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Original Article

Short-term treatment with low concentrations of L-arginine reduces allostatic load in aged Wistar rats

Fatemeh Lakzaei[1], Manizheh Karami[1*]

1. Department of Biology, Faculty of Basic Sciences, Shahed University, Tehran, Iran

***Corresponding author:**
Manizheh Karami, Department of Biology, Faculty of Basic Sciences, Shahed University, Tehran, Iran
Tel: +982151212243
Email: karami@shahed.ac.ir

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Abstract

Background and Objective: The allostatic load score is assessed based on functional and structural dysregulation of the brain and endocrine system, including markers of pro-inflammatory factors, such as nitric oxide (NO). This score increases with age, and one of the criteria for measuring this score is the examination of the status of endocrine glands, including the pituitary and ovaries. To investigate allostatic load, we studied the feedback efficacy of L-arginine supplementation, a precursor of NO, at a dose of 5 mg/kg for short- to long-term periods on the ratio of steroid hormone (estrogen)/gonadotropin (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]) levels in aged female Wistar rats.

Materials and Methods: Animals (weighing 250 to 300 g) were divided into control and experimental groups and received saline (1 mL/kg) and L-arginine (5 mg/kg) intraperitoneally once a day for 3 to 21 days, respectively. After treatment, blood samples were taken from the hearts of rats under deep anesthesia, and ovarian feedback on gonadotropins was analyzed. In addition, the gonads were examined histopathologically. The body weight index of all animals was compared between the pre- and post-treatment periods. The data were analyzed using analysis of variance (ANOVA) with an error coefficient (α=0.05).

Results: Treatment with L-arginine over 3 days significantly ($P< 0.05$) improved ovarian feedback function, as measured by the steroid/gonadotropin level ratio. However, the ovaries became swollen during the long-term treatment period, indicating an inflammatory response, and body mass index decreased.

Conclusion: Low-dose L-arginine over a few days may correct allostatic overload by improving ovarian function and improving steroid feedback on pituitary gonadotropins.

Keywords: Allostatic load, Feedback, Gonadotropins, L-arginine, Ovarian function

Background

Ovarian aging occurs in postmenopausal women due to the permanent cessation of ovarian function [1]. As a result, ovarian steroid hormone levels decrease, but gonadotropin hormone levels increase due to the absence of a negative feedback loop from sex hormone levels to gonadotropin hormones [2, 4]. Under normal conditions, follicles grow and secrete sex steroid hormones due to normal circulating levels of gonadotropin factors, such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [5], and a feedback loop is established between them [6]. However, during menopause, these processes stop, and the ovaries become cystic due to the disruption of the feedback mechanism, a disruption resulting from the dependence of sex steroid (estrogen) levels on gonadotropins [7].

Allostatic load results from chronic exposure to neural or neuroendocrine fluctuations in response to prolonged or chronic stress. It is a physiological measure of the cumulative stress load on the body, assessed by markers of functional dysregulation [8]. This feature, as defined by other authors, involves the interaction of different physiological systems at different levels of activity and can be determined with the help of markers, such as brain structure and function, structural brain age, clinical stress composition, and telomere length [9]. As previously shown by researchers, it depends on the human race [10] and includes 10 major markers that can be classified into four main biological systems: (1) cardiovascular, (2) metabolic, (3) inflammatory, and (4) neuroendocrine [7, 10].

The neuroendocrine system plays a role in ovarian development and aging, during which the ovary remains in the diestrus stage with a temporary or permanent cessation of follicular growth [5]. The loss of normal ovarian function due to menopause and the subsequent decline in ovarian sex hormone secretion disrupts the feedback relationship between

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estrogen and gonadotropin levels.

Disruption of the physiological effects of estrogens—including changes in circulating lipid levels and proinflammatory factors—likely contributes to increased allostatic load. Therefore, part of our rationale was to investigate the mechanism of steroid/gonadotropin feedback regulation in rats with ovarian aging to reduce allostatic load.

L-arginine is a semi-essential amino acid that is usually obtained from the diet and regulates the reproductive axis at the molecular level by acting on gonadotropin-releasing hormone (GnRH) in the hypothalamus, as well as through GnRH receptors and FSHβ receptors in the pituitary gland [11]. It acts as a substrate for nitric oxide synthase (NOS) and arginase, which convert it to L-citrulline and L-ornithine, and urea, respectively [12]. Age-related NO deficiency can lead to increased arginase activity, as arginase and NOS compete for the common L-arginine substrate [13].

During menopause, when menstrual periods no longer occur, the ovaries stop producing eggs and ovarian steroids [14]. Therefore, many postmenopausal women use hormone therapy to treat menopausal complications, including estrogen deficiency, which is a high risk factor for cardiovascular disease [15] and increased serum cholesterol and triglyceride concentrations [16].

Steroid hormones can regulate NO production in women, and studies have shown that low estrogen levels in postmenopausal women may reduce NO levels during this period [17].

To investigate menopausal problems, such as low NO levels, we administered L-arginine to older female rats that were in the diestrus stage. We also examined the characteristics of the rats' ovaries biometrically to detect possible signs of NO-related inflammation. In addition, we compared the body weights of all animals before and after treatment.

Methods

Animal subject

Virgin female Wistar rats, weighing 250-300 g, aged 15 to 16 months, and kept completely separate from males since weaning, were housed under standard conditions following the Guide for the Care and Use of Laboratory Animals (temperature 21±3°C and 12-h light/dark cycle with free access to food and water *ad libitum*). All experiments were conducted in accordance with the principles of the Declaration of Helsinki (DoH) [18] and approved by the Local Ethics Committee (IR.SHAHED.REC.1399.106).

Pap smear and female cycle

The menstrual cycle of female rats was determined using a Pap smear test. Animals in the diestrus stage were carefully selected and grouped for this study, including a control group (8 rats) and groups treated with L-arginine (5 mg/kg) for 3 and 21 days (8 rats per time period).

Treatments

As mentioned above, animals were divided into two groups: control and L-arginine treatment. The control group received saline (1 mL/kg) intraperitoneally (i.p.) [19], and the L-arginine treatment groups (i.p.) received the amino acid L-arginine (5 mg/kg) once daily for 3 and 21 days.

Serology

Sampling large volumes of blood in rats (more than 1.5 mL) is only possible from the heart according to animal care rules (DoH), and blood sampling from the heart should be performed only under deep anesthesia [18]. After 24 h post-treatment, blood samples were collected transcardially from rats under deep anesthesia with ketamine-xylazine, and after 1 h, the samples were centrifuged at 3000 rpm for 15 min to separate the blood clot. Serum samples were stored in the freezer (-20 °C) for two weeks. These samples were then evaluated with ELISA kits. In this study, the ratio of serum concentrations of ovarian steroids to pituitary gonadotropins was evaluated.

Pathology

After abdominal surgery on rats under deep anesthesia with ketamine-xylazine and biometric examination of the ovaries, the ovaries were collected in 10% formalin. Then (after 48 to 72 hours), sections of the ovaries were prepared and stained with hematoxylin-eosin (H&E). All data were compared with the control group.

Allostatic load measurement

To measure allostatic load, we worked on a set of physiological markers related to stress, specifically the dysregulation of the hypothalamic-pituitary-gonadal (HPG) axis. The biomarkers were categorized into neuroendocrine, biometric, serological, and pathological. To calculate it, all biomarkers were weighted equally to form a composite score equal to the sum of all measured biomarkers, where each marker is coded as "0" for normal findings and "1" for abnormal findings. We limited the markers to those that were important to us, and coded each normal state as "0" and each abnormal state as "1." The total allostatic load score was calculated by summing all 4 biomarker scores;

therefore, the total allostatic load score can range from 0 to 4.

Statistical analysis

All findings were computed after the analysis of normality using one-way or two-way analysis of variance (ANOVA). The error coefficient was set at 0.05. In cases where ANOVA confirmed a significant difference, further *post hoc* analysis was used to examine the differences between the groups. Data were presented as mean data points. Qualitative findings, such as images, were analyzed using ImageJ (version 1.41) software.

Results

Pap smear

According to the Pap smear test, the female rats in this study were in diestrus (Figure 1). At this stage, the predominant cell population is small leukocytes. Other types of cells, such as epithelial cells, are rarely seen on smears.

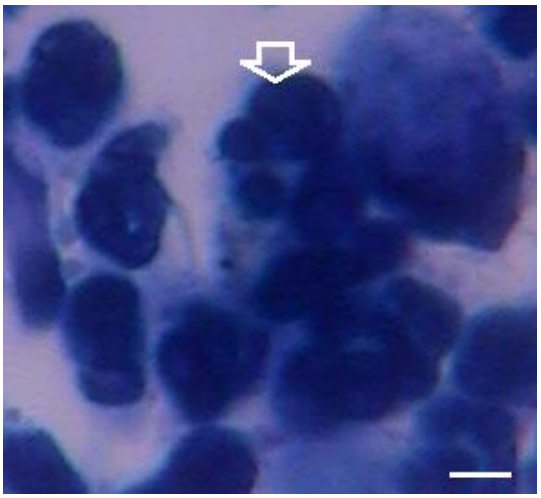


Figure 1. Papanicolaou smears. Vaginal smear showed the diestrus stage due to the predominant population of small leukocytes. The arrow indicates a leukocyte. The scale bar is 50 µm.

Histology of ovarian specimens

In the group treated with L-arginine for a long time, significant changes in ovarian tissue characteristics were observed compared to the group receiving saline solution, indicating vascular infiltration and hyperemia (image not shown). Furthermore, follicular cysts—averaging five or more per ovary—were observed (Figure 2). According to these results, there is a statistically significant ($P<0.05$) correlation between the duration of L-arginine treatment and the health index.

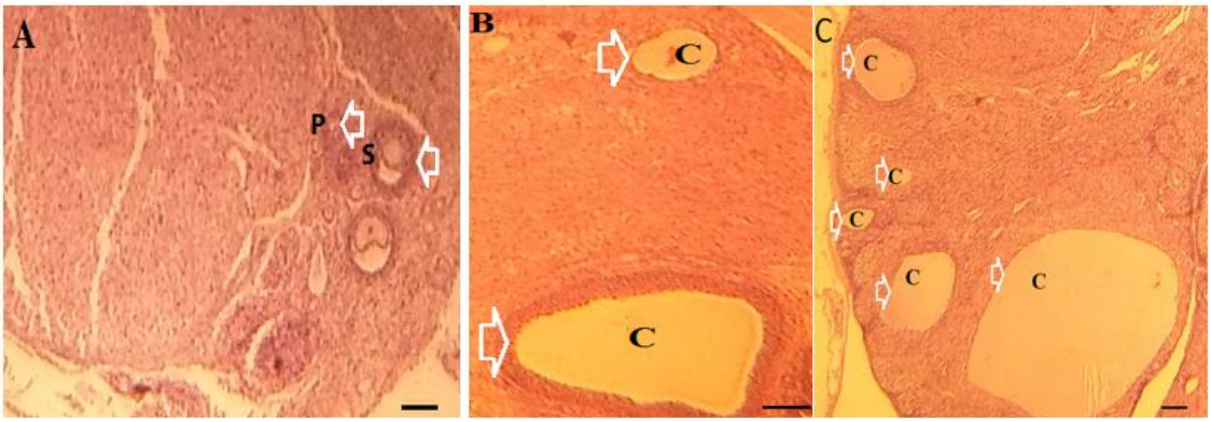


Figure 2. H&E staining of the ovary: evidence of follicular cysts compared with a healthy ovary. Arrows indicate follicular development in healthy rat ovary labeled A (P: primary follicle and S: secondary follicle) and cysts in ovaries of aged virgin rats (B). The number of these cysts was significantly increased with long-term treatment (C) compared with the control sample (B). The scale bar is 50 µm.

Findings of ovarian biometry

A significant relationship ($P<0.05$) was observed between the duration of L-arginine treatment and ovarian swelling, with long-term treatment resulting in a 25% relative increase in mean ovarian diameter. Regardless of the L-arginine dose (since it was the

same), they became inflamed due to prolonged treatment, suggesting the possible involvement of high NO levels. Given that L-arginine is a precursor to NO (a pro-inflammatory factor), ovarian swelling during long-term treatment with this substance is likely caused by excessively high levels of NO–a condition that is inversely correlated with health status.

Animal weight change

The group treated with the amino acid for a long period (21 days) demonstrated a weight loss (Figure 3) that was inversely correlated with ovarian swelling. Therefore, there was a relationship between the duration of L-arginine treatment and weight loss. Again, the animal's weight loss was significantly correlated with the allostatic load score.

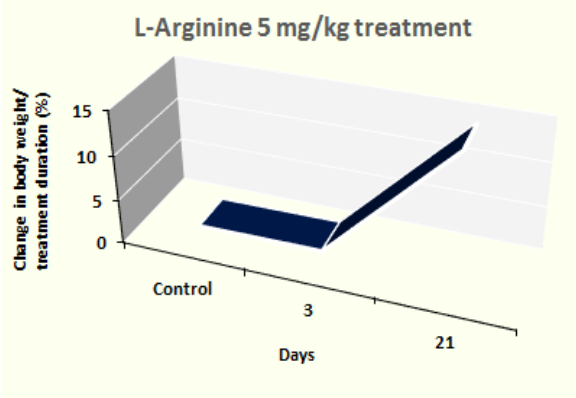


Figure 3. Percentage change in body weight/treatment duration in L-arginine-treated groups compared to the saline control group. The level of change in the short-term L-arginine-treated group was normal compared to the saline control group.

Estrogen/gonadotropins feedback

Steroid/gonadotropin levels, which reflect ovarian feedback in women with normal reproductive activity, returned to normal levels significantly ($P<0.05$) within a short time with L-arginine treatment compared with the control group receiving saline (Figure 4).

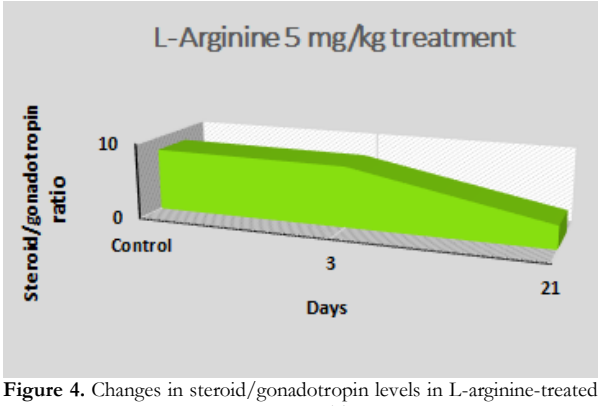


Figure 4. Changes in steroid/gonadotropin levels in L-arginine-treated groups compared to the saline control. The situation was normal in the short-term L-arginine-treated group compared to the saline control group.

Allostatic load score

A large number of parameters were studied to determine the allostatic load score in this study, and it was concluded that this score yielded the best results with 3 days of L-arginine administration (Figure 5).

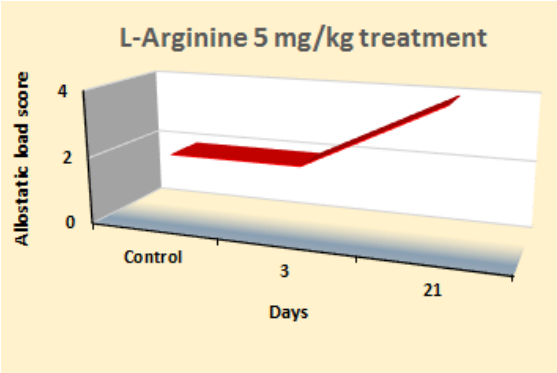


Figure 5. Allostatic load score. As shown, this score yields the best results with a 3-day administration of L-arginine. Note that the rats are aged and, even in the best state (*i.e.*, the control group), suffer from some form of age-related physiological dysregulation.

Discussion

The present research aimed to evaluate the effect of L-arginine treatment in aged rats with menopausal features. This study revealed that L-arginine administration is beneficial in improving ovarian aging. According to the available evidence, normal ovarian function was facilitated by low-dose L-arginine during short-term treatment, and the allostatic index was improved. However, with increasing treatment duration, the indices changed and inflammatory effects became apparent, as ovarian dimensions increased and body weight decreased.

Menopause occurs with ovarian aging, during which follicular development and ovulation cease. The decline in the levels of steroid hormones secreted by the ovaries, the main source of these hormones, leads to the symptoms of aging. In this condition, the levels of gonadotropin factors increase due to the lack of feedback from steroid hormones to regulate gonadotropin levels [20]. Estrogen is involved in the aging process, and any age-related changes in the concentration of this hormone in the blood and its binding to the receptor, as well as changes in its signaling pathways or related transcription factors, can affect the allostatic load. Estrogen can improve neuronal function and plasticity and also acts as a neuroprotective agent [21].

L-Arginine is synthesized from glutamine, glutamate, and proline via the intestinal-renal axis in humans and most other mammals (including rats)

and is metabolized by several metabolic factors, including arginase and NOS [22].

As noted elsewhere, allostatic load is associated with the function of several major systems, including the endocrine system [7, 10], which is part of the hypothalamic-pituitary-gonadal (HPG) axis. The gonads are the terminal endocrine organs in this axis. In this study, we specifically examined the ovaries of aged rats and found that the duration of low-dose administration of this amino acid was associated with allostatic load. In the longer treatment period, changes in ovarian size (inflammation) and a decrease in body weight were reported, which may indicate stimulation of the inflammatory system [23, 25]. However, as a limitation of this study, we were unable to observe macrophage infiltration and did not have histological findings to confirm it. In the short-term treatment, no significant changes in animal weight were observed ($P>0.05$). The weight loss in long-term treatment can be attributed to the long-term stimulation of the NO inflammatory system.

Steroid/gonadotropin levels were normal in the short-term group compared with the saline control group, which may reflect a relative increase in NO levels following i.p. injection of L-arginine, a known precursor of NO. In addition, NO can regulate ovarian hormone levels and lead to a natural feedback on gonadotropin levels [25], which is consistent with the present findings.

There is clearly a relationship between the neuroendocrine system and allostatic load score, and ovarian steroids are one of the main determinants of women's physiological health [7, 10, 19]. Ovarian aging in postmenopausal women occurs due to permanent cessation of ovarian function (egg production and sex hormone secretion). Menopause is a natural decrease in sex hormones and occurs as a natural part of the aging process, when female sex hormones decrease naturally and the ovaries stop releasing eggs. However, ovarian aging can also occur in women of childbearing age with low stored eggs, as in poorly responsive women [26, 27]. Wei et al. (2014) [28] have previously indicated that ovarian steroids protect against the harmful effects of repeated stress on glutamatergic transmission and prefrontal cortex-dependent cognition. On the other hand, as seen in the sources, to reduce the risk of repeated use of estrogen supplements, the use of appropriate adjuvant medications is now considered a low-risk treatment approach for menopausal women [29]. Given the allostatic load, NO may mediate this effect. Therefore, this low-risk approach is likely to be an alternative to high-risk treatments such as estrogen therapy.

The role of ovarian steroids as one of the allostatic mediators in animals was also demonstrated in this study. We found a significant relationship between the levels of these factors and improved ovarian function in aged rats. As previously shown, there is a close relationship between ovarian dysfunction, a decrease in the number of primary and growing follicles, and ovarian aging [30]. Therefore, this study, while confirming the role of the gonads in physiological health, showed that normal steroid/gonadotropin levels can occur under short-term treatment with L-arginine at low doses.

Conclusion

Allostatic load scores are increased in postmenopausal women due to impaired ovarian function. L-arginine at low doses likely improves health symptoms by ameliorating ovarian aging. It appears that the amino acid L-arginine, at low doses and in short-term treatment courses, may alleviate some of the problems of menopause, possibly by increasing ovarian hormone levels, thereby correcting ovarian steroid feedback and normalizing steroid/gonadotropin levels.

Acknowledgment

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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Ethical Approval

This study was approved by the local Ethics Committee (IR.SHAHED.REC.1399.106).

Authors contributions

MK designed the study; MK & FL completed the research and collected and organized data; MK analyzed and interpreted data. All authors reviewed and approved the final draft of the article.

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