



Alpha-Lipoic Acid Ameliorates Impaired Steroidogenesis in Human Granulosa Cells Induced by Advanced Glycation End-Products

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What's Known

- Advanced glycation end-products could accumulate in the ovary and alter the function and metabolism of granulosa cells.
- Alpha lipoic acid has protective effects on the ovary due to its antioxidant properties.

What's New

- Advanced glycation end-products induce hormonal disturbance in human ovarian granulosa cells via enhancing their own receptor expression (receptor for advanced glycation end-products).
- Advanced glycation end-products disrupt the steroidogenic enzymes' gene expression and steroid hormone production in granulosa cells.
- Alpha-lipoic acid protects human granulosa cells against advanced glycation end-products-induced steroidogenesis disturbance.

Abstract

Background: Ovarian granulosa cells (GCs) are essential for follicular development. Ovarian advanced glycation end-products (AGEs) accumulation is related to GCs dysfunction. Alpha-lipoic acid (ALA) illustrates therapeutic capabilities for infertility-related disorders. Therefore, this study assessed the effects of ALA on AGEs-induced GCs hormonal dysfunction.

Methods: The study was conducted from October 2021 to September 2022 at the Department of Medical Genetics, Shiraz University of Medical Sciences. Isolated GCs (n=50) were divided into control, human glycated albumin (HGA), HGA+ALA, and ALA treatments. Steroidogenic enzymes and AGE receptor (*RAGE*) genes were assessed by qRT-PCR. Steroid hormones and RAGE protein were evaluated using ELISA and Western blotting. Data were analyzed using GraphPad Prism software (ver. 9), and $P < 0.05$ was considered significant.

Results: Our findings showed that HGA treatment significantly ($P = 0.0001$) increased *RAGE* (by 140.66%), *STAR* (by 117.65%), *3 β -HSD* (by 165.68%), and *17 β -HSD* (by 122.15%) expression, while it decreased *CYP19A1* (by 68.37%) expression. RAGE protein level (by 267.10%) was also increased in HGA-treated GCs. A significant decrease in estradiol (by 59.66%) and a slight and sharp elevation in progesterone (by 30.40%) and total testosterone (by 158.24%) levels was also observed. ALA treatment ameliorated the HGA-induced changes in steroidogenic enzyme mRNA levels ($P = 0.001$) and steroid hormone secretion ($P = 0.010$).

Conclusion: This work shows that ALA therapy likely corrects hormonal dysfunctions caused by AGEs in luteinized GCs. This effect is probably achieved by decreased *RAGE* expression. Clinical research is needed to understand how AGEs and ALA interact in the ovary, which might lead to a more targeted ovarian dysfunction therapy.

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Introduction

Granulosa cells (GCs), as somatic cells surrounding the oocyte, have a vital function in modulating the follicular microenvironment and maintaining oocyte competency. These cells supply essential

nutrients for oocytes and actively synthesize steroid hormones.¹ Steroidogenesis is critical for synchronizing folliculogenesis and oocyte development. Thus, any dysregulation in this process could adversely influence the follicle.² As oocyte quality is the most critical determinant in assisted reproductive technology (ART) outcomes, investigating influential factors on the GCs function might directly/indirectly improve these technical results.³

Various pathological conditions, including obesity,⁴ endometriosis,⁵ polycystic ovary syndrome (PCOS),⁶ and diabetes,⁷ could adversely impact GCs functions. Notably, an increased amount of pro-inflammatory molecules known as advanced glycation end products (AGEs) have a major role in the pathogenesis of all these disorders.⁸

AGEs are a group of over twenty different molecules that are non-enzymatically produced through the Maillard reaction. When reducing sugar interacts non-enzymatically with amino acids found in proteins, lipids, or DNA, the Maillard reaction takes place. This reaction has been studied for years in the food industry because its products provide a desirable color and flavor to foods. However, due to the link of AGEs with several chronic disorders, such as diabetes mellitus, cardiovascular diseases, and Alzheimer's disease, as well as throughout the aging process, the *in vivo* study of reaction byproducts has gained more interest recently.⁸ Moreover, cigarette smoking and modern lifestyle with fast-food consumption are the primary sources of exogenous AGEs. The detrimental effects of AGEs on ovarian tissues and cells occur through cross-linking of the proteins or via the receptor for AGEs (RAGE) on target cells. The binding of AGEs to its receptor activates several inflammation-related signaling pathways and increases reactive oxygen species (ROS) production.⁹ This state accounts for the emergence of different reproductive complications, such as PCOS with an increased concentration of AGEs in sera and ovaries^{10, 11} and ovulatory dysfunction, as illustrated in mice feeding high-AGE diets.¹²

Considering previous reports, the AGEs were detected in the follicular fluid¹³ and could accumulate in the GCs of human and mouse models of PCOS.¹⁴ Further, GCs show higher RAGE expression than theca cells in women with PCOS.¹⁰ Some studies have illustrated the correlation between the amounts of AGEs and GCs malfunction. In ovarian GCs, AGEs alter glucose metabolism¹⁵ and steroidogenesis, contributing to infertility-related disorders.¹⁶ In addition, the accumulation of AGEs in GCs results

in the production of inflammatory cytokines. This condition is associated with poor oocyte quality and impaired embryo development.¹⁷

Regarding the adverse effects of AGEs on ovarian tissue, especially GCs, appropriate inhibitors of these molecules should be considered. Antioxidant compounds, as ROS scavengers, have been found as appropriate inhibitors against AGEs.¹⁸ Among these compounds, alpha-lipoic acid (ALA) would be a suitable candidate. Some studies have demonstrated the suppression of AGEs formation and RAGE expression by ALA.^{19, 20} Moreover, ALA has been considered for infertility treatment and enhanced reproductive outcomes.²¹ It also has a positive influence on the developmental competence of follicles²² and ovarian function improvement in animal models of primary ovarian failure²³ and oxidative injuries.^{24, 25}

As there is no experimental report on the ALA effects against the AGEs-RAGE axis in human GCs, this investigation aims to evaluate how AGEs may alter the expression of enzymes involved in steroidogenesis and, consequently, steroid hormone production, and how ALA, as a water and fat-soluble antioxidant, can intervene in this process.

Patients and Methods

Patient Selection

The current study was conducted from October 2021 to September 2022 at the Department of Medical Genetics, Shiraz University of Medical Sciences (Shiraz, Iran). Ovarian GCs were collected from 50 patients' candidates of intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization (IVF) at Shiraz Ghadir Mother and Child Hospital. Based on sample size calculation in our previous studies,^{1, 2} the number of GCs needed to perform different tests (n=20 for ELISA, n=8 for RT-PCR, n=5 for western blot, and n=5 for MTT),²⁶ and the possible sample attrition due to high blood contamination, the fewer live cells, and possible contamination after culture, 50 women were selected based on the inclusion criteria and clinical record information. All participating women provided written informed consent before entering the investigation. All procedures performed in studies involving human participants were in accordance with the ethical standards of the local Medical Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1400.485) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

All studied subjects were 18-36 years old with a body mass index (BMI) of 18.5–30 Kg/m². Women with normal ovaries were diagnosed as tubal factor, male factor, egg donors, or patients with normal ovary reserve were included in this study. Exclusion criteria were cigarette smoking, alcohol consumption, and patients with diabetes, endometriosis, chronic metabolic syndrome, thyroid disorder, obesity, and PCOS.

In this regard, blood sampling was performed on the third day of the menstrual cycle to measure basal serum levels of luteinizing hormone (LH), estradiol (E2), follicle-stimulating hormone (FSH), anti-Müllerin hormone (AMH), and thyroid stimulating hormone (TSH). Moreover, hemoglobin level (Hb), fasting blood sugar (FBS), Na⁺, K⁺, and white/red blood cell (WBC/RBC) counts were also determined.

Controlled Ovarian Stimulation Protocol

The gonadotropin hormone-releasing hormone (GnRH) antagonist protocol was the primary approach to ovarian stimulation in all studied cases. Briefly, ovarian stimulation began on the second/third day of the menstrual cycle by daily injection of recombinant human FSH (Gonal-F, Merck-Serono, Germany) and/or Menogon (Ferring, Germany). The serial vaginal ultrasound scan was performed for follicular monitoring, and when observing at least one follicle with a diameter of 12-14 mm, GnRH antagonist (Cetrotide, 0.25 mg, Merck-Serono, Germany) was subcutaneously injected daily. Administration continued until at least three follicles of ≥17 mm in diameter were observed. The recombinant human chorionic gonadotropin (hCG) (Ovitrelle, 500 IU, Merck-Serono, Germany) was used for triggering oocyte maturation. After 34-36 hours, oocyte retrieval was done with the aid of vaginal ultrasound.

Human GCs Isolation

On the day of oocyte retrieval, the remaining follicular aspirates of participants were separately collected and transported to the lab at 4 °C. Then, the fluid was centrifuged at 1600 rpm for 10 min at 4 °C. Subsequently, the supernatant was removed, and the cell pellet was resuspended in a cold RBC lysis buffer (1X) and briefly vortexed. To remove the RBC lysate, the sample was centrifuged at 1600 rpm for 10 min at 4 °C. Then, the cell pellet was resuspended and washed in 5 mL phosphate-buffered saline (PBS) and centrifuged at 1200 rpm for 5 min (two to three times).

The percentage of viable cells was determined with 0.4% Trypan Blue (Sigma-Aldrich, USA) following trypsinization of the

pellet with 0.25% trypsin/EDTA solution (Sigma, USA). Finally, the cells were resuspended in the Dulbecco Modified Eagle Medium (DMEM/F12, Biosera, France) culture medium.

Flow-cytometric investigations were performed for distinguishing leukocytes from GCs using mouse anti-human CD45, conjugated with fluorescein isothiocyanate (antiCD45-FITC) (sc-1178 FITC, Santa Cruz, USA), and GCs purity was determined to be more than 95%.

Human GCs Culture and Experimental Groups

To find the non-toxic concentrations of ALA (Sigma, USA), an MTT assay (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Sigma, USA) was performed. In this regard, 1×10⁴ GCs/well in 96-well plates were exposed to 50, 100, and 200 μM of ALA for 48 hours. After aspirating the media, media containing 10 μL of MTT solution was replaced and incubated for 3 hours in the dark at 37 °C. After that, dimethyl sulfoxide (DMSO, Sigma, USA 100 μL) was added to solubilize the formazan crystals. Finally, the optical density of each well at 570 nm was evaluated using a microplate reader (Epoch 2™, BioTek Instruments, USA). Cell viability percentages were calculated according to the following formula:

$$\text{Cell Viability (\% of Control)} = (A_t - A_b / A_c - A_b) \times 100$$

Where A_t , A_c , and A_b are the absorbance of the test sample, the control, and the blank, respectively.

The non-toxic concentration of human glycated albumin (HGA, as an AGEs representative) was determined based on a previous study.¹⁶ Accordingly, 400 μg/mL HGA and 100 μM ALA were selected as non-toxic concentrations for the following study parts.

The isolated GCs of each participant were divided into four groups. The cells were seeded in 6-well plates (2×10⁶ cells/well) in DMEM/F12 media (3 mL/well) supplemented with 5% charcoal-stripped fetal bovine serum (FBS) (Gibco, USA), 1% L-glutamine (Sigma, USA), and 1% penicillin/streptomycin (Sigma, USA), and the corresponding treatments were performed for each group for 48 hours under standard culture conditions (37 °C and 5% CO₂ incubator). The experimental groups were defined as follows: a) control (DMSO only); b) HGA (400 μg/mL, dissolved in media); c) HGA+ALA [400 μg/mL+100 μM (dissolved in DMSO)]; and d) ALA (100 μM) treatments. The final DMSO concentration in the culture medium was 0.5%.² The schematic view of the cell culture and study procedures is shown in figure 1.

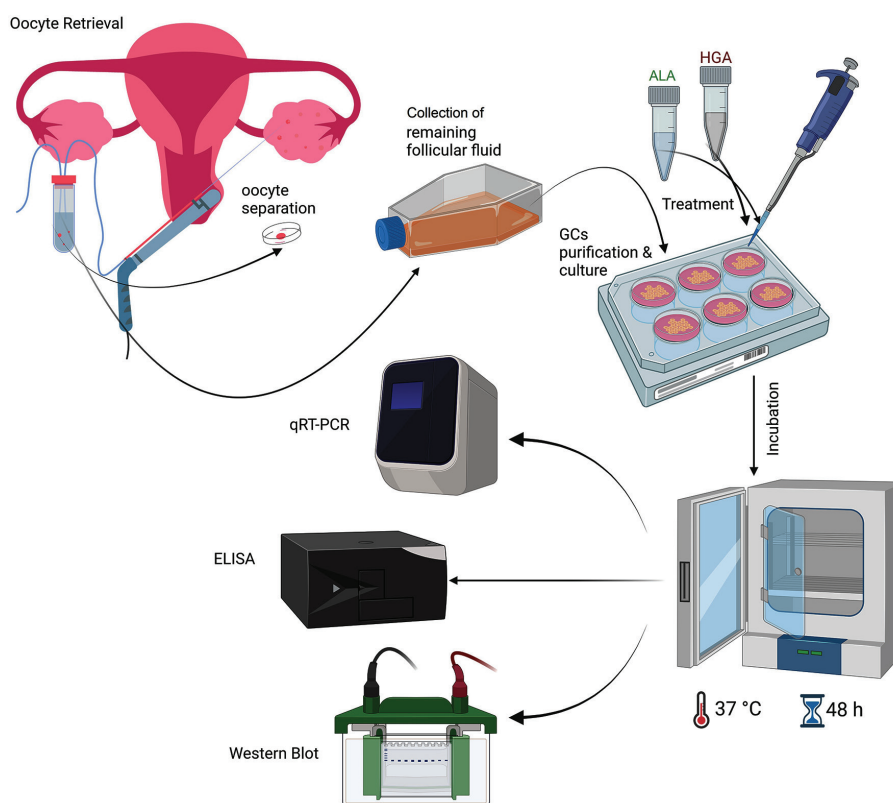


Figure 1: A schematic view of the cell culture and study procedures is shown (BioRender.com). HGA: Human glycated albumin; ALA: Alpha-lipoic acid; GCs: Granulosa cells

Quantitative Real-Time PCR (qRT-PCR)

To analyze the expression of the steroidogenic acute regulatory protein (*STAR*), 3β-hydroxysteroid dehydrogenase (*3β-HSD*), cytochrome P450 aromatase (*CYP19A1*), 17β-hydroxysteroid dehydrogenase (*17β-HSD*), and *RAGE*, total RNA extraction was performed using RNXTM-PLUS solution (SinaClon, Iran). Extracted RNA concentration and quality were evaluated using a nanodrop spectrophotometer (ThermoFisher Scientific, USA). Extracted mRNAs were reversely transcribed into first-strand cDNA using a cDNA synthesis kit (AddBio Co., South Korea) according to the manufacturer's instructions. Then, the quantitative real-time PCR (qRT-PCR) was done

by an Applied Biosystems StepOne system (ThermoFisher Scientific, USA) and a high-ROX SYBR Green PCR Master Mix (Ampliqon, Denmark). The comparative $2^{-\Delta\Delta Ct}$ method was utilized to quantify the samples where β-actin was the internal control. The Experiments were carried out in duplicate. In the present study, primers were designed using NCBI Primer-BLAST tools (Primer3 and BLAST), and their sequences are shown in table 1.

Measurement of E2, P4, and Total T Concentrations in Cell Culture Media

After 48 hours of cell culture, the media were collected to analyze E2, progesterone (P4), and total testosterone (T) concentrations released by

Table 1: Primer sequences of studied genes

Transcripts	Primer	Primer Sequences (5'-3')
<i>β-ACTIN</i>	Forward	GTGGGCATGGGTCAGAAG
	Reverse	GGGTACTTCAGGGTGAGGA
<i>STAR</i>	Forward	GGAGCTCTACTCGGTTCT
	Reverse	TTGCTAAGGATGCCCAAGG
<i>3β-HSD</i>	Forward	GGAGGGCTTCTGGGTCAG
	Reverse	AGTCAGCTTGGTCTGTCT
<i>17β-HSD</i>	Forward	TCCCAGAGCTTCAAAGTGTATG
	Reverse	GTCCAGCTGCAACGTCTC
<i>CYP19A1</i>	Forward	GTGGAATTATGAGGGCACATCC
	Reverse	CGGTTGTAGTAGTTGCAGGC
<i>RAGE</i>	Forward	GGCAGTAGTAGGTGCTCAA
	Reverse	GCCTGTGTTTCAGTTCCATTC

mural GCs. The assays were performed using commercially available ELISA kits (DiaMetra, Italy) by a microplate reader (Epoch 2™). The detection limits for P4, E2, and total T were 0.05 ng/mL, 5 pg/mL, and 0.2 ng/mL, respectively. All assays were done in duplicate.

Western Blot Assay

The western blotting technique was performed to analyze the RAGE protein level in human GCs. Briefly, after 48 hours of the experimental period, the GCs were lysed by a cold-RIPA buffer (ThermoFisher Scientific) containing protease inhibitors. Then, the lysates were centrifuged at 7000 rpm at 4 °C (10 min). After that, a Lowry assay was performed to determine the supernatant protein concentration. Following SDS-PAGE, protein transfer to the PVDF membrane was done (Bio-Rad, CA, USA), then it was blocked by skim milk in a Tris-buffered medium supplemented with tween 20 (Sigma, USA) for 120 min. After washing, the membrane was incubated with primary antibodies [anti-RAGE (SC-365154) and anti-GAPDH (GTX100118, as housekeeping protein)] (Santa Cruz, USA) at 4 °C overnight. Then, the PVDF membrane was incubated with secondary antibodies [(anti-rabbit IgG, BA1054-2) and (anti-mouse IgG, SC-516102)] for two hours at room temperature. Detection of protein bands was done using an enhanced chemiluminescence (ECL) kit (Bio-Rad) and X-ray film (Fuji-film, Tokyo) (n=5). The band intensity of RAGE protein was analyzed by densitometry readings/intensity ratio using ImageJ software (Developer: National Institutes of Health, USA) and was normalized to

the corresponding GAPDH value (as an internal control). The experiments were performed in duplicate.

The percent changes (gene, protein, and hormone assays) in treated samples over the control samples were calculated using the following formula:

$$\text{Percentage change} = (\text{Control-Treated}) / \text{Control} \times 100$$

Negative or positive percentage numbers illustrate a decrease or increase in amounts of change, respectively.

Statistical Analysis

To analyze data distribution, the Shapiro-Wilk test was done. In the case of normal data distribution, one-way ANOVA with *post-hoc* Tukey test was utilized to compare the statistical differences of experimental groups ($P < 0.05$). The Kruskal-Wallis test with *post-hoc* Dunn's test was done when data distribution was not normal. The GraphPad Prism software (version 9.0, Inc. La Jolla, California, USA) was used for statistical analyses, and the results were mentioned as mean \pm SEM.

Results

The demographic and clinical characteristics of participants in this study are presented in table 2. The mean age of the study participants was 30.64 \pm 0.55, and the mean of their BMI was less than 25 Kg/m². Most of them had regular menstruation (90%) and acceptable oocyte count (11.62 \pm 0.99) after ovarian stimulation.

Table 2: Demography and blood biochemical analysis of participants (n=50)

	Women with healthy ovaries	95% CI of mean	Acceptable ranges
Age (years)	30.64 \pm 0.55 (18–36)	29.52–31.76	—
BMI (Kg/m ²)	24.82 \pm 0.39 (19.9–30)	24.04–25.60	18.5–30
Infertility period (years)	4.40 \pm 0.45 (1–14)	3.49–5.31	—
No. of collected oocytes	11.62 \pm 0.99 (2–31)	9.62–13.62	—
Menstrual cycle	Regular	45 (90.00)	—
	Oligomenorrhea	5 (10.00)	—
FBS (mg/dL)	89.58 \pm 1.11 (70–106)	87.35–91.81	70–110
TSH (mIU/L)	2.51 \pm 0.13 (0.73–4.40)	2.24–2.78	0.4–4.2
FSH (mIU/mL)	6.39 \pm 0.24 (3.23–10.60)	5.90–6.87	3–10
LH (mIU/mL)	6.27 \pm 0.23 (3.09–10.30)	5.79–6.73	3–10
FSH/LH Ratio	1.03 \pm 0.02 (0.73–1.38)	0.99–1.07	1–1.2
E2 (pg/mL)	34.94 \pm 2.40 (3.01–96.10)	30.14–39.75	\leq 50
AMH (ng/mL)	2.85 \pm 0.18 (1.15–6.20)	2.48–3.21	2–6.8
Na ⁺ (mEq/L)	138.20 \pm 0.24 (135–143)	137.70–138.60	135–145
K ⁺ (mEq/L)	4.05 \pm 0.04 (3.50–4.90)	3.96–4.12	3.5–5.0
WBC (cells/ μ L)	7610.00 \pm 224.30 (4800–10600)	7159–8061	4500–10000
RBC (million cells/ μ L)	4.72 \pm 0.05 (3.80–5.50)	4.60–4.80	4.2–5.4
Hb (g/dL)	13.12 \pm 0.13 (10.90–15.00)	12.85–13.38	12.1–15.1

Data are presented as mean \pm SEM (range) and number (%). BMI: Body mass index; FBS: Fasting blood sugar; TSH: Thyroid-stimulating hormone; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone; E2: Estradiol; AMH: Anti-Müllerin hormone; WBC: White blood cells; RBC: Red blood cells; Hb: Hemoglobin

The serum levels of TSH (2.51 ± 0.13) and FBS (89.58 ± 1.11) in all participants were within the normal range on the third day of the menstrual cycle. Moreover, E2, FSH, LH, and AMH concentrations were within the acceptable range, indicating the normal endocrine and metabolic status of women participating in this study. The study participants also had normal conditions regarding electrolytes, blood cell count, and hemoglobin content.

The Viability of GCs after ALA Treatment

The maximum concentration of ALA with no toxicity was determined by using an MTT assay. The viability of GCs was significantly decreased by a 200 μM concentration of ALA, while 100 μM ALA was proved to be non-toxic ($P=0.0001$) (figure 2).

Effects of HGA with/without ALA on Steroidogenic Enzymes Gene Expression

The expression of the main enzymes involved in steroidogenesis was evaluated to shed light on how AGEs lead to hormonal abnormalities in human GCs. Accordingly, the *STAR*, *3 β -HSD*, *17 β -HSD*, and *CYP19A1* gene expression were measured by qRT-PCR. The expression of

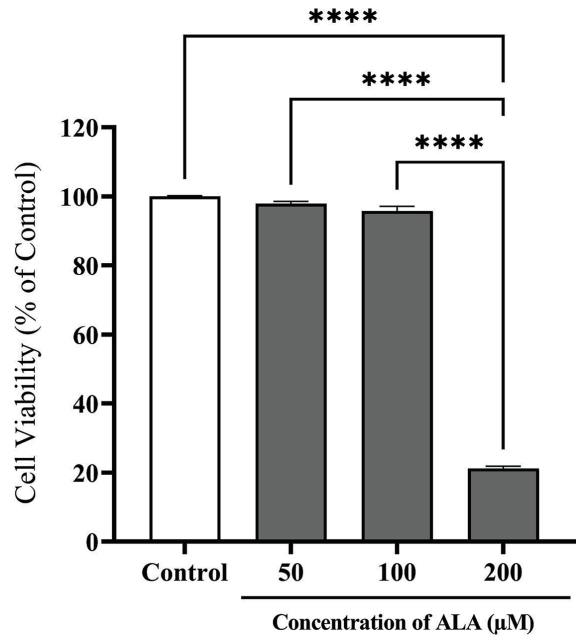


Figure 2: The effects of non-toxic concentrations of ALA on the viability of human luteinized GCs are shown using MTT assay. The experiments were performed in duplicate. The results were reported as mean \pm SEM (n=5). ****P=0.0001 represents significant differences between experimental groups. ALA: Alpha-lipoic acid; GCs: Granulosa cells; MTT: 3–4, 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide

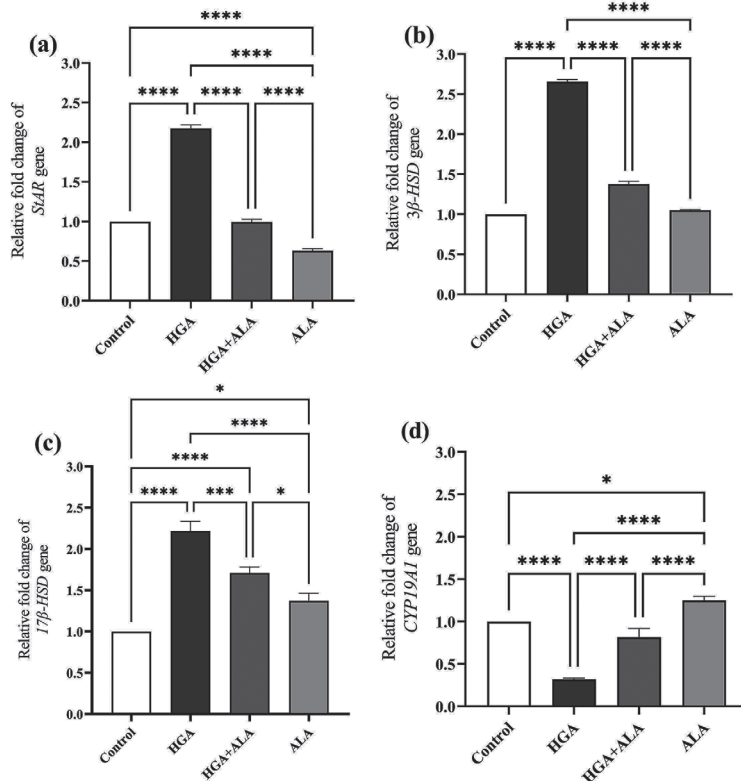


Figure 3: The effects of HGA (400 $\mu\text{g}/\text{mL}$) with/without ALA (100 μM) are shown on the expression of *STAR* (A), *3 β -HSD* (B), *17 β -HSD* (C), and *CYP19A1* (D) genes in human luteinized GCs. Quantitative real-time PCR was done for steroidogenic enzyme gene expression levels after 48 hours incubation. The experiments were performed in duplicate. The results were reported as mean \pm SEM (n=8). *P=0.022, ***P=0.001, and ****P=0.0001 represent significant differences between experimental groups. ALA: Alpha-lipoic acid; HGA: Human glycosylated albumin; GCs: Granulosa cells; *STAR*: Steroidogenic acute regulatory protein; *3 β -HSD*: 3 β -hydroxysteroid dehydrogenase; *17 β -HSD*: 17 β -hydroxysteroid dehydrogenase; *CYP19A1*: Cytochrome P450 aromatase

STAR, *3 β -HSD*, and *17 β -HSD* were significantly increased, while the *CYP19A1* gene expression was meaningfully decreased in the HGA group compared to controls. However, after treating the HGA-affected GCs with ALA (HGA+ALA group), the expression of *STAR*, *3 β -HSD*, and *17 β -HSD* exhibited significant reduction, and *CYP19A1* expression showed a significant increase compared to the HGA group and approximately returned to their normal values (figure 3).

In the HGA-treated group, a significant increase in the mRNA expression levels of *STAR* (by 117.65%), *3 β -HSD* (by 165.68%), and *17 β -HSD* (by 122.15%) were observed, while *CYP19A1* mRNA expression was decreased by 68.37% compared to controls ($P=0.001$). The addition of ALA to HGA treatment diminished the *STAR* (by 54.36%), *3 β -HSD* (by 48.17%), and *17 β -HSD* (by 22.97%) mRNA levels but enhanced the *CYP19A1* (by 157.91%) mRNA expression ($P=0.001$). Moreover, ALA treatment alone increased the *3 β -HSD* (by 4.87%), *17 β -HSD* (by 37.29%), and *CYP19A1* (by 24.98%) mRNA levels, while decreasing the *STAR* (by 36.86%) mRNA expression as compared to the controls (figure 3).

Effects of HGA with/without ALA on E2, P4, and total T Secreted by Human Luteinized GCs

Effects of HGA and ALA on the secretion of main steroid hormones by GCs were also investigated *in vitro*. According to figure 4A, E2 concentration in the media was significantly reduced after treatment of the GCs with HGA compared to the controls ($P=0.0001$). GCs treatment with HGA in the presence of ALA (HGA+ALA group) resulted in the elevation of E2 secretion compared to the HGA group, but the difference was not statistically significant ($P=0.516$). The levels of E2 secretion by GCs treated with ALA were similar to the control group. Furthermore, figure 4B shows that in HGA-treated GCs, the P4 secretion increased slightly but was statistically insignificant compared to the controls. ALA treatment significantly reduced P4 secretion levels in HGA+ALA and ALA-only treated groups compared to the HGA and control groups ($P=0.0001$). Figure 4C indicates that the concentration of total T in the media was significantly increased following HGA treatment, as compared to the other studied groups ($P=0.0001$). Treatment with ALA to the GCs culture in the presence or absence of HGA significantly decreased the production of total T compared to the HGA group ($P=0.0001$). No significant difference was observed between the control, HGA+ALA, and ALA groups.

Compared to the control, the cells treated with

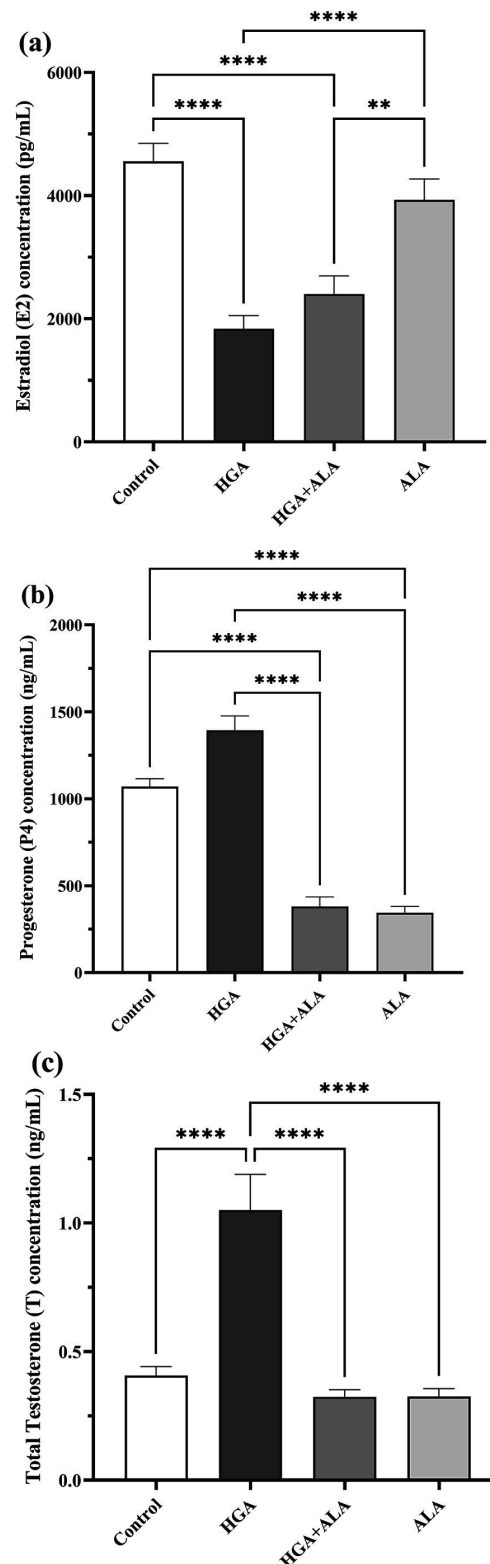


Figure 4: The effects of HGA (400 $\mu\text{g/mL}$) with/without ALA (100 μM) are shown on E2 (A), P4 (B) and total T (C) release by human luteinized GCs. After 48 hours incubation, cell culture media were collected for ELISA assay of released sex steroid hormones. The experiments were performed in duplicate. The results are reported as mean \pm SEM ($n=20$). ** $P=0.002$ and **** $P=0.0001$ represent significant differences between experimental groups. ALA: Alpha-lipoic acid; HGA: Human glycated albumin; GCs: Granulosa cells; E2: Estradiol; P4: Progesterone; T: Testosterone

HGA alone (for 48 hours) secreted significantly lower E2 (by 59.66%) ($P=0.002$), while total T production (by 158.24%) ($P=0.0001$) was considerably increased, and P4 release (by 30.40%) was slightly increased. The addition of ALA to the HGA-treated cells ameliorated this undesirable change through increased E2 (by 30.68%) and decreased P4 (by 72.70%) and total T (by 69.16%) concentrations (figure 4).

Effect of HGA with/without ALA on the RAGE Gene and Protein in Human Luteinized GCs

The HGA and ALA effects on the RAGE expression and its protein level in GCs are presented in figure 5. The results illustrated that the highest RAGE mRNA expression occurred in the HGA group ($P=0.0001$). However, treatment of the GCs with ALA in the presence of HGA (HGA+ALA group) could significantly downregulate its expression.

Moreover, the band intensity of RAGE protein was quantitatively analyzed by densitometry readings/intensity ratio. The results showed a significant increase ($P=0.0001$) in RAGE protein level under HGA treatment. Co-treatment of HGA+ALA significantly diminished RAGE protein level compared to the HGA group, whereas there was a significant difference ($P=0.008$) with controls. However, there were no significant differences between ALA and control groups regarding the RAGE gene and protein levels.

Adding HGA significantly enhanced RAGE gene and protein levels by 140.66% and 267.10%, compared to the control group ($P=0.0001$). Co-treatment of ALA and HGA significantly suppressed the HGA-induced increase in RAGE gene and protein levels by 45.12% and 14.83%, respectively ($P=0.003$) (figure 5).

Obtained results in the expression of enzymes, receptors, and hormone secretion as a result of the treatments used in the present study are schematically summarized in figure 6.

Discussion

Our findings indicated that HGA (AGEs) significantly upregulated *STAR*, *3 β -HSD*, and *17 β -HSD* mRNA levels but downregulated *CYP19A1* mRNA expression. The addition of ALA inhibited HGA-induced changes in these genes. HGA also altered E2, P4, and total T release by GCs in culture media, whereas ALA treatment overcame these undesirable changes. ALA downregulated RAGE mRNA and protein expression, which could be part of the mechanism by which ALA prevents HGA-induced changes in the steroidogenesis signaling pathway.

As the conditions of follicular atresia were not the aim of the current study, high concentrations of HGA, having toxic effects on GCs, were not applied. Thus, the administered HGA (400 $\mu\text{g}/\text{mL}$)¹⁶

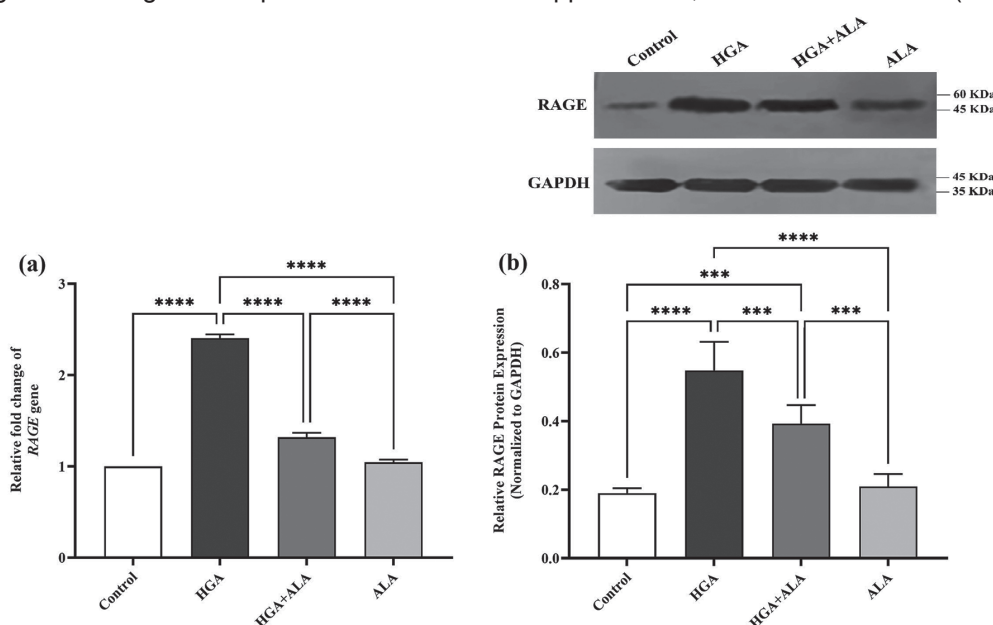


Figure 5: The effects of HGA (400 $\mu\text{g}/\text{mL}$) with/without ALA (100 μM) are shown on RAGE gene expression (A) and RAGE protein level (B) in human luteinized GCs. Quantitative real-time PCR was done for levels of RAGE gene expression after 48 hours incubation ($n=8$). Western blots and densitometry readings of RAGE protein bands were done using an enhanced chemiluminescence kit and X-ray film ($n=5$). The band intensity of RAGE protein was analyzed by densitometry readings/intensity ratio using ImageJ software and was normalized to the corresponding GAPDH value. The experiments were performed in duplicate. The experiments were reported as mean \pm SEM. *** $P=0.001$ and **** $P=0.0001$ represent significant differences between experimental groups. ALA: Alpha-lipoic acid; HGA: Human glycated albumin; GCs: Granulosa cells; RAGE: Receptor for advanced glycation end-products

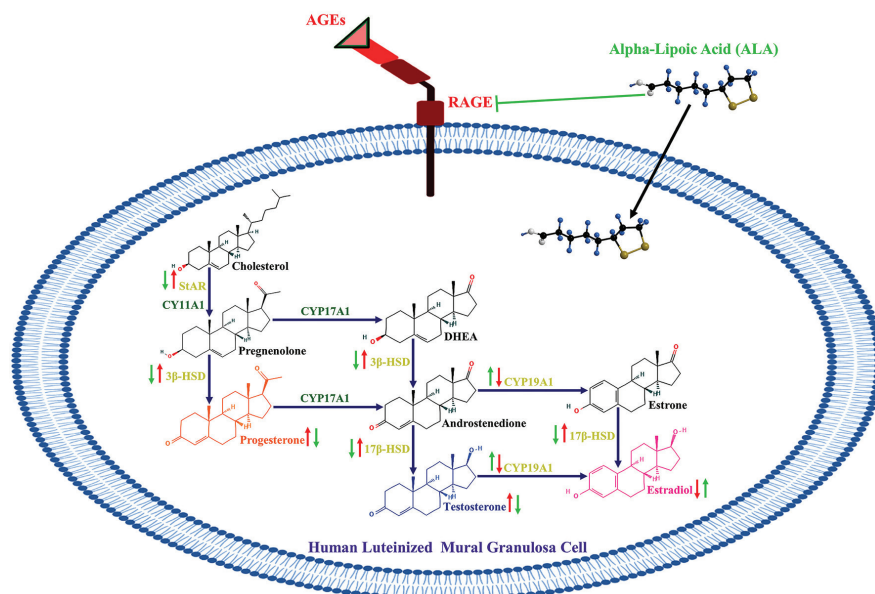


Figure 6: Schematic diagram represents the relationship between ALA and HGA in human luteinized GCs. HGA (as an AGEs representative) affects steroidogenesis by up-regulating *STAR*, *3β-HSD*, and *17β-HSD* (upward red arrows), and down-regulating *CYP19A1* (downward red arrows). ALA inhibits the expression of the *RAGE* gene and its protein. Moreover, it restores the effect of HGA on *STAR*, *3β-HSD*, *17β-HSD*, and *CYP19A1* (green arrows). ALA: Alpha-lipoic acid; HGA: Human glycated albumin; AGEs: Advanced glycation end-products; GCs: Granulosa cells; *STAR*: Steroidogenic acute regulatory protein; *3β-HSD*: 3β-hydroxysteroid dehydrogenase; *17β-HSD*: 17β-hydroxysteroid dehydrogenase; *CYP19A1*: Cytochrome P450 aromatase; *RAGE*: Receptor for advanced glycation end-products

was not toxic but was adequate to induce hormonal imbalance similar to a reported hormonal abnormality in other reproductive disorders, such as PCO patients who have a high level of AGEs.²⁷ Additionally, the GCs of normal ovulatory women were used to prevent interference or pre-exposure to high levels of AGEs. A nontoxic concentration of ALA (100 μM) was administered to assess whether it reduces the detrimental effects of HGA.

Our findings indicated that exposure of the GCs to the HGA significantly diminished E2 release, increased total T, and slightly raised P4 secretion *in vitro*. These alterations in hormone secretion profiles could be ascribed to the changes in the expression levels of steroidogenic enzymes in the luteinized GCs. HGA meaningfully increased *STAR*, *3β-HSD*, and *17β-HSD* mRNA levels that are involved in P4 and T production while decreasing *CYP19A1* mRNA expression, which converts androgens to estrogens. These undesirable changes in the expression of steroidogenic genes and production of hormones are along with the upregulation of *RAGE* mRNA and protein levels in HGA-treated GCs. Indeed, binding of AGEs to RAGE increases steroidogenic enzyme expressions, including *STAR*, *CYP17A1*, *3β-HSD*, and *17β-HSD*, which may lead to a hyper-androgenic condition.²⁷

In agreement with our results, Jinno and colleagues demonstrated a reverse correlation

between levels of E2 and follicular fluid/serum AGEs in infertile women.²⁸ Moreover, a positive correlation exists between serum levels of AGEs and T levels in female rats fed with a high-AGE diet.²⁹ An *in vivo* study showed that exposure of mice to a high-AGE diet prenatally altered the steroidogenic enzyme expression, such as a decreased expression of *CYP19A1*.³⁰ In another study, Merhi and others demonstrated that treatment of human cumulus GCs with HGA led to increased expression of *STAR*, *CYP11A1*, *3β-HSD*, *CYP17A1*, and *LHR* genes. They also demonstrated that E2 production was increased by HGA-treated cumulus GCs, whereas there was no report about the effect of HGA on P4 and total T secretion.¹⁶ It should be noted that Merhi and colleagues conducted their study on cumulus GCs, whereas the present study used mural GCs with different metabolomes. Cumulus cells produce more E2 than mural GCs, whereas their P4 release is not different.³¹

Our findings confirmed that AGEs are potential agents responsible for hormonal imbalance, such as aberrant increased P4 and reduced E2 secretion by GCs. It is known that P4 triggers ovulation in a narrow window at the end of the follicular phase, and its administration out of this window disturbs ovulation.³² Additionally, increased concentrations of P4 in the late follicular phase could be a reason for the reduced oocyte quality, embryonic development, and disrupted embryo-endometrial synchrony.³³

Previous studies have reported the adverse effects of AGEs on ovarian tissues, especially GCs.^{15, 16} During reproductive life, AGEs could accumulate in the ovary due to their long half-life.³⁴ Increasing concentrations of AGEs in the ovarian microenvironment may disrupt the normal function of female reproductive tissues, disrupt vascularization, and decrease the nutrient uptake by GCs that, in turn, lead to PCO-like syndrome³⁵ and ovarian aging.³⁴

In the present study, the potential of ALA in preventing the deleterious effects of HGA on the GCs functions was also investigated. ALA (1,2-dithiolane-3-pentanoic acid) is naturally produced in mitochondria by lipoic-acid synthase enzyme (LASY).³⁶ Furthermore, the body receives ALA from food and supplementary nutritional products. This anti-oxidant agent is soluble in water and oil; thus, it could pass through different cellular and organelle membranes.¹⁹ The findings of the current study showed that the ALA treatment (100 μ M) of HGA-exposed GCs considerably attenuated the adverse effect of HGA on steroidogenic enzyme expression and steroid hormone production.

ALA significantly reduced P4 and total T secretion by downregulating *STAR*, *3 β -HSD*, and *17 β -HSD* genes. Moreover, ALA significantly increased *CYP19A1* expression and, therefore, increased the E2 release of human GCs. Our result also indicated that ALA significantly decreases HGA-induced *RAGE* up-regulation. Other *in vitro* or *in vivo* studies confirmed the protective effect of ALA against AGE. Niu and colleagues³⁷ showed that ALA significantly reduced the cytotoxicity of AGEs on SH-SY5Y cells (a neuroblastoma cell line) and inhibited their *RAGE* expression. Additionally, topical administration of ALA nanoparticles in diabetic mice dramatically lowered *RAGE* expression and promoted wound healing.³⁸ It has been previously reported that androgens increase AGE accumulation and *RAGE* expression in luteinized GCs. Moreover, AGE in positive feedback increases *RAGE* expression.¹⁴ According to our results, the modulatory function of ALA in steroidogenesis could be attributed to the reduction of the *RAGE* expression, amplified by the androgenesis, which breaks this vicious cycle of AGE accumulation in the cell and subsequent adverse downstream effects. Besides, ALA may effectively restore AGEs deleterious effects by inhibiting AGE formation by blocking the amino and carbonyl groups on proteins and reducing sugars, respectively.¹⁹ Other possible protective mechanisms might be related to ALA insulin-sensitizing and antioxidant properties. The abnormal function of LASY in type II diabetic

mice leads to a decreased endogenous ALA synthesis. ALA deficiency induces redox imbalances and increased inflammation and may contribute to insulin resistance by disrupting glucose uptake.²⁴ Insulin resistance likely triggers AGE formation and, consequently, hormonal disturbances, hyperandrogenism, and LH elevation in PCO patients.³⁵ ALA significantly lowers serum LH levels and the LH/FSH ratio in PCO patients. ALA also improves lipid profile and insulin levels and decreases hyperandrogenism symptoms such as hirsutism and menstrual abnormalities.³⁶ Taken together considering the aforementioned studies, it is not unexpected that ALA supplementation might be effective for hormonal abnormality induced by high AGE levels.

It is true that different ovarian cells, such as theca cells and mural GCs, cooperate in steroidogenesis; simultaneously analyzing these two cell types in laboratory conditions is not easily possible. This issue was the main limitation of the present study.

Conclusion

The present study findings demonstrated that ALA (100 μ M) lowers *RAGE* mRNA and protein levels and protects human GCs against HGA-induced alterations in steroidogenesis. Based on our findings, ALA might be considered a possible option for treating AGE-induced ovarian dysfunction in infertile women with PCOS or diabetes, as well as those with unhealthy lifestyles and disrupted menstrual cycles who want to become pregnant. More research is needed, however, to investigate the molecular mechanism of ALA effects in the ovary. To the best of our knowledge, no research has been conducted on the effects of ALA on human female steroidogenesis as it is influenced by AGEs.

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Authors' Contribution

Z.D: contributed to performing experimental methods and data acquiring and analyzing, wrote

the manuscript; S.B: advised some experimental protocols and was involved in the critical revision of the manuscript; MH.N: participated in the study conception and design, data interpretation, and manuscript revision; F.M: contributed to performing experimental methods and data acquiring and analyzing, wrote the manuscript; M.M: contributed to performing experimental methods and data acquiring and analyzing; M.D: contributed to performing experimental methods and data acquiring and analyzing; S.M.T: participated in the study conception and design, data interpretation, and manuscript revision. All authors reviewed and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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