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Evaluation of the Effects Due to Injection of Biological Magnetic Iron Nanoparticles in Ovarian Tissue of Female Wistar Rats in Response to Electromagnetic Field

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ABSTRACT

Introduction: The use of different nanoparticles has recently found new dimensions in the treatment of many malignancies. Therefore, this study aimed to investigate the accumulation rate and tissue effects due to injection of biological magnetic iron nanoparticles in the ovarian tissue of Wistar rats in response to an electromagnetic field using inductively coupled plasma (ICP) and histopathological methods.

Methods: In this experimental study, animals were classified into four groups of six as follows: 1) group of healthy female rats without receiving nanoparticles in the absence of an electromagnetic field (control group), 2) group of healthy female rats receiving non-toxic dose of nanoparticles in the absence of an electromagnetic field, 3) group of healthy female rats receiving non-toxic dose of nanoparticles in the presence of an electromagnetic field, and 4) group of healthy female rats without receiving nanoparticles in the presence of an electromagnetic field. After grouping the rats, biological nanoparticles were injected intraperitoneally, and an electromagnetic field was created on the rats' skin at the site of the ovaries by a magnet, which was fixed on the skin using a tape. Then the presence of iron nanoparticles in the tissue was examined using ICP.

Results: Magnetic iron nanoparticles had low toxicity so that their half-maximal inhibitory concentration (IC50) in well Number 1 was 0.386. In the two groups receiving non-toxic doses of nanoparticles in the presence or absence of the electromagnetic field, no changes were observed in primary and secondary follicles as well as connective tissue and blood.

Conclusion: Magnetic iron nanoparticles have no destructive effects on ovarian tissue and have low cell accumulation; therefore, their use in this field is recommended to improve the future treatment of ovarian cancer.

Keywords: Biological iron nanoparticles, Histopathology, Ovary, ICP

Introduction

The prevalence of cancers is increasing in today's societies, and new methods have been recently developed to prevent, treat, and diagnose different types of cancer; in addition, many efforts have been made to increase the efficiency and reduce the side effects of treatments. Traces of nanotechnology could be found in many of these methods (1). The most important nanotechnology capability is the production of materials of nanometer (nm) dimensions. A group of these structures, called nanoparticles (NPs), have dimensions of at least less than 100 nm in size; among them metal nanoparticles are considered for use in various fields due to their unique physicochemical properties, including in the diagnosis and treatment of cancers and cardiovascular diseases, as well as in drug delivery into the cell. These nanoparticles show different optical and electronic properties in comparison with their homogeneous materials in large dimensions (2).

There are various methods for the production of nanoparticles, which are classified into three general methods: physical, chemical, and biological (3). Chemical production is simple, but toxic substances resulted from the reaction may remain on the produced nanoparticles. In the physical method, nanoparticles have little toxicity, but their production is time consuming (4). Due to these disadvantages and problems related to the use of physical and chemical methods, the biological production method has been currently considered by researchers due to its easiness, cost-effectiveness, low toxicity, and high compatibility with the human body (5). The production of nanoparticles by this method is called green technology. Enzymatic and non-enzymatic reactions (mainly through polysaccharides) are involved in the production of nanoparticles by living organisms, whether unicellular or multicellular (6). One of the best known techniques is the use of *Fusarium oxysporum* in the synthesis of these nanoparticles (7).

Ovarian cancer is the sixth most common cancer in the world and the second most common cancer of the genital tract with 200,000 new cases diagnosed annually, and since it is diagnosed in the late stage of the disease in only 75% of cases, it is the deadliest gynecological cancer in the first two decades of life (8). However, effective treatment is still possible in the early stages of the disease, so that more than 90% of patients could be treated in the early stages of the disease (9). Therefore, early diagnosis and timely treatment of this disease could reduce the incidence of ovarian cancer (10).

More than half of cancer patients undergo one of the cancer treatment methods, such as chemotherapy or radiation therapy. In most cases, combined methods of radiation therapy and chemotherapy are simultaneously used for patients (11). Unfortunately, treatment with invasive methods has side effects and in some cases, the side effects are severe. Endocrine activity and ovarian reproductive cycle are severely threatened following the use of toxins and ionizing radiation in the treatment of cancer. Patients undergoing chemotherapy are prone to problems such as premature ovarian failure, which is an important cause of infertility in these people (11).

Therefore, despite the presence of common methods such as chemotherapy, radiotherapy, and photodynamic therapy, 85% of patients need hybrid therapy, which has created an urgent need for new and innovative methods (12). The science of nanotechnology has a great potential for use in a wide range of cancer research, such as diagnosis, monitoring, and treatment strategies, and offers valuable ideas in this field. In recent years, researchers have tried to produce nanodrugs for the treatment of cancer with the help of many physical and chemical methods as intermittent and hybrid therapies (13). Iron oxide is considered an important candidate in the treatment of cancer due to its superparamagnetic properties and variable surface properties. Iron oxide synthesis in the presence of an oxidant forms several types of iron oxides, including hematite (a-Fe_2O_3) and magnetite (Fe_3O_4), among which iron oxide magnetite nanoparticles have been mainly used in medical research (14). Reducing drug resistance; reducing drug dose; providing greater efficiency in tumor diagnosis, targeting, and treatment; biocompatibility; and biodegradability are among the factors that make iron nanoparticles as suitable candidates for

clinical use (such as cell therapy, tissue regeneration, and drug delivery) (15).

Tan et al. (2016) found that magnetic liposome nanoparticles were better carriers for delivery of paclitaxel to a2780cp ovarian cancer cells than liposomal nanoparticles (14).

The results of a study by Lui et al. (2017) proved that the use of anti-cancer drugs coated by iron oxide not only is effective but also has more anti-cancer effects, which is due to the targeted drug delivery in the presence of nanomaterials (10).

Therefore, it seems that the use of iron nanoparticles, in addition to providing the possibility of diagnosing ovarian cancer, could also be effective in carrying drugs in these patients. Because nanocarriers are capable of carrying large amounts of drug and are highly biocompatible with the biological environment of the body, they could be directed to the target tissue and deliver drugs by an external magnetic field due to their magnetic properties. The use of these nanocarriers also reduces the destructive side effects of the drug on healthy tissues; in addition, lower doses of the drug are required for inducing the desired effect, which reduces treatment costs (1, 16).

Since the discovery of high-performance drugs with low side effects in the treatment of chemotherapy-resistant ovarian cancer is the main goal of research in the field of ovarian cancer treatment, and since reducing drug resistance is one of the properties of iron nanoparticles (17, 18), this study aimed to investigate the accumulation rate and tissue effects of intraperitoneal injection of biological magnetic iron nanoparticles in the ovarian tissue of female Wistar rats in response to an electromagnetic field using inductively coupled plasma (ICP) and histopathological methods in order to determine iron nanoparticles production method as well as the rate of their entry and performance in drug delivery into cancer cells.

In general, studies have shown that the production of biological nanoparticles is easier, less expensive, and less risky, they are not only harmless to body tissues but sometimes even useful and practical (19, 20, 21).

A study by Bai et al. (2017) investigated the toxic effect of these nanoparticles on ovarian cancer cells. The cytotoxic effect of these nanoparticles was investigated on A2780cp cancer cells by MTT assay, acridine orange/propidium iodide staining, and caspase test. The results showed that iron oxide metal nanoparticles coated with brown algae extract exhibited cytotoxic effect on chemotherapy-resistant ovarian cancer cells by inducing caspase 3 and 9-dependent apoptosis (internal pathway) within 24 h at about 250 μg/mL IC50 concentration (half-maximal inhibitory concentration) and within 48 and 72 h at 125 and 62.5 μg/mL, respectively. They concluded that brown algae extract is a suitable option for increasing stability and decreasing colloidal solution of iron oxide nanoparticles, which make these nanoparticles a suitable option in the treatment of ovarian cancer by inducing apoptotic effect on A2780cp cell line (1).

Materials and Methods

Production of biological magnetic iron nanoparticles

Production of magnetic iron nanoparticles was performed by fungi. In this way, *F. oxysporum* strain was first purchased and cultured in Sabouraud dextrose broth medium. The culture supernatant was exposed to an equal volume of 0.1 M $FeCl_3 \cdot 6H_2O$ salt, and the nanoparticles were produced by incubating the culture medium in a shaker incubator at 37 °C for 24 h.

Cytotoxicity test

Non-cancerous fibroblast cell line was used in this study. First, the cells at a specific concentration (104×4) with 200 μL of DMEM culture medium and 5% of bovine embryo serum were inserted into 96-block plates. After 24 h of incubation in the cell culture incubator and reaching 80% accumulation, the cells were exposed to different concentrations of iron nanoparticles and reduced by half in iron nanoparticles solution, respectively. The positive control of wells was a well without iron nanoparticles. Plates were placed in a 25% CO_2 incubator, depending on the percentage of sodium bicarbonate in the culture medium, for 24 h; then 10 μL of 5 mg/mL solution of MTT dye was added to them and kept at 37 °C for 4 h. After separating the solution, 100 μL of DMSO

dye was added to the wells by a sampler, and their light absorption was measured at 550 nm by an ELISA reader to determine the minimum cytotoxic concentration of iron nanoparticles.

Steps and procedures for nanoparticle injection in rats

In order to investigate the effect of iron nanoparticles on the ovarian histology of experimental rats, 24 rats aged 8-12 weeks with equal average weight were purchased and quarantined, and adaptation conditions were applied for one week so that the rats were adapted to the new environment. Rats were then weighed, randomly selected, grouped, and maintained. They were weighed weekly until injection.

The animals were grouped as follows:

1) Group of healthy female rats without receiving nanoparticles in the absence of an electromagnetic field (N = 6)
2) Group of healthy female rats receiving non-toxic dose of nanoparticles in the absence of an electromagnetic field (N = 6)
3) Group of healthy female rats receiving non-toxic dose of nanoparticles in the presence of an electromagnetic field (N = 6)
4) Group of healthy female rats without receiving nanoparticles in the presence of an electromagnetic field (N = 6)

After grouping the rats and ensuring sterilization of the nanoparticle solution by tyndallization method, biological nanoparticles were injected intraperitoneally based on the interventions. To create an electromagnetic field on the rat skin at the site of the ovary, a 5×5 mm square neodymium magnet was used, which was fixed on the skin of rats using a tape. The trial period was 24 hours and 7 days later.

At the end of the experiment, the rats were killed, and tissue sampling was performed. Isolated samples from all rats were immediately placed in containers containing 10% formalin solution for tissue fixation and sent to histology laboratory for preparing histological sections. After preparation and staining with H&E (Hematoxylin and Eosin), the samples were interpreted.

ICP-MS analysis

Using the ICP technique, the presence of iron within the cells could be examined. For ICP-MS analysis, tissue samples were first placed in vials containing ethanol at 100 °C for 10 min. The tissues were then placed in sterile petri dishes. The vials were placed in an oven at … °C for 3 days, and after adding 2 mL of nitric acid and 1 mL of hydrogen peroxide, the samples were placed in a microwave and digested. They were then diluted with 10 mL of distilled water and examined by ICP-MS. The amount of iron nanoparticles in the tissue was investigated, and the solution of iron nanoparticles produced was examined as a control. Known standard concentrations of iron were used as controls to prepare the standard graph.

Results

Production of biological magnetic iron nanoparticles

Magnetic iron nanoparticles were produced by fungi as follows: *F. oxysporum* strain was initially purchased and cultured in Sabouraud dextrose broth medium. The culture supernatant was exposed to an equal volume of 0.1 M $FeCl_3 \cdot 6H_2O$ salt, and nanoparticles were produced by incubating the culture medium in a shaker incubator at 37 °C for 24 h.

Cytotoxicity test results

Non-cancerous fibroblast cell line was used in this study. First, the cells at a specific concentration (104 × 4) with 200 µL of DMEM culture medium and 5% of bovine embryo serum were inserted in 96-block plates. After 24 h of incubation in the cell culture incubator and reaching 80% accumulation, the cells were exposed to different concentrations of iron nanoparticles and reduced by half in iron nanoparticles solution, respectively. The positive control of wells was a well without iron nanoparticles. Plates were placed in a 5% CO_2 incubator, depending on the percentage of sodium bicarbonate in the culture medium, for 24 h; then 10 µL of 5 mg/mL solution of MTT dye was added to them and kept at 37 °C for 4 h. After separating the solution, 100 µL of DMSO dye was added to the wells by a sampler, and their light absorption was measured at 550 nm

by an ELISA reader to determine the minimum cytotoxic concentration of iron nanoparticles (Figure 1).

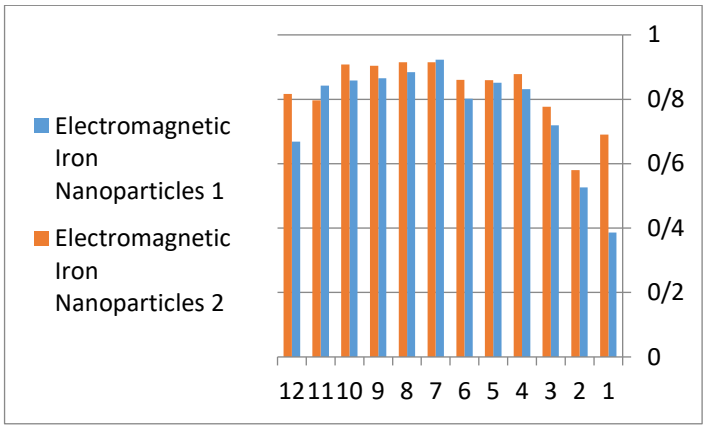

Figure 1. As shown in the figure, magnetic iron nanoparticles had low toxicity so that their IC50 value in well Number 1 was 0.386.

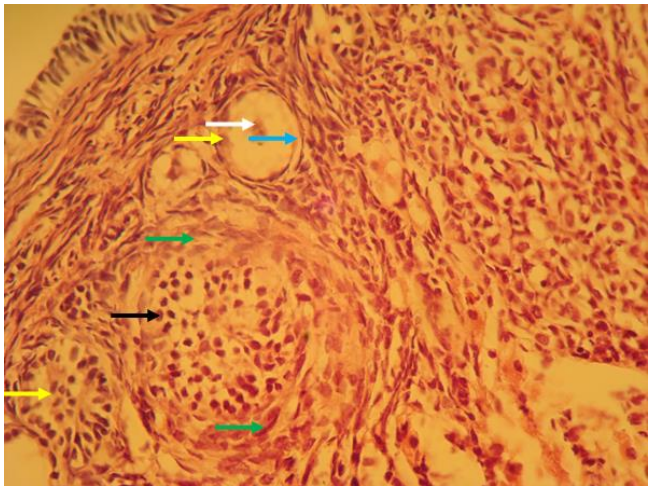

Figure 2. Photomicrograph prepared from the ovaries of rats in the control group (Hematoxylin and Eosin × 400 staining)

Histological results

As shown in Figure 2. in the sections prepared from the ovarian tissue of rats in the control group, all follicles, including the primary and secondary follicles (yellow arrow), were completely healthy and had a proper order and size, and the cells of the follicular layer were identified and observed in an appropriate number and regular manner. Oocytes inside the follicle were also observed with distinct nuclei and appropriate size and color (white arrow), and ZP3 was also visible around them (blue arrow). Granulosa cell layers (black arrow) and single cell layers (green arrow) were observed with normal number and color. In the ovarian stroma, a large number of primitive follicles were visible, and there was no abnormal accumulation of blood or irregularity in the tissue (Figure 2 and Table 1).

In the sections prepared from the ovarian tissue of rats in the electromagnetic field group with no injection, the tissue characteristics were similar to those of the control group, and no significant change was observed in the tissue. Ovarian follicles, including the primary multilayered follicles were of appropriate shape and size; although the oocyte was slightly indistinct (white arrow), it's ZP3 (blue arrow) was visible. Granulosa cells (black arrow) and the single cell layer and its cells number (green arrow) were normal and without any changes. There were no signs of changes such as hyperemia or cellular and tissue disruption (Figure 3 and Table 1).

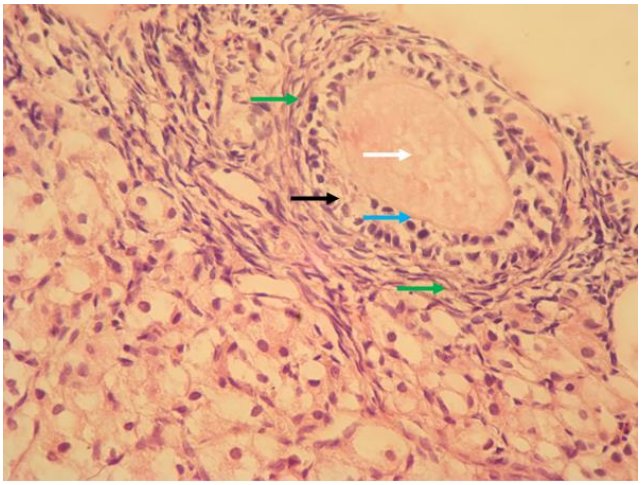

Figure 3. Photomicrograph prepared from the ovaries of rats in the electromagnetic field group (Hematoxylin and Eosin × 400 staining)

As shown in Figure 4, in the sections prepared from the ovarian tissue of rats in the non-toxic dose group in the presence of an electromagnetic field, the number and distribution of primitive and primary follicles as well as secondary follicles were similar to those of the control group. In the primary monolayer follicle, the granulosa cells were visible in a cubic shape with a distinct nucleus and cytoplasm (black arrow). The oocyte had a right size and shape (white arrow), and the ZP3 layer was also visible (blue arrow). The layer of single cells (green arrow) was normal and clear. There were no traces of tissue changes (Figure 4 and Table 1).

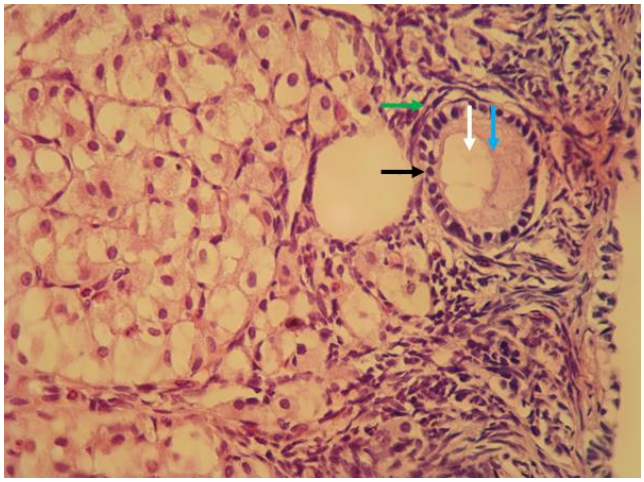

Figure 4. Photomicrograph prepared from the ovaries of rats in the non-toxic dose group of nanoparticles in the presence of an electromagnetic field (Hematoxylin and Eosin × 400 staining)

In the sections prepared from the ovarian tissue of rats in the non-toxic dose group in the absence of an electromagnetic field, the cellular and histological characteristics were the same as the previous group, and no specific and noticeable change was observed in the tissue. In the primary follicle, which was multilayered, granulosa cells were observed with an appropriate number and shape (black arrow), and the cells in the single layer (green arrow) were being formed and expanded. The oocyte had a suitable shape with a distinct nucleus (white arrow), and ZP3 was observed around it (blue arrow) (Figure 5 and Table 1).

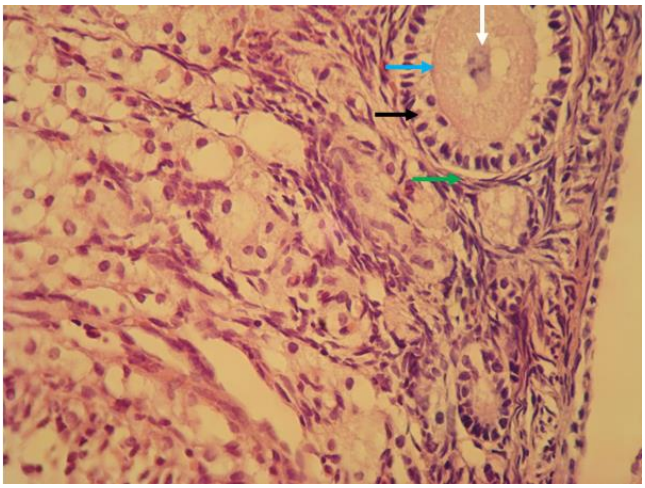

Figure 5. Photomicrograph prepared from the ovaries of rats in the non-toxic dose group of nanoparticles in the absence of an electromagnetic field (Hematoxylin and Eosin × 400 staining)

Table 1: Separation and grading of quantitative changes in the variables evaluated by ovarian tissue❋

Groups	Primordial Follicle	Primary Follicle	Secondary Follicle	Connection and Blood Changes
Control	0	0	0	0
Electromagnetic field	0	1	0	0
Non-toxic dose of nanoparticles in the presence of electromagnetic field	0	0	0	0
Non-toxic dose of nanoparticles without electromagnetic field	0	0	0	0

❋Grade 0 indicates no change, Grade 1 indicates mild change, Grade 2 indicates moderate change, and Grade 3 indicates severe change.

ICP-MS analysis

Table 2 shows the results of the ICP-MS element measurement test in ovarian tissue.

Table 2: Results of ICP-MS analysis❋

Test Results		
Sample Labels	Element	Wavelength
	Fe	238/204
Blank (ppm)	0/00	
Iron nanoparticles in tissue in the presence of electromagnetic field(ppm)	0/26	
Iron nanoparticles in tissue without electromagnetic field(ppm)	0/11	
Iron nanoparticles in solution (ppm)	1/15	

❋Elements are in milligrams per liter (ppm).

Discussion

Nanotechnology as a powerful modern technology is able to revolutionize the medical system worldwide. Nanotechnology involves the purposeful research and development of nanoparticles to understand, manipulate, and measure materials at the level of atoms, molecules, and supermolecules. Given the different physical, biological, and chemical properties of materials, they are fundamentally different from each other and often have unexpected actions. This system, i.e.

nanotechnology, is currently used for drug delivery to tissues (6).

The discovery of high-performance drugs with low side effects in the treatment of chemotherapy-resistant ovarian cancer is the main goal of research in the field of ovarian cancer treatment, and since reducing drug resistance is one of the properties of iron nanoparticles, this study aimed to investigate the accumulation rate and tissue effects due to injection of biological magnetic iron nanoparticles in ovarian tissue of female Wistar rats in response to an electromagnetic field using ICP and histopathological methods in order to determine iron nanoparticles production method as well as the rate of their entry and performance in drug delivery into cancer cells. In this study, magnetic iron nanoparticles had low toxicity, and no positive effects were observed in primitive, primary, and secondary follicles as well as connective tissue and blood tissue in two groups receiving non-toxic doses of nanoparticles with and without an electromagnetic field.

In the present study, it was found that the production of magnetic iron nanoparticles by biological methods is more economical and less risky, and their production time is shorter. The results showed that magnetic iron nanoparticles had low cytotoxicity and tissue toxicity with no destructive effects on ovarian tissue. Also, according to the ICP analysis results, they caused no density in the tissue, and the ovarian follicles were in a normal shape. But the presence of an electromagnetic field had a devastating effect on the primary follicles.

Bai et al. (2017) conducted a study to investigate the toxicity of these nanoparticles on ovarian cancer cells, the cytotoxicity of these nanoparticles was investigated by MTT method, acridine orange/propidium iodide staining, and caspase test on A2780cp cancer cells. The results showed that iron oxide nanoparticles coated by brown algae extract had a cytotoxic effect on chemotherapy-resistant ovarian cancer cells by inducing caspase 3 and 9-dependent apoptosis (internal pathway) within 24 hours at a IC50 value of about 250 μg/mL and within 48 and 72 h at 125 and 62.5 μg/mL, respectively. They concluded that brown algae extract is a suitable option for increasing stability and decreasing colloidal solution of iron oxide nanoparticles, which make these nanoparticles a suitable option in the treatment of ovarian cancer by inducing apoptotic effect on A2780cp cell line, (1). However, in the present study, the toxic effects of iron nanoparticles were mainly investigated, and good safety was observed.

A study by Tan et al. (2016) evaluated the efficacy of magnetic iron liposome nanoparticles as carriers of paclitaxel in ovarian cancer, and drug encapsulation rate in magnetic liposome nanoparticles and liposomes was estimated to be 97 and 96%, respectively. The results showed continuous and controlled drug release from magnetic liposome nanoparticles. MTT results also showed that magnetic liposome nanoparticles containing paclitaxel exerted higher cytotoxicity against a2780cp cells than drug-containing liposomal nanoparticles. Nanoparticles loaded with anticancer drugs could easily reach the cell membrane and increase the drug concentration at the cell surface compared to the standard drug, which increases the concentration of the intercellular drug and the effect of the drug. Their study results showed that magnetic liposome nanoparticles were a suitable carrier for paclitaxel delivery to a2780cp ovarian cancer cells, compared to liposomal nanoparticles (14), with increased efficiency and no tissue complications, confirmed in our study.

In a study by Lui et al (2017), the effect of iron oxide nanomaterials containing doxorubicin was investigated on ovarian A270 cancer cells. The results showed that nano-complex had less coverage, and doxorubicin had less toxicity and more anti-cancer effects compared to doxorubicin alone; also, a high percentage of this compound attacked only to cancer cells. In general, the results showed that the use of anti-cancer drug using iron oxide coating not only was effective but also had more anti-cancer effects, which was due to targeted drug delivery in the presence of nanomaterials (10). This finding increases the importance of our results in terms of non-histological toxicity of iron nanoparticles.

A review study was conducted to investigate diagnostic and therapeutic applications of

nanostructures in ovarian cancer in 2017 by reviewing various articles and their results, it was found that nanoparticles could be generally considered as one of the suitable candidates for diagnosis and treatment of cancer. Among these nanostructures, iron oxide nanoparticles were the most widely used NPs in the field of cancer diagnosis, which caused patients to have non-invasive MRI (magnetic resonance imaging) results due to their super paramagnetic properties. It was also found that common drugs used in this field along with suitable nanostructures such as PLGA were used to target and accumulate the drug in the target tissue. Finally, they concluded that in general, superparamagnetic iron oxide nanoparticles could be considered as a good candidate for simultaneous diagnosis and treatment of ovarian cancer (17), which could be further explored in future studies in Wistar rats.

A study by Namvar et al. (2015) investigated the cytotoxicity of iron oxide nanoparticles coated with algae extract on ovarian cancer cells and showed that the cytotoxicity of iron oxide nanoparticles on ovarian cancer cells was dose dependent and induced by apoptosis, which make these nanoparticles a suitable option in the treatment of ovarian cancer (18). This method could be studied and compared with the methods used in this research to achieve the best and most accurate method in this field.

Conclusion

In general, based on the results of this and other studies, it was inferred that biological magnetic iron nanoparticles had low toxicity with no adverse effects on ovarian tissue; therefore, their use in this field is recommended to improve the process of ovarian cancer treatment in the future.

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