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Germanium citrate improves ovarian granulosa cells viability and antioxidant defense system in aging female mice during endotoxemia

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The study aimed to investigate the vitality of ovarian granulosa cells (GCs), metabolic activity of neutrophils, as well as the antioxidant system state in aging female mice subjected to experimental endotoxemia, as well as the influence of germanium (Ge) citrate on the studied parameters under these conditions. Treatment with Gramm-negative bacteria lipopolysaccharide caused pathological changes in mouse ovaries: a decrease in GCs viability by increasing necrosis, an enhancement of metabolic activity of peripheral blood neutrophils, an increase in lipid peroxidaxion products and a violation of the antioxidant defense system (as evidenced by an elevation of the content of reactive products of 2-thiobarbituric acid and a reduction of reduced glutathione in liver homogenate, as well as a decrease in the concentration of ceruloplasmin in blood serum of aging female mice). Pretreatment of mice with Ge citrate was effective to reduce GCs death and improve their viability, decrease the degree of disruption of the redox balance and weaken the activity of cells of non-specific immune protection in aging endotoxemic animals. Our results suggest that Ge citrate may offer promising therapeutic benefits. Its cytoprotective effects and regulatory role in the antioxidant defense system, combined with its potential to reduce the intensity of gram-negative bacterial toxins induced inflammation, imply its usefulness. This multifaceted action may help prevent ovarian cell aging and, consequently, improve reproductive function.

Key words: reproductive aging; endotoxemia; germanium citrate; granulosa cell viability; TBA-active products; reduced glutathione; ceruloplasmin.

INTRODUCTION

Aging is usually characterized by a mild chronic proinflammatory state that may be complicated by the presence of bacterial or viral infections [1, 2]. Acute inflammation is known to be a physiological response to injury or infection with a chain of reactions that eventually lead to the recruitment of immune cells to clear the invading pathogens and heal the wounds. However, chronic inflammation resulting from the persistent presence of the initial trigger or dysfunction of signaling and/or effector pathways is detrimental to health [3]. Among the reasons for the development of age-associated chronic inflammation, cell aging and accumulation of cell debris are highlighted. Variety of stressors contribute to cellular senescence, including age-related telomere shortening, DNA damage, oxidative stress, mitochondrial dysfunction, proteotoxic stress, and oncogene activation [2-4]. Local changes in DNA methylation and global chromatin rearrangements that occur in aging cells lead to changes in gene expression and the secretion of a number of chemokines (such as IL-8, MCP-1), cytokines (IL-6, IL-1, S100), amphoterin, soluble receptors (for example, sTNFR), as well as enzymes for tissue remodeling. Therefore, the accumulation of senescent cells likely contributes to inflammation. During infection, inflammation is the primary response to infection, however pro-inflammatory mediators released during the immune response can also contribute to cellular senescence. The pro-inflammatory secretion

produced by senescent cells is associated with age-related diseases. Thus, senescent cells underlie a multifaceted vicious cycle that leads to increased inflammation, age-related diseases, and ultimately death [3]. Elevated levels of inflammatory cytokines have been found to play a critical role in ovarian dysfunction, and long-term oxidative stress decreases ovarian reproductive function by reducing follicle quality and progesterone production [2]. Ovarian follicular depletion determines the process of reproductive aging in women and is responsible for age-related decline in sex steroid levels and impaired fertility [5]. It has been revealed that infection with gram-negative bacteria leads to a deterioration in the morphological and functional state of the reproductive system cells in female sexually mature young mice [6]. In the present work, we investigated the vitality of ovarian granulosa cells (GCs), metabolic activity of effector cells of inflammation, as well as the antioxidant system state in aging female mice subjected to bacterial endotoxin-induced inflammation, as well as the influence of germanium (Ge) citrate on the studied parameters under these conditions. Ge citrate was obtained by the method of electric pulse nanotechnology [7]. This drug exhibits antioxidant properties that improve the immunobiological indicators and fertility, and is also characterized by environmental safety, high purity and bioavailability [8-10].

METHODS

Female Albino mice aged 8-9 months (weighing 30-34 g) were used for the research. During the work, the International Principles of the European Convention on the Protection of Vertebrate Animals of the Council of Europe (Strasbourg, 1986) were followed. To study the effect of Ge citrate (Nanotechnologies and Nanomaterials LLC, Kyiv, Ukraine) under the conditions of endotoxemia, mice were divided into 3 groups: 1) animals that received intraperitoneally (i.p.) lipopolysaccharide (LPS; *E. coli* 0111: B4, " Sigma-Aldrich", USA), 3 mg/kg of body weight; 2) group 2 was treated i.p. with Ge citrate at a dose of 100 mg/kg, twice: 24 h and 1 h before LPS administration (aqueous solution of Ge citrate was adjusted to the required volume with physiological solution: 0.4 ml/20 g of murine weight); 3) vehicle injected mice (control). After that, the animals were subjected to an ether anesthesia and the material for research was removed. Follicles were separated from the ovaries under a stereoscopic microscope and punctured with a needle in Dulbecco's modified Eagle's medium (DMEM, "Sigma", USA) to release cumulus-oocyte complexes and GCs. Oocytes were mechanically denuded from the cumulus cells by repeated pipetting with the glass pipette. GCs were sampled into sterile eppendorfs for further investigation of their viability. The assessment of cell death pathways was carried out immediately after their selection by the method of intravital double staining with fluorescent DNA dyes Hoechst 33342 and propidium iodide ("Sigma-Aldrich", USA) [11]. A video system for transmitting images to a computer from a Lumam I-1 fluorescent microscope (immersion objective 90×) was used. In order to assess the intensity of lipid peroxidation (LPO), the concentration of LPO end products in liver homogenate was measured. The method is based on their ability to interact with 2-thiobarbituric acid (TBA). Liver homogenate was obtained by grinding 500 mg of tissue in a porcelain mortar on ice in 5 ml of PBS and centrifuged at 4°C for 7 min at 12,000g. The content of TBA-active products (TBA-AP) in the obtained supernatants was determined by the colorimetric method [12] and expressed in nmol of malondialdehyde (MDA) per 1 gram of tissue, using the molar absorption coefficient of 1.56×10^5 $M^{-1} \cdot cm^{-1}$. Reduced glutathione (GSH) content in homogenized liver supernatants was determined by a standard spectrophotometric method using the Ellman reagent [13]. The level of ceruloplasmin (CP) in blood serum was estimated by the Ravin's colorimetric method using a test kit (PJSC «Reagent», Ukraine) according to the

manufacturer's instructions. The functional state of neutrophils was assessed according to the data of the oxygen-dependent and oxygen-independent metabolism of granulocytes. Nitroblue tetrazolium (NBT) test was applied for the evaluation of oxygen-dependent metabolism. It based on the ability of NBT to reduce to insoluble formazan under the influence of reactive oxygen species (ROS) produced by activated cells [14]. We used the semiquantitative lysosomal cation test (LCT) and calculated the average cytochemical coefficient (ACC) to assess the oxygen-independent metabolism of neutrophil granulocytes [15].

The results were analyzed with GraphPad Prism 5.00 software for Windows ("GraphPad Software", USA). After checking for normality of the distribution according to the Kolmogorov-Smirnov test, statistical analysis was performed using one-way ANOVA followed by multiple comparisons using the Newman-Keuls test. If samples were not normally distributed (when GCs viability and death were analysed), we used the non-parametric Kruskal-Wallis test with the Dunn pairwise multiple comparisons. Results are presented as m ± s.e.m. for normal samples and median [quartiles] for non-normal ones. Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Investigation of the Ge citrate effect on GCs viability and death in aging mice subjected to experimental endotoxemia. The results showed an increase in the death of GCs and a decrease in the number of live cells in aging endotoxemic mice compared to the control. The percentage of necrotic cells increased from 6.0 [5.0; 8.5] % to 15.0 [11.8; 17.8] % ($P < 0.05$). At the same time, the number of apoptotic GCs did not change significantly: 52.0 [51.0; 58.0] % versus 59.5 [58.5; 65.5] %. The percentage of living cells decreased from 42.0 [33.0; 43.5] % to 23.5 [21.8; 26.3] % ($P < 0.05$).

The use of Ge citrate improved GCs viability: the percentage of living cells increased to 36.0 [33.5; 37.8] % ($P < 0.05$) and their necrotic death decreased to 8.5 [6.8; 10.3] %; ($P < 0.05$). We previously showed that administaring of Ge citrate to aging non-endotoxemic mice significantly improved the viability of GCs: the percentage of living cells notably increased and their death by both necrotic and apoptotic pathways decreased [16].

Investigation of the Ge citrate effect on the pro- and antioxidant system state in aging mice subjected to experimental endotoxemia. The exposure to LPS led to an increase in the level of TBA-AP in liver homohenate compared to control mice ($P < 0.05$). The introduction of Ge citrate caused a decrease in the content of TBA-AP ($P < 0.05$) (Fig. 1).

When studing the antioxidant protection, a decrease in the content of GSH in liver homogenate in endotoxemic mice was revealed ($P < 0.05$). The introduction of Ge citrate did not cause any significant changes in the concentration of GSH (Fig. 2)

Another important component of the antioxidant system is CP. The endotoxin treatment led to a decrease in its content in blood serum ($P < 0.05$). The use of Ge citrate contributed to the increase in the level of CP ($P < 0.05$) (Fig. 3).

In a previous study, we found that Ge citrate reduces the degree of redox imbalance in aging animals without LPS-induced inflammation [16].

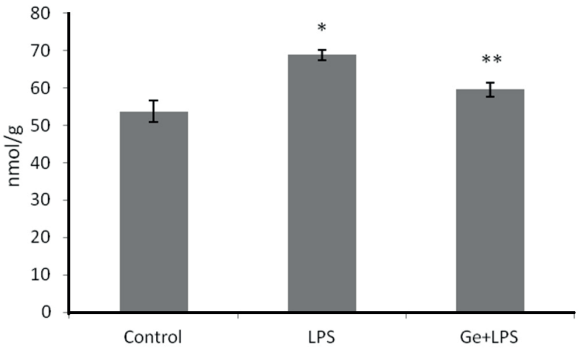

Fig. 1. Effect of Ge citrate on the content of TBA-AP in liver homogenate of aging endotoxemic mice. *$P < 0.05$ – relative to control; **$P < 0.05$ – relative to LPS action

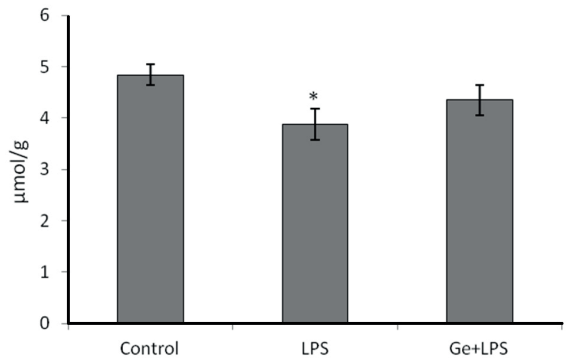

Fig. 2. Effect of Ge citrate on the content of GSH in liver homogenate of aging endotoxemic mice. *P < 0.05 – relative to control.

Investigation of the Ge citrate effect on the functional and metabolic activity of neutrophils in aging mice subjected to experimental endotoxemia. Analysis of the oxygen-dependent metabolism of peripheral blood neutrophils in aging endotoxemic mice showed that the infection with gram-negative bacteria toxin enhansed the intensity of reactive oxygen species (ROS) production. The percentage of formazan-positive cells increased to 69.7 ± 3.6% compared to 43.3 ± 3.6% in the control. The administration of Ge citrate contributed to a decrease in the percentage of formazan-positive cells to 55.0 ± 2.5%, this indicates a reduction of ROS generation in granulocytes. Evaluation of the cytochemical index (CCI) showed its decrease from 1.14 ± 0.08% in endotoxemic mice to 0.76 ± 0.05% in the group of infected animals that received Ge citrate. In control mice, the CCI value was 0.71 ± 0.07%.

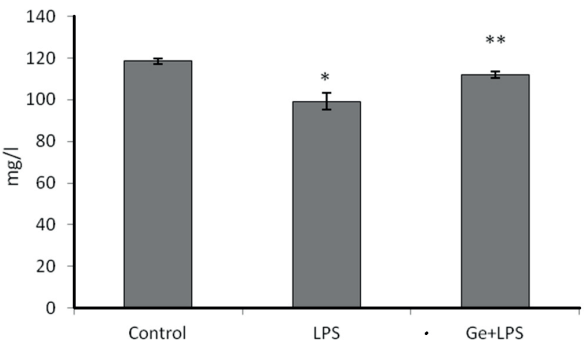

Fig. 3. Effect of Ge citrate on the content of CP in blood serum of aging endotoxemic mice. *P < 0.05 – relative to control; **P < 0.05 – relative to LPS action

According to the LCT data, administaring of LPS to aging mice promoted the activation of lysosomal factors synthesis. The use of Ge citrate contributed to the inhibition of this synthesis. As a response to the content of cationic proteins, the ACC decreased from 0.44 ± 0.03 (aging endotoxemic mice) to 0.31 ± 0.02 (if Ge citrate was administered). In control mice, the ACC value was 0.19 ± 0.02. Thus, the use of Ge citrate reduced the functional and metabolic activity of peripheral blood neutrophils under the conditions of experimental endotoxemia.

The results of our study showed that exposure to the endotoxin of gram-negative bacteria induced an increase in the formation of secondary LPO products. It has been demonstrated that the intensity of LPO processes increases with age [16]. However, infection in aging mice results in a more pronounced formation of TBA-AP, secondary LPO products, predominantly composed of MDA. It is known that MDA is considered as a marker of the development of oxidative stress and cell damage. Its accumulation can cause changes in the structure of the cell membrane and its rupture, which testifies to the cytotoxicity of MDA [17]. At the same time, there is a decrease in the content of GSH in liver homogenate and CP in blood serum in aging endotoxemic animals. This indicates a significant decrease in the ability of antioxidant defense components to counteract the development of oxidative stress. Thus, the obtained results suggest an increase in oxidative stress due to infection. According to the literature [3], oxidative stress is one of the factors contributing to cell aging. The latter, as has already been mentioned, plays a central role in inflammation. A high activity of cells of nonspecific immune protection was also revealed, which indicates a systemic inflammatory process. Excessive production of cytotoxic factors, in particular due to LPS-induced activation of phagocytes, especially together with insufficient compensatory capabilities of antioxidant defense, can lead to the development of new or

cause prolongation and complication of existing pathology (inflammation).

The introduction of Ge citrate caused a decrease in the content of TBA-AP in the liver and contributed to an increase in the level of CP in the blood serum, but did not cause significant changes in the concentration of GSH during experimental endotoxemia. In addition, when exposed to Ge citrate, a reduction in the functional and metabolic activity of peripheral blood effector cells was observed, which was characterized by a decrease in the release of specific aggressive substances into the extracellular space by neutrophils, in particular cationic proteins and ROS. Thus, the obtained results confirm our previous conclusion that Ge citrate exhibits antioxidant properties [16].

Our study of ovarian GCs viability and death in aging mice demonstrated increased necrotic death of these cells. Necrosis is an immunogenic type of death that promotes inflammation. Rising levels of proinflammatory factors during the immune response can also contribute to the aging of ovarian cells and, accordingly, the deterioration of reproductive function. It should be noted that statistically significant changes in apoptotic death after LPS infection were not detected, although we previously showed an increase in apoptosis in GCs of aging mice without endotoxemia compared to young animals [16]. It is possible that exposure to LPS induces the occurrence of numerous damages, which may include DNA breaks (this effect of LPS was found on GCs of young mice [6]) and various modifications of nucleotides, in particular DNA methylation. Such changes contribute to the aging of cells, which become resistant to apoptosis [3]. It is also likely that LPS administering promotes mitochondrial dysfunction, leading to reduced energy production and increased oxidative stress. As a result, GCs lacks the energy resources to complete apoptosis, and the cell dies due to necrosis (i.e., postapoptotic necrosis). Apoptosis is known to eliminate damaged senescent cells. The accumulation of cell debris due to disruption of apoptotic processes may contribute to the development or maintenance of inflammation and its intensification. The introduction of Ge citrate increased the viability of GCs and decreased their necrotic death. This indicates the cytoprotective properties of the studied drug. Additionally, the findings suggest that Ge citrate exhibits anti-inflammatory effects, as the reduction in necrosis contributes to a decrease in inflammation intensity.

An increase in LPO generation and a violation of the antioxidant defense system in aging female mice subjected to experimental endotoxemia were established, as evidenced by an increase in TBA-AP levels and a decrease in the content of GSH in the liver tissue, as well as a decrease in the CP concentration in blood serum. Heightened functional and metabolic activity of peripheral blood effector cells was revealed. There was an increase in the necrotic death of ovarian GCs. The use of Ge citrate reduced the degree of disruption of the redox balance in aging animals, weakened the activity of cells of non-specific immune protection and improved the viability of GCs, reducing their necrotic death. Our results suggest that Ge citrate may offer promising therapeutic benefits. Its cytoprotective effects and regulatory role in the antioxidant defense system, combined with its potential to reduce the intensity of gram-negative bacterial toxins induced inflammation, imply its usefulness. This multifaceted action may help prevent ovarian cell aging and, consequently, improve reproductive function.

The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of co-authors of the article.

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ЦИТРАТ ГЕРМАНІЮ ПОКРАЩУЄ ЖИТТЄЗДАТНІСТЬ ГРАНУЛЯРНИХ КЛІТИН ЯЄЧНИКІВ ТА СТАН СИСТЕМИ АНТИОКСИДАНТНОГО ЗАХИСТУ У СТАРІЮЧИХ САМИЦЬ МИШЕЙ ПІД ЧАС ЕНДОТОКСЕМІЇ

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Вивчали життєздатність гранулярних клітин (ГК) яєчників, метаболічну активність нейтрофілів, а також стан антиоксидантної системи у старіючих самиць мишей за умов експериментальної ендотоксемії, а також впливу цитрату германію на досліджувані показники. Введення ліпополісахариду (ЛПС) спричиняло патологічні зміни в яєчниках мишей через 24 год після ін'єкції: зниження життєздатності ГК через посилення некрозу, підвищення метаболічної активності нейтрофілів периферичної крові, збільшення інтенсивності перекисного окиснення ліпідів та порушення системи антиоксидантного захисту (про що свідчать зростання вмісту реактивних продуктів 2-тіобарбітурової кислоти та зниження вмісту відновленого глютатіону у тканині печінки, а також зменшення концентрації церулоплазміну у сироватці крові старіючих самиць мишей). Попередня обробка тварин цитратом германію (внутрішньоочеревинно 100 мкг/кг, 2 ін'єкції: за 24 год та 1 год до введення ЛПС) викликала зменшення загибелі ГК та підвищення їх життєздатності, зниження ступеня порушення окисно-відновного балансу та послаблення активності клітин неспецифічного імунного захисту у тварин з ендотоксемією. Отже, наші результати свідчать про те, що цитрат германію може бути корисною терапевтичною мішенню завдяки його цитопротекторній дії, а також ролі в регуляції системи антиоксидантного захисту та зниженні інтенсивності запальних процесів, пов'язаних з дією токсинів грам-негативних бактерій, що, у свою чергу, запобігатиме старінню клітин яєчників і, відповідно, сприятиме покращенню репродуктивної функції.

Ключові слова: репродуктивне старіння; ендотоксемія; цитрат германію; життєздатність гранулярних клітин; ТБК-реактивні продукти; відновлений глутатіон; церулоплазмін.

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