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مقاله پژوهشی

یافته‌های نوین در علوم زیستی

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اثر حفاظتی اسپیرولینا پلاتنسیس، بر تخمدان موش‌های تیمار شده با نانوذرات نقره

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چکیده. نانوذرات نقره بواسطه توانایی ایجاد سمیت در تخمدان‌ها، قادرند باروری زنان را تحت تأثیر قرار دهند. هدف از این مطالعه، بررسی نقش حفاظتی جلبک اسپیرولینا پلاتنسیس، در برابر سمیت ایجاد شده با نانوذرات نقره، در تخمدان موش‌های نژاد NMRI است. در این مطالعه، ۲۴ رأس موش نژاد NMRI به چهار گروه شش‌تایی تقسیم شدند: کنترل، نانوذرات نقره (۵۰۰mg/kg/day)، اسپیرولینا (۳۰۰mg/kg/day) و نانوذرات نقره + اسپیرولینا (با همان دوزهای تعریف شده). ۳۰ روز پس از تیمار دهانی از طریق گاواژ، پارامترهای بیوشیمیایی اندازه‌گیری شد و تخمدان‌های چپ از نظر استریولوژیکی بررسی شدند. در گروه نانوذرات نقره، مقدار ظرفیت آنتی‌اکسیدانی تام سرم، غلظت هورمونی، حجم جسم زرد و تعداد فولیکول‌های سالم در مقایسه با گروه کنترل، به‌طور معنی‌داری کاهش یافت (P<0/05). در گروه اسپیرولینا، غلظت مالون‌دی‌آلدئید و فولیکول‌های آترزی‌شده به‌طور معنی‌داری کمتر از گروه کنترل بود (P<0/05). میانگین حجم کل تخمدان، کورتکس، مدولا، انواع فولیکول‌ها و هسته آن‌ها، همچنین ضخامت منطقهٔ شفاف در هیچ‌کدام از گروه‌ها اختلاف معنی‌داری نسبت به گروه کنترل نداشت (P>0/05). اگرچه اسپیرولینا در گروه نانوذرات نقره + اسپیرولینا، نتوانست پارامترهای فوق را تا سطح گروه کنترل جبران کند، اما با کاهش استرس اکسیداتیو، توانست آسیب تخمدانی ناشی از نانوذرات نقره را به میزان قابل توجهی بهبود بخشد.

واژه‌های کلیدی. استرس‌اکسیداتیو، استریولوژی، فولیکول، منطقه شفاف، هورمون‌های جنسی

The protective effect of *Spirulina platensis* on the ovary of silver nanoparticle-exposed mice

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Abstract. Silver nanoparticles (Ag NPs) can affect female fertility because they can cause toxicity in the ovaries. The aim of this study is to see if *Spirulina platensis* (SP) can protect mouse from Ag NPs-exposed toxicity in its ovary. Twenty-four female Naval Medical Research Institute (NMRI) mice were divided into four groups (n = 6 per group): control; Ag NPs (500 mg/kg daily); SP (300 mg/kg daily) and Ag NPs + SP (With the same defined doses). 30 days after oral gavage treatment, biochemical parameters were measured and ovary compartments were estimated stereologically. The Ferric Reducing Antioxidant Power (FRAP) values, hormonal concentrations, corpus luteum volume, and the number of healthy follicles were all significantly lower (p<0.05) in the Ag NPs group compared with the control group. in the SP group, malondialdehyde concentration and atretic follicles were significantly lower (p<0.05) compared with the control group. There was no significant difference in the mean total volume of ovary, cortex, medulla, oocyte and its nucleus, and the thickness of the zona pellucida in any group. Although, SP in the Ag NPs + SP group cannot compensate the above parameters to the control level, it considerably improves ovarian damage caused by Ag NPs through reducing oxidative stress.

Key words. follicles, hormones, oxidative stress, stereology, zona pellucida

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INTRODUCTION

Nanotechnology is rapidly expanding today as a result of its wide range of applications in industry and science (Mafakheri et al., 2017). Silver nanoparticles (Ag NPs) are a type of material that is widely used in fields such as medicine, pharmacy, health care, agriculture, and industry (Zhang et al., 2016).

Ag NPs are usually smaller than 100 nanometers and contain 15 to 20 thousand silver atoms. In fact, these particles are produced in nanometer dimensions and remain in the same dimensions (Agarwal et al., 2013; Kumar et al., 2021). They show toxic effects on pathogens such as microbes, viruses and fungi (Ramezani et al., 2016).

Despite the widespread use of nanoparticles and the many efforts that have been made to demonstrate the desirable properties of Ag NPs in medicine, the biological effects of these particles on cells and organs are still not fully understood (Yavari et al., 2015). Ag NPs with a diameter of less than 50 nm can enter cells. After inhalation, ingestion, contact, or injection, these particles can deposit in the skin, lungs, or gastrointestinal tract, then move to secondary sites such as the liver, spleen, kidneys, muscles, brain, ovaries, and testes to cause toxicity in them (Seyedmehdi Shariatzadeh et al., 2016; Shojaeifard et al., 2017).

The use of Ag NPs in uncontrolled amounts can cause toxicity. This toxicity induces oxidative stress, which has detrimental effects on mitochondrial function. Furthermore, it accumulates in the cytoplasm and cell nucleus that causes cell damage (Seyedmehdi Shariatzadeh et al., 2016). The use of nanoparticles in home and industrial products is increasing rapidly. It is estimated that about 320 tons of Ag NPs are used in the world every year. As a result, the study of safety and awareness of the toxicity of them is essential to protect the environment and health of society (Sajid et al., 2014).

The ovary is one of the organs that Ag NPs induce toxicity in it. So, these particles can affect fetal development. As a result, it is important to study the effects of Ag NPs on the female reproductive system (Shojaeifard et al., 2017).

Spirulina platensis (SP) is a blue-green, photosynthetic, multicellular, filamentous, and microscopic alga that is grown in many tropical countries to feed humans and animals (about 2,500 tons per year). This product is used in industry, pharmaceuticals, food and chemicals due to its valuable nutrients (Evaz-Zadeh Samani et al., 2017; Mousavi Tometri et al., 2018).

SP contains of glucose, glycerol, sucrose, a variety of vitamins such as tocopherol, niacin, beta-carotene, thiamine, and cyanocobalamin, rich in protein, has eight essential amino acids in appropriate proportions and small amounts of sulfur-containing amino acids, rich in nucleic acid, rich in essential minerals such as magnesium, Potassium, iron, calcium, zinc, selenium and manganese. SP is the source of fatty acid, gamma-linolenic acid, and linoleic acid (Evaz-Zadeh Samani et al., 2017; fard et al., 2017; Mousavi Tometri et al., 2018). This alga contains antioxidant pigments such as carotenoids, Myxoxanthophyl, Zeaxanthin, Oscillaxanthin, and Diatoxanthin. SP is a rich source of the pigment Phycocyanin particularly, which is a very powerful antioxidant and has the property of scavenging free radicals (hydroxyl and peroxyl radicals) (Mousavi Tometri et al., 2018). Also, It contains of defense antioxidant system enzymes that play a key role in eliminating free radicals (Evaz-Zadeh Samani et al., 2017).

SP is able to create different biological functions such as eliminating free radicals and anti-cancer activity, anti-tumor, wound healing, increased immune system, blood glucose regulation, reduced kidney toxicity, increased intestinal lactobacilli, osteoarthritis and radiation protection, Obesity, zinc deficiency, and heart disease due to its antioxidant role (Glaser & Microsc, 2005; Goletiani et al., 2007; Grigorova et al., 2017).

This alga significantly reduced the level of oxidative stress by reducing lipid peroxidation and increasing glutathione. As a result, it can protect the ovaries against reactive oxygen species and reduce the level of free radicals (Mousavi Tometri et al., 2018).

In this study with ethical code, IR.ARAKMU.REC.1399.048, the protective role of *Spirulina platensis* in eliminating the harmful effects of Ag NPs on the ovaries of Naval Medical Research Institute (NMRI) mice has been considered.

MATERIALS AND METHODS

Laboratory animals: In this experimental study, 5-6 week old female NMRI outbred mice with a mean weight of 32 ± 4 g were used. Mice were obtained from Pasteur Institute of Tehran. One week before the experiment, they kept in the animal house of Arak University under standard conditions of light–dark cycles for 12 h at 21±2 ° C with free access to enough water and food. Female mice were divided into 4 groups (n=6) based on equal weight average and treated by gavage for 30 days:

Group 1: Control;

Group 2: Treatment with 500 mg/kg/day Ag NPs;

Group 3: Treatment with 300 mg/kg/day SP powder;

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Group 4: Treatment with Ag NPs + SP (with the same doses)

Preparation of treatment solutions: Ag NPs used in this study with a purity of 99.9% and a diameter of 20 nm were provided by Pishgaman Nanomaterials Company of Iran. The authenticity of these nanoparticles was confirmed by X-ray, Scanning Electron Microscope (SEM), and Transmission Electron Microscopy (TEM) by the company.

To prepare a solution of Ag NPs for the treatment of mice, first the concentration of 500 mg/kg/day Ag NPs (since doses more than 200 mg/kg Ag NPs cause free radicals in all tissues of the body and lead to cell damage (Ghorbani, 2014).) was calculated and weighed based on the weight of each mouse and then 100 µl of deionized water was added to it. After 10 minutes of vortex and 15 minutes of sonication, the solution was ready for treatment.

The SP powder used in this study was also obtained from Arian Gostar Research Services Company in Tehran. Its physicochemical compositions included 64% protein, 15% phycocyanin, 0.7% chlorophyll, 8.5% lipid, 6.9% moisture, and 4.2% ash. This powder was free of aerobic microorganisms, mold, yeast, Escherichia coli, Salmonella, and Staphylococcus.

To prepare SP solution, first the concentration of 300 mg/kg/day SP powder based on the weight of each mouse (Since the recommended dose for a 60 kg person is 1-11 g/day, and it has also been found that the use of 30% SP in the diet of mice for 13 weeks and 5% for 6 months has no adverse effect on mice (Jody Braverman, 2020; Yang et al., 2011).) was calculated. Then, 100 µl of deionized water was added to it and after 10 minutes of vortex, the desired solution was prepared for the treatment of mice.

It should be noted that the solution of SP and Ag NPs were prepared daily as needed.

Blood sampling and preparation of microscopic ovarian samples: After 30 days of treatment, a microscopic analysis of the predominant cell types obtained from vaginal smears was used to determine the estrous cycle phase of mice. Then, mice were anesthetized with Diethyl Ether and blood samples were taken from their hearts. After blood centrifugation (10 min, 13000g), the resulting blood serum was stored in a freezer at -80 °C for biochemical and hormonal studies. Left ovaries were fixed in Bouins solution for 24 hours for stereological studies (Sarma et al., 2020). Then the tissue passage steps including dehydration, clarification and penetration of paraffin on them were performed. At the end of the tissue passage, the ovaries were blocked, then 5 and 20 micron sections were prepared sequentially by Isotropic Uniform Random Sampling (IUR), respectively. 8-12 sections were selected by systematic random sampling method (k number) and stained by Hematoxylin and Eosin (H&E) method (Sayed Mohamad Ali Shariatzadeh et al., 2012).

Ovarian weight and percentage of body weight changes: The weight of the right ovaries was measured to compare the groups. The percentage of weight changes in mice were also calculated using the formula $\frac{w_2 - w_1}{w_1} \times 100$, where W_1 is the initial weight of the animal at the beginning of the experiment and W_2 is the weight of the animal at the end of the experiment (Khoushravesh et al., 2015).

Ovarian histological studies: For ovarian histological studies, in all four groups, the 5 micron sections were carefully examined using a magnification of 40× and the differences and textural similarities of the groups were recorded.

Ovarian stereological studies: Ovarian stereological examinations were performed according to the following methods:

Estimation of total volume of ovary, cortex, medulla and corpus luteum: The Cavalieri method was used to estimate total volume of ovary, cortex, medulla and corpus luteum; 10 sections were randomly selected of 5 microns thick sections from each ovary; Then a point probe was randomly placed on the image of the sections with a magnification of 40× and the points that coincided with the image of the ovary were counted (Figure 1) (Karbalay-Doust & Noorafshan, 2012).

The total volume of the ovary was calculated according to the following formula:

$$V_{total\ \ ovary} = \sum_{i=1}^{n} P \times a(P) \times T$$

$\sum_{i=1}^{n} P$ shows the sum of the counted points, T shows the distance of each section to the next selected section, and $a(P)$ shows the area of the point probe. Since the purpose of this method is to estimate the average actual volume of tissue, $a(P)$ was divided by the magnification square M^2 as follows (Chand & Legge, 2011):

$$a(P) = \frac{\Delta x \times \Delta y}{M^2}$$

Also, to estimate the volume of ovary compartment, first the volume density (V_v) for each ovary compartment was calculated as follows:

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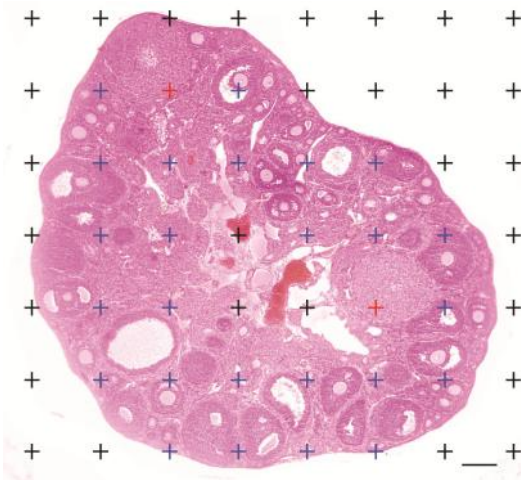

Figure 1. Estimation of the total volume of the ovary, cortex, medulla and corpus luteum based on Cavalieri method. The blue, black, and red dots on the ovary show the cortex, medulla, and corpus luteum, respectively, and the sum of all these dots represents the total volume of the ovary (H&E staining, magnification 40×) Scale bar = 300 µm

$$V_{v_{ovary\ compartments}} = \frac{\sum_{i=1}^{n} P_{ovary\ compartments}}{\sum_{i=1}^{n} P_{total}}$$

where $\sum_{i=1}^{n} P_{ovary\ compartments}$ is the sum of the points counted in each section and $\sum_{i=1}^{n} P_{total}$ is the sum of the counted points of the whole ovary. Then, by multiplying the volume density of each section in the total volume of the ovary, the total volume of each ovary compartment was obtained (Soleimani Mehranjani et al., 2010).

$$V_{cortex} = V_{total} \times V_{v_{ovary\ compartments}}$$

Calculation of coefficient error (CE) in volume estimation by Cavalieri method: The coefficient error for volume (V) estimation was obtained from the following equation on the basis that A is $\sum_{i=1}^{n} P^2$, B is $\sum_{i=1}^{n-1} P \times (P+1)$, C is $\sum_{i=1}^{n-2} P \times (P+2)$ and $\sum_{i=1}^{n} P$ is the sum of points in the counted sections (Glaser & Microsc, 2005).

$$CE(V) = \frac{\sqrt{\frac{3A + C - 4B}{3}}}{2 \times \sum_{i=1}^{n} P}$$

It should be noted that when the CE is less than 0.05% it is an excellent criterion and between 1-20% is an acceptable criterion. If CE is more than 20%, re-sampling is needed for more accuracy (Dezfulian & Shariatzadeh, 2006).

Estimation of the average number of ovarian follicles: Optical disector method was used to calculate the number of follicles in the ovary. Also, mikrokator and special counting frame were used. 20 micron thick sections were used to calculate the number of different types of follicles due to their different sizes; In this way, 10 sections were randomly selected. Then the field of view of each section was examined with 100× objective.

130 follicles were counted according to the counting method. In the counting frame, follicles were selected whose nuclei were inside the counting frame or on acceptable lines, and follicles that were in contact with the forbidden lines were not counted (Figure 2).

Also, atretic follicles were identified by the appearance of two or more of the following factors: cell debris within the antral cavity, more than two pyknotic nuclei in the granulosa cell layer, degenerating oocyte, granulosa cells pulling away from the basement membrane, swelling in the theca cells, and degenerated zona pellucida.

Thus, the number of follicles ($\sum_{i=1}^{n} Q$) in the disector height (h) and the area of each frame (a/f), and the number of frames ($\sum_{i=1}^{n} P$) were counted. Then, according to the formula ($N_v = \frac{\sum_{i=1}^{n} Q}{a/f.h.\sum_{i=1}^{n} P}$) the numerical density of the follicles (N_v) was estimated.

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Multiplying N_v by the total volume of the ovary, the total number of primordial, primary, secondary and mature follicles was estimated as follows (Sayed Mohamad Ali Shariatzadeh et al., 2012):

$$N_{total} = N_v \times V_{ovary}$$

Estimation of the mean oocyte volume and its nucleus in different types of ovarian follicles: Nucleator method was used to calculate oocyte volume and its nucleus. In this method, each oocyte whose nucleus reaches maximum focus while moving in the optical incision depth in the counting frame was selected.

According to Figure 3, the calculation of oocyte volume and its nucleus was performed using Motic 2000 images according to the following formula (Sayed Mohamad Ali Shariatzadeh et al., 2012):

$$V_n = \frac{4}{3}® \times \overline{L_n^{\,3}}$$

where L_n is the distance from the nucleolus center to the oocyte membrane or the nucleolus center to the nucleus membrane.

Estimation of the zona pellucida thickness: To calculate the zona pellucida thickness, 10 sections were randomly selected from 5 micron thick sections. Then all fields of every 10 sections were examined with 100× objective and the selected follicles (secondary and mature) were photographed. It should be noted that for the ovaries of each mouse, an average of 100 to 200 measurements were made to calculate the harmonic mean thickness of the zona pellucida. To measure the thickness, according to Figure 4, the probe for measuring the thickness was placed on the image without bias, and from the intersection of the probe lines with the inner surface of the zona pellucida, a vertical line is considered, tangent to the outer surface and its length is provided by Motic software was measured. These measurements were denoted as Orthogonal Intercept (Oi) and were given in the harmonic formula as follows (Sayed Mohamad Ali Shariatzadeh et al., 2012; Soleimani Mehranjani & Mansoori, 2016):

$$\text{Harmonic mean layer thickness} = \frac{8}{3}\pi \frac{\text{number of measurments}}{\text{sum of the reciprocal of orthogonal intercepts lengths}}$$

Afterwards, from the above formula, the average thickness of the zona pellucida was obtained.

Biochemical studies: Serum of mice was used to measure malondialdehyde (MDA) and the Ferric Reducing Antioxidant Power (FRAP), as follows:

Measurement of MDA concentration: This was done using the Buege and Aust methods. In this method MDA molecules react with Thiobarbituric acid (TBA) in acidic conditions and at high temperatures to produce a combination of MDA-TBA with purple dye that absorbs rays with a wavelength of about 532-535 nm. To do this, 200 µl of TCA-TBA-HCl reagent was first added to 100 µl of serum. The samples were centrifuged at 1000 g for 10 minutes after boiling for 15 minutes at boiling temperature. The supernatant created was read at 532 nm by the spectrophotometer. Finally, the concentration of MDA in the sample was expressed using the extinction coefficient (Shahsavari et al., 2015).

Measurement of FRAP concentration: This was provided by Benzey et al. In this method, the ability of plasma to reduce ferric ions is measured. At acidic pH, when the Ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the form of Fe^{2+}, a blue dye is produced which has a maximum absorption at 593 nm (Gupta et al., 2019). To perform this method, FRAP reagents including HCl, TPTZ and FeCl3 were prepared. Then 1.5 ml of reagent was added to 50 µl of serum. After 4 minutes, at a temperature of 37 °C, the supernatant was read at 593 nm by a spectrophotometer. Finally, the concentration of each sample was obtained by a regression equation obtained by adsorption at specific concentrations of ferrous sulfate (Piroozi et al., 2013).

Hormonal tests: Serum from proestrus-phase mice was used to assess the sex hormones, Estradiol (E2), progesterone, Luteinising hormone (LH), and Follicle-Stimulating Hormone (FSH), as follows:

E2 and progesterone hormones: E2 and progesterone ELISA kit, based on competitive enzyme immunoassay, from the Monobind company (USA) with a sensitivity of 8.2 pg/ml and a range of 10-4300 pg/ml for E2 and a sensitivity of 0.105 ng/ml And a range of 0.15–128 ng/ml was prepared for progesterone. The E2 and progesterone hormones in the samples compete with enzyme-bound E2 and the progesterone to bind to the coated antibody on the wells. After the incubation time, the wells were drained and washed. The enzyme substrate was then added to each well. Enzyme activity is inversely proportional to the concentrations of E2 and progesterone in the samples. Finally, based on the standard light absorption curve for specific concentrations, the concentrations of E2 and progesterone were obtained (Goletiani et al., 2007).

LH and FSH hormones: LH and FSH ELISA kits based on sandwich enzyme immunoassay. These hormones were prepared from Padten Gostar Isaar Company (Iran) with a sensitivity of 1 mIU/ml and a range of 0.9-60.4 mIU/ml for LH and 0.5-44.0 mIU/ml for FSH. The LH and FSH in the samples bind to their two specific antibodies as antigens. Both

are mouse monoclonal antibodies, one coated on the solid phase (wells) and the other bound to the Horseradish peroxidase (HRP) enzyme. The test sample, which contained the hormone, was exposed to two antibodies. After incubation, the wells were drained and flushed to remove antibodies bound to the excess HRP enzyme. The enzyme substrate was then added to each well. Enzyme activity is directly proportional to the concentration of FSH and LH in the samples. Finally, based on the standard light absorption curve for certain concentrations, the concentration of the samples was obtained (Nedresky & Singh, 2020).

Data analysis: The obtained data were statistically analyzed using Statistical Package for Social Sciences (SPSS) software (version 26, IBM, USA), one-way analysis of variance and Tukey test, and the difference between the means was considered significant at the level of $P < 0.05$. Kolmogorov-Smirnov test was used to check the normality of data distribution (SMA Shariatzadeh et al., 2020).

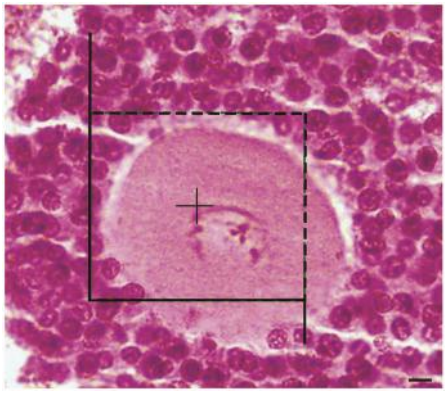

Figure 2. Counting the number of follicles by disector method. An adult follicle whose nucleus is inside the counting frame is counted in the follicle count (H&E staining, 1000×) Scale bar = 20 µm

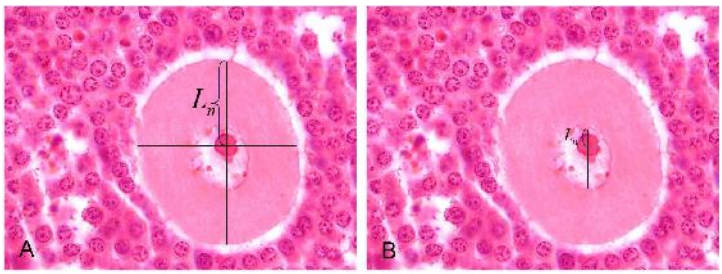

Figure 3. Oocyte volume measurement by Nucleator method. In this method, 20 micron sections were used. A. To calculate the volume of oocytes, from the center of the nucleolus to the oocyte membrane was measured. B. To calculate its nucleus volume, it was measured from the center of the nucleolus to the nucleus membrane (H&E staining, 1000×) Scale bar = 20 µm

Figure 4. Measuring the thickness of the zona pellucida (H&E staining, 1000×) Scale bar = 20 µm

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RESULTS

Mean right ovarian weight and percentage of body weight changes in mice: No significant difference was observed in any of the groups compared with the control group. (p > 0/05) (Table 1).

Ovarian histology: According to Figure 5 (A, a) in the control group, different types of follicles and corpus luteum were observed.

According to Figure 5 (B, b) in the group treated with Ag NPs, a significant decrease in the number of follicles and the mean volume of the corpus luteum was observed; although the mean total volume of the ovary, cortex and medulla decreased, but this decrease was not significant.

According to Figure 5 (C, c) in the group treated with SP, a significant increase in the number of follicles and the mean volume of the corpus luteum was observed. Although the mean total volume of the ovary, cortex and medulla increased, but this decrease was not significant.

According to Figure 5 (D, d) in the group treated with Ag NPs + SP, the number of follicles and the average total volume of the ovary, cortex, medulla and corpus luteum, compared with the group treated with Ag NPs approached the control group.

Estimation of total volume of ovary, cortex, medulla, corpus luteum and coefficient error rate: According to Table 2, the mean total volume of ovary, cortex and medulla in none of the groups compared with the control group did not show a significant difference (p > 0.05). However, the corpus luteum volume in the group treated with Ag NPs and the group treated with SP decreased and increased significantly compared with the control group (p < 0.05), respectively. Also, the corpus luteum volume in the group treated with Ag NPs + SP did not show a significant difference compared with the control group (p > 0.05).

It should be noted that the error coefficient of volume calculations was less than 0.05 in all cases.

The mean number of ovarian follicles: Comparison of the mean number of healthy follicles (primordial, primary, secondary and mature) showed a significant decrease and increase in the group treated with Ag NPs and the group treated with SP compared with the control group (p < 0/05). Also, the number of atretic follicles increased in the group treated with Ag NPs and in the group treated with SP showed a significant decrease, compared with the control group (p < 0/05). However, the number of healthy and atretic follicles in the group treated with Ag NPs + SP compared with the control group was not significantly (p > 0/05) (table3).

The mean oocyte volume and nucleus in different types of follicles: Comparison of the mean oocyte volume and nucleus in primordial, primary, secondary and mature follicles in none of the groups compared with the control group did not show a significant difference (p > 0/05) (Table 4 and 5).

Zona pellucida thickness: Comparison of the mean zona pellucida thickness in secondary and mature follicles did not show a significant difference in any of the groups compared with the control group (p > 0/05) (Table 6).

MDA and FRAP levels: MDA and FRAP in the group treated with Ag NPs compared with the control group showed a significant increase and decrease, respectively (P < 0.05). also, decrease and increase significant (p < 0.05) were observed in MDA and FRAP serum levels of mice treated with spirulina compared with the control group, respectively, and MDA and FRAP levels in the treated group. The amount of MDA and FRAP in the group treated with Ag NPs + SP did not show a significant difference compared with the control group (p > 0/05) (Table 7).

Levels of Estradiol, Progesterone, LH and FSH: The mean levels of Estradiol, Progesterone, LH, and FSH in the group treated with Ag NPs compared with the control group showed a significant decrease (p < 0/05). The levels of these hormones in the serum of mice treated with SP and the group treated with Ag NPs + SP, did not show a significant difference compared with the control group (p > 0/05) (table 8).

DISCUSSION

In the present study, the protective effect of SP on the ovaries of NMRI mice following toxicity induced by Ag NPs was investigated and the following findings were obtained:

Ovarian weight and percentage of body weight changes: There was no significant difference in ovarian weight and percentage change in body weight in none of the groups compared with the control group. This result is in line with previous research. (James et al., 2006; Parang et al., 2019).

Changes in body weight and ovaries are dose-dependent (Ramezani et al., 2016). So probably the dose, the duration of treatment and the size of Ag NPs and SP were not sufficient to affect physiological factors such as animal weight and ovarian weight. It only affected the function of body tissues.

Total volume of ovary, cortex, medulla, and corpus luteum: As compared with the control group, the group treated with Ag NPs and the group treated with SP showed a significant decrease and increase in corpus luteum volume, respectively, which was consistent with Shariatzadeh and Khagavi Jafarabad's findings (Seyed Mohammad Ali Shariatzadeh & Khagavi Jafarabad, 2018). However, no significant difference was observed in the total volume of ovaries, cortex and medulla in any of the groups compared with the control group. This result was also in line with Grigorova's results in 2017 (Grigorova et al., 2017). Probably, since

Table 1. Comparison of percentage of changes in body weight and right ovarian weight (g) in different groups of mice, 30 days after treatment with silver nanoparticles (500 mg/ kg/day) and spirulina (300 mg/kg /day).

Group	Control	Silver Nanoparticles	Spirulina	Silver Nanoparticles + Spirulina
percentage of body weight changes	1.96% [a]	-1.81% [a]	-2.17% [a]	-1.32 % [a]
ovary weight(g)	0.023 ± 0.003 [a]	0.020 ± 0.003 [a]	0.022 ± 0.004 [a]	0.021 ± 0.002 [a]

The values are means ± SD and the p-value indicates the probability of being significant. Averages with dissimilar professional codes are significantly different from each other. (one way ANOVA, Tukey's test, p <0/05).

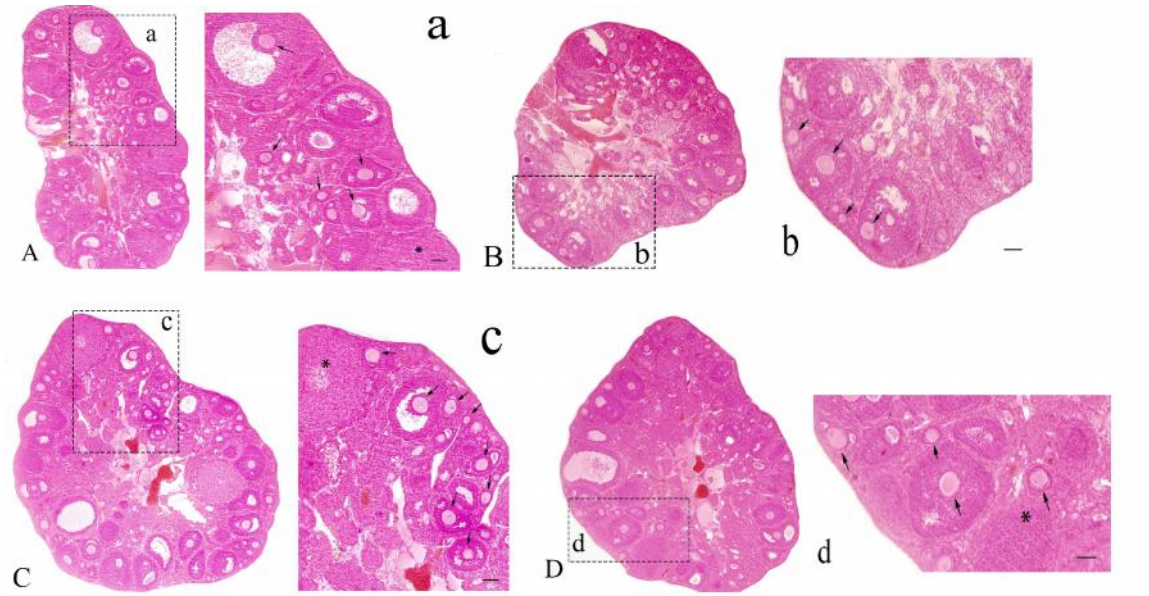

Figure 5. Microscopic images of 5 micron thick sections of ovarian tissue of mice in different groups, after 30 days of treatment. The stars and arrows show the corpus luteum and the types of follicles, respectively. (A) control, including corpus luteum and healthy follicles, (B, b) treated with Ag NPs, the number of healthy follicles and corpus luteum decreased, (C) treated with SP, the number of healthy follicles and corpus luteum increased, (D, d) Treated with Ag NPs + SP, the number of healthy follicles and corpus luteum approached the control group (H&E staining, magnification for A, B, C, and D image is 40×), Scale bar = 100 µm.

Table 2. Comparison of average total volume of ovary, cortex, medulla and corpus luteum (mm^3) in different groups of mice, 30 days after treatment with silver nanoparticles (500 mg/ kg/day) and spirulina (300 mg/kg /day).

Group / Volume (mm^3)	Control	Silver Nanoparticles	Spirulina	Silver Nanoparticles + Spirulina
Ovary	2.34±0.034 [a]	1.97±0.2 [a]	2.83±0.38 [a]	2.25±0.37 [a]
Cortex	1.87±0.32 [a]	1.55±0.16 [a]	2.24±0.26 [a]	1.84±0.34 [a]
Medulla	0.48±0.16 [a]	0.41±0.08 [a]	0.59±0.19 [a]	0.42±0.13 [a]
Corpus Luteum	0.50±0.11 [a]	0.30±0.06 [b]	0.64±0.12 [c]	0.44±0.05 [a]

The values are means ± SD and the p-value indicates the probability of being significant. Averages with dissimilar professional codes are significantly different from each other. (one way ANOVA, Tukey's test, p <0/05).

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Table 3. Comparison of the average number of types of follicles in different groups of mice, 30 days after treatment with silver nanoparticles (500 mg/ kg/day) and spirulina (300 mg/kg /day).

Group / Numbers	Control	Silver Nanoparticles	Spirulina	Silver Nanoparticles + Spirulina
Primordial	2090.26±189.06[a]	1837.47±150.55[b]	2320.85±64.40[c]	2036.82±138.06[a]
Primary	648.61±66.58[a]	598.20±43.87[b]	780.26±20.51[c]	665.70±43.20[a]
Secondary	347.63±41.56[a]	294.20±24.34[b]	395.11±13.34[c]	330.16±22.45[a]
Mature	165.90±22.20[a]	137.59±12.63[b]	192.75±9.96[c]	161.14±14.64[a]
Total healthy follicles	3271.71±308.47[a]	2867.45±220.42[b]	3688.97±70.22[c]	3193.82±214.82[a]
Atretic	337.04±16.97[a]	369.22±23.03[b]	308.48±9.01[c]	322.99±15.40[a]

The values are means ± SD and the p-value indicates the probability of being significant. Averages with dissimilar professional codes are significantly different from each other. (one way ANOVA, Tukey's test, p <0/05).

Table 4. Comparison of oocyte volume in different types of ovarian follicles (μm^3) in different groups of mice, 30 days after treatment with silver nanoparticles (500 mg/kg/day) and spirulina (300 mg/kg/day).

Group / Volume	Control	Silver Nanoparticles	Spirulina	Silver Nanoparticles + Spirulina
Primordial	1676.21±226.70[a]	1362.24±178.28[a]	1738.31±267.77[a]	1464.60±195.83[a]
Primary	6812.02±1316.11[a]	5121.43±756.88[a]	7542.34±1356.15[a]	5735.51±730.41[a]
Secondary	59140.80±9460.41[a]	47318.25±3132.99[a]	62859.95±9110.89[a]	54298.71±6198.47[a]
Mature	143141.24±7845.42[a]	126895.48±8421.13[a]	157581.83±16013.52[a]	131202.124±8653.92[a]

The values are means ± SD and the p-value indicates the probability of being significant. Averages with dissimilar professional codes are significantly different from each other. (one way ANOVA, Tukey's test, p <0/05).

Table 5. Comparison of oocyte nuclear volume in different types of ovarian follicles (μm^3) in different groups of mice, 30 days after treatment with silver nanoparticles (500 mg/kg/day) and spirulina (300 mg/kg/day).

Group / Volume	Control	Silver Nanoparticles	Spirulina	Silver Nanoparticles + Spirulina
Primordial	483.98±60.63[a]	399.72±24.67[a]	510.40±77.10[a]	408.52±33.21[a]
Primary	1319.33±175.17[a]	1124.21±92.64[a]	1442.09±145.50[a]	1184.45±106.52[a]
Secondary	2167.39±270.67[a]	1800.09±124.99[a]	2334.03±309.37[a]	2044.24±294.59[a]
Mature	4676.48±378.01[a]	4342/36±240/24[a]	4949.54±264.08[a]	4454.68±149.14[a]

The values are means ± SD and the p-value indicates the probability of being significant. Averages with dissimilar professional codes are significantly different from each other. (one way ANOVA, Tukey's test, p <0/05).

silver nanoparticles cause serious angiogenesis disorders, there is a significant difference in corpus luteum volume. This causes extensive cell damage in both theca and granulosa cells, as well as cell apoptosis, follicle destruction, and finally, no corpus luteum formation (Mirzaei et al., 2017). On the other hand, SP prevents angiogenesis inhibition among granulosa cells due to its antioxidant properties. As a result, it increases the number of mature follicles and consequently the corpus luteum (Ghaeni & Roomiani, 2016).

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Table 6. Comparison of zona pellucida thickness in secondary and mature follicles (µm) in different groups of mice, 30 days after treatment with silver nanoparticles (500 mg/ kg/day) and spirulina (300 mg/kg /day).

Group / Numbers	Control	Silver Nanoparticles	Spirulina	Silver Nanoparticles + Spirulina
Secondary	11.67 ± 0.43^a	11.10 ± 0.42^a	11.87 ± 0.31^a	11.14 ± 0.42^a
Mature	17.03 ± 0.65^a	16.67 ± 0.55^a	17.19 ± 0.49^a	16.82 ± 0.58^a

The values are means ± sd and the p-value indicates the probability of being significant. Averages with dissimilar professional codes are significantly different from each other. (one way ANOVA, Tukey's test, p <0/05).

Table 7. Comparison of MDA and FRAP (µM) in different groups of mice, 30 days after treatment with silver nanoparticles (500 mg/ kg/day) and spirulina (300 mg/kg /day).

Group / Numbers	Control	Silver Nanoparticles	Spirulina	Silver Nanoparticles + Spirulina
MDA (µM)	2.98 ± 0.50^a	4.65 ± 0.85^b	1.92 ± 0.34^c	3.47 ± 0.74^a
FRAP (µM)	0.63 ± 0.12^a	0.46 ± 0.09^b	0.88 ± 0.09^c	0.68 ± 0.07^a

The values are means ± SD and the p-value indicates the probability of being significant. Averages with dissimilar professional codes are significantly different from each other. (one way ANOVA, Tukey's test, p <0/05).

Table 8. Comparison of the average levels of Estradiol (pg/ml), Progesterone (ng/ml), LH and FSH (mIU/ml) in different groups of mice, 30 days after treatment with silver nanoparticles (500 mg/ kg/day) and spirulina (300 mg/kg /day).

Group / Numbers	Control	Silver Nanoparticles	Spirulina	Silver Nanoparticles + Spirulina
E_2 (pg/ml)	44.62±1.94 [a]	40.57±1.58 [b]	46.79±0.82 [a]	42.48±1.25 [a]
Progesterone (ng/ml)	2.13±0.30 [a]	1.54±0.86 [b]	2.58±0.18 [a]	2.08±0.19 [a]
LH (mIU/ml)	5.26±0.84 [a]	2.67±0.86 [b]	6.39±0.50 [a]	4.35±0.67 [a]
FSH (mIU/ml)	1.21±0.28 [a]	0/77±0.17 [b]	1.48±0.21 [a]	1.05±0.17 [a]

The values are means ± SD and the p-value indicates the probability of being significant. Averages with dissimilar professional codes are significantly different from each other. (one way ANOVA, Tukey's test, p <0/05).

Furthermore, the lack of significant differences in the total volume of the ovaries, cortex, and medulla is probably due to the fact that when oocytes and follicular cells begin to autolysis, they eventually leave a space that is immediately filled by ovarian scaffold cells, consequently, leaving no trace of it. (Mescher, 2016).

Mean number of ovarian follicles: The average number of healthy follicles in the group treated with silver nanoparticles was significantly reduced compared with the control group. However, a significant increase in their number was observed in the spirulina-treated group compared with the control group. These results were shown to be the opposite for atretic follicles. The results were in line with previous research (Ghorbanzadeh et al., 2012; Grigorova et al., 2017; Mousavi Tometri et al., 2018; Seyed Mohammad Ali Shariatzadeh & Khagavi Jafarabad, 2018; Yener et al., 2013). Because Ag NPs, after entering the ovaries, increase oxidative stress in these cells. As a result, probably, it will lead to apoptosis and a decrease in the number of follicles (Samani Jahromi et al., 2017; Zeweil et al., 2016).

Ag NPs also reduce antioxidants and sex hormones as they increase oxidative stress. These factors may also be a reason for the decrease in the number of healthy follicles and the increase in atretic follicles (Ghorbanzadeh et al., 2012).

On the other hand, Ag NPs disrupt angiogenesis due to the aromatic correlation between ovarian estrogen levels and angiogenesis. To determine the relationship between Cyp19 and estrogen, it is important to note that in the ovaries, key genes involved in coding cytochrome p450 aromatase are expressed by E2, indicating paracrine or autocrine

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effects induced by E2 (Hou & Zhu, 2017; Mirzaei et al., 2017). Additionally, it has been shown that the primordial and primary follicles receive their nutrients and oxygen from the blood vessels in the stroma. However, this condition varies in later stages of follicle development, such as late secondary follicles and graph follicles. At this stage, the vascular network that is needed for follicle growth is formed. In other words, angiogenesis begins at the early stage of secondary follicle formation and extends to the outer theca layer of this type of follicle (Exbrayat et al., 2015; Rosner et al., 2013).

Under physiological conditions, antral and mature follicles are involved in estrogen biosynthesis. Estrogen mediates the expression of specific adhesion molecules in endothelial cells. So, it leads to indirect control of ovarian angiogenesis. Therefore, any change in estrogen levels leads to significant changes in the expression pattern of adhesion molecules and ultimately affects the angiogenic activity of the ovaries and consequently, the number of follicles (Mirzaei et al., 2017).

The significant increase and decrease in the number of ovarian healthy and atretic follicles in the spirulina-treated group compared with the control group is most likely due to the alga's chemical composition, which includes phenolic acid, tocopherol, vitamin C, beta-carotene, and phycocyanin, all of which indicate antioxidant properties (Prahalathan et al., 2006). SP may be able to reduce oxidative stress and thus prevent follicular atresia as a result of this property.

Oocyte volume, its nucleus, and zona pellucida thickness: Changes in oocyte volume and nucleus in different types of ovarian follicles, as well as zona pellucida thickness were not significantly observed in any group compared with the control group, which was somewhat consistent with the results of Syrvatka et al. (Syrvatka et al., 2015). But they disagreed with Shariatzadeh and Khagavi Jafarabad's results (Seyed Mohammad Ali Shariatzadeh & Khagavi Jafarabad, 2018). Probably the reason for this result can be stated according to the works of Hou and Zhu (Hou & Zhu, 2017). Nanoparticles can accumulate in secretory cells and directly affect hormone secretion in the ovary. However, so far there is no evidence that nanoparticles can penetrate into oocytes and accumulate inside them. The reason for this, may be attributed to the multi-layered follicle and the specific structure of the zona pellucida. In fact, nanoparticles accumulate in the cytoplasm and nucleus of theca and granulosa cells, leading to ovarian cell apoptosis and accelerating antrum formation (Hou & Zhu, 2017). On the other hand, because Ag NPs lead to disruption of angiogenesis (Mirzaei et al., 2017), As a result, the damaged follicles become atresized, and the cells that are less exposed to these lesions continue to develop and are studied.

On the other hand, SP has the potential to be a therapeutic agent for the treatment of oxidative stress-related diseases (Ghaeni & Roomiani, 2016).

As a result, it is likely that the average volume of oocytes and nuclei of different types of follicles, as well as the thickness of the zona pellucida, have not changed statistically.

MDA and FRAP: MDA levels in the Ag NPs treated group and the SP treated group increased and decreased significantly when compared with the control group, respectively. Furthermore, the levels of FRAP in these two groups decreased and increased significantly when compared with the control group, which was consistent with the findings of the other studies (Mirzaei et al., 2017; Nasirian et al., 2019; Seyed Mohammad Ali Shariatzadeh & Khagavi Jafarabad, 2018; Yener et al., 2013). Ag NPs disrupt the balance between the production of oxygen free radicals and the body's antioxidant systems by inducing oxidative stress in body tissues. Free radicals are constantly circulating in the body due to the presence of single electrons and cause a lot of damage to macromolecules such as DNA, proteins, lipids, and carbohydrates (Seyedmehdi Shariatzadeh et al., 2016). Lipids are one of the most important molecules that are attacked by free radicals. Stress oxidation in the cellular environment leads to the formation of unstable and reactive lipids of unsaturated fatty acids, the breakdown of which leads to the formation of MDA (Shahsavari et al., 2015).

SP is able to increase the activity of glutathione peroxidase and glutathione reductase enzymes in the liver, thereby reducing the damage caused by oxidative stress. Therefore, it has a high ability to reduce free radicals and increase serum total antioxidant levels (Orlowski & M. S., 2018).

Hormone assay: The levels of estradiol, progesterone, LH and FSH in the group treated with Ag NPs and the group treated with SP showed a significant decrease and increase, respectively, when compared with the control group. It was in line with the results of Ebrahim in 2020 and Ramezani et al. in 2017 (Ebrahim, 2020; Ramezani et al., 2016). According to Hou and Zhu, Ag NPs may affect the secretion of hormones in the following two ways:

1) Ag NPs may enter the hypothalamus and pituitary secretory cells through the blood-brain barrier and alter the secretion of GnRH, LH, and FSH (Hou & Zhu, 2017). For example, one of the factors that increases GnRH secretion from

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hypothalamic neurosecretory neurons is potassium ion concentration. Ag NPs alter the permeability of cell membranes to potassium and then sodium, disrupting the activity of the Na-K ATPase and mitochondrial pump. As a result, the concentration of potassium ions in the blood decreases and this ion cannot stimulate potassium ligand-dependent calcium channels. Finally, it reduces GnRH release (Ramezani et al., 2016).

2) Ag NPs can enter the ovaries through the bloodstream and accumulate in theca and granulosa cells. This affects steroidogenesis. These changes can weaken the natural positive and negative feedback of the hypothalamic-pituitary axis and affect the natural secretion of sex hormones (Hou & Zhu, 2017).

Due to its strong antioxidant properties, SP may regulate the hormones LH and FSH by acting on the hypothalamus and pituitary gland. On the other hand, this alga can greatly reduce the free radicals produced during the ovulation process (Pankaj, 2015). So, it considerably regulates hormonal concentrations in the Ag NPs + SP group.

Conclusion

In the present study, although, spirulina cannot compensate the parameters tested in this study to the control level, it considerably improves ovarian damage caused by Ag NPs through reducing oxidative stress. As a result, SP may be useful as an antioxidant in the treatment of female infertility, and as an antioxidant, SP, along with fruits and vegetables, can be beneficial.

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