Journal of the Hellenic Veterinary Medical Society

Vol 71, No 3 (2020)

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doi: 10.12681/jhvms.25078

To cite this article:

SIGÜENZA, S., ÁLVAREZ, I., & MATILLA, E. (2020). Effect of N-acetyl cysteine on the quality of blastocyst formation rate using cultured vitrified murine embryos. *Journal of the Hellenic Veterinary Medical Society*, *71*(3), 2315–2322. https://doi.org/10.12681/jhvms.25078

J HELLENIC VET MED SOC 2020, 71(3): 2315-2322
ΠΕΚΕ 2020, 71(3): 2315-2322

Research article
Ερευνητικό άρθρο

Effect of N-acetyl cysteine on the quality of blastocyst formation rate using cultured vitrified murine embryos

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ABSTRACT: Vitrification is the best method for embryo cryopreservation although it increases endogenous reactive oxygen species (ROS) production. N-acetylcysteine (NAC) a free radical scavenger may be used for reducing ROS toxic effects. The aim of the present study is to investigate potential beneficial effects of NAC on the developmental embryo competence applying different culture conditions in vitrified-warmed 2-cell embryos derived *in vivo* or *in vitro*.Thus, 2-cell embryos were vitrified or cultured fresh in presence or absence of 1 mM of NAC during: a) the entire embryo culture, b) for 24 hours with NAC at days 1.5 (G1) or 2.5 (G2) and returned to basal embryo culture (KSOM) or c) cultured in the presence of NAC for 12 hours at day 3.5 (G3). Despite NAC addition to fresh or vitrified embryos produced *in vivo* or by IVF, blastocyst rates remained unchanged. In vitrified-warmed IU or IVF-derived embryos, total cell number varied when NAC was added at day 1.5 although differences were not significant (60.1 ± 1.9 vs. 59.4 ± 1.3 for IU G1 and control respectively; and 59.3 ± 1.6 and 52.6 ± 3.0 IVF G1 and control respectively; mean cell number ± SEM, $p > 0.05$). It seems that the embryo culture medium supplementation with 1 mM of NAC in the first day after vitrification of development improves blastocyst quality of murine embryos and does not exert any beneficial effect at oyher culture points.

***Keywords*:** N-acetylcysteine, vitrification, early embryos, mouse, blastocyst quality.

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Date of initial submission: 24-10-2019
Date of revised submission: 13-04-2020
Date of acceptance: 05-05-2020

INTRODUCTION

Embryo cryopreservation is an assisted reproductive technology that has been extensively used in conservation programs (Somoskoi et al. 2015; Mandawala et al. 2016). Vitrification is the safest and most rapid method for embryo cryopreservation as it avoids the formation of intracellular and extracellular ice crystals (Rall W.F. 1985; Vajta 2000). This method consists in the solidification of the cell components at low temperatures reaching an amorphous-ice state by combining: a) extremely rapid cooling rates (over 1000ºC/min) and b) high concentrations of permeable cryoprotectants (typically ethylene glycol or EG and dimethyl sulfoxide or DMSO) and an non-permeable cryoprotectants agents (a carbohydrate) (Rall 1987). This method expedites the freezing process reducing the exposure to the toxic effect of the cryoprotectants (Tsang W.H. and Chow L.K. 2009) although embryo survival and the subsequent development is lower than when compared to fresh embryos (Kuleshova and Lopata 2002; Azadbakht and Valojerdi 2008). It has been previously shown that cryopreservation causes alterations in the embryos by affecting the integrity of the cell membrane and cytoskeleton, inducing mitochondrial depolarization and increasing the production of reactive oxygen species (ROS) (Yan et al. 2010; Liang et al. 2012). Among these insults, oxidative stress derived from mitochondrial damage is known to trigger the apoptotic cascade leading to a decrease in the survival rate and developmental competence of embryos after thawing (Somfai et al. 2007; Tatone et al. 2010). Additionally, *in vitro* culture of mammalian embryos further enhances free radical production overwhelming the embryos' endogenous antioxidant capacity (Ali et al. 2002), being especially notable in vitrified embryos. For this reason antioxidant addition to the embryo culture medium has been tried and has shown to improve gamete quality and embryo development (Silva et al. 2015). N-acetylcysteine (NAC) is a potent free radical scavenger that can be considered as a supplement to alleviate glutathione (GSH) depletion and free radical formation during oxidative stress in mice (Silva et al. 2015). GSH is one of the major non-enzymatic antioxidants present in oocytes and embryos and is essential for their protection against oxidative stress (Marí et al. 2009). Based on a recently published work that described that addition of NAC to murine oocytes after vitrification improves the mitochondrial status of the oocytes and the quality of the blastocyst obtained by IVF (Matilla et al. 2019), in the present work the effect of NAC at 1 mM in vitrified-warmed 2 cell embryos produced in vivo and in vitro, was assessed. This embryonic stage was chosen because it is known that two-cell embryos are more sensitive to vitrification than other stages. To do this, two cells embryos are more sensitive to vitrification than other stages (Ghandy, N., Karimpur, M., Abbas, A. 2017). Also, we want check the effect of NAC addition during a long time of culture. Two-cell embryos were recovered *in vivo* or after *in vitro* fertilization (IVF), vitrified and incubated for 24 hours with 1 mM of NAC during different time points (day 1.5, day 2.5 or day 3.5) or during the entire culture to the expanded blastocyst stage. The percentage of embryos reaching the expanded blastocyst stage as well as the total cell number was assessed using a stereomicroscope.

MATERIAL AND METHODS

Reagents

Unless otherwise stated, all the reagents were purchased from Sigma-Aldrich (Barcelona, Spain).

Animals and superovulation protocol

All the experimental procedures were reviewed and approved by the Ethical Committee of the Junta de Extremadura (Spain; Ref. Exp-20190103-2). B6D2F1/OlaHsd mice were housed in the Animal housing of University of Extremadura under a 12 h light/12 h dark cycles at a controlled temperature (19-23ºC) with free access to food and water. Females between 20-25 gr. of weight were intraperitoneally (IP) injected with 8 international units of equine chorionic gonadotropin (eCG, Veterin Corion, Divasa Farmavic) followed 47 h later by 8 international units of IP human chorionic gonadotropin (hCG, Foligon, MSD) to trigger ovulation.

In Vitro Fertilization

Male B6D2F1/OlaHsd mice aged 7-10 weeks and weight around 30 gr. were euthanized by cervical dislocation and ventrally dissected to remove the cauda epididymis. Once located, the epididymis and attached *vas deferens* were sectioned and transferred to a Petri dish containing 500 µl of pre-equilibrated human tubal fluid (HTF; at 37°C in a 5% CO_2/ 95% air atmosphere at 100% humidity) covered with mineral oil. Sperm were obtained by gently pressing the cauda epididymis through the vas deferens and were allowed to capacitate for 45 minutes at 37°C in a 5% CO_2/ 95% air atmosphere at 100% humidity. At the end of the incubation, sperm concentration was mea-

sured using a Makler chamber (Sefi-Medical instruments LTD, CA, USA). Cumulus-oocyte complexes (COCs) were recovered from oviducts following female euthanasia and placed in a Petri dish containing 500 µl of pre-equilibrated HTF covered with mineral oil; COCs were inseminated using 1.5 x 10^6 sperm/ml and were co-incubated for 6 hours and then transferred to equilibrated potassium-supplemented simplex optimization medium (KSOM). The day at which IVF was performed was considered as day 0. The next morning, cleaved embryos in two cell stage were retrieved and allocated as described in the experimental design section.

In vivo embryo recovery

Female mice were hormonally stimulated to trigger ovulation as previously described; after hCG injection, females were paired with B6D2 males in a 1:1 ratio. After 24 hours, females were sacrificed by cervical dislocation and the embryos were collected from the oviducts; these 2-cell embryos were allocated into an experimental group (see the experimental design section).

Vitrification and warming

In vivo and *in vitro* produced two cell embryos were equilibrated in M2 medium added with 7.5% of DMSO (v/v), 7.5% ethylene glycol (v/v) and 20% (v/v) fetal bovine serum (FBS) for 3 min. Afterwards, the embryos were transferred to a vitrification solution consisting of M2 supplemented with 20% FBS added with 15% ethylene glycol (v/v), 15% DMSO (v/v) and 0.5 M sucrose for 1 minute. An average of 15 embryos were loaded in 0.25 ml French straws (IMV, L'Aigle, France) at room temperature and sealed by ultrasounds (Superultrasonic Co, Taiwan). After that, the straws were plunged into liquid nitrogen and stored for at least 7 days. Embryos were warmed at 37ºC for 3-4 minutes in M2 medium added with 0.5 M sucrose and 20% FBS (v/v) and washed in M2 medium drops for further 3 minutes.

Experimental design

Fresh or vitrified-warmed *in vivo* and *in vitro* produced two cell embryos (1.5 days of development) were separately allocated to one of the following experimental groups:

Control: embryos were cultured in KSOM to the blastocyst stage; G1: embryos were cultured for 24 hours in KSOM supplemented with 1 mM NAC (day 1.5 to 2.5), after this incubation embryos were transferred to KSOM until day 4; G2: embryos were cultured in KSOM, transferred for 24 hours to KSOM supplemented with 1 mM NAC (day 2.5 to 3.5), and returned to KSOM until day 4; G3: embryos were cultured in KSOM for 12 hours supplemented with 1 mM NAC (day 3.5 to 4); C-NAC: embryos were cultured in KSOM supplemented with 1 mM NAC to the blastocyst stage. The number of embryos reaching the blastocyst stage was recorded visually by a stereomicroscope. All the embryos were moved to a new droplet of medium each day in presence or absence of NAC depending on the treatment group (Figure 1).

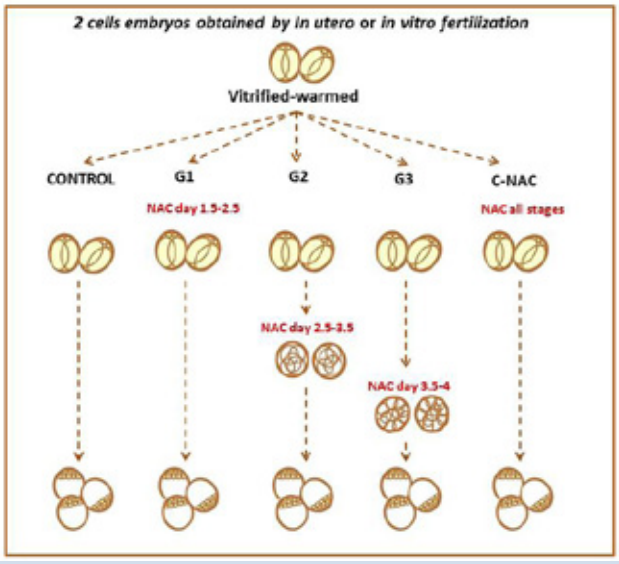

Figure 1. Two cell experimental design

Development to the blastocyst stage

To assess the development to the blastocyst stage, the embryos were followed *in vitro* for 3 days. The dish containing the embryos was placed under a stereomicroscope with a pre-heated stage (37°). The embryos showing a completely formed inner cell mass and expanded blastocele were assumed to have reached the blastocyst stage.

Total cell number

The number of cells in an embryo is the most critical indicator of embryo quality (ESHRE 2011). Therefore, in view of the previous data, expanded blastocysts were fixed in 4% formaldehyde in PBS added with 0.01% of polyvinyl alcohol (PVA; w/v) at 4ºC for 12 hours and stained with 2.5 µg/ml of Hoechst 33342 (Eugene, OR, USA) in PBS added with PVA for 10 minutes at 37ºC. Then, the blastocysts were mounted on glass slides with glycerol, covered with coverslips and sealed using nail polish. The embryos

were then visualized using a fluorescence microscope (Nikon Elipse TE2000-S) equipped with an ultraviolet lamp. Cell number was analyzed using the Fiji Image-J Software (1.45q, Wayne Rasband, NIH, USA).

Statistical analysis

Data were tested for normality using a Shapiro–Wilk test; the results are reported as mean ± standard error of the mean (SEM). Treatment groups were compared using ANOVA on ranks due to their non-Gaussian distribution. Between groups, all pair wise comparisons were made using a Holm-Sidak post-hoc test. All statistical analyses were performed using Sigma Plot software version 12.3 for Windows (Systat Software, Chicago, IL, USA). Differences among values were considered as statistically significant when $p < 0.05$.

RESULTS

Embryo development

Differences were found between treatments in the development to the blastocyst stage but were not significant ($p > 0.05$; Table 1). Homologous treatments *in vivo* and *in vitro* were compared in a second evaluation (control, G1, G2, G3 or C-NAC) but statistically significant differences were not found ($p > 0.05$).

Total cell number determination

Significant differences were found between Control group (74.2 ± 2.3; mean cell number ± SEM) compared with G2 (63.2 ± 2.7) and G3 (61.3 ± 3.2) in fresh *in utero* retrieved embryos ($p < 0.05$; Table 2). Significant differences were found comparing G1 group (74.1 ± 1.8) *vs* G2 (63.2 ± 2.7) and G3 (61.3 ± 3.2) in fresh *in utero* retrieved embryos ($p < 0.05$; Table 2).

Despite NAC addition in fresh IVF group significant differences were not found ($p > 0.05$; Table 2).

When *in utero*-derived embryos were vitrified and warmed significant differences where obtained in presence of 1mM of NAC (G2 and G3) compared to the control group (59.4 ± 1.3; mean cell number ± SEM *vs.* 45.7 ± 2.2 and 46.8 ± 2.2 respectively; $p < 0.05$; Table 2

2). Also, significant differences were found in embryos supplemented with 1 mM NAC in 1.5 day group compared to days 2.5 and 3.5 (60.1 ± 1.9 mean cell number ± SEM*; vs.* 45.7 ± 2.2 and 46.8 ± 2.2 respectively; $p < 0.05$; Table 2). Embryos cultured in presence of NAC during all stages showed significant differences between NAC added in 2.5 day (55.6 ± 3.0 vs. 45.7 ± 2.2; $p < 0.05$; Table 2).

Conversely, when IVF derived embryos were vitrified and warmed, NAC addition at day 1.5 increased the blastomere number in G1 (59.3 ± 1.6; mean cell number ± SEM) compared to day 2.5 and 3.5 of embryo development (59.3 ± 1.6; mean cell number ± SEM, vs. 49.6 ± 2.6 and 46.5 ± 2.6 (G2 and G3 respectively); $p < 0.05$, Table 2 and Figure 2).

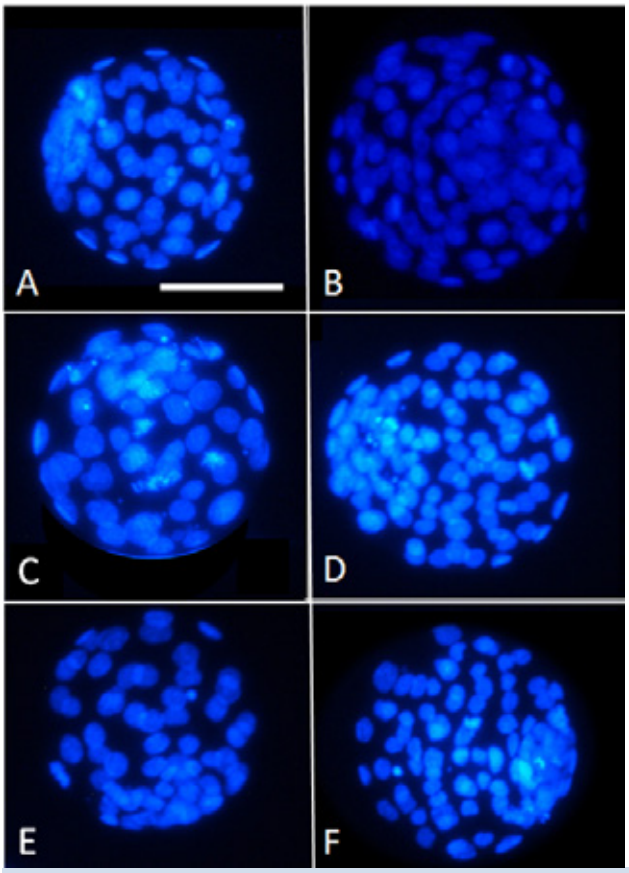

Figure 2. Blastocyst experimental design

In vivo or IVF-derived murine 2-cell embryos were obtained, vitrified and cultured to the blastocyst stage in the presence or absence of 1 mM NAC. Representative micrographs are shown of blastocysts derived from 2-cell embryos that were obtained after **A)** IVF (fresh); **B)** *In utero* harvesting (fresh); **C)** 2-cell embryos produced *in utero* followed by vitrification; **D)** produced *in utero* followed by vitrification, cultured in presence of 1 mM NAC for 24 hours (day 1.5) and allowed to develop; **E)** IVF derived 2-cell embryo subjected to vitrification and warming; **F)** IVF derived 2-cell embryo subjected to vitrification, cultured in presence of 1 mM NAC for 24 hours (day 1.5) and allowed to develop. White bar represents 100 μm; the micrographs were taken using a 40x objective.

Table 1. Embryo development to the blastocyst stage

Embryo source	Treatment	n	Blastocyst rate
Fresh *In utero* (IU)	Control	40	92.0 ± 2.7
	NAC G1	40	92.9 ± 1.8
	NAC G2	40	93.0 ± 3.1
	NAC G3	40	89.0 ± 3.5
	C-NAC	40	93.6 ± 3.9
IU- vitrified embryos	Control	40	83.8 ± 4.1
	NAC G1	40	85.1 ± 3.2
	NAC G2	40	82.5 ± 4.2
	NAC G3	40	81.1 ± 2.6
	C-NAC	40	84.3 ± 3.3
Fresh IVF	Control	40	84.3 ± 1.6
	NAC G1	40	85.3 ± 1.0
	NAC G2	40	81.2 ± 3.5
	NAC G3	40	80.7 ± 4.3
	C-NAC	40	83.4 ± 3.3
IVF – Vitrified	Control	40	84.9 ± 3.2
	NAC G1	40	85.0 ± 1.6
	NAC G2	40	78.4 ± 4.2
	NAC G3	40	76.4 ± 4.1
	C-NAC	40	83.2 ± 4.5

Blastocyst rates of fresh and vitrified mouse embryos obtained by IVF or *in vivo* in presence or absence of NAC. The groups studied were: Control: embryos cultured in the absence of NAC; NAC G1: 1 mM NAC was added for 24 hours to the embryo culture medium at day 1.5 of embryo development; NAC G2: 1 mM NAC was added for 24 hours to the embryo culture medium at day 2.5 of embryo development; NAC G3: 1 mM NAC was added for 24 hours to the embryo culture medium at day 3.5 of embryo development; C-NAC: culture medium was supplemented with 1 mM of NAC during the entire embryo culture. Statistically significant differences were not found between treatments in the same group or between homologous treatments in the different groups studied. Values are expressed as the mean percentage ± SEM ($p > 0.05$).

Table 2. Cell number of murine blastocyst from two cells embryos.

Embryos	Treatment	n	Cell number
Fresh *in utero* (IU)	Control	20	$74.2 \pm 2.3^{a,b}$
	NAC G1	20	$74.1 \pm 1.8^{c,d}$
	NAC G2	20	$63.2 \pm 2.7^{a,c}$
	NAC G3	20	$61.3 \pm 3.2^{b,d}$
	C-NAC	20	71.2 ± 2.1
IU- vitrified embryos	Control	20	$59.4 \pm 1.3^{a,b}$
	NAC G1	20	$60.1 \pm 1.9^{c,d}$
	NAC G2	20	$45.7 \pm 2.2^{a,c,e}$
	NAC G3	20	$46.8 \pm 2.2^{b,d}$
	C-NAC	20	55.6 ± 3.0^{e}
Fresh IVF	Control	20	55.4 ± 1.2
	NAC G1	20	55.4 ± 1.5
	NAC G2	20	52.8 ± 2.3
	NAC G3	20	50.8 ± 2.7
	C-NAC	20	55.1 ± 1.8
IVF - Vitrified	Control	20	52.6 ± 3.0
	NAC G1	20	$59.3 \pm 1.6^{a,b}$
	NAC G2	20	49.6 ± 2.6^{a}
	NAC G3	20	46.5 ± 2.6^{b}
	C-NAC	20	55.8 ± 1.4

Total cell number of vitrified mouse embryos obtained by IVF or *in vivo* in presence or absence of NAC. Control: embryos cultured in the absence of NAC; NAC G1: 1 mM NAC was added for 24 hours to the embryo culture medium at day 1.5 of embryo development; NAC G2: 1 mM NAC was added for 24 hours to the embryo culture medium at day 2.5 of embryo development; NAC G3: 1 mM NAC was added for 24 hours to the embryo culture medium at day 3.5 of embryo development; C-NAC: culture medium was supplemented with 1 mM of NAC during the entire embryo development. Values are expressed as the mean percentage ± SEM. Values bearing different letters in the same group differ statistically ($p < 0.05$).

DISCUSSION

Embryo vitrification is an important hallmark of the assisted reproductive technology industry. The murine model has been extensively used to test and develop vitrification protocols due to its high capacity to withstand the process. However, depending upon the developmental stage, their tolerance to vitrification notably varies (Ghandy, Nasibeh and Karimpur Malekshah 2017). For example, it has been described that vitrified 2-cell embryos exhibit a similar survival rate after warming compared to 4-cell, 8-cell, morulae and blastocysts, although their development to the blastocyst stage is significantly lower compared to vitrified-warmed embryos at the 8-cell stage (Zhang *et al.* 2009; Ghandy, N., Karimpur, M., Abbas, A. 2017; Ghandy, Nasibeh and Karimpur Malekshah 2017). This difference has been attributed to a lower cryoprotectant permeability of the zona pellucida at the earlier embryo stages and to increased ROS production after vitrification, being this stage the less suitable for cryopreservation (Pedro et al. 2005; Gao et al. 2012). Thus, in our setting, vitrified 2-cell embryos produced *in vivo* and *in vitro* were used to study the effect of NAC supplementation during the entire culture or at different time points. In our setting no statistically significant differences were observed in the developmental competence of the 2-cell embryos despite NAC addition, *in vitro* or *in vivo* embryo production and/or cryopreservation (Table 1; $p > 0.05$). However, it has to be mentioned the high development to the expanded blastocyst stage obtained after embryo vitrification in our setting (84.3 ± 1.6 and 84.9 ± 3.2; blastocyst rate for vitrified-warmed *in vivo vs.* IVF produced embryos respectively; Table 1). Previous works have reported up to 69.4% blastocyst rates after *in utero* retrieved 2-cell embryo vitrification using the Cryotop method (Zhang et al. 2009; Ghandy, N., Karimpur, M., Abbas, A. 2017; Ghandy, Nasibeh and Karimpur Malekshah 2017) or a 97.3% blastocyst rate when the embryos were produced by IVF and vitrified using the Kitasato System (Momozawa et al. 2017). Although the mouse strain used and the vitrification protocol (close in our setting *vs.* open in the mentioned reports) might influence the results obtained, our results suggest that *in vivo* and *in vitro* produced 2-cell embryos similarly withstand vitrification when a closed system is used.

As similar blastocyst rates were found among groups, we decided to compare the number of blastomeres/embryo between groups, as it has been shown to be a reliable indicator of embryonic quality (Mallol et al. 2013; Kong et al. 2016). Coinciding with our findings it has been reported that the mean cell number in mouse blastocysts recovered from uterus is 74.5 ± 2.3 (Sawicki and Mystkowska 1990) and that total cell number significantly drops in IVF-derived embryos (Van der Elst J et al. 1998). The lower cell number of *in vitro*-derived embryos has been linked to a higher cell death compared to *in utero*-derived embryos (Jurisicova et al. 1998) and to an enhanced ROS production occurring during *in vitro* embryo production (Guérin et al. 2001).

However, despite NAC addition during the entire embryo culture, total cell number did not improve in fresh or vitrified embryos disregarding their source (*in vitro* or *in utero*; Table 2). Similar findings have been described in vitrified *in vitro*-derived porcine embryos in which addition of L-ascorbic acid to the embryo culture medium ameliorated ROS production but did not result in enhanced total cell number (Castillo-Martín et al. 2014).

The results by Castillo-Martín et al. (2014) and our own results suggest that, antioxidants added during the entire embryo culture can exert effective ROS scavenging that is not reflected by an enhanced total cell count in the resulting embryos. Interestingly, addition of 1 mM of NAC to the embryo culture at the different time points tested (day 1.5, 2.5, 3.5 or the entire embryo culture) to vitrified-warmed 2-cell embryos produced *in vitro* or *in vivo* exerted different effects. The total cell number in vitrified-warmed embryos (*in vitro* or *in vivo)* increased when NAC was added at days 1.5 compared to the control although differences were not significant (Table 2 and Figure 2). Conversely, in all groups total cell number significantly decreased at day 2.5 (G2) and 3.5 (G3) compared to control; also, we found significant differences between G1 (1.5 Day) and day 2.5 (G2) and 3.5 (G3) from fresh and vitrified in utero embryos and IVF vitrified and warmed embryos. Our results suggest that NAC addition exerts its maximum beneficial effect right after embryo warming (*in vitro* and *in vivo* produced embryos). It is known that physiological ROS production is required for correct embryo division and pre-implantation development (Covarrubias et al. 2008). Thus, our data suggest that after vitrification and warming the NAC scavenging properties might be ameliorating the increased ROS production triggered by the cryopreservation process. This effect was observed only when NAC was added during the first third of the embryo culture (*in vivo*

produced embryos), but in the second and last third of embryo development, the damages induced seem to be irreversible. This effect can be attributed to the addition of the antioxidant in the correct moment, as its addition in an non-ideal stage of development can be harmful (Guerin et al. 2001). The fact that continuous NAC addition did not result in an enhanced total cell number suggests that excessive ROS scavenging could be interfering with blastomere cytokinesis explaining why total cell number does not increase (Bedaiwy et al. 2004).

CONCLUSION

NAC addition enhances total cell number and embryo development of vitrified murine 2-cell embryos obtained *in vivo* or *in vitro* when added at day 1.5 of culture. For IVF- vitrified embryos significant differences were found depending upon the day of NAC supplementation. The vitrification process detrimentally affects *in vivo*-derived 2-cell embryos more vividly than *in vitro* produced embryos, as the number of blastomeres is significantly lower after reaching the blastocyst stage. NAC supplementation during the entire culture to the blastocyst stage does not improve the quality of fresh 2-cell embryos and in utero vitrified-warmed embryos. NAC addition to 2.5 and 3.5 day has and toxic effect decreasing embryo quality of IU derived embryos ($p < 0.05$). More studies are necessary to clarify the optimal concentrations of NAC that improve the quality of vitrified murine embryos during early embryo development.

ACKNOWLEDGEMENTS

The authors wish to thank their accessibility to the rest of member of the Department of Biochemistry and Molecular Biology from the School of Life Sciences, University of Extremadura, Badajoz, Spain.

CONFLICT OF INTEREST

None declared by the authors.

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