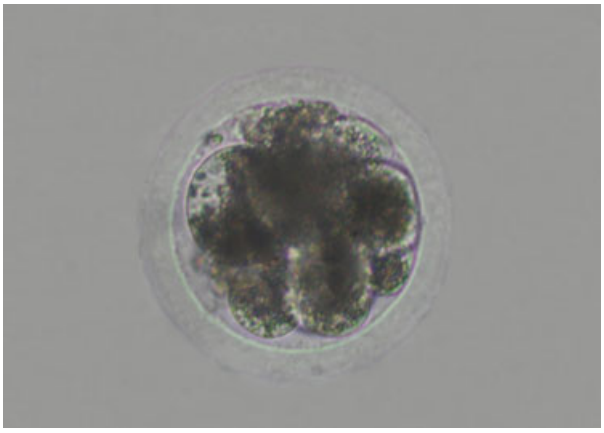

Fig. 1 *In vitro* cultured embryo produced by ICSI with air-dried spermatozoa.

When evaluating the sperm chromatin dispersion test on the day of ICSI, the percentage of spermatozoa having fragmented chromatin did not show correlation with cleavage rate ($P > 0.05$) or with embryo recovery rate ($P > 0.05$).

Discussion

This study represents one more step on the way to developing a simple method of gamete conservation for use in the field. In experiment I, cleavage rates were higher in all groups of oocytes activated with sperm extract. Nevertheless, this increase was not observed in the following experiment, where some of the sperm extract groups showed a lower cleavage rate. This difference cannot be attributed to stallion variation, because the sperm extract used in all the experiments was from the same stallion. The dose of sperm extract delivered in each injection is estimated to be 2 to 4 picoliters. Possibly small variations in the amount of this volume could induce changes in activating effect. A lower blastocyst production was reported in an equine cloning study, when the amount of injected sperm extract was increased (Choi *et al.*, 2009). Beyond its participation in oocyte activation, sperm extract would seem to provide factors that contribute to embryo development, which perhaps have been lost or altered in the air-drying process. Further studies are necessary to develop an efficient and repeatable protocol in the use of sperm extract.

When *in vitro* embryo culture was carried out, embryo development was interrupted at the 8–16 cell stage. In equines, the main activation of the embryo genome occurs at this stage (Ball *et al.*, 1993; Betteridge, 2011); thus, perhaps the dry state in sperm cells has consequences that are evidenced after maternal-embryo transition, when main genome activation occurs (Schultz, 2002).

In the first experiment of this study, cleavage rate was higher using cooled semen. This could be due to the spermatozoa being less damaged and also because it is possible to select a motile spermatozoon in these samples. Lazzari *et al.* (2002) reported a lower cleavage rate and no blastocyst production when injecting oocytes with spermatozoa that were nonmotile at the time of ICSI.

Equine oocytes have proved to be particularly resistant to parthenogenetic activation (Choi *et al.*, 2001); however, in our study, sperm extract showed some parthenogenetic activity (22% of cleavage).

In experiment II, working with *in vivo* embryo culture, intrauterine stage embryos were obtained. However, the total embryo recovery rate when using air-dried spermatozoa was much lower than in the control group (4%, 2/51 versus 33%, 7/21). This difference shows the greater

Table 2 ICSI using air-dried spermatozoa with *in vivo* embryo culture. Sperm DNA evaluation results using the sperm chromatin dispersion test (SCD), carried out at semen collection and before ICSI

Group	Sperm conservation	Activation[a]	Injected oocytes (n)	Cleavage[b](%)	Transferred embryos (n)	Recovered[b] (%)	% SCD fresh semen	% SCD at ICSI
Control	Cooled (24 to 28 h)	Ionomycin	90	25.5 (23/90)	21	33.3 (7/21)	17.7	18.5
1a	Air-dried (2 days storage)	Ionomycin	35	28.5 (10/35)	7	0	29	32.5
1b		SE	31	32.2 (10/31)	9	0	14.7	18.5
2a	Air-dried (2 weeks storage)	Ionomycin	33	42.4 (14/33)	12	0	29	40
2b		SE	32	31.2 (10/32)	10	0	17	27.7
3a	Air-dried (4 weeks storage)	Ionomycin	29	31 (9/29)	7	28.5 (2/7)	10.2	32.2
3b		SE	33	24.2 (8/33)	6	0	29	53

[a]No significant differences observed between two activation methods ($P = 0.39$ for cleavage rate and $P = 0.44$ for embryo recovery rate).
[b]No significant differences among treatments, nor with the control ($P = 0.44$ for cleavage rate and $P = 0.30$ in embryo recovery rate).
SE, sperm extract; % SCD fresh semen: percentage of spermatozoa in fresh semen having fragmented DNA; % SCD at ICSI: percentage of spermatozoa having fragmented DNA on the day of ICSI.

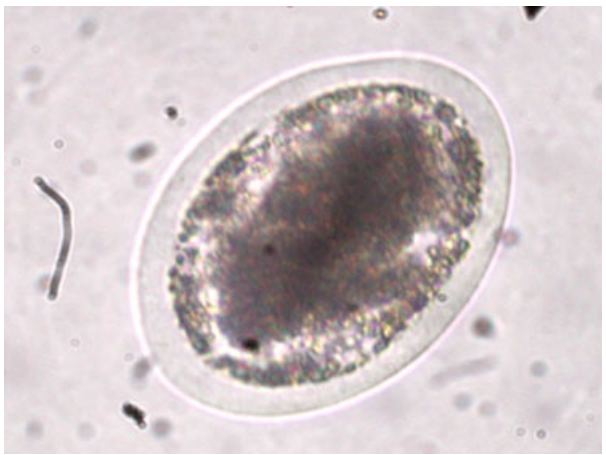

Fig. 2 Equine embryo produced by ICSI with cooled spermatozoa, recovered after *in vivo* culture.

developmental competence in embryos produced with a conventional sperm preservation method such as refrigeration.

Results in fertilisation and embryo development did not seem to be affected by the length of time of sperm preservation. In a study using lyophilised equine spermatozoa, Choi *et al.* (2011) used samples preserved at room temperature for 7 days and at 4 °C for 15 weeks, obtaining a similar blastocyst production in both groups (3%–6%) but lower than the control group (26%–28%). In our study, cleavage rate, embryo development rate and embryo recovery rate also did not show differences between storage periods and in most cases were likewise different to the control groups.

With regard to sperm DNA evaluation, except for one ejaculate which showed a 29% chromatin fragmentation in fresh semen, the values observed before air-drying were similar to those reported by Love (2005) in stallions of normal fertility when evaluated using the sperm chromatin structure assay (SCSA) (average 17%). Nevertheless, DNA fragmentation would seem to increase over time. Therefore, it is possible that the air-drying process induces sperm DNA fragmentation and that this is manifested only after several weeks of storage. In humans, spermatozoa with DNA damage are able to fertilise oocytes (Aitken *et al.*, 1998). Gene expression studies suggest that the oocyte and early embryos are equipped with mechanisms destined to rectify some degree of paternal DNA alterations (Wells *et al.*, 2005; Gasca *et al.*, 2007). The ability to repair this damage would seem to be related to the genomic and cytoplasmic quality of the oocyte, and second, to the amount of chromatin damage of the spermatozoon (Sakkas & Alvarez, 2010). It has been shown that the extent of damage in paternal DNA affects the embryo′s developmental competence to reach the blastocyst stage, a time when embryonic genome is activated, transcriptional activity has started and paternal genome plays an important role in embryo function (Seli *et al.*, 2004). In bovine *in vitro* fertilisation, no differences were seen in fertilisation rate between spermatozoa having intact DNA and those with irradiation-damaged DNA, but blastocyst rate was significantly lower in the second group (Fatehi *et al.*, 2006). Should a similar process occur in equines, the exact time after genome activation at which the embryo development is stopped would need to be defined. The lower embryo recovery rate obtained with air-dried spermatozoa in the second experiment could be because this process produces more DNA damage than the oocyte or early embryo is capable of repairing. Nevertheless, in this study, it was possible to obtain intrauterine stage embryos produced with air-dried spermatozoa, even when the sample was stored for the longest period evaluated and when oocytes were activated using a conventional method such as ionomycin incubation. Among

all the particularities of equine reproductive physiology, it is worth pointing out that the entrance of embryos into the uterus only occurs in the cases of fertilisation and embryo development. It is the embryo itself that sends signals to allow this event (Weber *et al.*, 1991). Therefore, it is necessary that embryos remain metabolically active throughout their time in the oviduct (Betteridge, 2011), a fact that gives us an idea of the viability of the embryos obtained in this study.

These results would indicate that it is possible to produce equine embryos by ICSI using air-dried spermatozoa stored for several weeks. Furthermore, *in vivo* culture of *in vitro* produced embryos allowed obtaining intrauterine transferable embryos. The use of sperm extract in oocyte activation would seem to increase cleavage rates, but that could be, at least partially, due to parthenogenetic effects. More studies are necessary to further elucidate this issue. The ability of air-dried spermatozoa to give origin to initial-stages embryos would not seem to be affected by storage time, despite observing an increase in DNA fragmentation over time. The development of a simple method of gamete conservation, with the main objective of preserving valuable genetics and of working with wild species under precarious conditions, is of great interest. Embryo production with air-dried spermatozoa would also be a potential alternative to avoid transmission of venereal viral diseases produced by virus that are sensitive to low humidity and light, such as equine viral arteritis.

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