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In vitro production of porcine zygotes using intracytoplasmic injection of vitrified sperm

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Contents

The objectives of this study were to evaluate if vitrified porcine spermatozoa are able to maintain their capacity to produce zygotes in vitro using intracytoplasmic sperm injection (ICSI) and to evaluate the zygote development in two in vitro atmospheric conditions: 5% CO_2 and tri-gas. A group of porcine oocytes maturated in vitro were injected with vitrified-warmed sperm (treatment group) and another group, with sperm diluted and conserved at 17°C (control group). To evidence parthenogenetic activation, some oocytes were submitted to a Sham test. The injected oocytes were cultured in G1 medium at 38°C, 100% humidity and 5% CO_2 or tri-gas. No significant differences ($p > .05$) were observed in embryo development between the oocytes injected with vitrified-warmed sperm (31.8%; 36/113), and those injected with semen diluted and conserved at 17°C (35.5%; 32/90), when cultured in 5% CO_2 or under tri-gas atmosphere (42.9%; 39/91 vs. 34.2%; 26/76, respectively). No significant differences ($p > .05$) were observed in the percentage of pronuclei (PN) obtained between 5% CO_2 and tri-gas, within each treatment either. Of the 52 oocytes submitted to the Sham test, only two presented a female PN (activation) indicating that the PN observed in the treatment group were a product of fertilization and not parthenogenetic activation. To conclude, porcine sperm vitrified using spheres, at a concentration of 5×10^6 spermatozoa/ml in TALP medium with 1% bovine serum albumin (BSA), conserve condensed and intact chromatin capable of producing early embryo development up to the pronuclear stage.

1 | INTRODUCTION

Vitrification is a process by which liquids modify their state at freezing temperatures without the formation of ice crystals (Jiménez-Rabadán et al., 2015; Liebermann et al., 2002; Sánchez et al., 2011) acquiring a glassy or vitreous state (Gao & Critser, 2000; Katkov et al., 2006; Luyet & Hodapp, 1938; Rall & Fahy, 1985). Moreover, it is a simple technique that requires less time and has a greater cost-benefit than conventional cryopreservation procedures. It does not require equipment or highly capacitated personnel to carry it out, and both vitrification and warming are carried out in a matter of seconds (Sánchez et al., 2011). The technique has been extensively studied ever since the first report of the successful vitrification of mouse embryos (Rall & Fahy, 1985) and has been applied to embryos and feminine gametes from different mammals, including humans. Nevertheless, the method has not been directly extrapolated to the male gamete because of the osmotic sensitivity of mammalian sperm when exposed to the high concentrations of cryoprotectants used in conventional vitrification (Isachenko et al., 2004). This exposition produces a deleterious osmotic effect (Sánchez et al., 2011), together with toxicity and possible chemical alterations in the spermatozoa (Isachenko, Isachenko, Katkov, Dessole, & Nawroth, 2003). All of this led to the idea of exploring vitrification methods that did not demand high concentrations of potentially toxic cryoprotectants. As a result, sperm vitrification using very high cooling and warming velocities in a very small sample emerged as an alternative to avoid these toxic concentrations (Isachenko et al., 2003).

It has been possible to conserve sperm chromatin condensation and integrity when vitrifying raw porcine spermatozoa in TALP medium

without cryoprotectants using the spheres method (Arraztoa, Miragaya, Pendola, Gambarotta, & Neild, 2012; Arraztoa et al., 2017). These results indicate that vitrification would seem to allow preservation of genetic resources; thus, this method appears as an alternative for cryopreserving porcine sperm, this being a species that is particularly sensitive to the changes suffered during conventional freeze-thawing.

Porcine embryo production by oocyte in vitro maturation and in vitro fertilization (IVF) using oocytes from abattoir ovaries is limited due to the high incidence of polyspermy (Coy et al., 2005). Therefore, intracytoplasmic sperm injection (ICSI) becomes a very useful alternative technique to in vitro produce monospermic zygotes in the porcine species (García Roselló, García Mengual, Coy, Alfonso, & Silvestre, 2008). In addition, provided that the sperm nucleus conserves its genetic integrity intact, ICSI allows one to obtain viable offspring without depending on sperm concentration, morphology or motility (Yanagimachi, 2005). Taking into account these statements, porcine sperm vitrification permits conservation of cells with an intact, condensed chromatin with the possibility of producing porcine zygotes in vitro using the ICSI technique.

Hence, the objectives of this study were as follows: (i) evaluate if porcine spermatozoa vitrified in spheres without cryoprotectants are able to maintain their capacity to produce zygotes in vitro using ICSI and (ii) evaluate zygote development in two in vitro atmospheric conditions: 5% CO_2 and tri-gas (5% CO_2, 5% O_2 and 90% N_2).

2 | MATERIALS AND METHODS

The Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires approved this study (protocol N° 2011/18).

All reagents were purchased from Sigma (St. Louis, MO, USA) except where stated otherwise.

2.1 | Semen processing

Raw porcine semen was obtained from three terminal cross-breed males, between 3 and 4 years old, lodged at the Faculty of Veterinary Sciences of the University of Buenos Aires, Argentina, situated 34°36′S and 58°26′W at sea level. Semen was collected using the gloved-hand method (King & Macpherson, 1973), and the sperm rich fraction obtained was either conserved at 17°C or vitrified.

Seminal samples conserved at 17°C for 24 hr, diluted 1:2 in Beltsville Thawing Solution (BTS) were centrifuged at 400 *g* for 5 min and re-suspended in 1 ml of TALP medium (NaCl 114 mmol/L; KCl 3,192 mmol/L; NaH_2PO_4 0.3416 mmol/L; $NaHCO_3$ 2 mmol/L; $CaCl_2$–$2H_2O$ 2 mmol/L; $MgCl_2$–$6H_2O$ 0.5017 mmol/L; lactate Na 10 mmol/L; pyruvate Na 0.2 mmol/L; gentamicin 25 μg/ml; glucose 11.99 mmol/L; HEPES 10 mmol/L) supplemented with 1% bovine serum albumin (BSA) for use in ICSI (control group).

For vitrification, raw semen samples were centrifuged at 400 *g* for 5 min to obtain a pellet, which was diluted in one step in TALP medium supplemented with 1% BSA to a concentration of 5×10^6 spermatozoa/ml. Vitrification was carried out using the spheres method reported by Arraztoa et al. (2017): briefly, small aliquots of 20–30 μl of diluted semen samples were dropped directly into liquid nitrogen. The microdroplets formed spheres upon contact with the liquid nitrogen and spontaneously sank after 4 s. The solid spheres were maintained in cryovials at−196°C for a minimum of 24 hr before being used. For warming, the spheres were submerged one by one (not more than five spheres per warming time) in a 15-ml tube with 5 ml of TALP + 1% BSA at 37°C for 30 s, accompanied by gentle vortexing for 5–10 s after each sphere addition. Warming was followed by centrifugation during 5 min at 300 *g*, and the pellet was re-suspended in TALP + 1% BSA for use in ICSI (treatment group).

2.2 | Semen evaluation

Before and after conservation, the following seminal parameters were evaluated according to Arraztoa et al. (2017): (i) total motility (progressive and circular) using a warm stage and phase-contrast microscopy (100×); (ii) viability using 6-carboxyfluorescein diacetate and propidium iodide (CFDA/PI stain) and epifluorescence microscopy (Leica® DMLS model, 400×); (iii) membrane function using the hypo-osmotic swelling test (HOS test) and a phase-contrast microscope (400×); (iv) acrosome integrity after fixing the samples in buffered formalin saline (BFS) and using a phase-contrast microscope (1,000×); (v) chromatin condensation using the toluidine blue (TB) stain and light microscopy (1,000×); (vi) chromatin susceptibility to acid denaturation using the acridine orange (AO) stain and epifluorescence microscopy (Leica® DMLS model, 1,000×).

2.3 | In vitro maturation of porcine oocytes

Cumulus-oocyte complexes (COCs) were obtained by aspiration of antral follicles (3–8 mm) from slaughterhouse ovaries. Maturation was carried out in TCM-199 medium supplemented with 57 mM cistein, 50 mg/L gentamycin sulphate, 0.5 mg/L porcine follicle-stimulating hormone (pFSH) (Folltropin-V®; Bioniche, Belleville, ON, Canada), 0.5 mg/L porcine luteinizing hormone (pLH) (Lutropin-V®; Bioniche) and 10% follicular fluid (Abeydeera, 2001), under mineral oil, in a humidified atmosphere of 5% CO_2 at 39°C during 48 hr. Oocytes were denuded in 0.1% hyaluronidase, evaluated under a stereomicroscope and those with homogeneous cytoplasm and a visible polar body were selected for ICSI.

2.4 | ICSI and in vitro culture of zygotes

Micromanipulation was carried out using an inverted Leica® DMIL microscope equipped with Narishige® micromanipulators. Briefly, a spermatozoon was immobilized by crushing the midpiece with the tip of the injection pipette and then aspirated from the flagellum. The mature oocyte was fixed by the holding pipette, placing the polar body in the 12 or 6 o'clock position to avoid damaging the metaphase plaque with the injection pipette. This pipette was pushed through the zona pellucida and subsequently through the oolemma into the cytoplasm at the 3 o'clock position. A small amount of ooplasm was aspirated

into the injection pipette to verify oocyte membrane penetration and subsequently, the immobilized spermatozoon was released. Randomly a group of mature oocytes was injected with vitrified-warmed sperm (treatment group), and another group was injected with sperm diluted and conserved at 17°C (control group). The microinjection was carried out in HEPES-TALP droplets supplemented with 0.3% BSA, while the semen suspension (both vitrified-warmed and liquid semen conserved at 17°C) was placed in drops of 10% polyvinylpyrrolidone (PVP). Temperature was maintained at 38°C throughout the process. As a control of the ICSI technique and to evidence parthenogenetic activation, mature oocytes were injected without depositing any sperm in the cytoplasm (Sham test).

2.4.1 | Experiment 1: culture of zygotes in an atmosphere with 5% CO_2

A total of 233 mature oocytes were injected, 90 of which were injected with vitrified-warmed sperm, 119 oocytes were injected with sperm conserved at 17°C, and 30 oocytes were submitted to the Sham test. In this experiment, the treatment and control groups were injected in different ICSI sessions.

The injected oocytes were washed and kept in a commercial culture medium without serum, media G1 (Gardner & Lane, 1997) at 38°C, 100% humidity and under 5% CO_2.

2.4.2 | Experiment 2: culture of zygotes in an atmosphere with 5% CO_2, 5% O_2 and 90% N_2 (tri-gas)

A total of 189 mature oocytes were injected, 91 of which were injected with vitrified-warmed sperm, 76 oocytes were injected with sperm conserved at 17°C, and 22 oocytes were submitted to the Sham test. In this experiment, the sperm from the treatment and control groups was injected during the same ICSI session to maintain the same conditions for both of them.

The injected oocytes were washed and kept in a commercial culture medium free of serum, media G1 (Gardner & Lane, 1997) at 38°C, 100% humidity and under tri-gas.

2.5 | Evaluation of pronuclei

The presence of pronuclei (PN) was evaluated 18 hr after the ICSI. The presumed zygotes were fixed for 15 min (2% glutaraldehyde in PBS), cultured with 1% Hoechst 33342 in PBS for 15 min, washed in PBS containing 1 mg/ml polyvinylpyrrolidone and mounted on glass slides. Oocytes were examined under an epifluorescence microscope using 330–380-nm (excitation) and 420-nm (emission) filters (Coy et al., 2005). The data collected from monitoring embryo development to the PN stage were analysed using comparisons of proportions and a $p < .05$ was considered significant.

3 | RESULTS

3.1 | Semen evaluation

Sperm motility, viability, membrane function and acrosome integrity were not maintained in the vitrified-warmed samples, whereas they were in the samples diluted with BTS and conserved at 17°C for 24 hr (Table 1). With regard to the sperm DNA quality, similar values were observed for both conservation methods (17°C and vitrification) in the 24-hr cooled samples and the vitrified-warmed samples (Table 1).

3.2 | Presence of pronuclei

3.2.1 | Experiment 1: culture of zygotes in an atmosphere with 5% CO_2

No significant differences ($p > .05$) were observed in embryo development to the PN stage (Figure 1) between the oocytes injected with

TABLE 1 Results obtained from the evaluation of porcine semen samples, before and after conservation at 17°C or vitrification (mean ± *SE*)

	Experiment 1 (CO_2 culture)				Experiment 2 (tri-gas culture)			
	Diluted in BTS		Vitrified		Diluted in BTS		Vitrified	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Total motility (%)	83.3 ± 3.3	62.5 ± 6.3	67.5 ± 8.3	0	78.6 ± 5.1	77.1 ± 4.7	67.5 ± 8.3	0
Viability (%)	76.7 ± 3.8	75.5 ± 3.3	63.3 ± 7.8	1.3 ± 0.5	70.1 ± 3.4	70.4 ± 7.2	63.3 ± 7.8	1.3 ± 0.5
Functional membranes (%)	61.2 ± 11.1	67.9 ± 9.9	51.3 ± 5.8	5.0 ± 1.1	67.6 ± 7.3	70.3 ± 6.0	51.3 ± 5.8	5.0 ± 1.1
Intact acrosomes (%)	97.3 ± 0.3	85.3 ± 9.7	94.3 ± 2.9	0	97.3 ± 0.6	96.3 ± 0.7	94.3 ± 2.9	0
Condensed DNA (%)	97.0 ± 1.5	94.4 ± 2.0	93.8 ± 1.7	91.6 ± 2.3	95.7 ± 0.6	94.6 ± 0.9	93.8 ± 1.7	91.6 ± 2.3
Non-denatured DNA (%)	99.3 ± 0.7	92.4 ± 1.2	98.9 ± 0.8	97.6 ± 1.3	98.0 ± 0.9	97.8 ± 0.8	98.9 ± 0.8	97.6 ± 1.3

Diluted in BTS Pre: semen samples diluted 1:2 in Beltsville Thawing Solution (BTS). Diluted in BTS Post: semen samples diluted 1:2 in Beltsville Thawing Solution (BTS) after 24 hr conservation at 17°C. Vitrified Pre: semen samples diluted in TALP + 1% BSA to a concentration of 5×10^6 spermatozoa/ml. Vitrified Post: warmed vitrified semen samples, centrifuged and re-suspended in TALP + 1% BSA.

vitrified-warmed sperm and those injected with semen diluted and conserved at 17°C, when cultured in a 5% CO_2 atmosphere (Table 2).

3.2.2 | Experiment 2: culture of zygotes in an atmosphere with 5% CO_2, 5% O_2 and 90% N_2 (tri-gas)

No significant differences ($p > .05$) were observed in embryo development to the PN stage (Figure 1) between the group of oocytes injected with vitrified-warmed sperm and those injected with semen diluted and conserved at 17°C, when cultured in a tri-gas atmosphere (Table 2).

No significant differences ($p > .05$) were observed in the percentage of PN obtained between 5% CO_2 and tri-gas, within each treatment (Table 2).

Of the 52 oocytes submitted to the Sham test and distributed among the different ICSI sessions, 50 evidenced no activation (Figure 2) and only 2, under the tri-gas culture conditions, presented a female PN (activation) (Table 2).

TABLE 2 Results of intracytoplasmic sperm injection using vitrified-warmed sperm and sperm diluted and conserved at 17°C

	Presence of PN (%)	
	5% CO_2	Tri-gas
Vitrified-warmed semen	31.8 (36/113)[a]	42.9 (39/91)[a]
Semen diluted and conserved at 17°C	35.5 (32/90)[a]	34.2 (26/76)[a]
Sham test	0.0 (0/30)[b]	9.0 (2/22)[b]

PN, pronuclei.
[a,b]Different letters, in the same column, indicate significant differences ($p < .05$).

4 | DISCUSSION

This study represents the first report of in vitro zygote production using vitrified-warmed porcine sperm for ICSI with in vitro matured porcine oocytes.

As no significant differences were observed between the treatment and control groups for the formation of PN, the results would seem to indicate that vitrified-warmed sperm conserve the same spermatic capacity for the formation of zygotes as sperm conserved at 17°C. This situation was maintained in both culture systems used (5% CO_2 and tri-gas atmosphere). In addition, the very low or absent percentage of PN obtained in the group of oocytes submitted to the Sham test would indicate that the PN observed in the treatment groups were a product of fertilization and not parthenogenetic activation arising from the ICSI technique itself.

With regard to the culture conditions used, if we compare the percentages of PN obtained in the treatment group with both 5% CO_2 and tri-gas atmosphere, although no statistically significant differences were detected, it is noteworthy that the number of PN was greater when using tri-gas atmosphere. This tendency could be indicating that tri-gas would be the culture condition of choice for future ICSI assays with vitrified-warmed porcine sperm. Im, Yang, Park, Chang, and Park (2000) reported that the oxygen concentration in the genital tract is lower than in atmospheric conditions; therefore, low oxygen concentrations may be beneficial for early embryo development. When bovine, porcine and ovine early stage embryos were cultured under low-oxygen concentrations (5% O_2), in vitro development was reportedly higher than those under high-oxygen concentration (5% CO_2 in air) (Im et al., 2000). In addition, embryos cultured under low-oxygen concentration (5% O_2) had higher total cell number and lower apoptotic cell number in bovine (Yuan et al., 2003) and porcine (Im et al., 2000) species. Perhaps the higher oxygen concentration, to which the in vitro cultured embryos are exposed, as compared to those present in the female genital tract, could be inducing the formation of reactive oxygen species (ROS), which are known to have deleterious effects on cells. Some of these effects include DNA damage, lipid peroxidation and oxidative modification of proteins (Johnson & Nasresfahani, 1994). Conversely, the presence of a greater number of zygotes in the treatment group compared with the number of zygotes of the control group under tri-gas atmosphere could be because the vitrified-warmed sperm in these culture conditions start the process of chromatin decondensation faster and easier than those conserved at 17°C. Binh, Van Thuan, and Miyake (2009), postulate that a certain level of membrane disintegration in the porcine sperm head region could be beneficial, allowing a rapid diffusion of the sperm factor "phospholipase C" within the ooplasm of the oocyte. This situation would cause a better oocyte activation and formation of the male pronucleus. We reported that vitrified porcine sperm present damaged plasma membranes and the absence of acrosomes (Arraztoa et al., 2012, 2017), therefore, this could perhaps promote pronuclei formation using vitrified sperm and

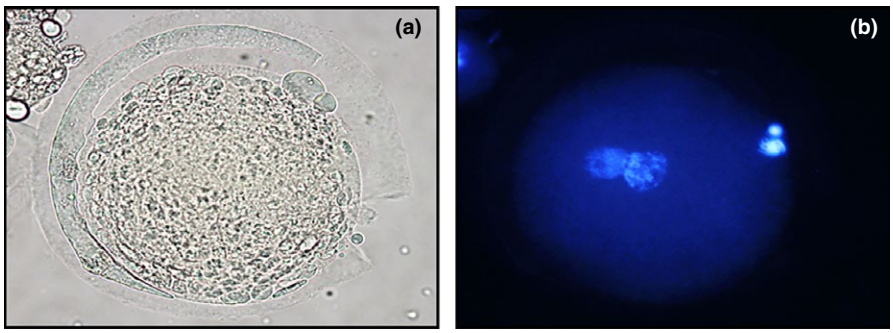

FIGURE 1 The presence of pronuclei in a porcine oocyte injected with a vitrified-warmed porcine spermatozoa. (a) Bright field. (b) Hoechst 33342 stain

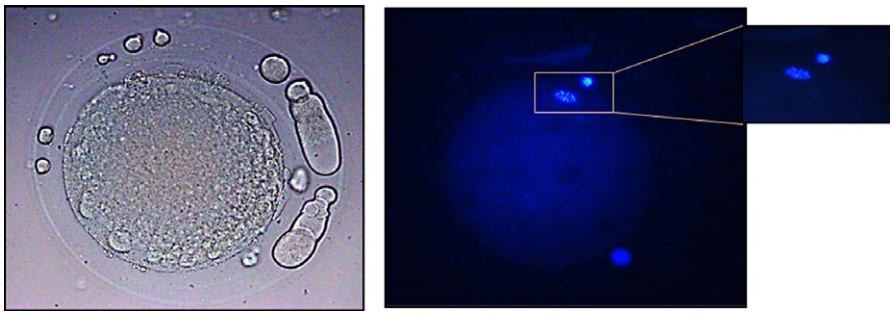

FIGURE 2 Metaphase plaque of an oocyte submitted to the Sham test (no activation). (a) Bright field. (b) Hoechst 33342 stain

thus we expected a higher PN formation with the vitrified-warmed samples vs. the control ICSI with cooled semen. Nevertheless, this was not the case when incubating in the CO_2 atmosphere, where ICSI results with vitrified-warmed sperm, although not significant, were lower than the control. Hence, perhaps the membrane damage suffered during cooling to 17°C is sufficient to be beneficial for oocyte activation, and beyond this damage there is no effect on PN formation. Possibly, the tendency to increase PN formation observed in the samples cultured in tri-gas using vitrified-warmed spermatozoa is due to synergism between the type of sample and the culture system used, but this would have to be confirmed by increasing the number of ICSI to ascertain if the tendency observed becomes significant.

In a previous study, we reported that the only seminal parameters of vitrified-warmed porcine sperm that showed no significant differences ($p < .05$) with the samples prior to vitrification were chromatin condensation (prior to vitrification: 89.9 ± 0.8% vs. vitrified-warmed: 93.3 ± 0.7%) and the sperm chromatin integrity (prior to vitrification: 97.2 ± 1.9 vs. vitrified-warmed: 91.2 ± 5.2%) (mean ± *SE*) (Arraztoa et al., 2012, 2017), while damaged sperm plasma membranes and the absence of acrosomes were observed. In these studies, both dimethylformamide and glycerol were assayed as the permeable cryoprotectants, and their use was compared to vitrification without any cryoprotectant. In conventional freeze-thawing protocols, both permeable and non-permeable cryoprotectants are used, in an effort to minimize their respective concentrations and thus their toxic effects. The use of sugars as non-permeable cryoprotectants has the added advantage of stabilizing cell membranes (Sánchez et al., 2011). Studies have been carried out using sucrose in human (Isachenko et al., 2008; Schulz, Muñoz, Risopatrón, & Sánchez, 2006), canine (Sánchez et al., 2011), rabbit (Rosato & Iaffaldano, 2013) and ram (Jiménez-Rabadán et al., 2015) sperm with encouraging results in sperm motility, membrane integrity and DNA quality in vitrified-warmed samples. Thus it would be interesting to evaluate the effectiveness of sucrose or other carbohydrates such as trehalose or raffinose for preserving porcine sperm motility, viability and acrosomes during vitrification. In this study, not only were we able to corroborate that vitrification of porcine spermatozoa allows conservation of cells with intact and condensed chromatin (97.6 ± 1.3% and 91.6 ± 2.3% respectively), but the percentage of PN obtained shows that it also permits conservation of cells with the capacity to produce zygotes using ICSI. Similar results have been reported by Kim, Lee, Jun, Lee, and Chung (1998) who obtained the initial stages of embryo development after using non-viable porcine sperm for ICSI (they injected isolated sperm heads) and by Alonso et al. (2014) who used air-dried equine sperm for ICSI. All these studies would support the suggestion made by García Roselló et al. (2008) that the presence of a viable sperm is not essential to obtain in vitro fertilization of an oocyte (using ICSI), rather, what is most important is the genetic material contained in the sperm head.

5 | CONCLUSION

According to the results obtained after using ICSI to produce porcine zygotes from in vitro matured oocytes injected with vitrified-warmed sperm, we could conclude that porcine sperm vitrification using the spheres method, at a concentration of 5×10^6 spermatozoa/ml in TALP medium supplemented with 1% BSA, allows conservation of sperm with condensed and intact chromatin capable of producing early embryo development up to the pronuclear stage.

Current studies are being carried out to evaluate further in vitro development of these zygotes and the possibility to obtain embryo stages transferable to the uterus.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research report.

AUTHOR CONTRIBUTIONS

The authors Arraztoa C.C and Baca Castex C. contributed with the ICSI sessions and the in vitro culture of the presumptive zygotes, while the author Alvarez G.M. did the in vitro maturation of the porcine oocytes. Arraztoa C.C, besides, cryopreserved and evaluated the porcine semen and analysed all the data obtained. The authors Cetica P.D and Neild D.M. have been the experiment directors, and all the authors together have contributed with the experimental design and the manuscript drafting.

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