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α-Lipoic acid prevents senescence, cell cycle arrest, and inflammatory cues in fibroblasts by inhibiting oxidative stress

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ABSTRACT

Senescence is a process characterized by an irreversible growth arrest in cells and induced by oxidative stress. In the current study, anti-aging potential of a well-known antioxidant, α-lipoic acid (α-LA), in rat embryonic fibroblast (REF) cells was assessed. In this regard, oxidative stress, inflammation, and apoptosis pathways were investigated on REF cells exposed to H_2O_2 as a senescence inducer and α-LA as a protective compound. In cells treated with α-LA and H_2O_2, level of β-galactosidase, as an aging marker, and oxidative stress biomarkers, were significantly lower than those exposed to H_2O_2 only. Furthermore, flow cytometry assay showed that α-LA caused a significant reduction in the number of apoptotic cells via the caspase-dependent pathway. In addition, it could neutralize the inflammatory effects of H_2O_2 and attenuated the concentration of inflammatory cytokines. In comparison to H_2O_2 group, a significant increase in G0/G1 arrest was observed during cell cycle analysis in cells exposed to H_2O_2 and α-LA. The results of this study show that α-LA has beneficial effects on H_2O_2-induced cellular senescence. α-LA works by attenuating the reactive oxygen species, subsiding inflammation, and affecting cell division.

1. Introduction

Aging is a set of complex changes taking place in an organism over time. It affects various functions of the body, which leads to diseases and ultimately death [1–3]. Aging process involves several factors that proceed gradually, therefore, despite of intense scientific advancement, the exact causes of aging are yet to be elucidated in detail [4,5]. Various factors like oxidative stress, glycation, telomere shortening, mutations, and aggregation of proteins are involved [6].

The role of reactive oxygen species (ROS) in the etiology of the aging process has been well documented. Thus, the "free radical theory" of aging, proposed by Denham Harman has gained significant attention in order to determine the dynamics of aging. There is sufficient evidence suggesting ROS as one of the main contributors in the aging process [2]. According to this theory, aging-associated disorders are the result of progressive and unrestrained production of ROS causing oxidative damage to body macromolecules, impairing physiological functions, and ultimately shortening the lifespan [1,7]. Free radicals and ROS are highly reactive chemical species. It has been proved that all of the above cause oxidative impairment by producing oxidative damage to macromolecules. In addition, among all, DNA is extremely susceptible to ROS action. ROS attack DNA, and cause DNA strand breaking, and this oxidative DNA damage leads to genomic abnormality [8].

In normal conditions, ROS are produced as byproducts during mitochondrial respiration [9]. The line of evidence revealed that ROS are also produced in the body as a result of exposure to certain chemical toxicants, chemotherapeutics, and toxins [10,11]. Other contributors causing the release of ROS in the body are inflammatory cytokines, and cytosolic enzymes [12]. When the balance of ROS production and antioxidant enzymes is disrupted with the overproduction of free radicals, mitochondrial damages, including damage to the mitochondrial

Abbreviations: Ac-DEVD-qNA, *N*-acetyl-Asp-Glu-Val-Asp-P nitroanilide; Ac-LEHD-qNA, *N*-acetyl-Leu-Glu-His-Asp-P-nitroanilide; DCFDA, 2′,7′–dichlorofluorescin diacetate; FRAP, ferric reducing antioxidant power; IL-Iβ, interleukin-1β; MTT, 3-4,5 dimethylthiazol-2-Yl-2,5-diphenyltetrazolium bromide; REF, rat embryonic fibroblast; ROS, reactive oxygen species; TBA, thiobarbituric acid; TERT, telomerase reverse transcriptase; TNF-α, tumor necrosis factor alpha; TPTZ, tri (2-pyridyl)-*S*-triazine; TTM, total thiol molecules; α-LA, alpha-lipoic acid

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respiratory chain, influencing Ca^{2+} homeostasis and alteration of membrane permeability have been observed [13]. On the other hand, in aging processes and various chronic diseases such as neurodegenerative diseases, the role of oxidative mitochondrial damage has been well-documented [14]. However, the exact mechanism of ROS-induced mitochondrial damage is yet to be revealed.

Cellular aging is a mechanism defined by an irreversible growth arrest in somatic cells, or in other words, it leads to cell cycle arrest. Cells undergoing senescence contain the high level of ROS, which causes oxidative damage to biological macromolecules [15]. Moreover, the rate of formation of senescent cells is related to the amount of oxidants present in the cell. Either increasing the level of oxygen species or lowering the quantity of antioxidant molecules increases the senescence process [2]. In addition, shortening of the telomere is also considered as one of the major causes of senescence [16]. Cellular oxidative stress, even under mild conditions, directly affects the rate of telomere shortening [17]. In addition, ROS also indirectly affect telomeres. They bind to the catalytic subunit of telomerase and telomerase reverse transcriptase (TERT) causing loss of their activity. *N*-acetyl cysteine has been reported to block ROS-induced loss of TERT activity, resulting in the delay of cellular senescence [18].

Variety of biochemical stresses arrest reversible growth ultimately developing cellular senescence. Accumulation of senescence-related molecules is responsible for aging or related diseases. Previous studies demonstrated contributing role of cellular senescence in tissue remodeling, aging, and related diseases. Despite harmful effects, cellular senescence also found to be beneficial in suppressing tumor, cancerous cells, tissue fibrosis, and wound healing. The senescence-associated secretory phenotype (SASP) molecules as the pro-regenerative therapeutic targets could help developing improved and cost-effective senolytic strategies for suppressing the negative effects of cellular senescence in ageing and related pathologies [19,20].

α lipoic acid (α-LA) is also known as thioacetic acid. It acts as coenzyme having sulfur. It is an organic compound involved in numerous metabolic functions and is both water and fat-soluble. Naturally, α-LA is found in wheat germ, beer yeast, and red meat. The physiological function of α-LA is very similar to vitamin B because its role is also critical in energy production at the cell level. As an essential component of some enzyme complexes like pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase, α-LA plays its major role in the metabolism of proteins, carbohydrates, and fatty acids. In addition, α-LA also regulates blood glucose levels by enhancing its intracellular transport. Besides the mentioned functions, α-LA acts as a scavenger of the free radicals produced during cellular respiration [21]. The thiol group present in α-LA reacts with free radicals, thus providing its antioxidant capacity. Moreover, it has been reported that α-LA also increases the antioxidant potential of superoxide dismutase and glutathione. In addition, α-LA has been found to cause the regeneration of vitamin E [22] (Fig. 1). α-LA is a biological antioxidant counteracting ROS and free radicals. Also, it has been reported that it plays a vital role in aging and regulating mitochondrial biochemical reactions by decreasing oxidative stress [21,23].

In vitro and *in vivo* studies show that α-LA has a key role in increasing the level of many natural thiol antioxidant and glutathion (GSH) [24,25]. α-LA supplementation in a dose range of 0.12 g/kg has been reported to reverse oxidative stress in rat hepatic and cardiac cells [23]. Moreover, previous experimental studies conducted that either on human lymphocytes [26], or diabetic rats [27], α-LA in 1,10,100,1000 μM and 10 mg/kg intraperitoneal for 14 days, respectively, exert substantial antioxidant properties. In addition, in numerous other chronic diseases involving oxidative stress, α-LA administration has been recorded to decrease ROS and prevent oxidative stress-induced changes [21,28]. Thus, enhancing body antioxidant mechanism against ROS may counteract the ROS associated aging process. By employing this approach, the incidence of some age-related disorders such as cancers, neurodegenerative diseases, and cardiovascular problems could be delayed up to some extent [29].

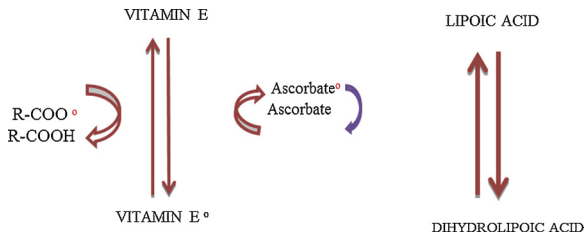

Fig. 1. Vitamin E recycling by α-LA in the presence of vitamin C.

Rat embryonic fibroblast (REF) cells exist in every organ of the animal. They enable remodeling and tissue repair and play a vital role in organ development, helps wounds heal and reduces inflammation. Thus, fibroblast cells are an attractive model for *in vitro* studies [30,31]. Free radicals and ROS have been well documented to contribute in the aging process, and "free radical theory" of aging, has gained considerable attention in determining the dynamics of aging [32]. Therefore, in the present study, our aim was to evaluate the mechanisms involved in the protective effect of α-LA on H_2O_2-induced ROS and premature senescence of rat embryonic fibroblast cells.

2. Material and methods

2.1. Chemicals

Inflammatory cytokines ELISA kits were bought from BenderMed Systems Inc. (Vienna, Austria). Rat specific β-galactosidase kit was used from (Cusabio, China). All chemicals and reagents for biochemical experiments were used from Sigma-Aldrich (Munich, Germany).

2.2. Isolation of REF cells

Ethical approval for isolation and culture of REF cells was obtained from the Tehran University of Medical Sciences (TUMS) ethics committee on medical research with code no. IR.TUMS.REC.1394.237. Isolation of cells from REF were done using the standard procedure as reported previously [30,33]. In brief, after inducing pregnancy, 55 mg/kg of pentobarbital was used for anesthetizing rats. After opening the abdomen, the removal of the uterine horn was done. The embryos were cleared. The cells were isolated both mechanically and with enzymatic treatment (0.25% Trypsin/EDTA). The enzymatic action was deactivated by adding Dulbecco's modified Eagle medium (DMEM)-HG, 10% FBS, 1% penicillin-streptomycin and 1% glutamine, and the tissue was pipetted up and down to get a single cell suspension. In this study, REF cells were used at third passage.

2.3. Treatment

2.3.1. First step: safety investigation and determination EC50

REF cells at passages 3 were seeded at a density of 5×10^5 cells in 24-well plates and preincubated for 2 h at 37 °C and 5% CO_2 humidified atmosphere. Initially, the effective concentration of α-LA was achieved by MTT (3–4, 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide) assay by pretreating the cells with various concentrations of α-LA (0, 1, 10, 100, 1000 μM) for 24 h. After determining the EC50 of α-LA anti-aging, anti-apoptotic, antioxidant and anti-inflammatory properties were evaluated using this concentration. The methodology of the experimental study is explained in Fig. 2. In the following, the REF cells were defined as four groups as follows: (1) Control group received only DMEM; (2) H_2O_2 group received 400 μM H_2O_2 in DMEM for 2 h [34,35]. (3) α-LA group received EC50 of α-LA in DMEM for 24 h (4) H_2O_2 + α-LA group received EC50 of α-LA acid for 24 h followed by 400 μM H_2O_2 for 2 h. Then, after 24 h cellular markers were assessed.

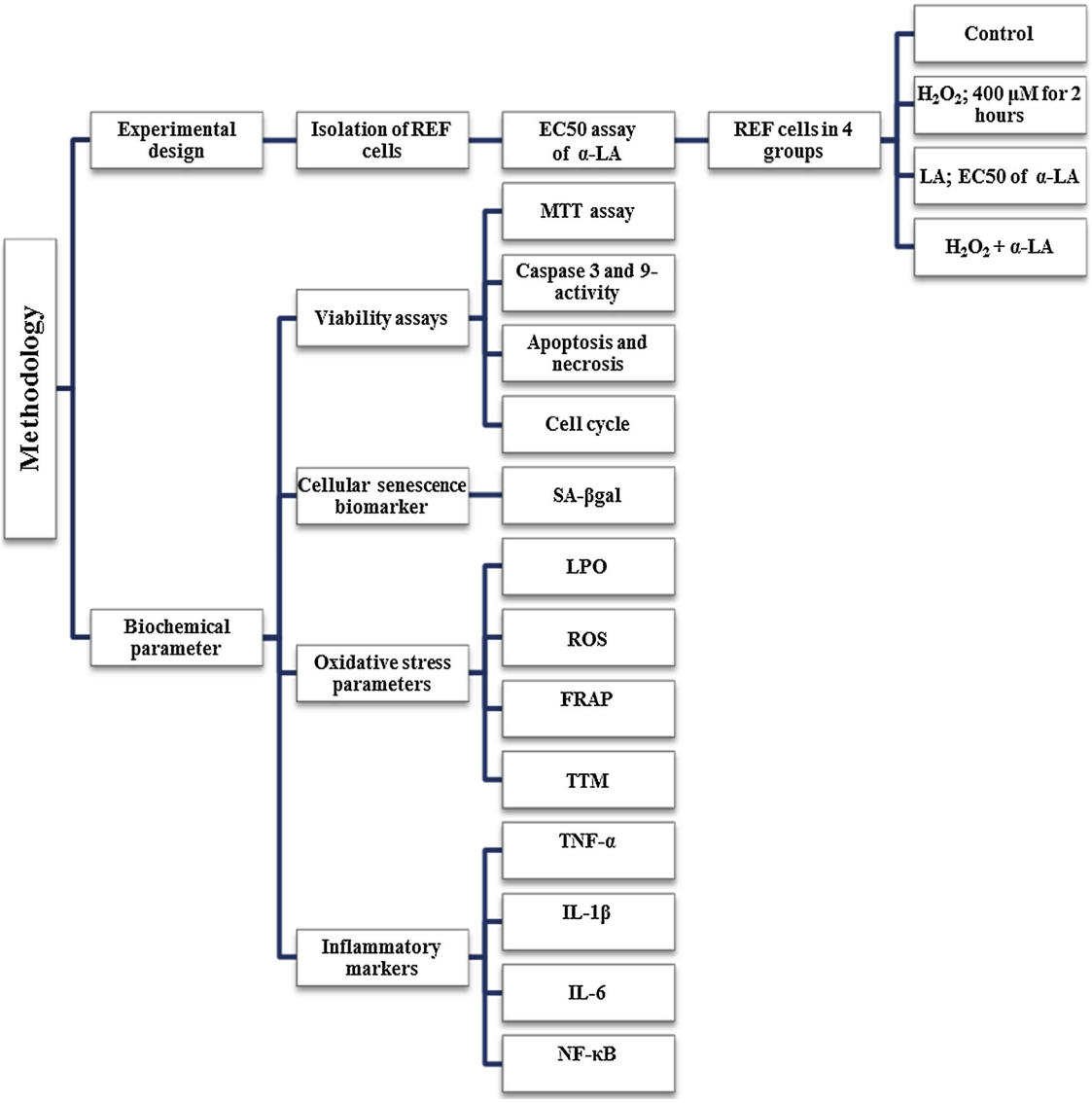

Fig. 2. The methodology of the experimental study.

2.4. Second step: senescence investigation biomarkers

2.4.1. Senescence-associated β-galactosidase staining assay

β-galactosidase activity at pH 6 is present only in senescent cells. For measuring the activity of β-galactosidase in REFs, the senescence galactosidase staining kit was used following the manufacturer's instructions. In brief, the REFs were cultured to get 70% confluency. After that, the cells were washed with PBS and fixed with fixative solution (20% formaldehyde, 2% glutaraldehyde in 10X PBS) for 10–15 min at room temperature. In the end, the cells were stained using β-galactosidase satin, they were then incubated overnight at 37 °C. The bluish-green color was measured with a light microscope (200X magnification). These colors show the number of senescent cells [30].

2.4.2. Quantitative identification of senescence-associated β-galactosidase assay

For assessing the β-galactosidase activity in REF cells, rat specific galactosidase kit was used following the manufacturer's instructions. In brief, after preparing all the reagents and solutions, samples were added to the wells and incubated for 2 h at 37 °C. Then, the liquid was removed, and 100 µL of biotin-antibody was added to each well and incubated the plates for 1 h at 37 °C. Then, after washing, 100 µL of HRP-avidin was added to each well and again incubated for 1 h. After complete aspiration, 90 µL of TMB was added to each well and incubated for 30 min avoiding direct sunlight. In the end, in order to stop the reaction, the solution was added to each well and the optical density was measured at 450 nm.

2.4.3. Cell cycle analysis by propidium iodide staining (flow cytometry)

For cell cycle analysis, trypsin was added to REF cells, and then fixation was done using ice-cold ethanol 70%, and centrifuged at 10,000 g for 5 min. Ice cold PBS was then used for washing and the pellets were redistributed in propidium iodide having RNAse. After that, incubation was done at room temperature. G1, S, and G2 phases were determined using flow cytometry assay [30].

2.5. Third step: oxidative stress parameters

2.5.1. Lipid peroxidation measurement

To determine the lipid peroxidation in REF cells, a common and inexpensive method of thiobarbituric acid (TBA) assay was used in spite of some consideration and limitations of this method in non-specificity of TBA reactivity on MDA and production of MDA from reactions other than lipid peroxidation [36]. Lipid peroxidation in REF cells was

measured by using thiobarbituric acid (TBA). In brief, the REF cells were homogenized and mixed with 800 μL trichloroacetic acid followed by centrifugation at 3500 g for 30 min. After this, the mixture of supernatant (600 μL) was done using 150 μL TBA (1% w/v). The resultant mixture was allowed to incubate in boiling water bath for 15 min and then n-butanol 400 μL was added. After cooling, the absorbance was recorded at 532 nm [30]. The MDA standard curve was made the concentration range of 0-120 μM. The lipid peroxides levels were reported as μM.

2.5.2. ROS assay

The production of ROS in REF cells treated samples can be determined by measuring the generation of 2′,7′–dichlorofluorescein (DCF) after treating by 2′,7′–dichlorofluorescein diacetate (DCFH-DA). To do this assay, after adding the DL-dithiothreitol (DTT, 50 μM) to REF cells, they were homogenized at 10,000 g for 10 min at 4 °C by lysis buffer solution, containing EDTA (1 mM), HEPES (5 mM), KCl (20 mM), and sucrose (0.25 M), pH = 7.4 into each vial. Following this, the supernatants of the samples were mixed with 80 μL of buffer assay and 5 μL of DCFH-DA (5 μM) for 15 min in 37 °C. Finally, changes in absorbance were detected by ELISA fluorimeter with excitation and emission spectra of 488 nm and 525 nm, respectively [26]. Finally, the results were defined as the percentage of control, which is assumed 100%.

2.5.3. Ferric reducing antioxidant power (FRAP) assay

To determine antioxidant capacity, FRAP test, which also quantifies the ability of biological fluids/tissues to reduce Fe3+ to Fe2+, was used. The procedure involved the preparation of FRAP reagent which includes 25 ml acetate buffer (300 mM, pH 3.6) with 16 ml acetic acid for one portion of buffer solution, 2.5 ml 2,4,6-tripyridyl-*S*-triazine (TPTZ) solution that obtained from TPTZ (10 mM) in HCl (40 mM) and 2.5 ml $FeCl_3.6H_2O$. To 246 μl of reagent recently prepared, 10 μl sample was added and then incubated at 37 °C for 10 min. The complex between Fe^{2+} and TPTZ forms a blue color with absorbance at 593 nm [37].

For preparing 100–1000 mM Fe^{2+}standard solution, ferrous sulphate ($FeSO_4.7H_2O$) was dissolved in distilled water. The results were expressed as mM ferric ions reduced to the ferrous per liter.

2.5.4. Assay of total thiol molecules (TTM)

In order to assay TTM in sample groups, Tris-EDTA buffer was added to the samples and the absorbance read at 412 nm (A1). Then 40 μl 5-5′-dithiobis-2-nitrobenzoic acid (DTNB) reagent was added to treated REF cells. After 15 min incubation at room temperature, the samples were centrifuged at 3000 g for 10 min, and finally, the absorbance of the supernatant was determined (A2). The absorbance of DTNB reagent was chosen as blank (B) [10]. The concentration of total thiol molecules (mM) was measured as follows: (A2-A1-B) × 1.07/0.05 × 13.6.

2.6. Fourth step: apoptosis and necrosis investigation

2.6.1. Flow cytometry evaluation of apoptosis and necrosis

Flow cytometry is a high-grade assay used to assess the biochemical and physical appearance of the cells, such as viability, apoptotic and necrotic percent of live cells in a fluid system through a laser beam [26]. For evaluation of apoptosis and necrosis, the REF cells were treated harvested and washed for staining with Annexin V-FITC antibody and PI according to the kit protocols (Apogee Flow System, UK). The REFs were studied for fluorescence potency in FL1 (FITC) and FL2 (PI) channels.

2.6.2. Measurement of caspase 3 and caspase 9 activity

The colorimetric measurement of caspase 3 and 9 activities assay is based on the addition of a caspase-specific tetrapeptide that is conjugated to the color labeled molecule p-nitroaniline (pNA). The cleavage of the peptide by the caspase releases the yellow chromophore pNA, which can be quantitated spectrophotometrically at 405 nm. The level of caspase enzymatic activity in the sample is related to the appearance of the yellow color upon the cleavage. Pretreated REF cells were collected and suspended in caspase lysis buffer (100 mM HEPES, 20% glycerol, 0.5 mM EDTA, 5 mM DTT) and incubated on ice for 10 min. Thereafter, caspase buffer (100 mM HEPES, 20% glycerol, 0.5 mM EDTA, 5 mM DTT) containing caspase 3 and caspase 9 substrates (Ac-DEVD-pNA and Ac-LEHD-pNA) was incubated with sample lysates for 4 h at 37 °C. The activities of caspase 3 and caspase 9 were stated as the percentage of control, assumed as 100% [26].

2.7. Fifth step: measurement of inflammatory cytokines

2.7.1. Determination tumor necrosis factor alpha

Quantitative measurement of tumor necrosis factor alpha (TNF-α) in the REF cells was done with a rat-specific TNF-α ELISA kit as per the manufacturer's instructions. In short, standards and all samples were added to the wells containing immobilized antibodies. After washing the walls, biotinylated anti-rat TNF-α antibody was added, followed by the addition of HRP conjugated streptavidin. The wells were again washed, and then TMB solution was added to each well. This produces a color related to the amount of present TNF-α. In the end, a stop solution was added. It causes a change in color from blue to yellow and the color intensity was measured at 450 nm.

2.7.2. Determination of IL-6

IL-6 in REF cells was evaluated using rat-specific IL-6 ELISA kit as per manufacturer's instructions. After preparing all the reagents, samples and standards were added to the wells, incubated at 37 °C for 90 min. Then biotinylated antibody (0.1 mL) was added required incubation was done, all the wells were washed three times using TBS buffer. Then Avidin-Biotin-Peroxidase Complex (ABC) working solution (0.1 mL) was added and then incubated at 37 °C for 30 min. After washing with TBS, TMB coloring agent (90 μL) was added and incubated for 30 min protected from light. Finally, stop solution (0.1 mL) was added and the absorbance was read at 450 nm.

2.7.3. Determination of IL-1β

IL-Iβ in REF cells was measured by using rat-specific IL-1β specific kit. According to the manufacturer's instructions, after making all the reagents, sample and standards were added to the wells. The incubation was done for 90 min at 37 °C and then biotinylated antibody (0.1 mL) was added and incubation was done for 60 min at 37 °C. After washing three times with TBS, ABC working solution (0.1 mL) was added and incubated for 30 min at 37 °C. After adding TMB coloring agent (90 μL) the strips were placed for 30 min protected from light. Finally, stop solution (0.1 mL) was added and the absorbance was recorded at 450 nm.

2.7.4. Determination of transcription factor (NF-κB)

NF-κB was determined using a specific transcription factor kit. In short, after preparing all the reagents and solutions, all samples were diluted to 50 μL using the buffer and centrifuged. Then 2–20 μg/μL nuclear fraction of samples were added to all microtube having 1 pmol biotinylated probe. After vortex mixing and incubation for 30 min, all 50 μL was transferred to wells and fixed with adhesive tape. After 1 h of mild agitation and a three-time, primary antibody (100 μL NF-κB) was added to each well. After that another incubation was done for 1 h without agitation, After 1 h, the plate was again washed for three times and secondary antibody (HRP) was added to each well, another incubation of 1 h at room temperature was done without agitation and the plate was again washed up. In order to measure colorimetric reactions, the developing solution was added, and the plate was incubated for 10 min avoiding direct sunlight. In the end, stop solution

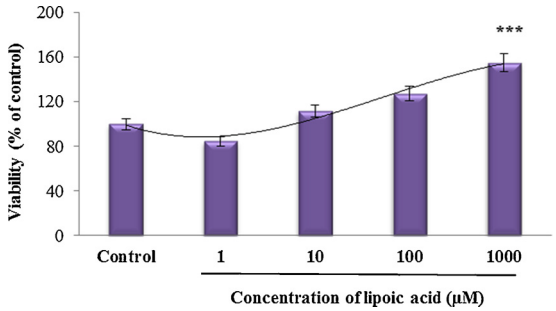

Fig. 3. Determining EC50 of α-lipoic acid on rat embryonic fibroblast cells, based on viability assay. Results are expressed as mean ± SEM, n = 6. Viability assay was done after 24 h incubation with α-lipoic acid and EC50 was determined as 947 μM. ***Significantly different from control group at $p < 0.001$.

(100 μL) was added and the absorbance was read at 450 nm.

2.7.5. Statistical analysis

Three independent experiments were carried out in duplicate. Data are expressed as mean ± SEM. One-way ANOVA and Tukey's multi-comparison tests were carried out by Stats-Direct 3.0.158. The p-value of < 0.05 was considered significant.

3. Results

3.1. First step: safety investigation and determination of EC50

3.1.1. EC50 determination of α-LA

In the first step of the study, EC50 of α-LA was assessed using MTT test and then analyzed by StatsDirect software. As shown in Fig. 3, all concentrations are safe and have no toxic effect. Also, α-LA in 1000 μM concentration significantly improved the viability of cells ($p < 0.001$). After evaluating the data, the EC50 of α-LA was calculated as 947 μM.

3.2. Second step: senescence investigation

3.2.1. β-Galactosidase activity

The quantitative results of β-galactosidase activity have been measured in nuclear by ELISA kit and are displayed in Fig. 4 (A, B, C, and D). Moreover, this activity has also been assessed by staining (qualification method), and the result is reported in Fig. 4 (E). The REF cells treated with H_2O_2 had 2.61-fold higher concentration of β-galactosidase than the control group ($p < 0.001$). On the other hand, REF cells treated with α-LA and H_2O_2 + α-LA showed 1.014 fold and 1.557 fold lower amount of β-galactosidase content, which was significantly different, compared to the H_2O_2 group ($p < 0.001$ and $p < 0.05$ respectively). This assay proved that α-LA as the anti-aging potential was able to suppress the β-galactosidase enzyme activity in REF cells.

3.2.2. Cell cycle analysis

As shown in (Fig. 5), the cell cycle distribution of α-LA and H_2O_2 alone and in combination on REF cells were defined in G0/G1, S, and G2/M phases. In the control group, 63% of REF cells were in G0/G1, 3.35 % in S phase and 13.8 in the G2/M phase. Compared with the control group, H_2O_2 induced cell apoptosis in sub G0/G1 ($P < 0.001$) and cell arrest in G0/G1 ($P < 0.001$), while the amount of G2/M cells was reduced ($p < 0.01$). In other words, the G1 population increased from 63% in the control to 80% in the H_2O_2 group. In comparison to cells treated with H_2O_2, a significant increment in G2/M arrest was observed in both α-LA group (15.7%, $p < 0.01$) and H_2O_2 + α-LA (13.7%, $P < 0.01$).

3.3. Third step: oxidative stress biomarkers determination

3.3.1. Oxidative stress as TBARS

Data on the effect of H_2O_2 and α-LA is shown in Table 1. As indicated, exposure of REF cells to H_2O_2 caused about 73% increment of lipid peroxidation level as compared to the control ($p < 0.001$). Moreover, there was a significant difference of 33% in the rate of lipid peroxidation in REF cells exposed to both α-LA and H_2O_2 compared to the H_2O_2 group ($p < 0.001$).

3.3.2. ROS level

In comparison to the control, REF cells exposed to H_2O_2 showed a higher amount of ROS ($p < 0.001$). While the amount of ROS produced in cells treated with α-LA was significantly lower compared to the H_2O_2 group ($p < 0.001$). As shown in Table 1, exposing REF cells to both α-LA and H_2O_2 resulted in a lower quantity of ROS compared to the H_2O_2 ($p < 0.001$).

3.3.3. Antioxidant power as FRAP

The effect of α-LA on FRAP is presented in Table 1. H_2O_2 exposure has significantly decreased the antioxidant potential in REF cells ($p < 0.01$). However, adding both α-LA and H_2O_2 demonstrated higher antioxidant potential ($p < 0.001$) in comparison to REF cells only treated with H_2O_2.

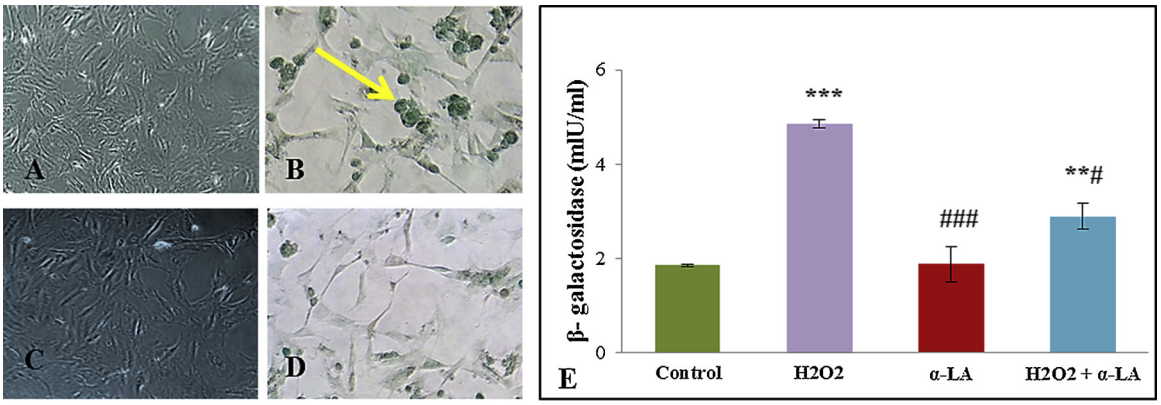

Fig. 4. β-galactosidase assay in rat embryonic fibroblast cells (200x magnification). Results are expressed as mean ± SEM, n = 6. Arrow indicates senescent cell. Control group received only DMEM (A), H_2O_2 group received 400 μM H_2O_2 in DMEM for 2 h (B), α-LA group received EC50 of α-LA in DMEM for 24 h (C), and H_2O_2 + α-LA group received EC50 of α-LA acid for 24 h followed by 400 μM H_2O_2 for 2 h (D).
***and **Significant differences from control group at $p < 0.001$ and $p < 0.01$, respectively.
and # Significant differences from H_2O_2 group at $p < 0.001$ and $p < 0.05$, respectively.

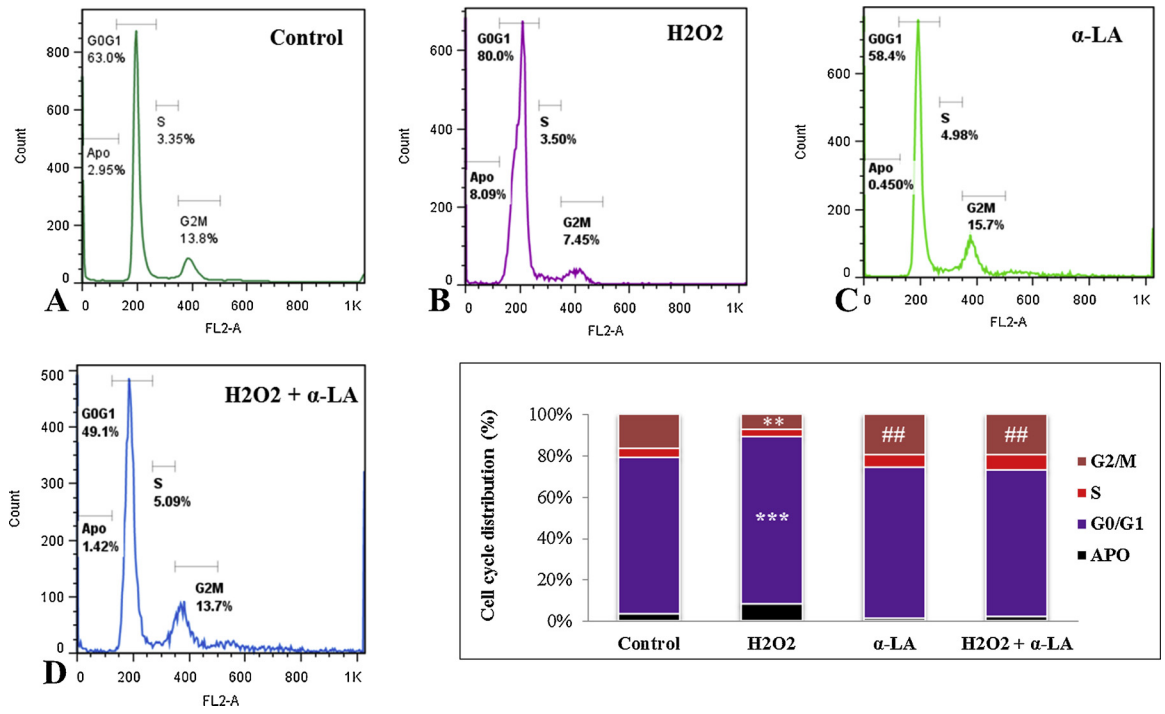

Fig. 5. The effect of α-lipoic acid on cell cycle distribution phases in control, H_2O_2, α-LA, H_2O_2 + α-LA groups.
Results are expressed as mean ± SEM, n = 6. Control group received only DMEM (**A**), H_2O_2 group received 400 μM H_2O_2 in DMEM for 2 h (**B**), α-LA group received EC50 of α-LA in DMEM for 24 h **(C)**, and H_2O_2 + α-LA group received EC50 of α-LA acid for 24 h followed by 400 μM H_2O_2 for 2 h (**D**).
*** and **Significant differences from control group at $p < 0.001$ and $p < 0.01$, respectively.
##Significant difference from H_2O_2 group at $p < 0.01$.

3.3.4. TTM level

The effect of α-LA on TTM is shown in Table 1. H_2O_2 exposure has significantly decreased the amount of TTM in REF cells in comparison to the control group ($p < 0.001$). It has to be mentioned that, simultaneous exposure of REF cells to α-LA and H_2O_2, demonstrated higher levels of TTM ($p < 0.01$) in comparison to REF cells only receiving H_2O_2.

3.4. Fourth step: apoptosis and necrosis investigation

3.4.1. Apoptosis and necrosis evaluation by flow cytometry

As demonstrated in (Fig. 6), the cell viability in the control group (96.93%) is the highest among all groups. The rate of late apoptotic cells in H_2O_2 group showed a significant increase compared to the control group (36.03%, $p < 0.001$). Moreover, population of live cells significantly increased in α-LA (89.82%, $p < 0.01$) and H_2O_2 + α-LA (84.4%, $p < 0.01$) groups, respectively compared to the H_2O_2 group (47.37%). This assay showed that cells treated with α-LA and H_2O_2 reduced the rate of late apoptosis and necrosis from 36% and 15.7% in the H_2O_2 group to 5.4% and 1.3%, respectively.

3.4.2. Caspase-3 and -9 activities

As shown in (Fig. 7), treating with H_2O_2 caused a noticeable rise in the activities of both caspase 3 and caspase 9 in REF cells ($p < 0.001$), as compared to the control. On the other hand, there was noted a significant difference in the level of both caspase 3 ($p < 0.001$) and caspase 9 ($p < 0.01$) in REF cells treated with both α-LA and H_2O_2, when compared to REF cells only receiving H_2O_2. This result indicates that α-LA possess a positive impact on caspase 3 and 9 and subsequent pathways proceeding to apoptosis.

3.5. Fifth Step: inflammatory cytokines response

The effects of α-LA on TNF-α, IL-1β and Il-6 and NF- κB, are presented in (Fig. 8I, 8II, 8III, 8IV). H_2O_2 exposure has significantly raised the quantity of IL-1β, IL-6, and TNF-α ($p < 0.001$) and NF- κB ($P < 0.01$) in REF cells compared to the control group. While treating cells with α-LA alone caused a significant reduction in the level of TNF-

Table 1
The effect of α-lipoic acid on oxidative stress biomarkers.

Parameter/unit	A Control (Mean ± SEM)	B H2O2 (Mean ± SEM)	C α-LA (Mean ± SEM)	D H2O2 + α-LA (Mean ± SEM)
LPO (μM)	105.57 ± 3.58	182.98 ± 6.65***	103.44 ± 3.7###	140.51 ± 1.58***###
ROS (u/mg protein)	1.85 ± 0.061	4.86 ± 0.24***	1.88 ± 0.92###	2.893 ± 0.66**###
FRAP (mM)	158.79 ± 5.85	96.79 ± 2.68**	146.57 ± 3.2###	118.79 ± 4.82**###
TTM (μmol/mg protein)	66.59 ± 4.54	32.17 ± 2.77***	70.4 ± 4.97##	56.78 ± 1.55*##

Results are expressed as mean ± SEM, n = 6. LPO (Lipid Peroxidation), ROS (Reactive Oxygen Species), FRAP (Ferric Reducing Antioxidant Power), TTM (Total Thiol Molecules). Control group received only DMEM (**A**), H_2O_2 group received 400 μM H_2O_2 in DMEM for 2 h (**B**), α-LA group received EC50 of α-LA in DMEM for 24 h **(C)**, and H_2O_2 + α-LA group received EC50 of α-LA acid for 24 h followed by 400 μM H_2O_2 for 2 h (**D**).
***, ** and *Significant differences from control group at $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively.
and ##Significant differences from H2O2 group at $p < 0.001$ and $p < 0.01$, respectively.

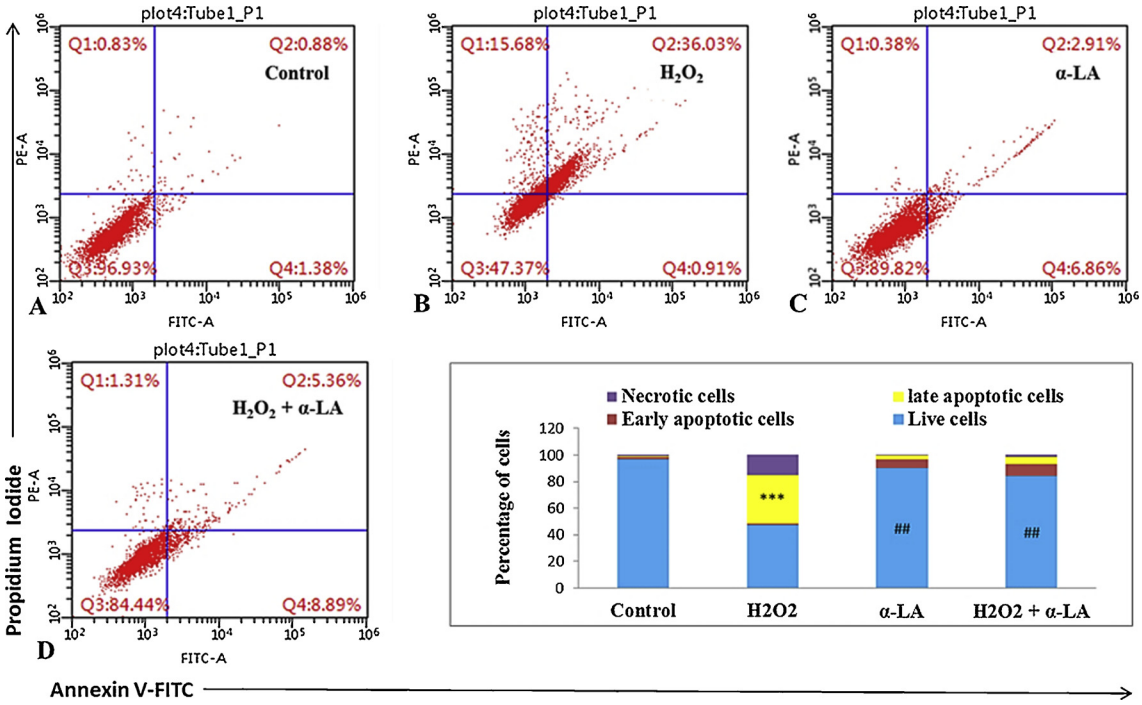

Fig. 6. Flow cytometry analysis of H_2O_2-induced and α-lipoic acid on rat embryonic fibroblast cells. Results are expressed as mean ± SEM, n = 6. The x-axis and y-axis demonstrate Annexin-V-FITC and PI fluorescence, respectively. In each plot, Q1 represents changes in percent of necrotic cells (FITC - and PI +), Q2 indicates changes in percent of late apoptotic cells (FITC + and PI +), Q3 displays changes in percent of live cells (FITC - and PI -) and Q4 shows changes in percent of early apoptotic cells (FITC + and PI -).

Control group received only DMEM (**A**), H_2O_2 group received 400 μM H_2O_2 in DMEM for 2 h (**B**), α-LA group received EC50 of α-LA in DMEM for 24 h (**C**), and H_2O_2 + α-LA group received EC50 of α-LA acid for 24 h followed by 400 μM H_2O_2 for 2 h (**D**).

***Significant difference from control group at $p < 0.001$.

$^{\#\#}$Significant difference from H_2O_2 group at $p < 0.01$.

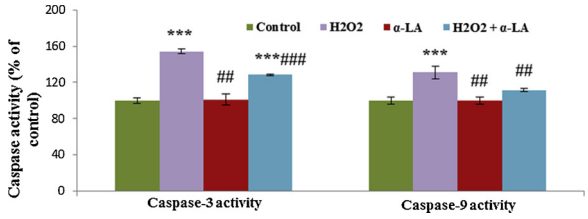

Fig. 7. The effect of α-lipoic acid on caspase-3 and caspase-9 in rat embryonic fibroblast cells.

Results are expressed as mean ± SEM, n = 6. Control group received only DMEM (**A**), H_2O_2 group received 400 μM H_2O_2 in DMEM for 2 h (**B**), α-LA group received EC50 of α-LA in DMEM for 24 h (**C**), and H_2O_2 + α-LA group received EC50 of α-LA acid for 24 h followed by 400 μM H_2O_2 for 2 h (**D**).

***Significant difference from control group at $p < 0.001$.

$^{\#\#\#}$ and $^{\#\#}$ Significant differences from H_2O_2 group at $p < 0.001$ and $p < 0.01$, respectively.

α and NF- κB ($p < 0.001$) compared to the H_2O_2 group. The concurrent treating of REF cells to α-LA and H_2O_2, resulted in decreasing the amount of all tested cytokines as TNF-α, IL-1β ($p < 0.01$), Il-6 ($P < 0.05$) and NF- κB ($P < 0.01$) compared to the H_2O_2 group.

4. Discussion

This is the first study evaluating the protective effects of α-LA on the aging process in (REF) cells along with inflammatory and apoptotic markers. Forasmuch as even a little change in the function of normal embryonic cells causes age-related diseases, therefore, in addition to measuring qualitative and quantitative β-galactosidase level, oxidative stress markers, which are important in the function of the cells, have been investigated.

Senescent associated β-galactosidase activity is present in H_2O_2 treated cells, permitting the identification of senescent cell in cell culture and mammalian tissues [38,39]. In this study, qualitative and quantitative β-galactosidase measuring showed that in REF cells treated with H_2O_2, the concentration of β-galactosidase was significantly increased. While, those treated with α-LA in combination with H_2O_2, had less amount of β-galactosidase content comparing H_2O_2 treated cells ($P < 0.05$). The lessening of β-galactosidase activity indicates that α-LA possesses the anti-aging potential. Previously, high senescence-associated β-galactosidase activity has been seen in H_2O_2 treated human umbilical vascular endothelial cells and subsequent use of α-LA has attenuated H_2O_2 induced senescence [39]. Moreover, in another report, α-LA has been found to reduce the activity of β-galactosidase and abrogate senescence in human fibroblast [40].

Caspase 3 and caspase 9 are a group of proteases regulating inflammation and apoptosis in mammalian cells. α-LA has been documented to have beneficial effects on reducing the rate of apoptosis [41,42]. In the present study, the use of α-LA resulted in a significant decline in the activities of both caspase 3 ($P < 0.001$) and caspase 9 ($P < 0.01$) in REF cells treated with H_2O_2. This result indicates that the α-LA has a positive impact on caspase 3 and 9 and subsequent pathways proceeding to apoptosis. Previously, α-LA has been reported to significantly reduce caspase 9 activity in human lymphocytes [26], and caspase 3 and 9 activities in cardiac myocytes along with the mitochondrial cytochrome c release [43]. Moreover, in retinal endothelial cells (RECs), α-LA has been found to exert a favorable effect on apoptosis by decreasing caspase 3 activity [44].

Moreover, for investigating the antiapoptotic effects of α-LA Annexin V-FITC and PI staining was done and investigated via flow cytometry. The results indicated that in cells treated with α-LA and H_2O_2 a reduction in the rate of late apoptosis and necrosis was seen

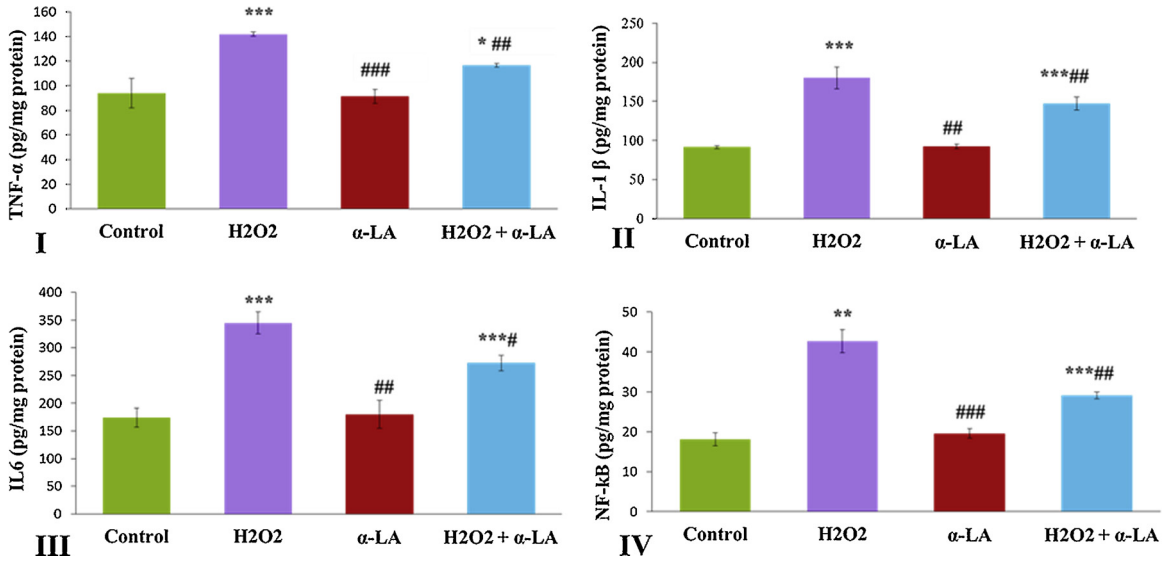

Fig. 8. The effect of α-lipoic acid on inflammatory cytokines and NF-κB. Results are expressed as mean ± SEM, n = 6. In this Fig. (**A**), (**B**), (**C**), and (**D**) show the effect of α-LA on TNF- α (I), IL-1β (II), IL-6 (III), and NF-Kb (IV) levels, respectively.
Control group received only DMEM (**A**), H_2O_2 group received 400 μM H_2O_2 in DMEM for 2 h (**B**), α-LA group received EC50 of α-LA in DMEM for 24 h (**C**), and H_2O_2 + α-LA group received EC50 of α-LA acid for 24 h followed by 400 μM H_2O_2 for 2 h (**D**).
***, ** and *Significant differences from control group at $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively.
###, ## and # Significant differences from H_2O_2 group at $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively.

(from 36% and 15.7% in H_2O_2 group to 5.4% and 1.3%, respectively). In a study using human lymphocytes exposed to H_2O_2, α-LA (1, 10, 100, and 1000 μM for 24 h) has been reported to cause an increase in the cell viability and to inhibit the overexpression of genes in apoptosis pathways. In this study, it was also demonstrated that increasing caspase enzyme activity and reducing mitochondrial activity are the key mechanisms to establish the pathway of apoptosis [26]. Caspase enzyme activity is described by assessing caspase-3 and -9 activities. Flow cytometry outcomes showed that α-LA in a concentration of 947 μM causes a reduction in both caspase-3 and -9 activities.

Oxidative impairment is an ultimate outcome in cells as a result of exposure to free radicals and followed by the imbalance between ROS and biological defenses in cells. These ROS have been well documented to exert a negative effect on many diseases [45]. In this study, after exposing cells to H_2O_2, TTM and antioxidant potential of REF cells were significantly reduced ($P < 0.001$ and $P < 0.01$) respectively while an increment in lipid peroxidation was seen ($P < 0.001$). However, exposing REF cells with α-LA significantly reduced oxidative stress markers including ROS and LPO, and improved TTM and antioxidant power.

The anti-oxidant activity of α-LA is not specific to H2O2-induced oxidative stress, this protection is also possible against other stressful and harmful conditions. From literature reviews, we found that α-LA supplementation could specially protect lipids and DNA against exercise-induced oxidative stress [46]. In addition, other studies on rat kidney show that oxidative stress-mediated apoptosis and DNA damage, which is induced by lead intoxication, can be reduced using α-LA and it could be applicable as a cytoprotective against oxidative stress of tissue damage caused by heavy metals [47]. The previous study showed that dietary antioxidants have positive or negative roles in endogenous antioxidant systems. It was described that lipoic acid as a supplement diminished oxidative stress profile, lipid peroxidation and malondialdehyde production and activation of glutathione peroxidase but in contrast, glutathione levels increased and affected the quality of life in murine models and reduced lifetime of SAMP8 mice as the murine model [48,49].

In fact, inflammation is a protective response of the cell to assaults, and by the inflammatory process, the cell gets rid of causative agents. However, the cellular inflammatory response to any toxic assault is coupled by secretion of various cytokines. Moreover, many age-related chronic diseases are characterized by high levels of inflammatory cytokines [50,51]. Results of present study performed that α-LA alone has no effect on the level of inflammatory cytokines including TNF-α, IL-1β, and IL-6. But interestingly, the concentration of these inflammatory cytokines which were enhanced significantly in REF cells treated with H_2O_2, were reduced in cells treated with H_2O_2 and α-LA together. Previously, the anti-inflammatory effect of α-LA has been studied in the incidence of Alzheimer's disease [52], cardiovascular diseases [53], osteoclastic bone loss [54], and type 2 diabetes [55,56]. These results clearly indicate that α-LA not only reduces oxidative stress, but also decreases cell inflammation, and effectively neutralizes ROS action. Transcription factor NF-κB regulates the production of many cytokines. Also, NF-κB has been reported to be upregulated in certain age-related chronic diseases [57]. In the current study, the level of NF-κB was high in REF cells as a result of exposure to H_2O_2. On the other hand, α-LA treated REF cells showed a significantly lower concentration of NF-κB ($P < 0.01$). Also, further studies have shown that α-LA has been described to modulate or suppress NF-κB activity in human monocytic and endothelial cells [57,58].

The members of NF-κB pathway are targets of several post-translation modifications (PTM) affecting its activation and transcriptional efficiency [59,60]. Activation of the NF-κB pathway mainly involves phosphorylation and ubiquitination in stress conditions and inflammatory responses. Moreover, increased protein acetylation can initiate cellular senescence while molecules involving cell survival such as sirtuin particularly SIRT1 and 6 repress NF-κB signaling via p65 deacetylation demonstrating the involvement of a huge number of defense factors and a mechanism involving entropic aging. Aforementioned stated facts proving that biochemical mechanisms of chronic inflammations are responsible for entropic aging and age-related diseases. Inflammation is a product of host defense response against pathogens and tissue injury. There established a recent opinion regarding linking of aging process during the evolution of host defense. These facts strengthen the potential of NF-κB signaling components as targets for future research and the development of effective therapies against aging or related diseases [59–61]. It has been shown that α-LA plays

role in inhibition of SIRT1, which means that lipid-lowering effect of α-LA depends on SIRT1 activity. Another possible mechanism for α-LA is that it activates SIRT1/LKB1/AMPK pathway, which leads to regulating SREBP-1, FoxO1, and Nrf2, and their downstream lipogenic targets [62].

Measurement and control of cellular senescence in G1 are major checkpoints in aging research [63,64]. Studies on H_2O_2 stimulated cellular senescence have shown that the role of the P53 transcription factor, P21, P16, and Rb proteins are crucial [64]. These factors damage DNA and shorten the telomere [64,65]. This process is critical in distribution and arresting of the cell cycle in the G1 phase.

Performed studies on H_2O_2-induced cellular senescence have shown that H_2O_2 caused cell cycle arrest in G1 phase [64,66,67]. In this study, the G1 population increased from 63% in control to 80% in H_2O_2 group. On the other hand, treatment of the cells with α-LA-induced cell cycle arrest in G2/M and S phases in comparison to the groups treated only with H_2O_2. All these investigations point toward the potential benefit of α-LA in constraining the activity of redox-sensitive transcription factors seen in the aging process or even in exposure to environmental toxicants [68].

It has to be mentioned that ROS is not always harmful to cells. ROS can cause beneficial effects on cells in many conditions. For example, it was shown that low calorie intake significantly slows the rate of ageing in mammals and lowers the onset of numerous age-related diseases. Another example of beneficial effects is increasing the lifespan of unicellular organisms following dietary restriction [69,70]. Another effect of low levels of ROS is that they play a vital role in synthesizing some cellular structures and defending against specific pathogens. This mechanism includes modifications in redox status, insulin sensitivity, and inflammation neuroendocrine function. On the other hand, as we all know, when ROS production exceeds a limitation, harmful effects start [48]. In summary, we can say that ROS in low or moderate concentrations can be beneficial to cells while in high concentrations can be harmful.

5. Conclusion

There is solid evidence on the association between the application of antioxidant and aging process in cells. While studies indicate that generation of the free radicals is one of the most important mechanisms underlying the aging process. This study has discussed the role of antioxidant and senescence on the in-vitro model. The most obvious finding is that free radicals trigger aging process and the results of this research confirm this theory. Focused on cell survival, oxidative stress indicators, inflammatory cytokines, cell cycle, apoptosis and necrosis during this investigation confirmed the positive effects of α-LA on regulation and reduction of cellular aging parameters. Such compound decreases the effects of aging on REF cells by suppressing oxidative stress, lowering inflammatory parameters and altering cell division. Additionally, α-LA has the capacity to be evaluated further, both in clinical and experimental studies regarding aging toxicity. The finding data of this research highlight the importance of α-LA treatment and its protective role on aged REF cells. This study was several limitations such as nature of in-vitro studies, which may not affect in-vivo models, needs more investigation on endogenous enzymes and biochemical pathways to find the exact role of α-LA activity on aging and cellular senescence. Altogether, cellular aging will be developed when some biochemical process arrests reversible growth. Aggregation of aging mediators affected aging and related disease. Previous investigations showed that aging is a double-edged sword such as tissue remodeling, inhibition of cellular malignancy, tissue fibrosis and wound healing.

Conflict of interest

The authors declare that there is no conflicting interest with respect to the contents of this research article.

Authors' contribution

MB gave the idea, did the study and contributed to draft the manuscript; HB participated in drafting and editing the manuscript. MR, MN, MAR, MG helped in performing the experiment and analysis the data. MA supervised the whole study. All authors read and approved the final version of this study.

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