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ORIGINAL ARTICLE

Alpha Lipoic Acid Impact on Apoptosis-Related Gene Expression in Mature Mouse Oocyte

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KEYWORDS

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ABSTRACT: Utilizing antioxidants offers a promising strategy for mitigating the effects of oxidative stress. This study was designed to assess the influence of alpha-lipoic acid (ALA) on the maturation of mouse oocytes and their apoptosis-related genes. Germinal vesicle oocytes, obtained from female mice aged 6-8 weeks, were subjected to in vitro maturation. Among these, 488 oocytes were exposed to 100 µM ALA, while 506 oocytes matured without ALA over a 16-hour duration. Subsequent evaluations were conducted to determine oocyte maturation rates. A portion of the mature oocytes at the metaphase II (MII) stage underwent in vitro fertilization, while the remaining oocytes were utilized to analyze the expression of *Caspase 3, Bad, Bax,* and *Bcl2* through real-time RT-PCR. To quantify cell numbers, resulting blastocysts were stained with DAPI (4',6-diamidino-2-phenylindole). The group treated with ALA exhibited significantly higher rates of MII oocytes (77.30%) and a greater proportion of embryos developing into the blastocyst stage (33.87%) in comparison with the control group (55.81% and 25.06%, respectively; $P<0.05$). Moreover, the average cell count within blastocysts significantly increased in the ALA-treated group (82.37) compared to the control group (68.5; $P<0.05$). Furthermore, the pro-apoptotic genes expression decreased significantly, while the expression of the Bcl2 gene exhibited a significant increase in the ALA-treated group in comparison to the control group ($P<0.05$). In conclusion, the supplementation of the maturation medium for mouse oocytes with ALA resulted in improvements in oocyte development and overall embryo quality. This effect was attributed to the downregulation of pro-apoptotic genes and the upregulation of the anti-apoptotic gene.

INTRODUCTION

In the realm of assisted reproductive technology, In vitro maturation (IVM) stands out as a recognized alternative approach, showing promise in enhancing oocyte maturation through the supplementation of culture media with growth factors and hormones [1]. Oocyte maturation is a multifaceted process, encompassing two pivotal stages: nuclear maturation, which involves meiotic resumption and the first polar body extrusion, and cytoplasmic maturation, characterized by alterations in cytoplasmic organelles that bestow the capacity to support fertilization and subsequent embryo development [2].

The occurrence of successful pregnancies following IVM of oocytes underscores its safety and effectiveness in augmenting fertility potential [3]. IVM offers distinct advantages, particularly for specific patient cohorts, including those with polycystic ovarian syndrome, individuals with limited ovarian reserves, recurrent IVF failures, cases where oocytes need retrieval before ovarian freezing, pre-implantation genetic diagnosis, and

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instances of resistant ovarian syndrome [4, 5].

Nonetheless, a significant limitation associated with IVM is its susceptibility to oxidative stress (OS). The high generation of reactive oxygen species (ROS) leads to OS, which can exert detrimental effects on oocyte maturation and subsequent embryo development [6]. OS can also act as a trigger for apoptosis in oocytes and embryos [7]. Multiple factors, such as elevated oxygen levels, oocyte manipulation, and various physical and chemical conditions, contribute to the heightened production of ROS, ultimately resulting in OS and potential oocyte death and apoptosis [8].

To mitigate the adverse effects of OS, the inclusion of antioxidant agents in culture media has emerged as an effective and time-tested strategy [9-13]. These agents engage with ROS and nitrogen species, either directly or indirectly, to counteract the effects of OS [12, 13]. Among the noteworthy non-enzymatic antioxidants is Alpha Lipoic Acid (ALA) and its derivative, dihydrolipoic acid (DHLA). ALA operates by scavenging ROS, chelating heavy metals, and inhibiting lipid peroxidation in cell membranes [11, 14-16]. Furthermore, ALA functions as a redox couple, facilitating electron transfer, recycling Vitamins C and E, and enhancing cellular glutathione peroxidase, superoxide dismutase, and catalase activities [11, 12, 17]. Prior research has demonstrated that the presence of ALA during in vitro culture leads to higher oocyte survival and maturation rates, as well as an increased blastocyst rate [11, 15]. Additionally, ALA has exhibited the ability to enhance the growth and development of follicles obtained from mouse ovaries [18-20]. These compelling findings, combined with a body of research confirming the positive impact of ALA on oocyte maturation [11, 14-16], suggest that its benefits may extend to the prevention of oocyte apoptosis.

As such, the principal aim of this study is to delve into the mechanisms underlying the action of ALA in oocyte maturation and its potential influence on the expression of genes associated with apoptosis in mouse oocytes during the process of in vitro maturation.

MATERIALS AND METHODS

Reagents

Unless otherwise indicated, we obtained all chemical reagents from Sigma Aldrich (England), and we prepared all culture media using Milli-Q water.

Study Design

In compliance with the animal care policies of our university, we used 50 adult female NMRI (National Medical Research Institute) mice, aged 6–8 weeks, for our experimental study. The mice were housed in climate-controlled environments with a 12-hour light and 12-hour dark cycle, 23±3°C temperature, and 44±2% relative humidity. They were allowed unlimited access to food and water.

Collection of GV Oocytes

In this study, 10 IU of pregnant mare serum gonadotropin (PMSG, Intervet, Australia) were injected intraperitoneally into mice. Once the mice had been alive for 48 hours, they were humanely killed by cervical dislocation, and their ovaries were taken out and mixed with α-minimal essential medium (α-MEM; Gibco, UK). 10% fetal bovine serum (FBS; Gibco, UK), 2.2 g L^{-1} sodium bicarbonate, 25 mM HEPES, 100 IU mL^{-1} penicillin, and 75 µg mL^{-1} streptomycin were added to this medium to enhance it.

Under a stereomicroscope, we extracted GV stage oocytes from antral follicles using a 31 G needle. Only oocytes with a centrally placed nucleus, a consistent perivitelline space, a uniform Zona Pellucida (ZP), and transparent cytoplasm made it through our rigorous selection process.

Oocyte Maturation in Vitro

10 IU mL^{-1} human chorionic gonadotropin (hCG), 100 IU mL^{-1} penicillin, 75 µg mL^{-1} streptomycin, 75 m IU mL^{-1} recombinant human follicle-stimulating hormone (rhFSH), and 2.2 g L^{-1} sodium bicarbonate made up the culture medium, which was enriched with 10% FBS (fetal bovine serum; Gibco, UK). Furthermore, an additional 100 µM of ALA was added to the experimental group's α-MEM medium [19]. The oocytes

were incubated in 20 µl droplets of the maturation media, covered with mineral oil, and maintained for 16 hours at 37°C, 100% humidity, and 5% CO_2 in the environment. With the aid of an inverted microscope, we counted the number of degraded, GV (germinal vesicle), GVBD (germinal vesicle breakdown), and MII (metaphase II) oocytes when the culture period was over. Each experiment was carried out at least three times, using the MII oocytes for in vitro fertilization and subsequent embryo culture.

In Vitro Fertilization and Embryo Culture

We collected sperm from the dissected cauda epididymis of mature male NMRI mice (n=5). The cauda epididymis was positioned in 300 µl drops of global total medium (Life Global, Belgium) supplemented with 5 mg mL^{-1} bovine serum albumin (BSA; Sigma, Germany) and was covered with mineral oil. Gently squeezing the epididymis released spermatozoa, which were then incubated at 37°C in an environment with 5% CO2 and 95% humidity for 90 minutes to facilitate capacitation.

The capacitated sperm suspension was introduced into 20 µl drops of global total medium, which was supplemented with 15 mg mL^{-1} BSA to reach a final concentration of motile sperm at 1×106/ml [10]. Meanwhile, MII oocytes from each study group were transferred to these drops and incubated at 37°C, under 5% CO2, and in 95% humidity for a period of 4-6 hours. Subsequently, we harvested presumptive zygotes, rinsed them three times in global total medium containing 5 mg mL^{-1} BSA, and cultured them in drops of the medium for a total duration of 120 hours. The progress of embryonic development towards the blastocyst stage was continuously assessed over the 120-hour period using an inverted microscope.

Blastocyst Staining

To stain the embryos, we took the collected blastocysts from both groups, with eight blastocysts in each group across three replicates. These blastocysts were subjected to staining using DAPI (4', 6-diamidino-2-phenylindole) to mark the cell nuclei. The procedure involved briefly immersing the blastocysts in phosphate buffer saline (PBS) containing 50 µ l^{-1} of DAPI and 5 µl of Triton-X100 for duration of 10 seconds. Following this staining step, the blastocysts underwent three consecutive rinses in PBS. Cell counting was then carried out using a fluorescent microscope [20].

Gene Expression Analysis

RNA was extracted from MII oocytes, with 45 samples in each group and three replicates, using Trizol reagent from Qiagen (USA) in accordance with the manufacturer's guidelines. To eliminate potential genomic DNA contamination, the RNA underwent DNase I treatment from Cinnagen (Iran). The quality of the RNA was assessed based on the density ratio between the 28S and 18S rRNA bands. 500 ng of the isolated RNA with an optical density ratio greater than 1.8 for 260/280 was used for cDNA synthesis. This was accomplished by following the manufacturer's instructions and using a cDNA Synthesis Kit from Fermentas (MD, USA). We then assessed the Caspase3, Bad, Bax, and Bcl2 gene mRNA expression levels. The QuantiFast SYBR Green PCR Kit from Qiagen (Germantown, USA) was used in conjunction with an ABI Step One real-time PCR machine from Applied Biosystems (ABI, USA) to conduct this assessment.

As shown in Table 1, the gene-specific primer sets were specially created using DBA Oligo, Inc.'s (USA) AlleleID software version 7.5. Primer 3 software, which is available on the NCBI website, was used to thoroughly verify the primer sequences in order to ensure their specificity for the mRNA of the target genes and the lack of genomic DNA replication. Quantitative Real-Time PCR Experiments (MIQE) provided guidelines that were followed in all real-time PCR protocols. In the analysis, the reference gene Ef1 was used.

A denaturation cycle at 95°C for 15 minutes was the first step in the thermal profile's programming. This was followed by 40 cycles at 95°C for 30 seconds, and then one cycle at 60°C for 45 seconds. Following that, the 2-ΔΔCT method was employed to calculate the relative expression fold. By using melting curve analysis, the specificity of each amplification reaction was confirmed. Every gene had a no-template control (NTC) tube in every experiment.

Table 1. Primer list.

Gene	Accession number	Primer pair sequence (5'-3')	PCR product size(bp)
Bcl2	NM-009741.5	F:5'-TGCTGCTATCCTGCCAAG-3' R:5'-GTCTGTGTTCTTCATCGTTACTTC-3'	108
Caspase3	NM-009810.3	F:5'-GCTGACTTCCTGTATGCTTA-3' R:5'-GTTGCCACCTTCCTGTTA-3'	164
Bad	NM-007522	F:5'-GGAGCAACATTCATCAGCAG-3' R: 5'-TACGAACTGTGGCGGACTC-3'	91
Bax	NM-007527.3	F:5'-GCGAATTGGAGATGAACT-3 R: 5'-CAGTTGAAGTTGCCATCA-3' '	129
Ef1	NM-007906	F:5'-AGTCGCCTTGGACGTTCTT R: 5'-CCGATTACGACGATGTTGATGTG	124

Statistical analysis

We conducted each experiment a minimum of three times, and data analysis was performed using SPSS version 22 software for Windows (SPSS Inc., Chicago, IL, USA), employing the Independent Samples t Test. The data is reported as the mean ± standard deviation (SD), and statistical significance was considered when $P < 0.05$.

RESULTS

Oocyte maturation rate

An overview of the oocyte maturation rates in the two study groups can be found in Table 2. Of the 506 oocytes in the control group, 55.81% had reached the MII stage, 7.14% had reached the GVBD stage, and 18.50% had been arrested at the GV stage by the end of the culture period. On the other hand, the group that received ALA treatment showed greater rates of GVBD, GV stage oocytes, and MII, at 10.84%, 4.50%, and 77.3%, respectively. Furthermore, 18.94% and 7.14%, respectively, of the degenerated oocytes were found in the control and ALA-treated groups. A statistical analysis showed that the group treated with ALA had a significantly higher rate of oocyte survival than the control group ($P < 0.05$). Additionally, the rates of MII and GVBD oocytes were significantly higher in the ALA-treated group than in the control group ($P < 0.05$), whereas the rate of arrested GV oocytes was significantly lower in the ALA-treated group ($P < 0.05$).

Table 2. The survival and developmental rates of mouse oocytes.

Groups	Total Number of oocytes	Survived No (Mean ± SD)	GV No (Mean ± SD)	GVBD No (Mean ± SD)	MII No (Mean ± SD)	Degeneration No (Mean ± SD)
Control	506	407 (81.06±5.84)	90 (18.50±4.87)	32 (7.14±4.49)	291 (55.81±7.64)	93 (18.94±5.84)
ALA	488	457* (92.86±3.90)	21* (4.50±5.44)	48* (10.84±4.45)	387* (77.30±7.31)	31* (7.14±3.90)

The data is presented as mean ± standard deviation and the percentage was computed in relation to the total oocyte count. Significant differences ($p < 0.05$) are indicated by an asterisk (*) in relation to the corresponding control groups in the same column. ALA is an acronym for Alpha Lipoic Acid-Mediated In Vitro Maturement. The germinal vesicle is represented by GV, germinal vesicle breakdown is denoted by GVBD, and metaphase II is denoted by MII.

Embryonic development

Table 3 displays the percentages of embryonic development up to 120 hours in in vitro culture. In the

control group, the mean percentages for two-cell, four-cell, eight-cell, morula, and blastocyst were 55.64%, 46.78%, 34.25%, 28.53%, and 25.06%, respectively. In the presence of ALA, these percentages significantly increased to 77.84%, 78.40%, 63.58%, 44.00%, and 33.87%, respectively. The rates in the ALA-treated group were significantly higher compared to the control group ($P < 0.05$).

Table 3. The developmental rate of embryos.

Groups	Total MII oocytes	2-Cell No (Mean ± SD)	4-Cell No (Mean ± SD)	8-Cell No (Mean ± SD)	Morula No (Mean ± SD)	Blastocyst No (Mean ± SD)
Control	171	95 (55.64±10.91)	43 (46.78±10.73)	30 (34.25±11.16)	26 (28.53±10.84)	21 (25.06±11.82)
ALA	247	192* (77.84±8.75)	148* (78.40±6.60)	122* (63.58±8.80)	84* (44.00±9.48)	64* (33.87±5.90)

We determined the percentages of 2-cell embryos in relation to the number of inseminated oocytes, and for 4-cell, 8-cell, morula, and blastocyst embryos, the percentages were calculated relative to the 2-cell embryos. The data is presented as both the quantity and as (mean ± standard deviation). * denotes significant variations when compared to the control groups in the corresponding column ($p < 0.05$).

Blastocyst staining and cell counting

Figure 1 presents the representative images of stained blastocysts. The mean cell count of blastocysts in the control group was 68.50, while in the ALA-treated group, it was 82.37. Significantly, the ALA-treated group exhibited a higher cell count compared to the control group ($p < 0.05$).

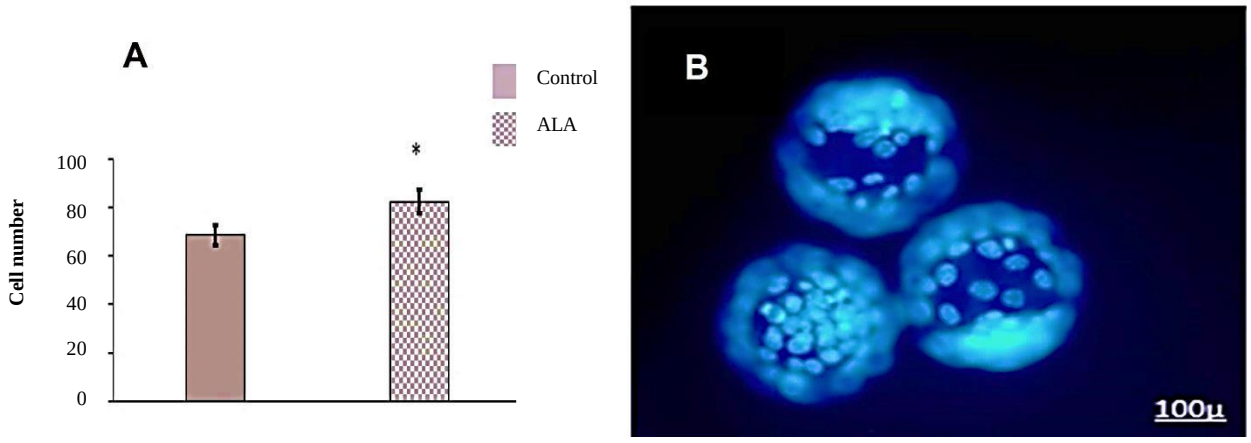

Figure 1. A: Blastocyst Cell Number, B: DAPI staining of blastocytes *: Indicates significant difference with control group P<0.05.

Gene expression analysis

Figure 2 provides a summary of the real-time RT-PCR analysis results. Within the control group, the housekeeping gene was expressed relative to the Caspase3, Bad, Bax, and Bcl2 genes at 1.02 ± 0.03, 0.95 ± 0.03, 1.08 ± 0.10, and 0.97 ± 0.11, respectively. The group that received ALA treatment, on the other hand, displayed values that were 0.49 ± 0.10, 0.55 ± 0.09, and 0.08 ± 0.04, respectively. Figure 2 ($P<0.05$) shows that the ALA-treated group had significantly lower relative expression of Caspase3, Bad, and Bax compared to the control group. On the other hand, the ALA-treated group exhibited significantly higher relative expression of Bcl2 mRNA (2.76 ± 0.65) and Bcl2/Bax ratio (53.57 ± 4.01) when compared to the control group (Bcl2: 00.98 ± 00.12 and Bcl2/Bax ratio: 00.91 ± 00.17; $P<0.05$).

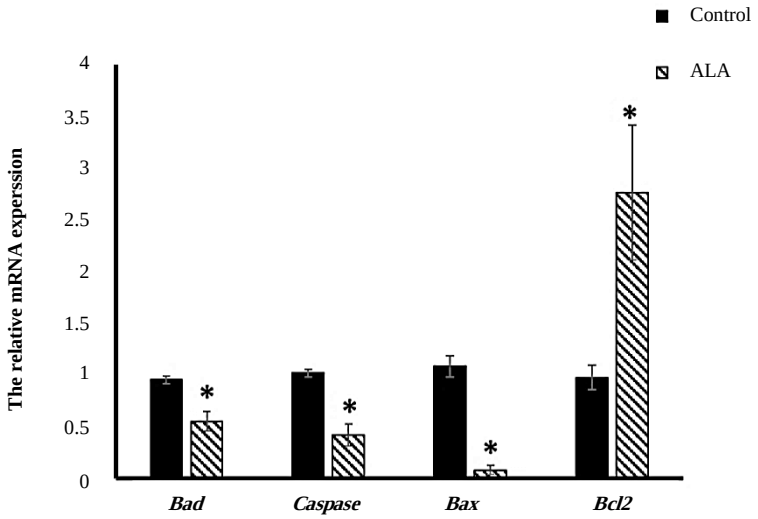

Figure 2. Relative expression of Bad, Bax, Caspase3 and Bcl2 mRNA Data is presented as mean SD.
*: Indicate significant difference with control group (P<0.05)

DISCUSSION

In this research, we explored the effects of Alpha Lipoic Acid (ALA) on the developmental competence and the expression levels of genes related to apoptosis in mouse GV oocytes. Our findings demonstrated a significant increase in the survival and maturation rates of GV oocytes when ALA was present, leading to improved embryonic development. While our study did not measure reactive oxygen species (ROS) levels, the positive outcomes can be attributed to the well-known antioxidant properties of ALA. Several prior studies have reported reductions in ROS levels following ALA treatment.

Earlier research has also highlighted the advantages of ALA in the in vitro maturation of oocytes or follicles in various mammalian species. For instance, ALA has been effective in mitigating oxidative stress during the in vitro culture of mouse and bovine oocytes, resulting in enhanced oocyte development and maturation. Similarly, ALA acted as an antioxidant during the in vitro culture of goat oocytes by reducing ROS levels and increasing total antioxidant capacity, ultimately improving embryo development and quality. Our study aligns with these findings, as we observed higher embryo cell counts and blastocyst rates in the ALA-treated group compared to the control.

However, it is important to note that some studies have reported varying results, which could be attributed to differences in culture media and supplements used. Furthermore, ALA has been shown to influence ovarian follicular development and oxidative profiles. It is believed that ALA upregulates the expression of genes related to folliculogenesis, further enhancing developmental competence.

Moreover, ALA serves as a scavenger of ROS and can chelate heavy metals. Its effectiveness in both reduced and oxidized forms makes it a versatile antioxidant. ALA has also been demonstrated to reduce the expression of inflammatory cytokines and interleukins, thereby reducing intracellular ROS levels.

During in vitro culture, apoptosis can be induced by various factors, with pathways involving pro-apoptotic genes like Bax and Bad affecting mitochondrial membrane permeability. In the present study, we observed a significant decrease in the pro-apoptotic genes (Caspase 3, Bad, and Bax) expressions in the ALA-treated group, while the expression of the anti-apoptotic gene (Bcl2) was significantly higher compared to the control group. Furthermore, the Bcl2/Bax ratio, which serves as an indicator of oocyte survival or apoptosis, was significantly higher in the ALA-treated group. This balanced ratio positively correlated with oocyte quality and developmental competence, ultimately leading to blastocyst development.

ALA functions as an antioxidant that inhibits the mitochondrial or intrinsic pathway of apoptosis. This effect has been observed in previous studies, where ALA

downregulated the pro-apoptotic genes expression such as C-Myc during the in vitro culture of bovine preantral follicles. Additionally, ALA supplementation in oocyte maturation media reduced the expression of Bad and Caspase as pro-apoptotic genes and increased the expression of Sod, Bcl as anti-apoptotic genes in blastocyst stage embryos. ALA has also been shown to alter the expression of various genes associated with apoptosis.

In conclusion, supplementing the culture medium of oocytes with ALA has the potential to enhance their development and improve embryo quality. This study highlights the promise of ALA as a valuable addition to assisted reproductive technology, offering favorable outcomes for oocyte development.

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ETHICAL CONSIDERATION

The protocol for this study was conducted in accordance with the guidelines and ethical standards of the animal ethics committee at Tarbiat Modares University (IR.MODARES.REC.1400.184).

Conflict of Interest

The authors declare that they have no conflicts of interest.

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