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Effect of chronic L-arginine consumption on albumin level and epidermal growth factor receptor expression in the ovaries of aged female Wistar rats

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Abstract

Background and Objective: Sex hormone levels decrease in ovarian aging, causing follicular cysts and low levels of the ovarian albumin/epidermal growth factor receptor (EGFR). L-arginine, a nitric oxide (NO) precursor, plays a role in preventing ovarian aging. We intended to show whether chronic L-arginine consumption has anti-aging benefits on the ovary.

Materials and Methods: Wistar rats (250 g, 36 weeks) were divided into two groups (control and experimental). The control group received 1 mL/kg saline intraperitoneally (i.p.). The experimental group received L-arginine (5, 25 and 50 mg/kg) and L-NAME (5 and 25 mg/kg) alone or together for 3, 5, 9 and 21 days, once a day. At the end, blood samples were taken from rats under deep anesthesia, and serum albumin levels were measured using an ELISA kit. The ovaries were then surgically removed and subjected to biometric, histopathological (Hematoxylin-Eosin and Evans blue) and immunohistopathological tests for EGFR. All data were analyzed using analysis of variance (ANOVA) at α=0.05.

Results: L-arginine reduced the number of follicular cysts in the shortest period (3 days) at the lowest dose (5 mg/kg) and improved serum and ovarian albumin levels as well as EGFR levels in ovaries compared to treatment with L-NAME/L-arginine. These phenomena indicate the involvement of NO. High-dose L-arginine (regardless of duration) increased the number of follicular cysts. Weight change was not statistically significant.

Conclusion: L-arginine in short periods and with low concentration can reduce ovarian aging problems by increasing the level of NO.

Keywords: Nitric oxide, L-NAME, Albumin, EGF receptor, Aging

Ovarian aging is a natural and physiological process characterized by the loss of the quantity and quality of the oocyte or follicular reservoir. It should be known that female babies have a limited follicular reserve that starts the process of permanent reduction without renewal. Follicular reduction along with reduced egg quality definitely brings difficult conditions for women at reproductive age (1).

We all know that the aging population threatens the world today. Studying about aging is important and the aging of the population has multiplied its importance. The issue of ovarian aging is also included in this category. One of the problems observed in ovarian aging, menopause, is the change in the level of the sex hormone estrogen. This hormone interacts with its receptors in the reproduction axis from the hypothalamus and other brain nuclei to the end organs such as the ovary and is effective in regulating reproductive and vital functions and is considered the main anti-aging factor (2). Apart from this factor, other factors such as the level of albumin in the blood circulation are considered indicators of ovarian aging and menopause. Circulating albumin levels and possibly ovarian albumin levels have been shown to decrease under the above conditions (3).

Epidermal growth factor (EGF) is known for its role in cell division and differentiation in developing

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animals, but EGF signaling plays an important role in lifespan and longevity in adults. EGF, acting through phospholipase Cγ and IP3 receptor signaling, preserves pharyngeal and body wall muscle function in the elderly and delays the accumulation of lipofuscin-enriched pigments (a marker of senescence) in cells (4).

L-arginine is an amino acid that, in addition to its role in the synthesis of nitric oxide (NO) and reducing the risk of vascular and heart diseases, also has anti-aging benefits (5). Arginine prevents the growth of tumors by strengthening the body's defense function. Also, this amino acid is able to reduce blood free radicals, release growth hormone, improve the function of muscle cell signals, support good cholesterol and regulate fat metabolism and salt levels in the body (6).

NO is a gaseous messenger molecule with a short half-life of a few seconds that is produced by enzymes with three different isoforms and is involved in a wide range of physiological processes in the body. For example, it is a vasorelaxant in the cardiovascular system and acts as a neurotransmitter in the central nervous system (CNS). Regulation of gonadotropin hormones, egg maturation and ejaculation are among the functions of this molecule in the reproductive system (6).

With the above explanations, the present study examined the effect of chronic consumption of NO precursor L-arginine on the albumin and EGF receptor levels, as well as ovarian status in female rats.

2. Materials and Methods

2.1. Aanimals

Aged virgin female Wistar rats with a weight of about 250 g and an age of 36 weeks were used. The animals were obtained from the animal breeding and care center of Shahed University and kept in groups of four in standard cages. All standard protocols were followed according to the Declaration of Helsinki (DoH) such as room temperature at 23 ± 2 °C and 55 ± 15% humidity, 12 h light/dark rhythm with free access to food and water. This proposal was approved by the Ethics Committee of the University and Code of Ethics was granted (IR.SHAHED.REC.1401.042).

2.2. Animal grouping

The virgin and intact rats were grouped according to a random-based design into the control and experimental groups:

1- The control group received saline (1 mL/kg) for 3, 5, 9, and 21 days as an intraperitoneal (i.p.) injection, once daily.

2- The experimental group was subdivided as follows:

a- Single L-arginine: This group received L-arginine (5, 25 and 50 mg/kg) intraperitoneally during treatment periods (3, 5, 9 and 21 days), once a day.

b- Single L-NAME: This group received L-NAME (5, and 25 mg/kg, i.p.) during treatment periods (3, 5, 9 and 21 days), once daily.

c- Cumulative L-NAME/L-arginine: L-NAME (5, 25 mg/kg) was administered 20 minutes before L-arginine (5, 25, 50 mg/kg) at treatment intervals (3, 5, 9 and 21 days).

2.3. Used materials

Substances used were L-arginine (Merck, Germany), NG-Nitro-L-arginine methyl ester (L-NAME) (Biochemical Inc., U.S.A.), Epidermal growth factor receptor (EGFR) antibody (RGD: 2543, U.S.A.), ketamine and xylazine (Veterinary Organization, Tehran with official permission), and Hematoxylin & Eosin (F Arman Co., Tehran, Iran).

2.4. Experimental procedures

At the end of the experiments, the rats were anesthetized with ketamine and xylazine, and blood samples were taken from the heart under deep anesthesia. After clotting, the samples were centrifuged and serum was taken and placed in a deep freezer (-80°C). Then, the target tissue was separated and placed in 10% formalin for at least 72 hours, and then histological examinations were followed. The level of serum factors such as albumin was determined using an ELISA kit. The levels of albumin (using Evans Blue) and EGFR (using immunohistochemistry) were determined in the ovaries. The ovarian tissue was stained with hematoxylin-eosin method and the ovary was examined biometrically.

2.5. Hematoxylin-Eosin (H&E) staining method

H&E is a common histological staining technique used to examine the structure of cells and tissues in a specimen. First, the tissue is stained with hematoxylin, a basic dye that stains acidic structures such as cytoplasm and nuclei blue. The tissue is then stained with eosin, an acidic dye that stains basic structures such as the cytoplasm and extracellular matrix pink. This staining is widely used in pathology and histology to examine biopsy tissues or postmortem specimens. It creates a clear contrast between different structures in the specimen and is done as follows:

After deparaffinization (by placing twice in xylene, 45 minutes each time) and hydration with decreasing degrees of alcohol, tissue sections were placed in 20% hematoxylin dye for 30 minutes, then washed with distilled water and then immersed in eosin for 20 minutes. In the next step, the samples were immersed in distilled water and dehydrated in alcohols of 50, 70, 80 and 90% (about 2 minutes each). At the end, thin slices (3-4 µm) were embedded in xylene twice for three minutes each. Finally, they were mounted using Entellan glue (Merck, Germany).

2.6. Evans blue coloring

Evans Blue is widely used in animal studies and histology research laboratories to estimate blood volume and vascular permeability, identify lymph nodes, and tumor sites. In recent years, a series of Evans Blue derivatives have been developed, which have a longer half-life in the blood and are released later. In addition, in some preclinical animal studies, its new feature is used to detect necrosis. To stain with Evans blue, first, the dye was prepared in phosphate buffered saline (PBS) with a suitable pH, and after mixing and cleaning with filter paper, it was placed in a dark container. For staining after deparaffinization by xylene twice and each time for 45 minutes and hydration with reducing alcohols each time for 5 minutes, a few drops of Evans blue color solution were poured on the slices and placed in a dark Bain-Maries (37 ºC) for 10 minutes. Then the slides were washed in distilled water and dehydrated with increasing degrees of alcohol (for 10 minutes), clarified by xylene for 10 minutes, and finally mounted by Entellan glue (Merck, Germany).

2.7. Epidermal growth factor receptor (EGFR) specific immunohistochemical stain

Immunohistochemistry, also called IHC, is a combination of histology and immunology. Like other immunoassay techniques, it consists of three important parts: antibodies, signal generation systems for tracking, and solid phase in which the reactions are carried out. In this method, the presence or absence of a specific antigen in the tissue can be checked by using an antibody that specifically binds to the antigen. This connection can be direct (one-step) or indirect (two-step). In the direct method, the specific antibody of the tissue antigen is attached to the enzyme. The enzymes produce a color in the vicinity of the substrate, which can be seen under the light microscope. Sometimes dye is used instead of enzyme. In the indirect method, the antigen-specific antibody is attached to the tissue, and after that, the secondary antibody is added, which adheres to the primary antibody. For IHC study, first, the samples were placed on slides charged with poly-L-lysine and after deparaffinization and hydration, they were placed in PBS (pH = 6.7) and then passed in 0.03% Triton X 100 solution. The slides were then exposed to specific antibody (RGD: 2543, USA.) for one hour in a Bain-Marie (37 ºC). After that, they were washed four times in buffer and then avidin was added to the samples and kept in the laboratory for 10 minutes. Then they were exposed to chromogen for 10 minutes and washed with buffer and then with distilled water. Then they were dehydrated and cleaned and finally assembled with Entellan (Merck, Germany).

2.8. Statistical analysis

All data were analyzed in SPSS software (version 22) and the results were expressed as mean ± standard error of the mean. Normal distribution was first performed using the Kolmogorov-Smirnov test and then analysis of variance (ANOVA) was followed. Tukey's HSD test was used to show differences between groups. $P<0.05$ level was considered significant. Descriptive analysis was quantified with the help of ImageJ (free Java software).

3. Results

3.1. The effect of chronic injection of L-arginine with different doses (5, 25 and 50 mg/kg) at different time intervals on the number of follicular cysts, serum albumin level and EGFR density

3.1.1. Follicular cyst status in treatment and control samples

Following an increase in the duration of injection as well as the prescribed dose, the situation worsened (the percentage of cysts increased) (Figure 1).

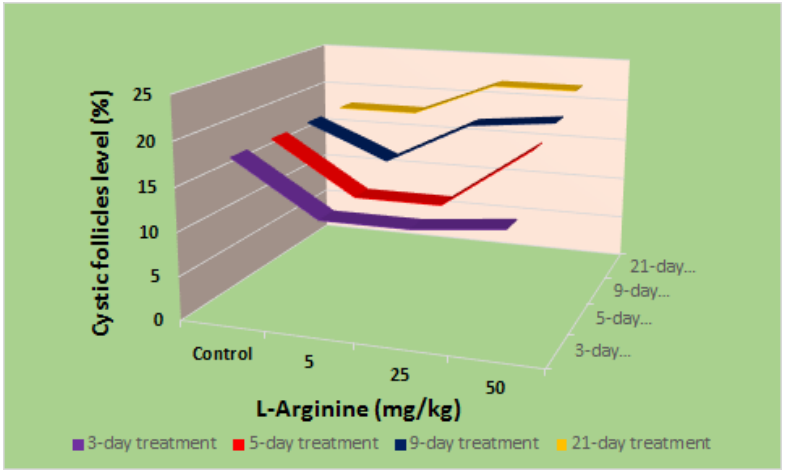

Fig. 1. State of follicular cyst. The percentage of cysts was proportional to the increase in the duration of injection as well as the prescribed dose.

3.1.2. Epidermal growth factor receptor (EGFR) status

The percentage of positive response was proportional to the increase in the duration of injection as well as the prescribed dose (Figure 2).

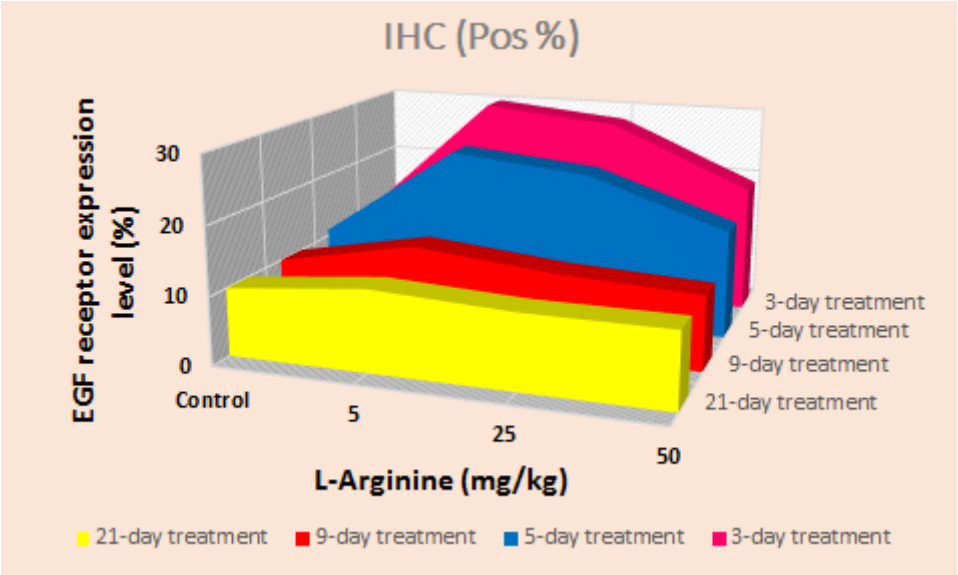

Fig. 2. Expression level of EGFR in treatment and control samples. In proportion to the increase in the duration of injection as well as the prescribed dose, the percentage of positive response decreased.

3.1.3. Tissue staining results: Results of hematoxylin-eosin (H & E) histological studies

Images were obtained from both control ovarian samples and samples treated with L-arginine (5, 25 and 50 mg/kg) during three, five, nine and 21 days. Histological examination of the ovaries of control rats shows ovarian aging (estrous cycle disturbance). But with short-time treatment and a lower dose of L-arginine (5 mg/kg), the condition improved (follicles in different stages of growth increased and the number of follicular cysts decreased). However, as the period and dosage increased, the conditions became unfavorable.

Intervention with NO synthase enzyme inhibitor, L-NAME, reversed the aforementioned protective conditions, indicating the involvement of NO in the protective effect of L-arginine. (Figure 3).

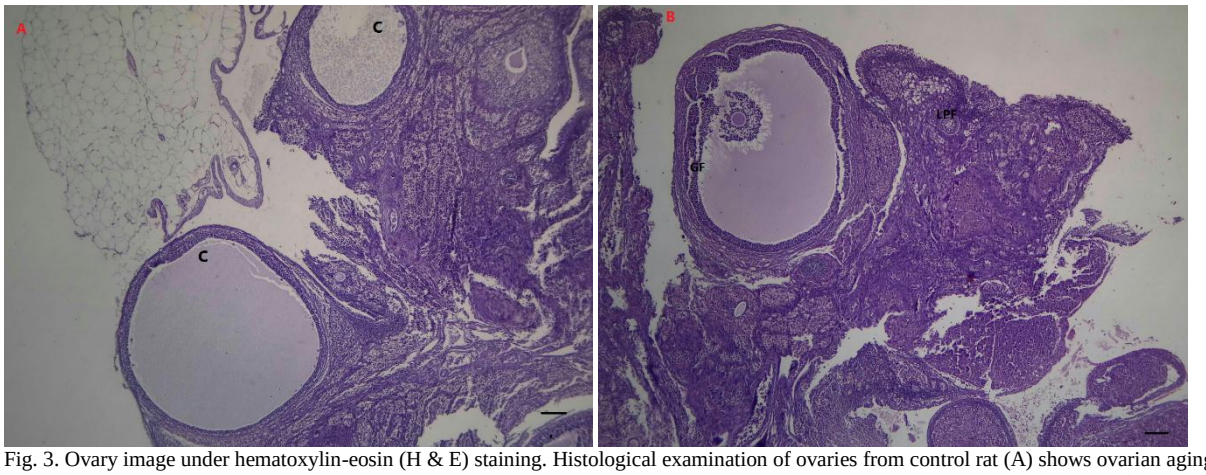

Fig. 3. Ovary image under hematoxylin-eosin (H & E) staining. Histological examination of ovaries from control rat (A) shows ovarian aging (C: cyst). But with a shorter duration (3-day treatment) and a lower dose of L-arginine (5 mg/kg), the condition improved (B) (follicles at different stages of development; GF: graafian follicle, LPF: late primary follicle). The scale bar is 50 µm.

3.1.4. Results of histological studies with Evans blue staining.

The results of the histological examination of rat ovaries show a decrease in tissue albumin. But only with short-term treatment and a lower dose of L-arginine, the condition improved (high intensity staining was observed) (Figure 4).

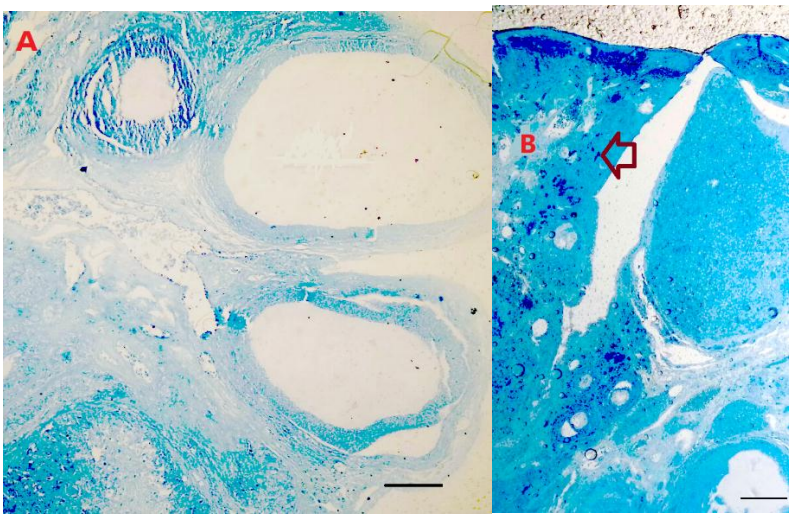

Fig. 4. Image of the histology examination of the animal's ovary with the Evans blue method. Ovaries of control rat (A) show decreased tissue albumin. But only with short-term treatment and a lower dose of L-arginine (B), the condition improved (the arrow indicates a positive reaction). Scale bar is 50 µm.

3.1.5. Results of immunohistochemical studies of EGFR

The results of immunohistochemical examination of the ovaries of control rats show a low level of EGFRs density, which confirms the ovarian aging process. With a shorter treatment and a lower dose of L-arginine, the situation improved (the percentage of positive response was increased) (Figure 5).

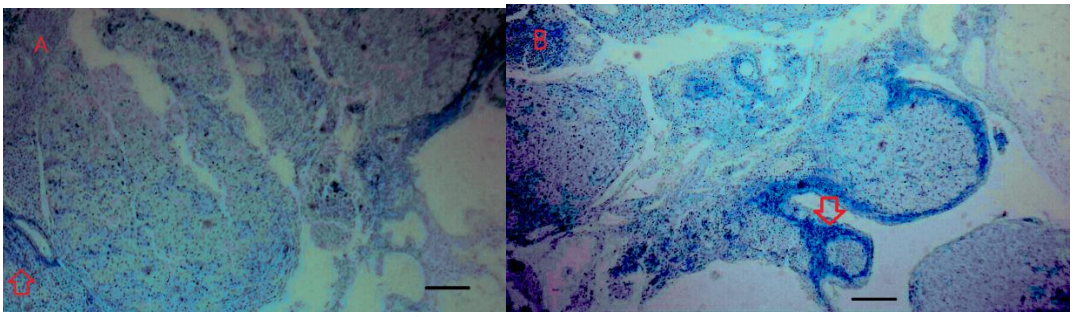

Fig. 5. EGFR immunohistochemical method. Ovaries of control rat (A) show reduced receptor density. With a shorter treatment and a lower dose of L-arginine (B), the condition has progressed towards improvement (the arrow indicates a high positive reaction). Scale bar is 50 µm.

4. Discussion

The aim of this study was to investigate the effect of chronic injection of L-arginine in improving ovarian aging in the reproductive system of old female Wistar rats, which was carried out based on 3-21 day injection of L-arginine and serum and tissue evaluation. The level of albumin in the ovary, as well as the expression level of epidermal growth factor (EGF) receptors in the ovary, as well as the biometric and histological examination of the ovary (to check the state of follicular cysts) were checked.

Undoubtedly, aging is not due to a sudden change in the physiological functions of the body, but is the result of gradual changes that occur over many years. During its growth and activity, the cell regularly receives genetic information from inside for the synthesis of protein materials. Any action or factor that disrupts the process of transferring this information causes cell dysfunction. Mistakes in genetic information can be made through mutations, misreading of codes, formation of aberrant chemical bonds, and other ways. Such genetic errors in the copying and translation of codes cause inactivation and immobilization of proteins, and ultimately cause cell inactivation. Ovarian aging is a natural and physiological aging process characterized by the loss of the quantity and quality of the oocyte or follicular reservoir (1). It is possible that this organ, the ovary,

becomes inflamed due to aging, which is a physiological response of the immune system against harmful internal and external stimuli. Inflammation involves a dynamic sequence of events that manifests itself in an intense vascular reaction. Despite the infinite variety of destructive factors, these phenomena have relatively constant characteristics, because they are determined not only by the damaging factor, but also by the release of endogenous substances, the chemical mediators of inflammation. The primary phenomena that constitute the inflammatory response include dilation of blood vessels and increased permeability, which leads to the passage of fluids from the vascular bed to the damaged tissue and the infiltration of leukocytes in the lesion area. Thus, inflammation removes, dilutes, and limits the injurious agent, but at the same time initiates a set of mechanisms that help to repair or replace the damaged tissue (7).

Arginine is one of the twenty essential amino acids in living cells that, as a precursor of NO, performs many metabolic actions such as balancing electrolyte levels by regulating biochemical and biological activities of the brain. It plays an important role in reducing stress and correcting risk factors, and it also slows down and treats various blood and tissue diseases (8-10). But what are the beneficial effects of this molecule during long-term administration of L-arginine (precursor of NO)? The fact that is discussed in this article. With chronic injection of L-arginine (from 3 days to 21 days), the result was that in a shorter period (3 days treatment) with lower doses, the number of follicular cysts decreased. However, albumin level and EGFR expression level increased. By increasing the dose of L-arginine and increasing the treatment period, the situation was reversed. Weight change was not statistically significant (not shown).

In this research, in order to clarify whether NO is a mediator or not? A preventive intervention with NO synthase enzyme inhibitor (i.e. L-NAME) was performed and it was observed that the protection of low doses of L-arginine was reversed by pre-injection of L-NAME. Therefore, the possibility that the NO system is responsible for mediating that ovarian anti-aging phenomenon was strengthened.

Another important point is the reduction in the number of cysts and the resumption of follicular growth, which usually leads to an increase in the level of circulating sex hormones, which corresponds to an increase in the level of albumin and the level of expression of EGFR (see 3-4). The most interesting point is the results of EGFR levels in the ovary. A logical relationship between EGFR levels and physiological conditions and stages of development, differentiation and repair has been previously reported (11). This factor has a homologous similarity with growth and differentiation factors such as growth hormone, and its signaling is a type of kinase activity cascade that leads to gene expression and anabolism processes (12-13). Therefore, the results related to this factor are interesting.

In this research, the assumption of the role and implication of this factor was consistent with the results, and this is also a good innovation. Although there are still limitations to definitive conclusions, even with these findings, it can be concluded that L-arginine in short-term periods and at low concentration can reduce ovarian aging problems and these effects can be caused by increased NO levels.

The limitations of the research should not be ignored: the lack of easy access to many materials and measurements, in addition, the cost of data analysis. As part of the limitations of this research, it should be noted that we did not measure the level of sex hormones and gonadotropins and the level of NO. But we found out an interesting fact. We are interested in developing future research. Useful suggestions are to measure the level of NO and gonadotropins and sex hormones and the level of antioxidants, which can be considered in future studies.

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