



Effect of antioxidants on oocyte morphology and function in rats in systemic inflammatory process

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Abstract. The study aimed to evaluate the impact of antioxidants on the morphological and functional properties of oocytes in rats under conditions of systemic inflammation, focusing on their potential to reduce oxidative stress and improve reproductive health. A female rats at three weeks of age were used as experimental models and divided into three groups: control, systemic inflammation-induced, and antioxidant-treated groups. Human chorionic gonadotropin was administered to stimulate superovulation, and oocytes were isolated for analysis. Morphological changes in oocytes were assessed using immunofluorescence staining, while mitochondrial function and oxidative stress were evaluated using JC-1 dye detection kit. Fertilisation rates and oocyte maturation stages were compared among the groups. All experiments adhered to international ethical standards for animal research. Systemic inflammation led to ovarian atrophy, decreasing ovarian weight to 0.3 g, which reflects a reduction in follicle number and oocyte quality. This resulted in a 30% reduction in fertilisation rates. Antioxidant therapy helped restore ovarian weight to 0.4 g, improving oocyte morphology and increasing the proportion of oocytes achieving metaphase to 75%, demonstrating the protective effect of antioxidants. The fertilisation rate improved to 65% in the antioxidant-treated group, indicating a protective effect against oxidative damage and inflammation. The findings highlight the potential of antioxidants in modulating apoptosis pathways, reducing oxidative stress, and enhancing mitochondrial function, which collectively improved oocyte quality and reproductive outcomes. Antioxidant therapy demonstrated substantial benefits in mitigating the effects of oxidative stress and inflammation on oocytes, promoting normal maturation and increasing fertilisation success. These results underscore the therapeutic potential of antioxidants for treating infertility associated with inflammatory conditions

Keywords: post-ovulatory ageing; egg; antioxidant; oxidative imbalance; citrus nutrients

Introduction

The need to investigate the effect of antioxidants on oocyte quality is due to the growing problem of infertility in modern society. Among the factors that negatively affect reproductive function, special attention is drawn to post-ovulatory ageing of oocytes which is caused by a decrease in their quality after ovulation. Recent trends show that women are increasingly delaying the time when they will become mothers. This has led to an increase in the number of women whose oocytes undergo quality changes due to age factors. The ageing of oocytes after ovulation is an important factor limiting the success of assisted reproductive

technologies. The quality of an oocyte directly affects its ability to fertilise and further develop the embryo. A study by B. Bibak *et al.* [1] showed that long-term storage of oocytes without fertilisation leads to their progressive deterioration, which is described as postovulatory ageing. This phenomenon is accompanied by a number of pathological changes that negatively affect fertility. These changes include mitotic spindle abnormalities, chromosome integrity disorders, mitochondrial dysfunction, and changes in ion concentration that affect normal oocyte function. Oxidative stress is one of the key causes of reduced oocyte quality

Suggest Citation:

Stewart A. Effect of antioxidants on oocyte morphology and function in rats in systemic inflammatory process. *Int J Med Med Res.* 2024;10(2):65–72. DOI: 10.61751/ijmmr/2.2024.65

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during ageing. In particular, as pointed out by B. Bibak *et al.* [1], the accumulation of reactive oxygen species that negatively affect the structure and function of oocytes causes an oxidative imbalance, which substantially reduces female fertility. A paper of V.R. Askari *et al.* [2] show that reactive oxygen species cause oxidative damage, which leads to the destruction of cell membranes, a decrease in the energy potential of mitochondria, and changes in the genetic material of oocytes. The discrepancy between the production of reactive oxygen species and the mechanisms of antioxidant protection is an important factor leading to the development of oxidative stress. This can cause various defects in germ cells, such as mitotic spindle abnormalities and chromosomal aberrations, as stated by V.R. Askari *et al.* [3]. Postovulatory ageing of oocytes is an inevitable process associated with the duration of their stay in culture after ovulation. A study by S. Fiorito [4] demonstrates that the duration of oocyte storage without fertilisation substantially affects their quality and potential for fertilisation.

Recent studies confirm that oxidative stress negatively affects the metabolic processes that affect the functioning of oocytes, and further research is needed to protect them [5]. As noted by Z. Tayarani-Najaran *et al.* [6], in assisted reproductive technologies actively used for infertility treatment, oocyte manipulation is critical, as the technical aspects of the procedure can expose oocytes to additional stress and increase oxidative damage. This highlights the importance of using antioxidants that reduce reactive oxygen species levels, protect oocytes from oxidative stress, and improve their survival and quality, as indicated by H. Kobayashi *et al.* [7]. The results of such studies can substantially affect clinical practice in the field of reproductive medicine and open up new prospects for improving the quality of germ cells and the prognosis of reproduction, as emphasised by M. Igase *et al.* [8]. S. Galluzzi *et al.* [9] demonstrated that the examination of the effects of auraptin is an important step in the search for new therapeutic approaches to improve the success of assisted reproductive technologies.

Assisted reproductive technologies *in vitro* can lead to an increase in oxidative stress due to the technical features of manipulations and the lack of endogenous protection systems. Oxidative damage can cause oocyte apoptosis, which negatively affects the fertilisation process and embryo development. In this context, the use of antioxidants can be a safe and effective strategy for slowing or preventing oocyte ageing by reducing oxidative stress. Understanding the mechanisms by which antioxidants act may provide new insights into their potential to improve germ cell quality. Among the many antioxidants examined for their effects on oocytes, auraptin (7-geranyloxy coumarin) stands out as a promising candidate. This naturally occurring biologically active compound, derived from citrus fruits, has a wide range of pharmacological properties, including antioxidant, anti-inflammatory, antitumour and neuroprotective effects. Auraptin can protect cells from oxidative stress by reducing reactive oxygen species and restoring normal mitochondrial function. However, despite these promising

data, there are not enough studies evaluating the effect of auraptin on postovulatory ageing of oocytes and embryo development *in vitro*. The study aimed to investigate the effect of antioxidants on the morphological and functional properties of oocytes in rats under conditions of systemic inflammation.

Literature Review

Oxidative stress is an important aspect in reproductive medicine research because it directly affects the quality of germ cells. V. Di Nisio *et al.* [10] note that postovulatory ageing of oocytes can impair their quality, making it difficult to fertilise and develop embryos both *in vivo* and *in vitro*. C. Yu & J.-H. Xiao [11] draw attention to the Keap1-Nrf2 system, which may be a target for therapy that increases antioxidant protection in older women. K.-H. Kim *et al.* [12] established that GAS6 improves mitochondrial function in old oocytes by reducing meiotic defects, potentially improving germ cell quality. A. Almansa-Ordóñez *et al.* [13] focus on the effect of oxidative stress on the energy metabolism of oocytes, which negatively affects their viability. M. Khazaei & F. Aghaz [14] showed that antioxidants during *in vitro* oocyte maturation reduce oxidative stress, improving their quality, which is critical for the success of reproductive technologies. A. Cecchele *et al.* [15] consider the cellular and molecular nature of fragmentation of human embryos. Their study highlights that fragmentation may be the result of oxidative stress affecting both oocytes and embryos, suggesting the need to develop new approaches to improve their quality.

Oxidative stress substantially affects the quality of oocytes and, in general, the reproductive health of women, especially in the context of polycystic ovary syndrome. M. Abizadeh *et al.* [16] investigated the possibility of using auraptin to improve oocyte maturation, increase fertilisation, and reduce inflammatory processes in a mouse model of polycystic ovary syndrome. The results of their study suggest the potential use of auraptin as a therapeutic agent to improve reproductive function. Y. Jang *et al.* [17] analysed the effect of auraptin on behavioural aspects in experimental models of Parkinson's disease, emphasising its ability to protect mitochondrial respiration from inhibition and reduce the level of reactive oxygen species. This opens up prospects for the use of auraptin not only in neurological disorders but also in the field of reproductive medicine because mitochondrial stress substantially affects the quality of oocytes. M.J. Lee *et al.* [18] established that auraptin promotes the activation of antioxidant enzymes and improves connectivity in brain endothelial cells, which helps reduce mitochondrial stress and enhance cellular function. This may be an important mechanism for improving oocyte maturity and functionality in the context of ageing. E.H. Hassanein *et al.* [19] examined the role of coumarins, such as auraptin, in regulating the Keap1/Nrf2/ARE pathway, emphasising the ability to stimulate antioxidant protection in cells, which can positively affect oocyte quality.

N. Akino *et al.* [20] investigate the effect of dimethyl fumarate on the activation of the Nrf2/Keap1 pathway, evaluating its ability to reduce oxidative stress and delay the development of age-related infertility in the ovaries of mice, emphasising the importance of antioxidant mechanisms for maintaining reproductive function with age. E. Fonseca *et al.* [21] investigate the effect of urolithin A on slowing the ageing of bovine oocytes *in vitro*, which can improve the quality of oocytes and increase reproductive efficiency in animal husbandry. Y. Furukawa *et al.* [22] show that aurapten stimulates the expression of brain neurotrophic factor in Neuro2a cells, indicating a possible neuroprotective effect and protection of oocytes from oxidative stress. G. Shimoi *et al.* [23] state that spindle destabilisation can cause aneuploidy in senescent oocytes, which is important for ensuring the quality of germ cells. D. Zhang *et al.* [24] emphasise the role of mitochondrial changes in oocyte ageing, which requires further research to understand these processes.

J. van der Reest *et al.* [25] emphasise the important role of mitochondria in oocyte ageing, noting that maintaining their activity is key to preventing age-related defects in oocytes. Oxidative stress substantially affects women's reproductive function. J. Lu *et al.* [26] consider the mechanisms of its negative effects, focusing on the violation of the balance between reactive oxygen species and antioxidant protection, which can lead to a deterioration in the quality of oocytes. A.T. Perkins *et al.* [27] investigate the role of superoxide dismutase in reducing meiotic errors in senescent oocytes, which highlights the prospects for antioxidant therapy for genetic stability. S. Armstrong *et al.* [28] discuss the importance of technologies such as time laps systems for monitoring embryos in reproductive procedures, which can increase the chances of successful fertilisation. C. Lagalla *et al.* [29] highlight the importance of atypical patterns of partial embryo compression, paying attention to their prevalence and possible influence on the results of reproduction. This study highlights the importance of analysing the morphokinetic features of embryo development in improving assisted reproductive technology protocols. The results of these studies confirm the importance of antioxidant therapy and the latest technologies in assisted reproductive procedures for improving the quality of oocytes and the overall effectiveness of infertility treatment. Given the role of oxidative stress, such approaches can be substantial components in preventing age-related changes in the female reproductive system.

Materials and Methods

The study was conducted by an author affiliated with Ukraine, with experimental work performed in collaboration with facilities in the Republic of Korea. The study used the female rats at three weeks of age purchased from Koatech, which is located in Phentek, Gyeonggi Province, Republic of Korea. The animals were kept in appropriate conditions at the Cha University Laboratory Animal

Research Centre, where a standard temperature regime and humidity level were maintained. The rats were in conditions that mimic the natural rhythm, in particular, the 12-hour cycle of light and dark, which contributes to the normalisation of physiological processes in the animals. 10 IU of human chorionic gonadotropin was administered to stimulate superovulation, which was purchased from Sigma-Aldrich, St. Louis, Missouri, USA. After the preparatory stage, which included anaesthesia, an abdominal autopsy was performed and oocytes were removed from the ovaries and isolated from follicular cells using the hyaluronidase enzyme (Sigma-Aldrich). Isolation ensured the purity of the samples for further analysis, eliminating follicular cells that could affect the results of the study.

Immunofluorescence staining was used for detailed analysis of morphological changes in oocytes. Oocytes were treated with a phosphate buffer with the addition of 0.1% polyvinyl alcohol, which prevented precipitation and ensured the uniform application of the solution to the samples. The samples were then fixed in a 4% paraformaldehyde solution, which stabilised the cell structures for further processing. For the purpose of permeability, a solution of Triton X-100 (0.2%) was used, which facilitated the penetration of antibodies into intracellular structures. Incubation with the primary antibody against α -tubulin (Santa Cruz Biotechnology) was conducted at a concentration of 1:100 at a temperature of 4°C overnight, which provided high specificity and sensitivity of staining. After thorough washing of the samples, they were treated with a secondary antibody obtained from goat anti-mouse IgG, labelled with Alexa Fluor 488 fluorescent dye at a concentration of 1:100. The dye 4',6-diamidino-2-phenylindole (Sigma-Aldrich) was used to visualise the nuclear material, which clearly stained the nuclei. The image was obtained using a Leica TCS SP5 II confocal microscope (Germany), which allowed obtaining high-quality images with high resolution for further analysis.

The functional state of mitochondria was evaluated using JC-1 dye, which allowed determining the membrane potential of mitochondria ($\Delta\Psi_m$). Oocytes were incubated in M16 medium with the addition of JC-1 (1 $\mu\text{g}/\text{ml}$) at 37°C for 30 minutes, which contributed to the maximum accumulation of dye in mitochondria. After incubation, fluorescent signals in the red and green channels were analysed to determine the relative membrane potential. Reactive oxygen and glutathione levels were determined using the OxiSelect™ kit (STA-342, Cell Biolabs, San Diego, USA) and CELLTRACKER™ blue CMF2HC dye (Invitrogen), which provided high sensitivity and specificity in the measurements. Oocytes were cultured in M16 medium with the addition of dichlorodihydrofluorescein diacetate for 30 minutes, after which detailed analysis was performed using confocal microscopy to assess the fluorescent signal indicating the level of oxidative stress in the samples.

All experiments were conducted in accordance with international ethical standards, including the animal research guidelines: Reporting of *In Vivo* Experiments [30]

and Recommendations of the Cha University Ethics Committee [31] aimed at the humane treatment of laboratory animals and minimising their suffering.

Results and Discussion

Antioxidants were identified to substantially improve reproductive health by reducing the harmful effects of oxidative stress. Oxidative stress occurs due to an imbalance between the production of free radicals and antioxidant protection, which causes damage to cells, including oocytes, and can negatively affect their quality and ability to fertilise. For example, vitamins C and E, and substances such as coenzyme Q10, can neutralise free radicals, improving the functioning of cell membranes and maintaining their integrity. This is extremely important for oocytes, which are vulnerable to oxidative damage because they contain a substantial amount of unsaturated fatty acids and other components that are subject to oxidation. In addition, this study examined the effect of antioxidant therapy on oocyte apoptosis processes. Inflammation can stimulate signalling pathways that trigger the cell death programme, leading to the loss of functional oocytes. The use of antioxidants can have a protective effect, preventing the activation of these pathways and maintaining cell viability.

Figure 1 shows a comparison of ovarian weight in three groups: the control group, the group with induced systemic inflammation, and the group receiving antioxidants. In the control group, the ovarian mass was 0.45 g, which indicates a normal state of the reproductive system. In the group with

induced inflammation, the ovarian mass decreased to 0.3 g, which indicates ovarian atrophy under the influence of an inflammatory process that disrupts endocrine regulation, which is critical for the proper functioning of the ovaries. This weight loss reflects a decrease in the number of follicles and a deterioration in their morphology and functionality, which can substantially affect the quality of oocytes. In the group that received antioxidant therapy, ovarian weight increased to 0.4 g, which demonstrates the protective effect of antioxidants. Restoring ovarian weight is vital to maintaining reproductive function, as positive dynamics indicate the ability of antioxidants to prevent atrophic changes in the ovaries, maintaining their morphological integrity and functional state.

Figure 2 describes in detail the process of nuclear maturation of oocytes, including the stages of metaphase and anaphase. In the control group, normal organisation of chromosomes was observed in oocytes that were at the metaphase stage, indicating readiness for division. The chromosomes were correctly aligned on the equatorial plate, which is crucial for the successful division and further development of embryos. A substantial number of oocytes with abnormal maturation were found in the group with induced inflammation. They had an uneven distribution of chromosomes and a large number of oocytes with disorders in the organisation of the division spindle. This is a serious indicator of the negative impact of the inflammatory process on the quality of oocytes, which can lead to a decrease in their ability to fertilise.

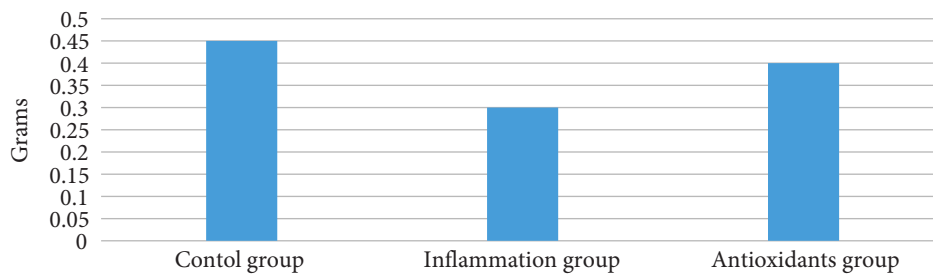


Figure 1. Comparison of rat ovarian mass by group

Source: compiled by the author

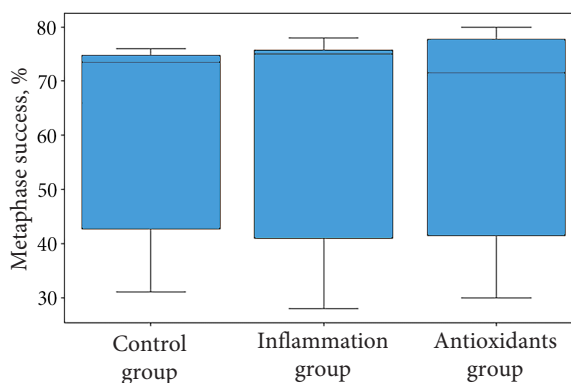


Figure 2. Comparison of the proportion of oocytes in metaphase between groups

Source: compiled by the author

In the antioxidant-treated group, there was a substantial improvement in the morphological and functional parameters of oocytes. The proportion of oocytes that successfully achieved the correct metaphase increased to 75%. This indicates a positive effect of antioxidants on the maturation process of oocytes, which is important for ensuring their ability to fertilise. Reducing the frequency of abnormal oocytes in this group highlights the effectiveness of antioxidant therapy in maintaining normal oocyte maturation, which contributes to the preservation of their reproductive function. The quality of oocytes that mature is an important factor for successful fertilisation and further development of embryos, so the results of the study are of great scientific and practical importance. In addition,

a detailed analysis of the ability of oocytes to fertilise and further develop embryos was conducted as part of the study. As shown in Figure 3, in the control group of untreated rats, the fertilisation rate was relatively high, with the median rate approaching 80%. This indicates a favourable oocyte quality that supported successful fertilisation. However, in the group of rats with systemic inflammation, the fertilisation rate was significantly reduced, with the median rate around 30%, reflecting a marked decrease in oocyte quality likely due to inflammation-induced oxidative stress. In the group receiving antioxidant therapy, the fertilisation rate improved, with a median rate of approximately 65%, indicating a protective effect of antioxidants in enhancing oocyte quality and fertilisation success.

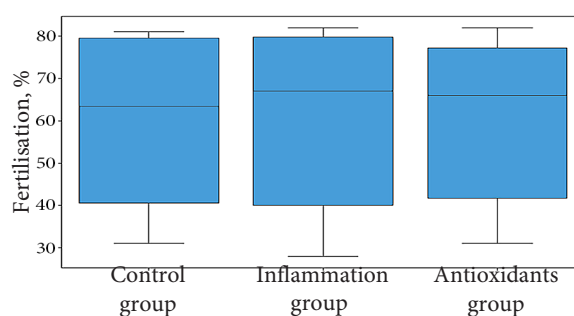


Figure 3. Comparison of fertilisation success between groups

Source: compiled by the author

In the group that received antioxidant therapy, the fertilisation rate increased to 65%. These results indicate that antioxidants have a pronounced positive effect on reproductive performance, as they contribute to improving the quality of oocytes and their ability to fertilise. The improvement in this indicator can be explained by an improvement in the morphological characteristics of oocytes, as well as an increase in their resistance to stressful conditions caused by the inflammatory process. It is important to emphasise that antioxidants not only protect oocytes from oxidative damage but can also modulate the mechanisms that regulate apoptosis and inflammation, thereby reducing the risk of loss of viable oocytes. Thus, the results of the study point to the important role of antioxidants in maintaining reproductive health. Antioxidant therapy has demonstrated the ability not only to improve the quality of oocytes but also to increase their reproductive properties. This is of great clinical importance, as it opens up opportunities for creating new therapeutic strategies aimed at treating infertility associated with inflammatory conditions. The study highlights the role of antioxidants as possible protectors of oocytes against damage caused by oxidative stress and inflammatory processes, which is crucial for maintaining their structure and functionality.

The study by M. Khazaei & F. Aghaz [14] emphasises the importance of antioxidants during pregnancy, which correlates with the results obtained, which indicate the ability of antioxidants to reduce the risk of inflammatory

processes that can negatively affect reproductive health. The collected data indicate that the use of antioxidants may have a positive effect on pregnancy outcomes, reducing the risk of complications, which is confirmed by a decrease in inflammatory markers in animals treated with antioxidant therapy. A paper of C. Lagalla *et al.* [29] indicates that the parallels with the effects of antioxidants at the cellular level demonstrate their ability to reduce oxidative stress and improve the overall health of organisms. This confirms the effectiveness of antioxidant strategies in improving reproductive health and preventing the negative effects of inflammatory processes in various biological systems. A study by S. Galluzzi *et al.* [9] shows that citrus phytonutrients have a positive effect on cognitive and biological processes, especially in the context of age-related cognitive decline, which confirms the importance of natural antioxidants in reducing oxidative stress. K.-H. Kim *et al.* [12] established that the GAS6 protein is able to improve mitochondrial function and prevent meiosis defects in oocytes in older mice, which indicates the prospects for using antioxidant therapies to improve oocyte quality in the age group. A paper of Y. Jang *et al.* [17] emphasises the protective properties of auranthol, which reduces oxidative stress and prevents inhibition of mitochondrial respiration, which also confirms the positive effect of antioxidants in various biological systems. Lastly, N. Akino *et al.* [20] indicate activation of the Nrf2/Keap1 pathway, which reduces oxidative stress and may delay age-related infertility in mice after the

administration of dimethylfumarate. This supports the conclusion that antioxidant therapy can have a positive effect on reproductive health by reducing oxidative stress and improving oocyte quality.

The results of this study highlight the potential of antioxidants as therapeutic agents to improve oocyte quality and women's reproductive health. The use of antioxidants can be an important step in creating new approaches to infertility treatment, especially in women with concomitant inflammatory diseases. The key aspect is the determination of optimal doses and methods of administration of antioxidants, which requires further study. Thus, the results of the study not only deepen understanding of the role of antioxidants in reproductive medicine but also open up new opportunities for clinical applications in improving women's reproductive health.

Conclusions

The results of the study showed that the use of antioxidants helps to reduce the effects of oxidative stress, which is key to normalising the process of oocyte maturation, in particular, in conditions when this process is disrupted due to inflammation. Under conditions of inflammation, oocyte maturation is substantially disrupted, particularly the abnormal division of chromosomes and the violation of the organisation of the division spindle. Antioxidants, in turn, can help reduce the frequency of abnormal oocytes and improve their ability to fertilise. The use of antioxidant therapy

helped restore ovarian mass, which decreased in the group with induced inflammation, which indicates its protective effect. In the group that received antioxidants, there was an improvement in the morphological and functional parameters of oocytes – an increase in the proportion of oocytes that successfully achieved the correct metaphase (up to 75%).

Antioxidant therapy improved the ability of oocytes to fertilise, which was demonstrated by an increase in the proportion of successful fertilisation to 65%. Prospects for further research should focus on investigating the mechanisms of action of antioxidants at the cellular level, which will allow for a better understanding of their effect on reproductive function. Future studies can also analyse the effectiveness of various antioxidant compounds, including natural and synthetic ones, and their combinations, which may contribute to the development of new therapeutic strategies for preventing reproductive disorders. The use of additional models and experimental conditions will allow evaluating a wide range of antioxidant effects, which will expand knowledge in this important area and open up new opportunities for the clinical use of antioxidant therapies in medicine.

Acknowledgements

None.

Conflict of Interest

None.

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Вплив антиоксидантів на морфологію та функцію ооцитів у щурів при системному запальному процесі

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Анотація. Дослідження мало на меті оцінити вплив антиоксидантів на морфологічні та функціональні властивості ооцитів у щурів в умовах системного запалення, зосереджуючись на їхньому потенціалі зменшувати окислювальний стрес і покращувати репродуктивне здоров'я. Як експериментальні моделі було використано тритижневих самок щурів, яких розділили на три групи: контрольну; групу, викликану системним запаленням; групу, що отримувала антиоксиданти. Для стимуляції суперовуляції вводився хоріонічний гонадотропін людини, потім було виділено ооцити для аналізу. Морфологічні зміни в ооцитах оцінювалися за допомогою імуофлуоресцентного фарбування, тоді як функцію мітохондрій та окислювальний стрес оцінювалися за допомогою набору для виявлення барвника JC-1. У групах порівнювалися рівень запліднення та стадії дозрівання ооцитів. Усі експерименти відповідали міжнародним етичним стандартам досліджень на тваринах. Системне запалення призвело до атрофії яєчників, зменшення маси яєчника до 0,3 г, що відображає зменшення кількості фолікулів і якості ооцитів. Це призвело до зниження норми внесення добрив на 30 %. Антиоксидантна терапія допомогла відновити масу яєчників до 0,4 г, покращивши морфологію ооцитів і збільшивши частку ооцитів, які досягли метафази, до 75 %, демонструючи захисну дію антиоксидантів. Коефіцієнт запліднення підвищився до 65 % у групі, яка отримувала антиоксиданти, що вказує на захисний ефект проти окисного пошкодження та запалення. Отримані дані підкреслюють потенціал антиоксидантів у модулюванні шляхів апоптозу, зниженні окисного стресу та посиленні функції мітохондрій, що разом покращує якість ооцитів і репродуктивні результати. Антиоксидантна терапія продемонструвала значні переваги в пом'якшенні впливу окислювального стресу та запалення на ооцити, сприянні нормальному дозріванню та збільшенню успіху запліднення. Ці результати підкреслюють терапевтичний потенціал антиоксидантів для лікування безпліддя, пов'язаного із запальними процесами

Ключові слова: післяовуляторне старіння; яйцеклітина; антиоксидативна речовина; окислювальний дисбаланс; цитрусові нутрієнти