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EFFECT OF RESVERATROL TREATMENT ON THE FEMALE REPRODUCTIVE FUNCTION UNDER CONDITIONS OF EXPERIMENTAL GLOMERULONEPHRITIS

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ABSTRACT

Premature ovarian failure affecting women under 40 years of age, is actively studied. This disease is becoming widespread due to the delay in maternity and is currently a medical and social problem. Until now, it is unclear whether the development of the autoimmune process is a primary cause of this disease or it is the result of chronic pathology. Glomerulonephritis represents a serious problem for reproductive health of women. The reproductive function may be affected by both, the glomerular disease itself and glucocorticoid and cytostatic therapy. Among the known antioxidants, resveratrol has received numerous approvals when used in various disease patterns, including oocytes. The effect of resveratrol on reproductive function in women under conditions of experimental glomerulonephritis has not been studied yet, which makes this research relevant today. The aim of the given study was to estimate the effect of resveratrol treatment on the female reproductive function under conditions of experimental glomerulonephritis. Experiments have been conducted on white laboratory mice: females and males. Experimental glomerulonephritis was achieved by immunization of mice of the first generation with a kidney antigen suspension derived from a parent. Resveratrol (50 mg/kg, Sigma-Aldrich, USA) was injected intraperitoneally 4 times. It has been shown for the first time that under conditions of experimental glomerulonephritis the treatment of resveratrol increase meiotic maturation, decrease apoptotic and necrotic death of the follicular environment of oocytes and a decrease of the post-implantation mortality rate of embryos in mice.

KEYWORDS: Experimental glomerulonephritis, autoimmune process, oocyte meiotic maturation, apoptosis, necrosis, resveratrol.

INTRODUCTION

Recent studies prove systemic inflammation to be a factor in autoimmunization and reproductive disorders in women.[1,2] Evidence show that oxidative stress is involved in reproductive disorders in women, including infertility, miscarriage, fetal growth retardation and premature labor, while excess of active forms of oxygen can lead to damage to proteins, lipids and DNA of cells.[3,4]

Among the known antioxidants, resveratrol has received numerous approvals when used in various disease patterns, including oocytes.[5-10]

Previously, under conditions of experimental glomerulonephritis (EG) it was investigated the oocyte meiotic maturation, the viability, and the DNA single-strand breaks of cells of the follicular environment of oocytes (FEO), thymic cells and lymph nodes cells. It was found that experimental immune glomerulonephritis occurs: oocytes damage, namely the suppression of meiotic maturation, reduction of the number of living cells of lymph nodes, thymic and FEO and nuclear DNAdamage of the FEO, thymic and lymph nodes cells.[11]

In current studies the effect of resveratrol on female reproductive function under conditions of EG has not been previously studied, which makes its investigation relevant today.

The aim of the given study was to estimatethe effect of resveratrol treatment on the female reproductive function under conditions of EG, namely the process of oocyte passage of meiotic maturation stages - metaphase I and metaphase II, on the viability and integrity of DNA of cells of the FEO as well as pre- and post-implantational embryonic mortality.

MATERIALS AND METHODS

Animals. Experiments (two series) have been conducted on white laboratory mice: 68 females (10 weeks, 20-22

g) and 12 males (25 weeks, 25-27g) in compliance with all requirements for work with laboratory animals (International European Convention for the Protection of Vertebrate Animals, Strasbourg, 1986). After the experiments, anesthetized by Nembutal animals were exterminated by cutting the spinal cord.

Experimental glomerulonephritis in mice was achieved by immunization of white laboratory mice of the first generation with a kidney antigen suspension derived from a parent. Animal immunization was carried out at the rate of 10 mkL of suspension per 10 g of body weight according to the following scheme: 3 times intra-abdominal 1 time per day; re-immunization was carried out after 3 weeks with a single intra-abdominal treatment of the same dose. Before the start and during the experiment, the animals were assessed by the objective status (appearance, general motor activity, need for food and water, body weight was checked 2 times a week) and the excretory kidney function (based on the number of spontaneous urinary tract disorders per day). Urine samples were determined using protein strips using a single dose of urine (diagnostic test strips for fast detection of protein, "Pharmaco", Ukraine).

In the ***first series*** animals were divided into the following groups
I- Control animals (N=8), treated with physiological solution (0.3 mL);
II - Animals under conditions of EG (immunized with an antigenic kidney suspension) (N=8);
III - Animals under conditions of EG (immunized with an antigenic kidney suspension) were treated with resveratrol (N=8);
IV - Animals under conditions of treatment with resveratrol (N=8).
N - Number of animals in the group.

The experimental material (ovaries) were taken under anesthetic anesthesia on the third day (after the last injection).

In the ***second series*** animals were divided into the following groups:
I - Control animals (N=6), treated with physiological solution (0.3 mL);
II - Animals under conditions of EG (immunized with an antigenic kidney suspension) (N= 12);
III - Animals under conditions of EG (immunized with an antigenic kidney suspension) were treated with resveratrol (N=12);
IV - Animals under conditions of treatment of resveratrol (N=6).

One day after the last injection, males were planted to females in a ratio of 1:3. Couple of male and female and further manipulation with the embryos were performed according to Mank.[12]

The experimental materials were collected (ovaries, tubes and uterus) under anesthetic anesthesia on the 10^{th} and 18^{th} days after males were planted to female.

The study was completed (24/25 days after males were planted), with birth live pups in control and experimental groups of animals.

The treatment was carried out in the following way: Kidney antigen suspension – intraperitoneal three times 1 time per day; the procedure was repeated in 3 weeks, one time intraperitoneally with the same dose (10 mkL of suspension per 10 grams of body weight of the animal). Resveratrol (R5010, Sigma-Aldrich, USA) was introduced intraperitoneally 3 times: 1 time per day for 1 hour before immunization of animals with suspension of kidney antigen; as well as in 3 weeks once with the same dose (50 mg/kg, 0.3 mL).

Oocytes cultivation. The oocytes were mechanically isolated from the ovaries of mice in a non-enzymatic way and units/one ovaries were counted. Then, oocytes from one group were collected and distributed into separate chambers 10-20 oocytes each. All control and experimental oocytes were cultured under the same conditions (a sterile box, cameras with 0.4 ml culture medium DME and 15 mM HEPES, Ca^{2+} concentration of 1.71 mM, temperature 37° C, duration 20 hours). Morphological study of oocytes was performed under a microscope MBS-10 after 2 hours of cultivation (% of total): the oocytes which restored the meiotic maturation (resumption meiosis) and were at metaphase I stage (germinal vesicle break-down) and after 20 hours of cultivation oocytes with the first polar body (were at metaphase II stage) were counted.

Method color fluorescent dyes. The apoptotic and necrotic death of cells of FEO was estimated by morphological characteristics using the method of in vivo dual-color fluorescent dye nucleic acids Hoechst 33342 and propidium iodide.[13] Morphological studies were performed using a fluorescent microscope LUAMAM I-1 (Russia) with water-immersion at x85. There has been used a video system sending the image from the microscope to the computer. The percentage of the living, apoptotic and necrotic cells has been determined by counting at least 1200 cells.[13]

The method of DNA comets (alkaline). To detect DNA single-strand breaks of cells of FEO, the method of alkaline gel electrophoresis of isolated cells was used (DNA-comet assay method).[14]

Electrophoresis of preparations (after stabilization for 20 minutes in alkaline electrophoresis buffer) was performed using the device MultiphorII ("LKB", Sweden) at a voltage of 24 V and a current of 100 mA for 30 minutes. The DNA comet analysis on electro-phoregrams painted by Hehest 33342 (700 μmol/L) was performed visually using a luminescent microscope

LUAMAM I-1 (Russia) using a water-immersion lens (×30). Each micropreparate was analyzed up to 400 separately located DNA comets. By the ratio of DNA in the "head" and "tail" comets were divided into 5 classes (0-4).[15]

Pre- and post-implantation embryonic mortality. Female control and experimental groups crossed with intact males. Counted: A - number of live embryos; B - number of seats of resorption (number of dead embryos); C - number of *corpora lutea* of pregnancy. Indicators of pre-and post-implantation death was calculated using the formula: ((C-A+B)/B)•100% and (B/(A + B))•100%.

Statistical analysis. For the statistical analysis of the results the software package Graph Pad Prism version 5.00 for Windows (Graph Pad Software, San Diego California, USA) have been used. The verification of the received data on normality of distribution was carried out on the Kolmogorov-Smirnov test. For a normal distribution, the statistical processing of the results when comparing the two data groups was performed using Student's t test, with more data groups using a single-factor ANOVA analysis, followed by a comparison of mean values between the groups according to the Newman-Keuls post hoc test; p <0.05 was considered statistically significant.

RESULTS

Data about the number of oocytes that restored meiotic maturation (Metaphase I) and formed the first polar body (Metaphase II) under conditions of EG and treatment of resveratrol are presented in Fig.1.

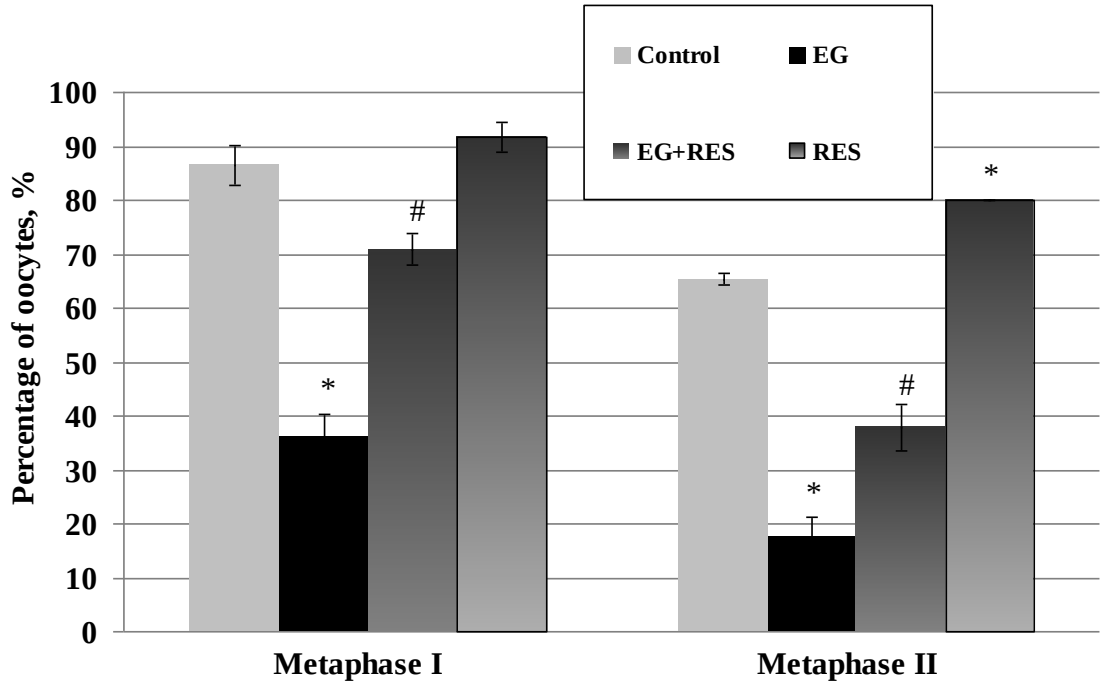

Fig. 1: The effect of resveratrol treatment on oocyte meiotic maturation under conditions of EG (n=8).

Notes: * p <0.05 - probability differences in the average group data with respect to these variables in the control group animals; # p <0.05 - to these variables in the group animals under conditions of EG. RES-resveratrol, n - the number of independent repetitions.

Thus, under conditions of EG and resveratrol treatment leads to an increase in the number of oocytes that restored meiotic maturation (Metaphase I) and formed the first polar body (Metaphase II).

Data about the number of cells of FEO with morphological signs of apoptosis and necrosis under conditions of EG and resveratrol treatment are presented in Fig.2.

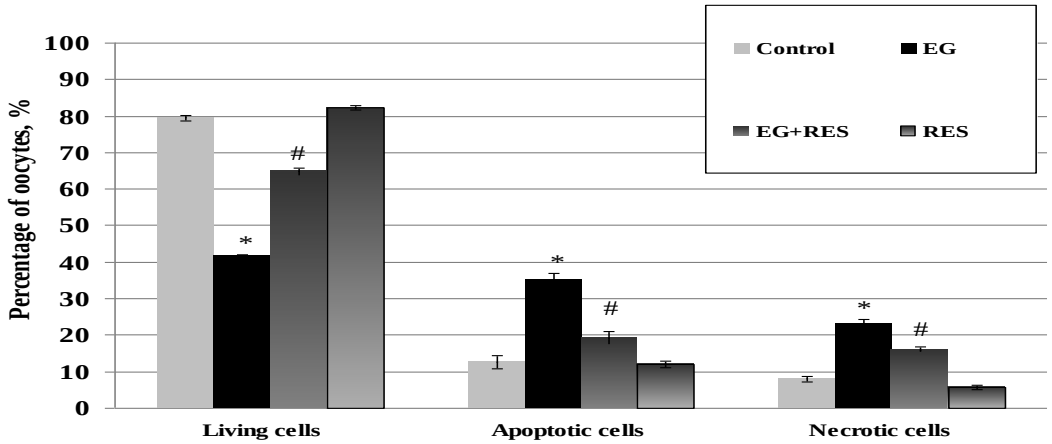

Fig. 2: The number of cells of FEO with morphological signs of apoptosis and necrosis under conditions of EG and treatment of resveratrol (n=8).

Notes: * p <0.05 - probability differences in the average group data with respect to these variables in the control group animals; # p <0.05 - to these variables in the group animals under conditions of EG. RES-resveratrol, n - the number of independent repetitions.

Thus, under conditions of EG and resveratrol treatment leads to an increase in the proportion of living and decrease in the proportion of apoptotic and necrotic FEO cells.

The data about the distribution of the DNA comets of FEO cells under the conditions of EG and resveratrol treatment are presented in Table 1.

Table 1: Distribution of DNA comets of nuclei of FEO cells under the conditions of EG and resveratrol treatment.

A group of animals	Classes of DNA comets (%)				
	0/1's	2's	3's	4's	0/1's
I (control) n=8	82,20±4,83	12,40±3,17	2,20±0,83	1,37±0,78	1,83±0,36
II (EG) n=8	1,20±0,57*	2,26±0,31*	4,20±1,18	19,80±1,14 *	72,54±5,32*
III (EG+ resveratrol) n=8	9,88±3,40 #	22,75±4,77#	32,88±3,87#	21,38±2,56	13,13±3,31#
IV (resveratrol) n=8	78,12±5,19	15,83±3,56	3,45±1,43	1,28±0,73	1,32±0,34
Notes: * p <0.05- relative to the corresponding values in the control; # p <0.05 - relative to the corresponding values under the conditions of EG; n - the number of independent repetitions.					

Thus, under conditions of EG and resveratrol treatment leads to reduction of the DNA damage FEO cells (due to the reduction of single-strand DNA breaks).

It has been established that the treatment of resveratrol does not affect the pre- and post-implantation mortality of embryos. Under conditions of EG and resveratrol treatment there were no effect on the volume of pre-implantation mortality of embryos but post-implantation embryonic mortality reduces to 2,63±0,91 (p<0.05, n=6), compared with 5,16±0,63 under the EG (Table. 2).

Table 2: Pre - and post - implantation embryonic mortality under the conditions of the of EG and the treatment of resveratrol.

A group of animals	Pre-implantation embryonic mortality, %	Post-implantation embryonic mortality,%
I (control), n=6	2,76±0,74	1,38±0,37
II (EG), n=6	2,88±0,43	5,16±0,63 *
III (EG+ resveratrol), n=6	3,33±0,37	2,63±0,91 #
IV (resveratrol) n=6	3,03±0,84	2,08±0,63
Notes: * p <0.05- relative to the corresponding values in the control; # p <0.05 - relative to the corresponding values under the conditions of EG; n - the number of independent repetitions.		

Thus, under conditions of EG and resveratrol treatment does not change the pre-implantation mortality but decreases the post-implantation mortality.

DISCUSSION

Premature ovarian failure (POF), a disorder of ovarian function affecting women under 40 years of age, is actively studied. This disease is becoming widespread due to the delay in maternity and is currently qualified to be a medical and social problem. According to contemporary research, in the POF development, the deletion of autoimmune pathology plays a leading role.[16,17]

Until now, it is unclear whether the development of the autoimmune process is the primary cause of this disease or it is the result of chronic pathology.[16,18]

Glomerulonephritis, in particular that of immune etiology, represents a serious problem for reproductive health of women. The reproductive function may be affected by both the glomerular disease itself as well as glucocorticoid and cytostatic therapy.[19,20] There is evidence of a significant percentage of preterm labor and perinatal fetal loss in patients with membranous glomerulonephritis and IgA-glomerulonephritis. Meanwhile, 90% of women with membranous glomerulonephritis give birth to healthy children.[21]

Previous research shows that under conditions of experimental immune glomerulonephritis it was found oocyte damage, increase of cell death of FEO, lymph nodes and thymic cells and DNA damage of target cells.[11] In this work we have established that under conditions of EG the treatment of resveratrol leads to an increase in the percentage of oocytes that successfully pass both phases of meiotic maturation, improves the parameters of cell viability- the proportion of living cells of the FEO increases, a quantity of the apoptotic and necrotic FEO cells decreases. Besides this we have obtained the decrease of DNA damage of FEO cells. There were no changes in pre-implantation, but post-implantation embryonic mortality decreases.

The data we receive is consistent with.[9,10,22-27] In addition, the data is consistent with to our findings obtained earlier, according to which under conditions of experimental immune complex-mediated failurethe treatment of resveratrol improves the parameters of meiotic maturation of oocytes, reduces the loss of FEO cells and damage to the DNA of their nuclei as well as the cells of the thymus and lymph nodes.[28,29]

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CONCLUSION

It has been shown for the first time that under conditions of experimental glomerulonephritis the treatment of resveratrol increase meiotic maturation, decrease apoptotic and necrotic death of the follicular environment of oocytes and a decrease of the post-implantation mortality rate of embryos in mice.

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