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SHORT COMMUNICATION

Impact of N-acetyl-L-cysteine on spindle morphology and reactive oxygen species in vitrified/warmed in vitro matured bovine oocytes

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Abstract

Low developmental potential of vitrified in vitro matured (IVM) bovine oocytes is frequently attributed to high levels of reactive oxygen species (ROS) and abnormal spindle assembly. This study aimed to evaluate the efficacy of N-acetyl-L-cysteine (NAC), a cell-permeating antioxidant, added to IVM medium in reducing ROS and preserving spindle configuration of vitrified/warmed IVM bovine oocytes. Oocytes collected from abattoir ovaries were either cultured in IVM medium or in IVM medium supplemented with 1 mM NAC for the initial 8 h of IVM. Half of the oocytes of each group were vitrified/warmed, and spindle morphology and ROS production were assessed at 24 h of IVM. Results indicated that fresh oocytes IVM with NAC improved spindle configuration, with significantly lower ROS levels compared to the control group. Vitrification resulted in lower percentages of bovine oocytes reaching the metaphase II stage but similar ROS levels to non-vitrified oocytes, regardless of NAC supplementation. However, the supplementation of NAC during maturation had no effect on spindle or chromosome configuration of vitrified oocytes. These findings emphasize NAC's potential in enhancing the quality of IVM bovine oocytes but its addition at 1 mM for 8 h to IVM medium did not decrease levels of ROS nor improve spindle assembly after vitrification.

KEYWORDS
chromosomes, cryopreservation, microtubules

1 | INTRODUCTION

Cryopreservation of mammalian oocytes is an essential component of assisted reproduction and animal production. However, despite recent developments, the optimal procedure for bovine oocyte cryopreservation has not been established yet. The impaired embryo development of vitrified oocytes has been attributed to abnormal meiotic spindle assembly and increased levels of reactive oxygen species (ROS), among others (reviewed by Mogas, 2018). Different exogenous antioxidants have been tested to reduce oxidative damage caused by oocyte cryopreservation (revised by Cao et al., 2022). N-acetyl-L-cysteine (NAC) is an antioxidant that exerts its effects through direct electron donation to reactive species and indirectly by serving as a precursor of glutathione (GSH), thereby enhancing the intracellular antioxidant system and protecting of the meiotic spindle against oxidative stress (Pei et al., 2018). In bovine oocytes, Sun et al. (2021) reported that supplementation of IVM medium with a specific duration (initial 8 h of IVM), and concentration

(1 mM) of NAC increased intracellular GSH synthesis and reduced ROS production, improving oocyte and embryo quality. NAC supplementation throughout vitrification/warming and IVM significantly reduced ROS levels and improved nuclear maturation and mitochondrial distribution pattern of murine oocytes vitrified at the germinal vesicle (GV) stage (Yue et al., 2016). This study examined the efficacy of 1 mM NAC added to IVM medium prior to vitrification/warming in terms of its capacity to reduce oxidative stress and protect the spindle configuration of IVM bovine oocytes.

2 | MATERIALS AND METHODS

2.1 | Experimental design

After collection, COCs were IVM for 24 h in a non-supplemented IVM medium (Control group), or cultured for the initial 8 h in an IVM medium supplemented with 1 mM NAC (NAC group). NAC concentration and duration of supplementation were chosen based on the findings of Sun et al. (2021). At 22 h of IVM, half of the oocytes of each IVM group were vitrified/warmed and allowed to recover for 2 additional hours, resulting in the vitrification groups VIT and NAC-VIT. Oocytes from each of the four treatment groups were collected at 24 h of IVM to assess spindle and chromosome configurations (three replicates) and ROS production (three replicates).

2.2 | In vitro maturation

The in vitro maturation procedure has been described in detail elsewhere (García-Martínez et al., 2022).

2.3 | Oocyte vitrification and warming

At 22 h of IVM, half of the oocytes of each IVM group were partially denuded and vitrified/warmed, as described in García-Martínez et al. (2022). After warming, oocytes were transferred back into a non-supplemented maturation medium to allow them to mature for 2 additional hours.

2.4 | Spindle and chromosome configuration

At 24 h of IVM, oocytes were denuded of cumulus cells, fixed and immunostained as described in García-Martínez et al. (2020). Configuration of the metaphase II (MII) spindle was considered as morphologically normal when a barrel-shaped structure formed by organized microtubules crossing the length of the spindle from pole to pole was observed. Chromosome configuration was regarded as normal when chromosomes were arranged on a compact metaphase plate at the equator of the spindle. Abnormal spindle structures included partial or total disorganization or decondensation of microtubules. Abnormal chromosome organization included dispersal of chromosomes or chromosomes with an aberrant, less-condensed appearance (Figure 1).

2.5 | Reactive oxygen species

Intracellular ROS levels in oocytes were quantified at 24 h of IVM by labelling with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) as described in García-Martínez et al. (2020).

2.6 | Statistical analysis

GraphPad Prism software 9.3.1 was used for data analysis. Normality and homogeneity of variances were checked using Shapiro–Wilk and Levene tests, respectively. Parametric results were compared by a one-way analysis of variance followed by the Bonferroni test. For non-parametric results, a Kruskal–Wallis test was used. Data are expressed as means ± standard error of the mean (SEM). Significance was set at $p \leq .05$.

3 | RESULTS AND DISCUSSION

Both vitrification groups had significantly lower percentages of bovine oocytes reaching the MII stage compared to the control group while fresh oocytes IVM with NAC did not differ from the fresh control (Table 1). Maturation of fresh oocytes with NAC for 8 h produced similar percentages of normal chromosome configurations but higher ($p < .05$) percentages of normal spindle configuration when compared to fresh control oocytes. Previous studies have shown that NAC reduces spindle anomalies of oocytes in patients with mild endometriosis (Giorgi et al., 2016) or during post-ovulatory oocyte ageing in vitro in mice (Wang et al., 2019). Also, 1.5 mM NAC supplementation during vitrification/warming and IVM has been shown to significantly improve the embryo developmental competence of vitrified GV murine oocytes (Yue et al., 2016). However, these positive effects of NAC were not noticeable in the NAC-VIT group, and similar percentages of normal spindle and chromosome organization were observed when compared to the vitrified control group. This lack of effect may be attributed to either the meiotic stage at which oocytes were vitrified (Yue et al., 2016) or to the timing of NAC supplementation. Matilla et al. (2019) found that adding NAC after vitrification of murine oocytes resulted in better oocyte quality.

In our study, supplementation of IVM medium with 1 mM NAC for 8 h significantly decreased ROS levels ($n = 43$; 0.477 ± 0.013) when compared to the control group ($n = 43$; 0.431 ± 0.009; Figure 2). However, no significant differences in ROS levels were observed between vitrified and fresh oocytes, regardless of the NAC supplementation (VIT $n = 38$; 0.438 ± 0.013; NAC-VIT $n = 32$; 0.445 ± 0.012).

Although the results on ROS production obtained in this study in fresh, non-vitrified oocytes IVM with NAC are consistent with those of Sun et al. (2021), 1 mM NAC during the initial 8 h of

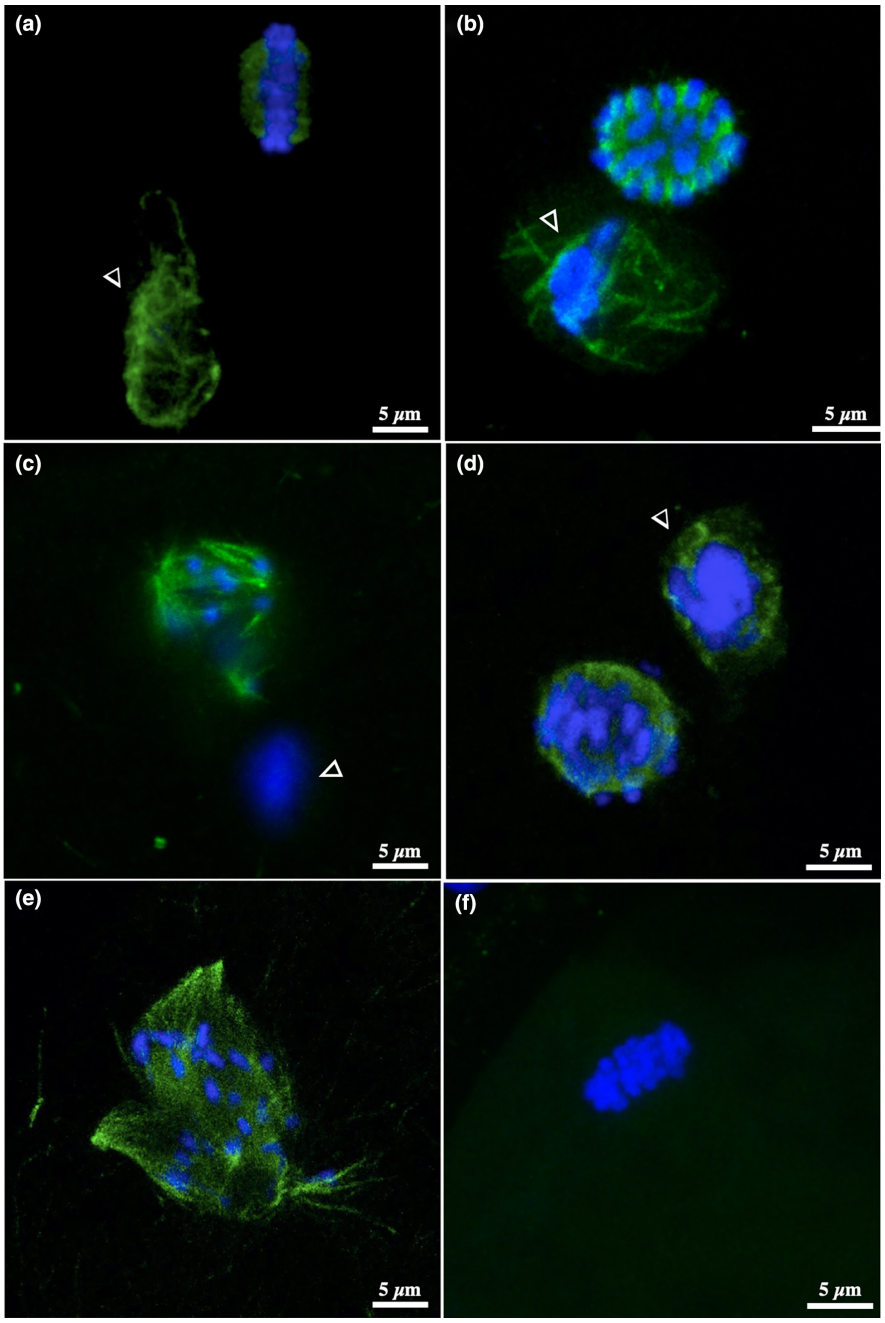

FIGURE 1 Representative confocal laser-scanning photomicrographs of spindle and chromosome configurations of vitrified IVM bovine oocytes. (a, b) Normal spindle and chromosome configurations; (c–e) Disorganized chromosomes; (f) Condensed chromosomes; (c, e) Disorganized spindle configuration; (d, f) Partial o tolal decondensed spindle configuration. Green: tubulin (Alexa Fluor™ 488); Blue: chromosomes (DAPI). White arrowheads point to polar bodies.

TABLE 1 Effect of 1 mM NAC supplementation of IVM medium on spindle and chromosome configurations after vitrification/warming.

			Spindle configuration (%)*		Chromosome configuration (%)*	
Treatment	***n***	**MII (%)**	**Normal**	**Abnormal**	**Normal**	**Abnormal**
Control	50	84.0 ± 1.75^{a}	83.33 ± 2.82 ^{a}	16.67 ± 2.82 ^{a}	80.95 ± 3.29 ^{ab}	19.05 ± 3.29 ^{ab}
NAC	43	81.4 ± 2.54^{ab}	94.29 ± 1.53 ^{b}	5.71 ± 1.53 ^{b}	85.71 ± 1.36 ^{b}	14.29 ± 1.36 ^{b}
VIT	33	78.7 ± 2.48^{b}	92.31 ± 1.86 ^{ab}	7.69 ± 1.86 ^{ab}	73.08 ± 1.69 ^{ac}	26.92 ± 1.69 ^{ac}
NAC-VIT	35	71.4 ± 2.80^{b}	84 ± 2.35 ^{a}	16 ± 2.35 ^{a}	64 ± 3.31 ^{c}	36 ± 3.31 ^{c}

Note: Data are given as mean ± SEM. ^{a,b,c}Different superscript letters indicate significant differences between treatments ($p < .05$).

*Rates of oocytes with the given morphology were calculated from the total number of oocytes reaching the MII stage. COCs were in vitro matured in Control: non-supplemented IVM medium; NAC: IVM medium supplemented with 1 mM NAC for the initial 8 h of IVM; VIT: non-supplemented IVM medium and vitrified/warmed; NAC-VIT: IVM medium supplemented with 1 mM NAC for the initial 8 h of IVM and vitrified/warmed.

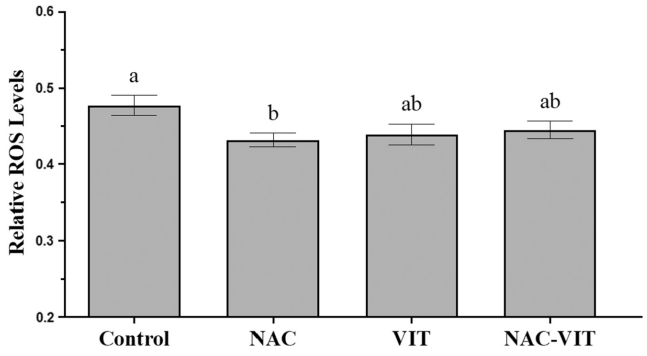

FIGURE 2 Effects of 1 mM NAC supplementation of IVM medium on relative ROS fluorescence intensity of vitrified/warmed bovine oocytes. The fluorescence intensity of the cytoplasm was quantified using ImageJ Software. [a,b]Different superscripts indicate significant differences ($p < .05$). Data are shown as mean ± SEM.

IVM did not ameliorate ROS production in vitrified oocytes. Yue et al. (2016) observed that NAC significantly reduced ROS activity and improved mitochondrial distribution patterns when murine oocytes were vitrified at the germinal vesicle stage, underlying the importance of the oocyte meiotic stage at vitrification or the timing of NAC supplementation (Matilla et al., 2019). In porcine oocytes, 2.5 and 3.5 mM NAC supplementation significantly protected the developmental potential of oocytes exposed to heat stress while 1.5 mM was not sufficient to rescue the developmental potential of heat-stressed treated oocytes (Hu et al., 2020). Therefore, higher concentrations may be necessary to detect a potentially beneficial protective effect of NAC on vitrified IVM bovine oocytes. Nevertheless, it is important to consider that concentrations as high as 10 mM of NAC during the IVM period were found to be harmful to the oocyte (Sun et al., 2021). In this sense, more experiments to assess NAC capacity to protect bovine oocytes against vitrification-induced damage are warranted.

AUTHOR CONTRIBUTIONS

S.G. and T.M. conceived and designed the experiments; S.G. and J.D-M. performed the experiments. T.M. provided the resources and S.G. and T.M. wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

None of the authors have any conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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