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The influence of alpha-lipoic acid on ovarian follicle growth in induced aging mice

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Abstract

The ovary contains follicles at various developmental stages. The present study aimed to investigate the antioxidant efficiency of alpha-lipoic acid (α-LA) on follicle growth in induced aging ovaries. Juvenile female mice (n=24) were allocated into four groups (n=6, each); the control group received distilled water, and the induced aging group (T1) received D-galactose (300 mg/kg). The co-administrated group (T2) was treated with α-LA (300 mg/kg) and D-galactose (100 mg/kg), while the fourth group (T3) was treated with α-LA (100 mg/kg). At the end of treatments (8 weeks), animals were sacrificed, and ovaries were processed for hematoxylin and eosin staining and immunostaining for proliferating cell nuclear antigen (PCNA). PCNA was detectable in oocytes but only in granulosa cells of activated follicles. The D-galactose treatment successfully induced ovarian aging as the proportion of primordial and growing follicles was significantly reduced accompanied by massively increased atretic follicles. Additionally, only numerous PCNA positively stained follicles were recognized. The co-administrated α-LA moderately rescued the characteristics of ovarian aging. Mice treated with α-LA demonstrated a substantial increase in the population of atretic follicles, antral follicles, and PCNA positively stained follicles. There was no change in oocyte size at any follicle growth stage among groups. In conclusion, α-LA moderately rescued the detrimental impacts of induced ovarian aging. For the first time, the expression of PCNA was linked with ovarian aging, where PCNA-staining has been recognized as a valuable tool to evaluate the proliferative activity of the granulosa cells.

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Introduction

The mammalian ovary is a multifunctional organ comprising follicles at various developmental stages. The initial stage is the primordial follicle, a relatively small dormant structure representing the total available population of follicles throughout the female reproductive life (1). Progressively, a constant process of follicular activation and atresia causes a decline in the proportion of the ovarian reserve (2). Consistently, assisted reproductive technology, e. g., in vitro fertilization, is influenced by numerous factors, among which generation of reactive oxygen species (ROS). ROS are a class of highly reactive oxygen-containing compounds (e. g. hydrogen peroxide) generated during various cellular activities (3). Additionally, in vitro cultivation of oocytes promotes the vulnerability to oxidative stress due to prolonged exposure to light and oxygen. Oxidative stress and the consequent increased ROS are among the principal causes of infertility or poorly developing embryos (4,5). For instance, in the ovary, the upregulation of ROS in granulosa cells negatively impacts oocyte quality, fertilization, and embryogenesis (6).

However, another study found that after the pre-ovulatory gonadotropin surge, there is a transitory increase in ROS levels accompanied by a decrease in antioxidant transcription, implying that the increase in ROS is a required trigger for ovulation (7). Several in vitro studies have indicated the crucial impact of alpha-lipoic acid (α-LA) supplementation on the development and maturation of cultured preantral follicles isolated from cows (5,8), mares (9), ewes (10), rats (11), and mice ovaries (12). As an antioxidant, alpha-lipoic acid ($C_8H_{14}O_2S_2$) is an organosulfur compound derived from octanoic acid, functions either directly by eliminating ROS or indirectly by reprocessing other intracellular antioxidants, such as vitamins C and E (13,14). The advanced stages of follicle growth tend to be more vulnerable to granulosa cell apoptosis mediated by oxidative stress; however, oxidative stress and antioxidant impact on the primordial and primary follicle growth remains controversial (15). Quantification and classification of the ovarian follicles is the current approach to evaluate the size of the ovarian reserve, which might accompany by immunohistochemical staining of specific proteins; For instance, the proliferating cell nuclear antigen, PCNA (16,17). A recent investigation stated that mice fed on an α-LA enriched diet increased the rate of activated primordial follicles by competing with ovarian oxidative stress (18). In ovine, recent work has indicated that in vitro supplementation of antioxidants produces a crucial implication on sperm motility, oocyst penetration rate, and enhanced embryo production (19). However, numerous investigations have suggested that an increased level of antioxidants, e.g., α-LA, can promote apoptosis, which results in programmed cell death (12,20). The induced ovarian aging by D-galactose has been widely used as a valuable tool to study follicular development in terms of oxidative stress (21,22), age, body condition, and reproductive phase (23).

Thus, we hypothesized that supplementation of α-LA might protect the ovaries against the induced aging changes mediated by D-galactose. The present study was designed to examine the implication of α-LA treatment on ovarian follicle development in induced ovarian aging. In addition, to determine the association of treatments with ovarian cell proliferative activity.

Materials and methods

Ethics approval

The experimental methodology and animal welfare were approved by the Institutional Animal Care and Use Committee (Ref; 2021.21), College of Veterinary Medicine, University of Mosul.

Experimental design and groups

Female mice (n=24) aged 21 days were used in this study. According to the Institutional Animal Care and Use Committee, mice were maintained and fed under the same conditions in the Animal House Unit at the College of Veterinary Medicine/ University of Mosul. The standard laboratory conditions included temperature (22±4ºC), humidity (55%), and a 12 h light /dark cycle. In addition to the untreated control group (n=6) that received normal saline (orally and subcutaneously), animals were randomly allocated into other three equal treated groups (n=6 animals each). In the first group (T1), to induce aging, mice were treated with subcutaneous injections of D-galactose (Santa Cruz, sc-202564) at the dose of 300 mg/kg BW. The second group (T2) received both D-galactose and alpha-lipoic acid (300 mg/kg (s.c) and 100 mg/kg (p.o.) BW, respectively). Mice in the T3 group were treated with alpha-lipoic acid (Santa Cruz, sc-202032) at 100 mg/kg (p.o) BW. At the end of the treatment period (8 weeks), all animals were euthanized with inhaled ether before being sacrificed by cervical dislocation and exsanguination.

Ovary dissection and processing

Ovaries (n=12, each group) were dissected and cleaned free from the adhering tissues. The freshly cleaned ovaries were immediately immersed in neutral buffered formalin 10% until being embedded in paraffin. Then, nonsequential midsections from each ovary were processed at 5µm using a manual microtome (24).

Hematoxylin and eosin staining

Ovary sections were stained with Gills hematoxylin II and 1% aqueous eosin (H&E) following a standard protocol (24). Sections were imaged with a digital camera (OMAX, A35180U3, China) fitted to an optical microscope (Kruuse, Primophot 290205, Denmark).

Follicle classification and counting

The hematoxylin and eosin-stained sections were utilized to determine the ovarian histology, estimation of oocyte size by ImageJ software (Fiji 1.46, 2012), and follicle classification. Follicles were classified into four categories depending on the oocyte diameter and follicle morphology. The non-growing primordial follicles where an oocyte (<17.1µm) is bounded with a single layer of flattened pre-granulosa cells. The activated, growing follicles where an oocyte (17.2- 29.6µm) is enclosed either with a monolayer of both the flattened and cuboidal granulosa cells or at least one layer of cuboidal granulosa cells. The multilayered follicles (29.7-38.9µm) with 1-2 small antrums are termed preantral. In comparison, follicles with a single large antrum are termed antral. Additionally, follicles with degenerative changes were counted and termed atretic (25,26).

Immunohistochemistry

Following a standardized procedure (26), ovary sections were stained with Rabbit PCNA antibody (Elabscience Biotech. Inc. USA catalog no. E-AB-70004) at a dilution rate

of 1:200. An immunostaining detection kit (Rabbit-Dap (Poly-HRP), catalog no. RDEIHC0007) and secondary antibodies (Goat anti-rabbit) were purchased from AL-Shkairate Estab. for Med. Supp., Jordan. For negative control, sections were incubated with equivalent concentrations of non-immune rabbit IgG. Slides were incubated in diaminobenzidine substrate (DAB) until a brown color appeared. Ovary sections were counterstained with hematoxylin, dehydrated, and cleared with xylene before being mounted for evaluation.

Statistical analysis

ImageJ software measured the oocyte diameter, and data were presented as mean (µm)±SEM. Differences in the mean oocyte diameter were statistically analyzed using one-way ANOVA with the Kruskal-Wallis test. Significant variations in follicle proportions among groups were determined using the Chi-square test. All analyses were performed using Sigma Plot 12.5 software, where differences between treated and untreated groups were considered significant if $P<0.05$.

Results

Ovarian morphology, follicle quantity, and classification

Hematoxylin and eosin-stained ovaries in the control group demonstrated the physiological sequential follicular growth stages. However, the induced aging group (T1) ovaries revealed an increased number of atretic follicles and corpora lutea; therefore, the T1 group demonstrated larger-sized ovaries. The ovarian feature described in the T1 group was modulated by the supplementation of α-LA (T2), where more follicular growing stages were observed, accompanied by a massive reduction in the population of atretic follicles and corpora lutea. Interestingly, ovaries in the T3 group demonstrated large-sized ovaries, riches with advanced stages of follicle development, mainly located in the peripheral ovarian region. Nevertheless, the central ovarian region in this group demonstrated both increased vascularization and a proportion of atretic follicles (Figure 1).

To test the possible impact of treatments on the proportion of various follicular growth stages, follicles were classified into non-growing (primordial), growing (primary and secondary), preantral, and antral stages. In addition, the proportion of atretic follicles was counted. By comparison with the control, statistics demonstrated a significant decline ($P<0.05$) in the proportion of the non-growing primordial follicles in both the induced aging (T1) and α-LA (T3) groups but not in the T2 group. Interestingly, the proportion of growing follicles was significantly decreased ($P<0.05$) in the T1 mice ovaries relative to the control group. Induced aging mice revealed the lowest proportion of preantral follicles; however, the decreased proportion was not enough to express a statistical difference ($P= 0.08$) against the control mice (14.6% and 17.3%, respectively). Treatment with α-LA (T3) revealed the highest proportion of antral follicles, where a significant difference was detected ($P<0.05$) relative to the control. Remarkably, by comparison with control, the induced aging and α-LA treated groups revealed a substantial increase ($P<0.0001$ and $P<0.05$, respectively) in the proportion of atretic follicles but not in the T2 ovaries (Table 1).

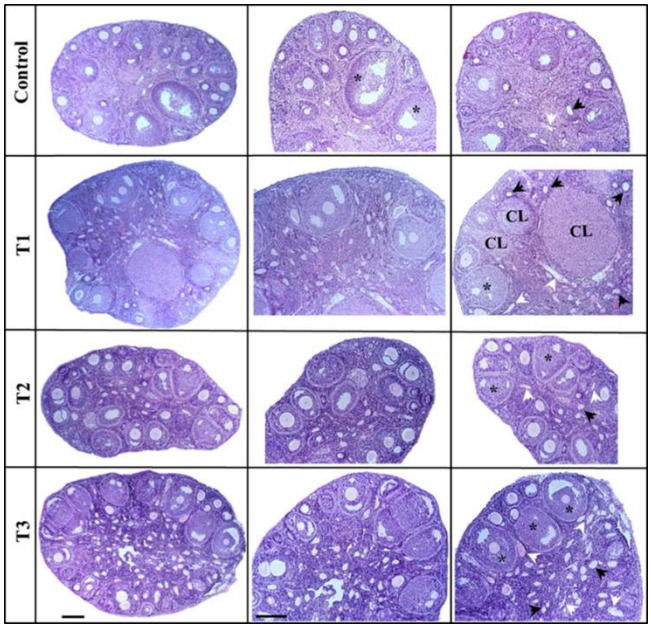

Figure 1: The impact of treatments on ovarian histomorphology. By comparison with the control, ovaries in the treated groups demonstrated substantial changes in the ovarian structure manifested by the presence of many corpora lutea (CL, T1) and atretic follicles (black arrows), particularly in T1 and T3. Many growing and advanced follicular growth stages(starred) were revealed in T2 and T3. The T2 group established regular changes in ovarian structure. White arrows demonstrate blood vessels. Sections were stained with H&E stain, scale bar:100µm.

The mean oocyte diameter in treated groups was estimated and statistically compared against the untreated control to determine the impact of treatments on the oocyte size. Data demonstrated that induced aging or supplementation of α-LA does not cause significant changes in the mean oocyte size at any stage of follicle growth (Figure 2).

Immunohistochemistry

Immunohistochemical labeling of PCNA protein was utilized to examine the effects of the treatments on ovarian cellular proliferation. PCNA was localized with a high intensity of staining in the nuclei of the oocytes (at all growth stages) and granulosa cells of the growing follicles, except for the pre-granulosa cells of the quiescent primordial follicles. Higher immunoreactivity to PCNA was determined

in multilayered growing follicles than in the earlier growth stages. However, low intensity of staining was recognized in the nuclei of the stromal and theca cells. Interestingly, PCNA was not detected in the atretic follicles, though only numerous granulosa cells were detected positive in follicles that initiated the process of atresia (Figure 3).

Table 1. Numbers and proportions of the counted and classified follicles

	Non-growing		Growing		Preantral		Antral		Atretic	
	No.	%	No.	%	No.	%	No.	%	No.	%
Control	395	34.7	222	19.5	197	17.3	107	9.4	217	19.1
T1	334	*30.8	171	*15.8	159	14.6	93	8.6	328	**30.2
T2	383	31.5	214	17.6	215	17.7	139	11.5	263	21.7
T3	424	*29.9	241	17.0	246	17.4	178	*12.6	327	*23.1

Follicles were classified according to the estimated oocyte diameter, granulosa cell layers, antral formation, and the presence of degenerative changes. Starred data in each column indicate a significant difference relative to the control group. Statistical variation between the control and treated groups was analyzed using Chi-square (*P<0.05, **P < 0.0001).

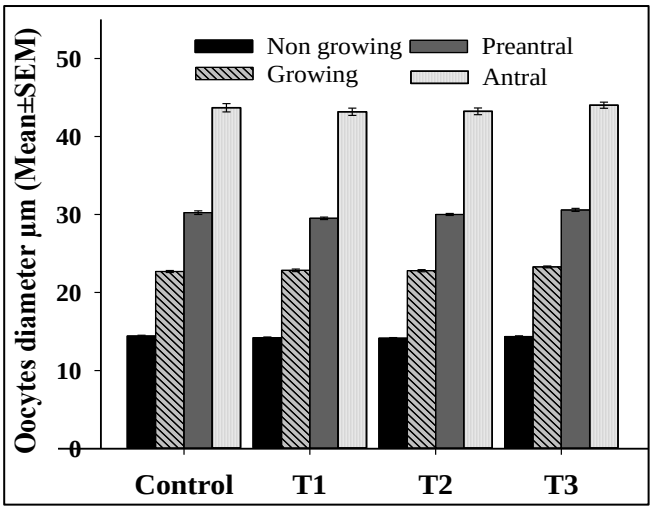

Figure 2: Effect of treatments on oocyte size. Statistical analysis using one-way ANOVA with the Kruskal-Wallis test showed no significant difference in the mean oocyte diameter at any growth stage relative to the control. Data are presented as mean oocyte diameter (µm) ±SEM.

Low-power microscopy demonstrated the localization pattern of PCNA in the whole ovaries. Compared with the control, the characteristics of ovarian aging were noticeably produced with the D-galactose treatment (T1), where only numerous large follicles were stained with PCNA, while many follicles appeared unstained. In addition, the ovary sections revealed a massive follicular depletion. Interestingly, the ovarian section in the co-supplementation group (T2) has restored the follicular activity with various growing stages, where a more significant number of follicles were detected positive. Supplementation of α-LA (T3) increased the number of the positively PCNA-stained antral follicles relative to control and other treated groups. The intensity and PCNA-staining pattern in the positively detected follicles were consistent among groups (Figure 4).

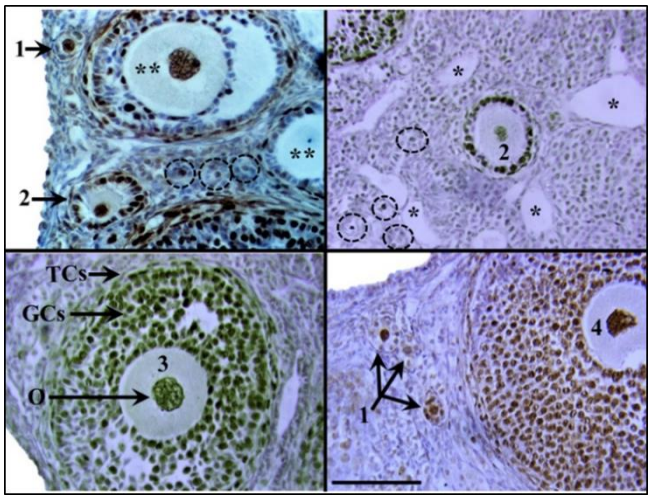

Figure 3: The localization pattern of PCNA in the ovary. PCNA was positively detected in primordial (circled) and transitional follicles (1) oocytes but not in the surrounded flattened pre-granulosa cells. However, primary (2), preantral (3), and antral follicles specifically expressed PCNA with a high intensity of staining in the oocytes (o), granulosa cells (GCs), and weakly in theca cells (TCs). PCNA was detectable in several endothelial cells of the enlarged blood vessels (starred). Only numerous granulosa cells were detected positive in follicles undergoing atresia (double starred). All images were obtained from the α-LA treated group. Scale bar: 50µm.

High-power imaging revealed that PCNA is not detectable in the atretic follicles. It is somewhat surprising that treatment with α-LA (T3) revealed the presence of numerous follicles that specified a premature antrum formation, where its relative oocytes are only surrounded by 3-4 layers of granulosa cells. In contrast, some lutein cells of the corpora lutea expressed PCNA protein, but only with a weak intensity of staining (Figure 5).

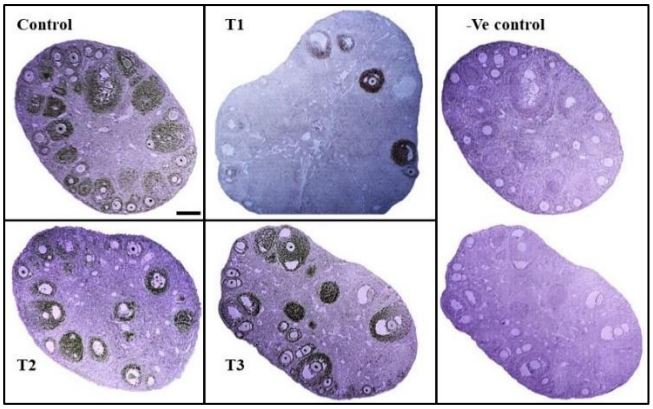

Figure 4: Impact of the treatments on the localization of PCNA in the ovary. Compared with other groups, the induced aging (T1) caused a massive reduction in the positively stained follicles, which was moderately restored in the T2 group. The highest number of antral follicles detected positive was in the T3 group. For the negative control, sections were incubated with equivalent concentrations of a non-immune rabbit IgG, scale bar: 200µm.

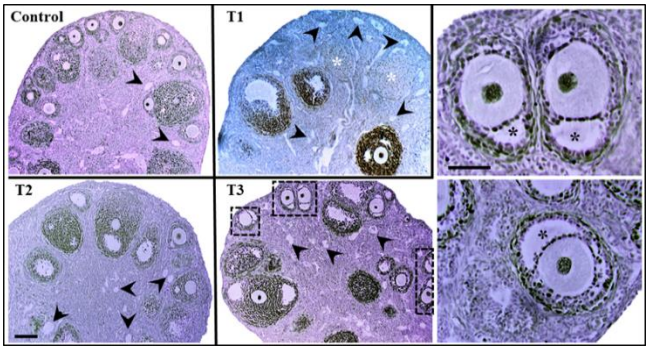

Figure 5: High-power imaging of the PCNA-stained ovaries. Ovary sections in all groups (Control-T3) expressed PCNA protein. Atretic follicles (black arrows) do not express PCNA. In the induced aging ovary (T1), corpora lutea cells (white stars) demonstrated weak PCNA staining. In the T3 group, several follicles (T3- boxed) demonstrated a pre-mature antral formation (black stars). The magnified right-sided panel show only several layers of granulosa cells surrounding the oocyte, where many relative granulosa cells were unstained. Scale bar Control-T3: 100µm, right-side panel:25µm.

Discussion

For decades, several pieces of literature have investigated factors involved in ovarian aging and tested various protocols that might rescue both the follicular reserve and growth. The present work tested the hypothesis that the administration of α-LA might modulate accelerated age-related ovarian changes. To date, there is no study signifying the impacts of α-LA supplementation or induced aging on the localization of a cellular proliferative marker, PCNA protein; Therefore, the second objective was to determine the possible association of the α-LA /D-galactose treatments on the expression of PCNA. It has been suggested that treatment with D-galactose can directly promote oxidative stress and subsequently induces ovarian toxicity (27-30). The present study successfully demonstrated ovarian aging as the principal age-associated ovarian change. By comparison with the control, ovary sections of the induced aging mice established a significant reduction in the proportion of both the primordial and growing follicles. This observation might be attributed to the dramatically increased rate of follicle atresia, possibly as a consequence of the elevated levels of ROS (15,22).

In mice, it has been indicated that D-galactose treatment causes destructive changes in the zona pellucida, which trigger a reduced number of healthy growing antral follicles, and increased atretic follicles (31). An additional reason might be attributed to the downregulation in the expression of growth differentiation factor-9 (GDF9), an oocyte growth factor (28). This statement was supported by another study where the expression of Anti Mullerian Hormone (AMH), a multi-layered granulosa cells marker, was significantly reduced in the induced aging mice (32) and was detected with a high level in the ovaries of α-LA treated rats (33). This outcome is consistent with previous induced aging works using D-galactose (21,27,32). In contrast to other groups, ovaries in the induced aging mice demonstrated an increased number of corpora lutea and the lowest proportion of antral follicles. This observation might be associated with the upregulated pituitary hormones (FSH and LH), where a previous work declared that levels of these hormones were significantly increased in the D-galactose-treated mice (31). However, four-week-old rats fed on a high-D-galactose diet demonstrated a reduced rate of corpora lutea relative to the control (27). The disagreement with our results might be attributed to many circumstances, e.g., the animal type, administrated dose, and duration of administration. In addition, the decreased number of PCNA-stained follicles in the induced aging mice might reflect the declining number of the growing follicle and the proliferative activity in the granulosa cells. In previous work, treatment with D-galactose increases the expression of the P16 protein in granulosa cells and oocytes, where the P16 protein is directly associated with the downregulation of cellular proliferation and inducing apoptosis (30).

In contrast to the induced aging group, the co-administration with α-LA (T2) exhibited a reduction in the quantities of atretic follicles and an increased proportion of the non-growing and antral follicles. In addition, more PCNA-stained follicles were specified. These data might indicate that α-LA has effectively overcome ovarian toxicity mediated by D-galactose treatment (8,11,12). For instance,

supplementation of α-LA protects against the ovary from the increased ROS level and the upregulated pro-apoptotic gene, TNF-α (33). In rat males, treatment with alpha-lipoic acid consistently functioned against the induced testicular toxicity manifested by enhanced sperm quality-associated parameters and increased proliferative activity of spermatogonia (34).

It has been suggested that α-LA exerts its effects in a dose-dependent manner; for example, compared with a dosage of 600 mg/kg, induced aging mice that received a daily dietary dosage of α-LA at 150 mg/kg significantly rescued higher rates of primordial, growing, and antral follicles from atresia (18). Unexpectedly, α-LA-treated mice demonstrated a reduced proportion of primordial follicles. This effect can explain that α-LA promotes the process of primordial follicle activation to initiate growth (18); in contrast to the T1 group, a higher proportion of growing follicles was estimated (15.8% and 17.0%, respectively). This statement is consistent with another study as the rate of activated primordial follicles in α-LA treated ewes was significantly higher relative to control, manifested by more FOXO3a and Ki67 immunoreactivity, cell activation, and proliferation markers (10). However, the most unanticipated effect of the α-LA treatment was demonstrated by the increased proportion of atretic follicles and premature antrum formation. There is no available information about the possible toxic impact of α-LA on the ovary. Nevertheless, as α-LA treatment caused a significant elevation in the proportion of growing follicles, the increased proportion of atretic follicles might be attributed to the process of autophagy. Autophagy is a physiological process where unnecessary or malfunctioning cellular components are eliminated. It has been reported that autophagy favors cellular degeneration and death, acts on energy regulation, and enhances cellular survival, differentiation, proliferation, and resistance against stress, including aging (35-37).

The present study suggests that α-LA might accelerate the growth of the growing follicles as the significantly highest proportion of antral follicles was estimated in the α-LA group. Exclusively, ovary sections of α-LA -treated mice demonstrated increased and enlarged blood vessels. This observation agreed with another study on ewes treated with α-LA, as more CD31, endothelial cells marker, staining was revealed (10). Even though the variation in the proportion of the classified follicular growth stages, there was no significant difference in the oocyte size relative to the control. An in vitro previous study indicated that treatment with D-galactose produces no effects on the oocyte size relative to the control (38). Proliferating cell nuclear antigen (PCNA) has been used as a biomarker of progressive follicular development in immature mice (39), rats (16,40), and adult ewes (41). In the T1 group, although oocyte count and the H&E staining demonstrated a moderate proportion of growing follicles, immunostaining revealed that only numerous large follicles expressed PCNA. This observation indicates the significance of PCNA staining in distinguishing between follicular activity among groups. In addition, the staining pattern in the T1 might be specified that unstained follicles are either inactive or undergoing atresia. Regardless of the treatment groups, the PCNA-staining pattern in the ovary sections was identical in all growing follicles. In Inconsistence with previous work (16), although PCNA was initially detected in the oocytes of primordial follicles, its expression in the relative pregranulosa cells coincided with its morphological changes from flattened into cuboidal cells. This observation might indicate the value of PCNA staining to determine the rate of follicular activation, where only proliferative granulosa cells were detected positive (23). However, another study in rats indicated that PCNA was not detectable in the oocytes of primordial follicles (40). The distinction from our observation might be attributed to the different staining protocols, specificity of the utilized PCNA antibody, and animal species. Another work confirmed our results, as PCNA was localized in oocytes of primordial follicles of prenatal and neonatal mice (39). Nevertheless, because the oocyte is meiotically arrested (1), the expression of PCNA in oocytes cannot be qualified for cell proliferation; instead, it might be associated with damaged DNA repairment (40).

Conclusions

Treatment with α-LA moderately rescued the detrimental effect of induced ovarian aging by decreasing the proportion of atretic follicles, conserving primordial follicles, and promoting follicle growth. Interestingly, for the first time, the association of PCNA expression was linked to ovarian aging and has been recognized as a valuable protocol for evaluating the proliferative activity of granulosa cells in growing follicles.

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Conflict of interest

The author declares no conflict of interest.

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تأثير حامض الالفا ليبويك على نمو الجريبات المبيضية في الفئران المستحدثة الشيخوخة

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الخلاصة

يحتوي المبيض على جريبات في مختلف مراحل النمو، تم دراسة كفاءة حامض ألالفا ليبويك كمضاد للأكسدة على نمو الجريبات في المبايض المستحدثة الشيخوخة. قسمت إناث الفئران الفتية (العدد الكلي=٢٤) إلى أربع مجاميع (٦ حيوانات لكل منها). أعطيت مجموعة السيطرة ماء الملح الفسلجي، فيما حقنت مجموعة الشيخوخة المستحدثة بسكر الكالاكتوز (٣٠٠ ملغم/ كجم). عوملت مجموعة بكل من حامض الألفا ليبويك (٣٠٠ ملغم/ كجم) والكالاكتوز (١٠٠ ملغم/ كجم)، في حين تلقت الفئران في مجموعة حامض الالفا ليبويك (١٠٠ ملغم/ كجم). بانتهاء فترة التجربة (٨ أسابيع)، قتلت الحيوانات وعولجت المقاطع المبيضية للصبغ بالهيماتوكسيلين والايوسين لدراستها نسيجيا ولغرض التلوين النسيجي المناعي باستعمال الأجسام المضادة لمستضد تكاثر الخلية النووي. كشف بروتين مستضد تكاثر الخلية النووي في البويضات والخلايا الحبيبية للجريبات النامية. كما نجح الكالاكتوز في استحداث شيخوخة المبيض حيث انخفضت نسبة الجريبات البدائية والنامية بشكل كبير مصحوبًا بزيادة كبيرة في الجريبات الرتقة. فضلا عن التعرف على عدد قليل من الجريبات الموجبة لصبغة مستضد تكاثر الخلية النووي. عولجت علامات الشيخوخة المستحدثة في المبيض بشكل معتدل من خلال المعالجة بحامض الليبويك. في المجموعة الثالثة، على الرغم من ارتفاع نسبة الجريبات الرتقة، أظهر العلاج بحامض الليبويك زيادة في عدد الجريبات الناضجة والموجبة لصبغة مستضد تكاثر الخلية النووي. لم ينتج أي من المعالجات فرقًا إحصائيًا في حجم البويضة في أي مرحلة من مراحل النمو مقارنة بمجموعة السيطرة. بالخلاصة، اظهر حامض الليبويك القدرة على تغيير معتدل للآثار السلبية لشيخوخة المبيض. لأول مرة، تم ربط اصطباغ مستضد تكاثر الخلية النووي مع شيخوخة المبيض، كما اعتبر الاصطباغ بمستضد تكاثر الخلية النووي كأداة قيمة لتقييم النشاط التكاثري للخلايا الحبيبية.