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Effects of *N*-acetylcysteine on growth, viability and reactive oxygen species levels in small antral follicles cultured *in vitro*Efigênia B. Cordeiro, Bianca R. Silva, Laís R. F. M. Paulino, Pedro A. A. Barroso, Laryssa G. Barrozo, Miguel F. de Lima Neto, José R. V. Silva 

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ABSTRACT

Objective: To investigate the effects of different concentrations of *N*-acetylcysteine on follicular growth and morphology, as well as on viability, levels of reactive oxygen species (ROS) and meiotic progression of oocytes from *in vitro* cultured bovine early antral follicles.

Methods: Isolated early antral follicles (about 500 μ m) were cultured in TCM-199⁺ alone or supplemented with 1.0, 5.0 or 25.0 mM *N*-acetylcysteine at 38.5 °C with 5% CO₂ for 8 days. Follicle diameters were evaluated at day 0, 4 and 8 of culture. At the end of culture, the levels of ROS, chromatin configuration and viability (calcein-AM and ethidium homodimer-1 staining) were investigated in the cumulus-oocyte complexes. Comparisons of follicle diameters between treatments were performed. Data on percentages of morphologically normal follicles, growth rates and chromatin configuration in different treatments were compared.

Results: An increase in follicular diameters after culture in all treatments was observed, except for follicles cultured with 25.0 mM *N*-acetylcysteine. Fluorescence microscopy showed that oocytes cultured in all treatments were stained positively with calcein-AM, and that 5.0 mM *N*-acetylcysteine reduced fluorescence for ethidium homodimer-1. Intracellular levels of ROS in oocytes from follicles cultured with 1.0 mM *N*-acetylcysteine showed a significant reduction compared to other treatments. The presence of *N*-acetylcysteine in culture medium did not influence the rates of oocyte at the germinal vesicle stage.

Conclusions: *N*-acetylcysteine at concentrations of 1.0 and 5.0 mM reduces ROS levels and staining for ethidium homodimer-1 in *in vitro* cultured follicles, respectively, while 25.0 mM *N*-acetylcysteine decreases follicular growth and the percentages of continuously growing follicles.

KEYWORDS: Antioxidant; *N*-acetylcysteine; Antral follicles; Bovine; Reactive oxygen species

1. Introduction

The growth of preantral and early antral follicle is a key step of follicular development associated with intense transcriptional activity and acquisition of oocyte competence[1]. Some *in vitro* studies report that oocytes from small antral follicles, between 1 and 2 mm, have significantly reduced competence when compared to those from larger antral follicles (>3 mm), which have the ability to complete nuclear maturation[2,3]. Several molecular events and the bidirectional communication between the oocyte and surrounding granulosa cells by transzonal projections coordinate the development of early antral follicles up to ovulation[4].

Significance

The *in vitro* development of early antral follicles up to maturation opens new perspectives to use of their oocytes for *in vitro* fertilization. However, oxidative stress during *in vitro* culture of these follicles can result in accumulation of reactive oxygen species (ROS) and consequently oocyte degeneration. To minimize the damages caused by oxidative stress, the results of this study show that supplementation of culture medium with 1.0 mM *N*-acetylcysteine reduces ROS levels and improves oocyte viability in cultured early antral follicles. Thus, the presence of this substance in culture media has great potential to improve *in vitro* culture systems for follicles and oocytes, which can bring positive impacts for assisted reproduction technologies.

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In the last decades, *in vitro* culture of ovarian follicles has contributed to a better understanding of the roles of hormones and growth factor during follicular development[5,6], but *in vitro* growth of follicles up to maturation was still not reported for human and domestic animals[7,6]. In domestic animals, the *in vitro* development of secondary follicles during 18 days of culture is generally associated with increased degeneration rate at early antral follicle stages[8]. Under normal physiological conditions, the cells produce variable levels of reactive oxygen species (ROS), but their excess can cause oxidative stress and compromise cell development[9,10]. This is one of the main factors associated with the low quality of *in vitro* cultured ovarian follicles[6,11]. Therefore, adding substances with antioxidant activity in the culture medium can prevent or mitigate these damages[12].

N-acetylcysteine (NAC) is a precursor of cysteine and reduced glutathione that has an important role in the control of oxidative stress[13]. Fabbri *et al*[14] reported that NAC and follicle-stimulating hormone (FSH) improved preantral follicle growth and viability in cultured ovarian tissues. Furthermore, NAC increased the content of glutathione and reduced ROS levels in mice cumulus-oocyte complexes (COCs) matured *in vitro*[15]. In porcine COCs, NAC also reduced the levels of ROS, and increased cumulus cell expansion during *in vitro* maturation[16]. However, the effects of NAC during the *in vitro* growth of bovine early antral follicles are still unknown. Studying early antral follicle growth *in vitro* can contribute to the elucidation of the mechanisms that regulate their growth, and consequently enable the use of their oocytes in *in vitro* maturation protocols.

This study aims to investigate the effects of different concentrations of NAC on the growth and morphology of early antral follicles and on the viability, ROS levels and meiotic progression of oocytes from small antral follicles cultured *in vitro*.

2. Materials and methods

2.1. Source of ovaries

Ovaries ($n=150$) from adult cows were collected from a local slaughterhouse immediately after slaughter. The ovaries were washed in 70% ethanol, followed by two rinses in tissue culture medium 199 (TCM-199) buffered with 2-hydroxyethyl (HEPES) and supplemented with penicillin (100 IU) and streptomycin (0.1 mg/mL). The ovaries were transported within 1 h to the laboratory in TCM-199 at 4 °C.

2.2. Follicle isolation and *in vitro* culture

In the laboratory, the ovarian cortex (1–2 mm) was fragmented with a sterile scalpel blade and placed in TCM-199 medium supplemented with HEPES. All procedures were aseptically performed in a safe

laminar flow station. Antral follicles of approximately 500 μm in diameter were manually dissected from the strips of cortical tissues using 26-gauge needles under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan). After isolation, follicles with a visible oocyte surrounded by granulosa cells, an intact basement membrane and the presence of an antral cavity were selected for culture. The follicles were individually cultured in 150 μL of culture medium under mineral oil in Petri dishes (60 mm \times 15 mm, Corning, USA). The control culture medium, called TCM-199⁺, consisted of TCM-199 (pH 7.2–7.4) supplemented with FSH (50 $\mu\text{g}/\text{mL}$), insulin (50 $\mu\text{g}/\text{mL}$), transferrin (50 $\mu\text{g}/\text{mL}$) and selenium (50 $\mu\text{g}/\text{mL}$) (ITS), 0.015 mg/mL of bovine serum albumin (BSA), penicillin (100 IU), streptomycin (0.1 mg/mL), glutamine (50 $\mu\text{g}/\text{mL}$), and hypoxanthine (50 $\mu\text{g}/\text{mL}$)[17,18]. For the treatments, the follicles were randomly cultured in TCM-199⁺ alone or supplemented with 1.0, 5.0 or 25.0 mM NAC[19,20]. Follicles were cultured at 38.5 °C with 5% CO₂ in air for 8 days[19]. At day 4 of culture, 75 μL of medium was replaced by fresh medium.

2.3. Assessment of follicular morphology and growth

The morphological evaluation of follicles was performed with the aid of a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) at days 0, 4 and 8. Morphologically normal, follicles had spherical oocyte surrounded by homogeneous granulosa cells, intact basement membrane and external stromal-thecal layer. Follicles with dark oocytes and cumulus cells were considered degenerated. In addition, perpendicular measurements were performed on normal follicles at days 0, 4 and 8.

2.4. Assessment of oocyte viability by fluorescence microscopy

After culture, COCs were extruded with the aid of 25 G needles and incubated with 100 μL of TCM-199 containing 4 mM calcein-AM and 2 mM ethidium-1 homodimer (Molecular Probes, Invitrogen, Karlsruhe, Germany) at 37 °C for 15 min. Then, they were washed three times in TCM-199 and examined under fluorescence microscope (Nikon, Eclipse, TS 100., Japan). Oocytes and cumulus cells were considered viable if cytoplasm was stained positively with calcein-AM (green) and the chromatin not labeled with ethidium homodimer-1 (red)[21].

2.5. Evaluation of ROS levels in COCs from cultured follicles

Oocytes were washed in 0.1% polyvinyl alcohol in phosphate-buffered saline (PBS-PVA) and incubated with 6-carboxy-2,7-dichlorodihydrofluorescein diacetate (H2DCFDA, Molecular Probes®, Eugene, OR) at 38.5 °C for 30 min, in the dark. Then, oocytes were washed with PBS-PVA and placed on glass slides with ProLong® Gold (Molecular Probes, Eugene, OR). The slides were evaluated under an epifluorescence microscope (Nikon, TS100)

at a wavelength of 460 nm. The fluorescence intensity of ROS saturation was analyzed individually using Image J software (version 1.46; National Institutes of Health, Bethesda, MD). The relative fluorescence intensity was considered directly proportional to the ROS concentration.

2.6. Evaluation of chromatin configuration

To assess chromatin, cumulus cells were removed by vortexing and the oocytes fixed in 4% paraformaldehyde for 15 min and transferred to 0.1% Triton X-100. The chromatin configuration was evaluated after adding 10 µg/mL of Hoechst 33342 on an inverted epifluorescence microscope (Nikon, TS100). Oocytes were classified according to the stages of nuclear maturation, *i.e.*, as germinal vesicle (GV), germinal vesicle breakdown (GVBD) or degenerated.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 9.0 (<https://www.graphpad.com/scientific-software/prism/>). Data of follicular diameter and the levels of ROS, calcein and ethidium homodimer-1 staining were initially subjected to normal distribution analysis using D'Agostino & Pearson Test. After passing the normality test, comparisons between treatments were performed by analysis of variance (ANOVA) and Tukey test. These data are expressed as mean±SD. For data that did not pass normality test, like those of follicular growth in the different periods (days 0 to 4 or days 4 to 8), comparisons were performed by Kruskal-Wallis test. They were presented as median [interquartile range (IQR)].

Data of percentages of morphologically normal follicles, growth rates and chromatin configuration were compared using *Chi-square* or Fisher's exact tests. Differences were considered significant when $P < 0.05$.

2.8. Ethics statement

This study was approved and carried out in accordance with the rules and guidelines of the Ethics and Animal Welfare Committee of the Federal University of Ceara (number 02/21).

3. Results

3.1. Effects of NAC on follicular morphology and growth

A progressive and significant increase in follicular diameters was observed with the increase of culture period from 0 to 4 and 8 days in all treatments, except for follicles cultured with 25.0 mM NAC that did not have significant growth between days 4 and 8 (Table 1). After 8 days of culture, follicles cultured with 25 mM had significantly smaller diameters compared to follicles cultured with TCM-199⁺ (Table 1).

Table 2 shows follicular growth in the different periods of culture (from day 0 to day 4 and from day 4 to day 8). Different from follicles cultured with 1.0 or 5.0 mM NAC, 25.0 mM NAC significantly reduced follicular growth rate between the periods of 4 and 8 days of culture (Table 2).

Table 1. Diameters of early antral follicles day 0, 4 and 8 after *in vitro* culture in TCM-199⁺ alone or supplemented with different concentrations of *N*-acetylcysteine.

| Days | TCM-199 ⁺ | NAC 1.0 mM | NAC 5.0 mM | NAC 25.0 mM |
|-------|----------------------------|-----------------------------|-----------------------------|----------------------------|
| Day 0 | 534.23±47.25 ^{Aa} | 531.08±69.70 ^{Aa} | 530.87±68.42 ^{Aa} | 529.62±60.19 ^{Aa} |
| Day 4 | 634.68±86.04 ^{Ab} | 620.76±81.63 ^{Ab} | 625.89±80.90 ^{Ab} | 609.50±76.44 ^{Ab} |
| Day 8 | 711.69±89.69 ^{Ac} | 687.45±77.95 ^{ABc} | 696.63±82.92 ^{ABc} | 653.98±83.79 ^{Bb} |

Data are expressed as mean±SD. Comparisons between treatments are performed by ANOVA and Tukey test. Lowercase letters (a, b, c) represent statistically significant differences between culture days, within each column ($P < 0.05$). Capital letters (A, B, C) represent statistically significant differences between treatments, within each line ($P < 0.05$). NAC: *N*-acetylcysteine.

Table 2. Follicular growth in the different periods (days 0 to 4 or days 4 to 8) after culture *in vitro* TCM-199⁺ alone or supplemented with different concentrations of *N*-acetylcysteine.

| Days | TCM-199 ⁺ | NAC 1.0 mM | NAC 5.0 mM | NAC 25.0 mM |
|---------|----------------------|---------------------|----------------------|----------------------------------|
| Day 0-4 | 96.89 (6.27-323.61) | 82.02 (5.42-288.48) | 87.56 (20.60-254.56) | 65.18 (3.37-200.77) |
| Day 4-8 | 65.57 (5.24-271.75) | 50.89 (6.20-175.32) | 55.22 (5.05-271.16) | 31.63 (7.02-120.18) [*] |

Data are expressed as median (IQR). Comparisons between treatments are performed by Kruskal-Wallis test. ^{*}Significant difference between culture intervals (day 0-4 and day 4-8) ($P < 0.05$). NAC: *N*-acetylcysteine.

Table 3. Percentage of continuously growing follicles after 4 and 8 days of *in vitro* culture in TCM-199⁺ alone or supplemented with different concentrations of *N*-acetylcysteine.

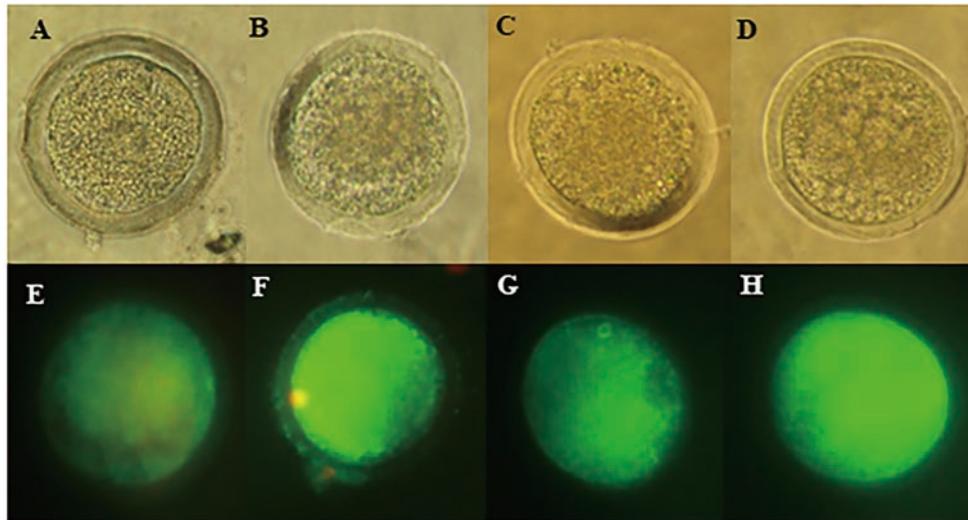
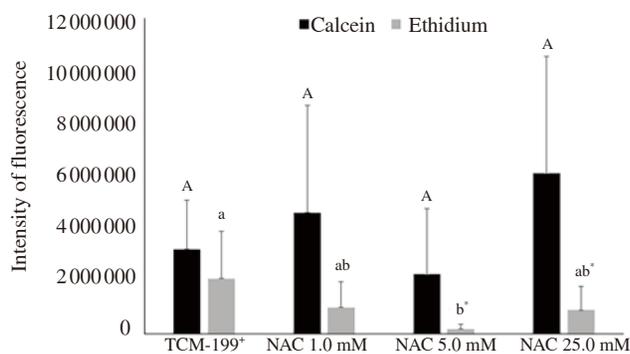
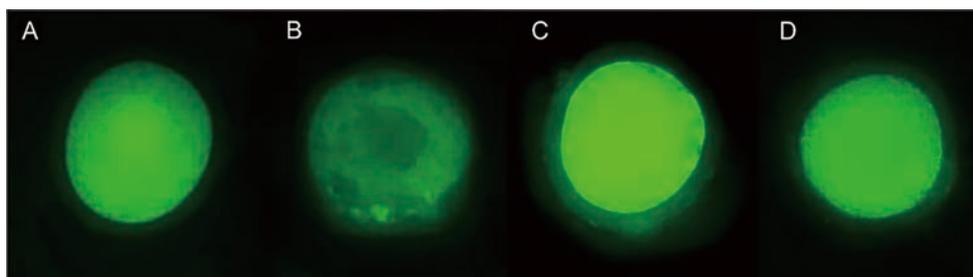
| Days | TCM-199 ⁺ , n=100 | NAC 1.0 mM, n=110 | NAC 5.0 mM, n=106 | NAC 25.0 mM, n=104 |
|-------|------------------------------|-------------------------|-------------------------|-------------------------|
| Day 4 | 90 (90.0) ^a | 95 (86.4) ^a | 91 (85.8) ^a | 77 (74.0) ^b |
| Day 8 | 60 (60.0) ^{a*} | 70 (63.6) ^{a*} | 64 (60.4) ^{a*} | 41 (41.3) ^{b*} |

Data are expressed as *n*(%). Comparisons between treatments are performed by *Chi-square*. Lowercase letters (a, b) represent statistically significant differences between treatments ($P < 0.05$). ^{*}Represents statistically significant differences between the culture periods, within each column (day 4 and day 8). NAC: *N*-acetylcysteine.

Table 4. Percentages of oocytes at germinal vesicle and germinal vesicle breakdown stages and with degenerated chromatin after culture of antral follicles in TCM199⁺ alone or supplemented with different concentrations of *N*-acetylcysteine.

| Parameters | TCM-199 ⁺ , n=32 | NAC 1.0 mM, n=30 | NAC 5.0 mM, n=31 | NAC 25.0 mM, n=24 |
|---|-----------------------------|------------------------|------------------------|------------------------|
| Germinal vesicle (GV), n(%) | 25 (78.12) ^a | 19 (63.3) ^a | 21 (67.7) ^a | 15 (62.5) ^a |
| Germinal vesicle breakdown (GVBD), n(%) | 5 (15.6) ^b | 11 (36.6) ^a | 6 (19.3) ^b | 7 (29.2) ^a |
| Degenerated chromatin, n(%) | 2 (6.2) | 0 (0.0) | 4 (12.9) | 2 (8.3) |

Comparisons between treatments are performed by Fisher's exact test. Lowercase letters (a, b) represent statistically significant differences between GV and GVBD in the same treatment ($P < 0.05$). NAC: *N*-acetylcysteine.

**Figure 1.** Oocytes from bovine antral follicles cultured for 8 days after staining with calcein-AM (green) and ethidium homodimer-1 (red). Oocytes from all treatments are mainly stained with calcein-AM. Oocytes from antral follicles cultured in TCM-199⁺ alone (A, E) or supplemented with 1.0 (B, F), 5.0 (C, G) or 25.0 mM *N*-acetylcysteine (D, H).**Figure 2.** Fluorescence intensity staining shows that oocytes cultured with 5.0 mM *N*-acetylcysteine (NAC) have lower fluorescence intensity for ethidium homodimer-1 (grey columns) than those cultured in the control medium. Within each treatment, lower fluorescence for ethidium-1 homodimer in relation to calcein-AM is observed in oocytes cultured with 5.0 and 25.0 mM NAC. No differences are observed in fluorescence intensity of calcein-AM in oocytes cultured in the different treatments. Data are expressed as mean ± SD. The same letter A represents no significant differences. Lowercase letters (a, b) represent statistically significant differences in fluorescence intensity for ethidium homodimer-1 (grey columns) between different treatments ($P < 0.05$). *Represents the significant difference in fluorescence intensity between calcein-AM and ethidium homodimer-1 in each treatment.**Figure 3.** Oocytes stained with 6-carboxy-2, 7-dichlorodihydrofluorescein diacetate (H2DCFDA) for detection of reactive oxygen species. Oocytes cultured in TCM-199⁺ alone (A), or with 5.0 (C) or 25.0 mM (D) *N*-acetylcysteine are more intensely stained than those cultured with 1.0 mM *N*-acetylcysteine (B).

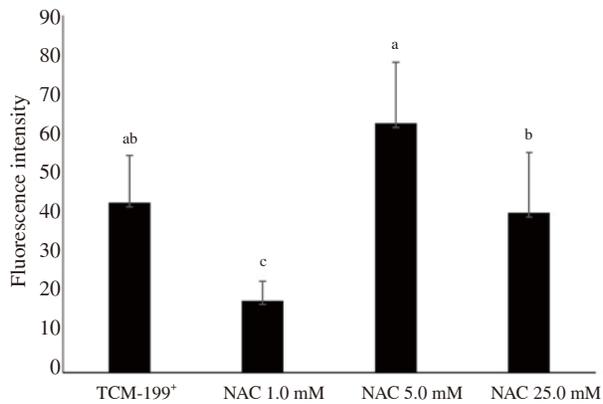


Figure 4. Fluorescence intensity stained with 6-carboxy-2, 7-dichlorodihydrofluorescein diacetate (H2DCFDA) for detection of reactive oxygen species in oocytes from follicles cultured in TCM-199⁺ alone or supplemented with different concentrations of *N*-acetylcysteine (NAC). Lowercase letters (a, b, c) represent significant differences between treatments. Data are expressed as mean±SD.

The presence of 25 mM NAC in culture medium significantly reduced percentage of growing follicles after 4 and 8 days, when compared to other treatments. In presence of 25.0 mM NAC, the percentages of growing follicles were reduced, when compared with growing follicles in the first 4 days of culture (Table 3).

3.2. Viability of oocytes from cultured follicles

Oocytes from follicles cultured with 5.0 mM NAC showed a significant reduction in fluorescence intensity for ethidium homodimer-1 when compared to those cultured in the control medium. In each treatment, significant lower fluorescence for ethidium-1 homodimer in relation to calcein-AM was observed in oocytes from follicles cultured with 5.0 and 25.0 mM NAC (Figure 1 and 2). However, when comparing the fluorescence intensity of calcein-AM in oocytes from follicles cultured in the different treatments, there was no significant difference.

3.3. Evaluation of ROS levels in oocytes from cultured follicles

Oocytes from follicles cultured with 1.0 mM NAC had significantly lower levels of ROS than those cultured in the control medium alone or with 5.0 and 25.0 mM NAC (Figure 3 and 4).

3.4. Evaluation of chromatin configuration in oocytes from cultured antral follicles

Most of the oocytes from antral follicles cultured in TCM-199⁺ alone or supplemented with different concentrations of NAC were in GV stage, being their percentage higher than those at GVBD. The percentages of oocytes with degenerated chromatin did not differ among treatments. Oocytes from follicles cultured in TCM-199⁺

alone and with 5.0 mM NAC had lower percentages of oocytes at GVDB stage than at GV stage (Table 4).

4. Discussion

This study shows that 1.0 mM NAC reduces the levels of ROS in oocytes from early antral follicles cultured *in vitro*. It is known that the accumulation of ROS is one of the limiting factors during *in vitro* culture, causing oxidative stress and various harmful effects to cells[22,23]. Likewise, Sun *et al*[24] showed that 1.0 mM NAC attenuated ROS levels during *in vitro* oocyte maturation. In addition, many other studies have also demonstrated the antioxidant effects of NAC[24–27]. The antioxidant potential of NAC is mainly due to its ability to easily penetrate the cell membrane and be deacetylated producing cysteine, for the intracellular synthesis of glutathione[28,29].

The presence of 5.0 mM NAC in culture reduced the fluorescence intensity for ethidium homodimer-1 in oocytes, showing that NAC helps maintain oocyte membrane integrity during culture. When there is loss of integrity, the ethidium homodimer-1 can easily enter the cell, and due to the high affinity with nucleic acids, it binds to the DNA emitting red fluorescence[30], allowing verifying the physical and chemical changes in the cell membrane. In contrast, calcein can passively cross the cell membrane, and within cells it is converted by intracellular esterases to a lipid-insoluble polar product (calcein) that is retained by cells with intact membranes, producing intense green fluorescence[31], thus allowing the verification of cell viability. In caprine granulosa cells, 5.0 and 10.0 mM NAC reduced the genotoxicity caused in response to exposure to methoxychlor[17], while in fibroblasts, 5.0 and 10.0 mM NAC reduced the production of ROS and the toxicity induced by 2-hydroxyethyl methacrylate, consequently reducing cell death and restoring mitochondrial activity[32].

In our study, 25.0 mM NAC reduced follicular growth and the percentages of continuously growing follicles. Recently, Sun *et al*[24] reported that NAC at concentrations above 10.0 mM causes a reduction in the pH of the culture medium and is harmful to oocyte growth during *in vitro* maturation. The reduction in the pH of the medium may explain the lower follicular growth of early antral follicles cultured with 25 mM NAC. Clinical studies have shown that the plasma concentration of NAC ranges from 300 to 900 mg/L, which is equivalent to 1.8–5.5 mM[33]. Furthermore, previous studies have suggested that NAC at higher concentrations may have a pro-oxidant effect[34]. Sprong *et al*[35] showed that high doses of NAC (550 and 950 mg/kg in 48 h) in rats increase oxidative stress and toxicity induced by lipopolysaccharide, but low doses of NAC are highly effective against lipopolysaccharide toxicity. Moraes *et al*[36] also observed that the increase in the plasma concentration of NAC, as a result of its association with dapson, potentiated the adverse effect of dapson in rats.

Regarding to chromatin configuration, NAC did not influence the percentages of oocytes that remained in the GV stage. The experimental model used with the culture of the intact antral follicle maintains communication between the oocyte and granulosa cells, allowing the supply of follicular components to the oocyte and the conservation of high levels of cyclic adenosine monophosphate (cAMP), consequently preventing the spontaneous resumption of meiosis[36,37], and guaranteeing the time necessary for the oocyte to acquire the developmental competence to proceed with the following stages of its growth[38]. In addition, evidence suggest that the control of meiotic resumption or arrest can be influenced by ROS levels, and the presence of antioxidants in the culture medium is very important to inhibit the spontaneous resumption of meiosis under *in vitro* culture conditions[23].

In conclusion, supplementation of culture medium with 1.0 mM NAC reduces ROS levels, while the presence of 5.0 mM NAC reduces the fluorescence intensity for ethidium homodimer-1. NAC at concentration of 25 mM, however, reduces follicular growth and percentages of continuously growing follicles during *in vitro* culture of bovine early antral follicles. In future studies, *in vitro* culture of early antral follicles for long term periods is still needed to promote complete oocyte cytoplasmic maturation.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

Efigênia B. Cordeiro performed *in vitro* culture of bovine early antral follicles and statistical analysis of data. Bianca R. Silva isolated the follicles and measured their diameters before and after culture. Laís R. F. M. Paulino isolated the follicles and stained them with fluorescent markers. Pedro A. A. Barroso collected the ovaries in slaughterhouses and prepared *in vitro* culture media. Laryssa G. Barroso contributed to the acquisition and analysis of data. Miguel F. de Lima Neto evaluated follicular viability after staining with fluorescent markers. José R. V. Silva designed the work and critically revised the manuscript.

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