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EFFECT OF SILVER NANOPARTICLES ON THE OVARY OF PCO-INDUCED FEMALE MICE: A HISTOPATHOLOGICAL STUDY

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ABSTRACT
This study was conducted to investigate the effect of two concentrations (0.5 and 1ppm) of silver nanoparticles on the histological structure of PCO-induced female mice. The 100 PCO-induced mice were divided into two main groups according to the duration of the treatment (30 and 45 days). Each group included five subgroups, these were: PCO-induced mice treated with (0.5 and 1ppm) of silver NPs solution which, was administrated by intraperitoneal injection (i.p) twice a week. Other PCO-induced mice were treated with metformin at a dose of (1mg/day) orally three days a week. At the end of each experiment these mice were sacrificed and ovaries were isolated and preserved for histological study. The histopathological changes in the ovaries of the treated groups in both experiments were ranging between reversible damage and irreversible. Reversible damages included different types of degeneration (hydropic, hyaline and amyloid deposition) and were more obvious in the ovaries of the PCO-induced treated with low dose of silver NPs. But, irreversible damages like necrosis appeared in the ovaries treated with high dose of silver NPs. The administration of silver NPs with low dose and in a discontinuous manner revealed minor damage in the ovaries of the PCO-induced mice. This feature might be advantage in case of applying silver NPs for drug delivery. More studies are needed to assure this opinion with different sizes of silver NPs and different modes of administrations.

KEYWORDS: PCO, silver NPs, metformin, ovary.

INTRODUCTION

In general, nanomaterials have unique physiochemical properties, such as ultrasmall size, large surface area to mass ratio, and high reactivity, which are different from bulk materials (in microscale) of the same composition. These properties can be used to overcome some of the limitations found in traditional therapeutic and diagnostic agents (El-Ansari and Al-Diahan, 2009). The potential of inorganic nanoparticles has been explored worldwide in nanomedicine, drug delivery and biomedical devices, cosmetics, electronics, energy sector and environmental protection. Silver NPs or nano-silver occur among these inorganic NPs (Chaudhuri and Paria, 2012). Silver NPs is widely recognized for its capacity to kill bacteria, researchers and companies alike have long incorporated its use in various applications. Specifically, the lack of cytotoxicity to mammalian cells has resulted in its use in antibacterial and antibiotic treatment for a long time (Seo *et al*., 2014).These nanoparticles has been extremely popular in a diverse range of consumer materials, including plastics, soaps, pastes, metals, textiles and, food packaging. This is because of the exceptional broad spectrum bactericidal activity of silver and relatively low cost of manufacturing (Ranjbar *et al*., 2013; Haider and Kang, 2015). Moreover, it has anti-fungal, anti-inflammatory, anti-viral, anti-angiogenesis, and anti-platelet activity. In addition, it can accelerate wound healing and dressing of wound, ointments and implant coatings (Lee *et al*., 2016; Nilforoushzadeh *et al*., 2012; Ciftci *et al*., 2013; Soenen *et al*., 2013). Nevertheless, it has been shown that nanoparticle is comparatively over toxic than other sizes besides; different size and shape of silver-NPs have different toxicities. These nanoparticles can be ingested directly via water, food, cosmetics, drugs, drug delivery devices etc (Sardari *et al*., 2012). Ovarian polycyst (PCO) may be defined as the presence of 12 or more follicles measuring (2-9) mm in diameter, this definition was approved according to the joint ASRM/ESHRE consensus meeting on the polycystic ovarian syndrome (PCOS), in Rotterdam (Chang *et al*., 2000; Balen *et al*., 2004). Later, the ovarian volume (OV) was added to the definition which might exceed 10 cm^3 (Lee *et al*., 2012).The histopathological criteria for the PCO include: observation of atretic follicles and/or degenerating granulosa cells, hypertrophy and luteinization of the inner theca cell layer and thickened ovarian tunica (Balen, 2004). Furthermore, two main histological features were added to the definition, these included: the excessive number of follicles or murltifollicularity and stromal hypertrophy (Jonard and Dewailly, 2003). Insulin can augment ovarian steroidogenesis directly by reducing the biosynthesis of hepatic sex hormone –binding globulin(SHBG) which, is the key circulating protein that controls the bioavailability of testosterone or indirectly, by augment LH-mediated androgen production (Mukherjee and Maitra, 2010). Although, there are no specific treatments available for the PCOS patients, in general, treatments aimed at decreasing insulin and androgen levels. This encompasses drug therapy including insulin lowering and anti androgen

medications or oral contraceptives, and life style interventions (Mansour *et al*., 2016).

Women with PCOS had higher basal and glucose-stimulated insulin levels than weight-matched controls (Pasquali *et al*., 2002). Insulin increases the testosterone bioavailability in PCOS women by reducing hepatic production of sex hormone binding globulin (SHBG).Therefore, insulin lowering drugs like, metformin (1, 1-dimethylbiguanid) can improve insulin action by increasing insulin sensitivity, thereby decreasing hyperinsulinaemia (Tang *et al*., 2006). Further, it has been suggested that metformin reduces hyperandrogenism through its effect on both the ovary and adrenal gland suppressing their androgen production, reducing pituitary luteinizing hormone and increases the production of sex hormone binding globulin by the liver (Bailey and Turner, 1996). Metformin can modulate the reproductive axis affecting the release of GnRH and LH as a result; serum levels of LH, LH/FSH, and rostenedione, DHEA-s and progesterone would decrease (Valija-Asimi, 2013). In a clinical study by Sanoee *et al*. (2011) and according to their results they put a possible conclusion that metformin therapy, even in a relatively short time such as three months, in patients with PCOS may cause decrease in the ovarian volume by decreasing intraovarian stromal androgens. Studies by Duleba and Dokras, (2012) demonstrated that, women with PCOS have altered circulatory levels of some markers of inflammation, which may reflect a state of chronic low grade inflammation. Silver-NPs on the other hand, have shown anti-inflammatory properties in both animal models and in clinic (Ge *et al*., 2014). Therefore, this study was designed to examine the histopathological effects appeared in the ovaries of the PCO- induced mice which were treated two concentrations (0.5and 1ppm) of silver NPs by intraperitoneal injection (i.p) and, along two durations (30 and 45 days).

MATERIALS & METHOD

Animals

A number of mature female mice (100), weighting about (20-25) gm were purchased from Animal House of the High Institute for Infertility Diagnosis and Assisted Reproductive Technology/Al-Nahrain University. Animals were retained under standard conditions of temperature (25-28)°C and 12 hours light dark cycle throughout the period of experiments.

Induction for polycystic ovary (PCO)

In order to induce polycystic ovaries (PCO), female mice were intraperitoneally injected (i.p) with human Chorionic Gonadotropin hCG (Choriomon) at a dose of (10I.U.) three times a week for two weeks (Bogovich, 1991). The stage of estrous cycle in this step, was not determined because, many of the mechanisms involved in the induction of ovarian follicular cysts are thought to be independent of the stage of development of the ovary (Bogovich, 1992).

Solution of silver nanoparticles (silver NPs)

Silver nanoparticles solution was prepared by Iranian Nanoparspanda Company. The concentration of this solution was (4000) ppm and the size of these particles were about (50-100) nanometer. The characteristics of this solution were confirmed by using Scanning Probe Microscope (SPM) at department of Chemistry /College of Science /University of Baghdad. Then the solution was activated by using ultrasound sonicator at department of Physics/ College of Science /University of Baghdad every two weeks. In this study, two concentrations (0.5 and 1 ppm) were prepared from this stock. According to a study conducted to assess the effect of silver nanoparticles on ovarian features via intraperitoneal injection (Ghorbanzadeh *et al*., 2012) and according to the following formula: $C_1 V_1 = C_2 V_2$ (Skoog *et al*., 2004).

Metformin drug solution

One pill of metformin (500) mg was crashed using a manual mortar, the powder then was dissolved in (25)mL of normal saline .This suspension was considered as Stock 1.One mL of Stock 1 was taken and diluted with (19) mL normal saline and this solution was considered as Stock 2. Preparation of stock 2 was repeated every time when animals treated with the drug. However, (0.1)mL was taken from stock 2 and administrated orally to each female three times a week throughout the duration of each experiment. The metformin dose for each female mouse was (1) mg / day (Elia *et al*., 2006). After PCO induction, a number of PCO-induced mice were sacrificed and the ovaries were isolated from the surrounding adipose tissue and preserved in Boun's solution which, was then replaced by ethanol 70% for histological study. The rest of the PCO-induced mice were divided into two main groups according to the period of treatment (30 and 45 days) then, each group was divided into five subgroups according to the experimental design below:

Experimental design

The PCO Induced animals (100 in number) were divided into two main groups according to days of treatment (30 and 45 days) respectively. Each group has the following subgroups: the first and second subgroups were treated with (0.5 and 1ppm) respectively by i.p. injection twice a week while, the third subgroup was treated orally with metformin three days a week in addition to the control and PCO subgroups.

Histological preparation

Ovaries for all the treated groups were fixed with boun's solution which, was then replaced by ethanol 70%. Processing of the samples was done by routine method (Bancroft and Stevens, 2010).

RESULTS

Figure (1) represents section in an ovary of the control showing a secondary follicle with two growing follicles and a corpus lutium.

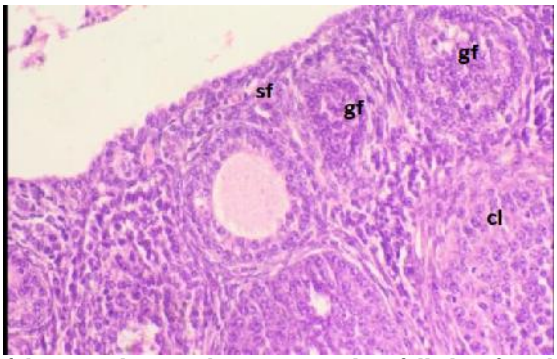


FIGURE 1: section in an ovary of the control group showing secondary follicle (sf) with two growing follicles and a part of the corpus lutium (cl).400X.H&E.

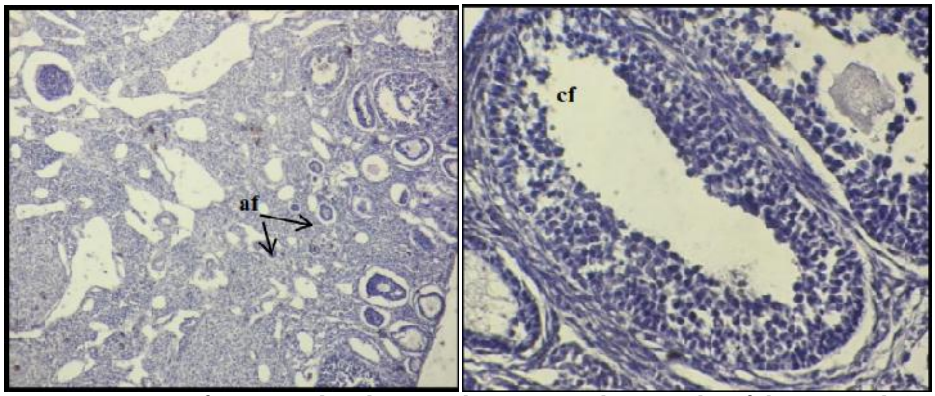


FIGURE 2: sections in an ovary of a PCO-induced mouse showing in a- hypertrophy of the stroma (hp) with increase in number of atretic follicles (af), 100X. Higher magnification (400X) in b-showing large cystic follicles(cf), thin undistinguishable theca layer (T). H&E.

Figure 2 represents section in an ovary of a PCO-induced mouse, treated with hCG hormone for two weeks compared to the control, revealing in -A- on the low power, hypertrophy of the stroma, absence of the corpus lutium and increase in number of atretic follicles with small cysts. Whereas, another section with higher magnification demonstrated large cystic follicle and with thin theca layers. Histological structure of the ovary from the PCO-induced mice treated with silver NPs (0.5ppm) for (30 day) is represented in figure (3). Necrosis appeared in the granulosa layer of one growing follicle which caused completely losing of this layer but, the oocyte seemed normal, there was pyknosis (p) in granulosa cells of the neighboring follicle, vacuolation (V) appeared in the theca layer while edema (E) was seen in the stroma and, a few fatty degeneration were also seen among the granulosa cells. Higher magnification revealed corpus luteum for the same treated group, lutein cells, collagen bundles appeared as pinkish color with fusiform shaped fibrocytes in some areas of the corpus luteum, this indicated the transformation of the corpus luteum to the functionless corpus albuginea, as in figure (4). Deposition of silver NPs was obvious in some spots of the corpus luteum. Moreover, deposition of amyloid (a) protein could be seen in black arrow the neighboring blood vessel, figure in blue arrow (4).

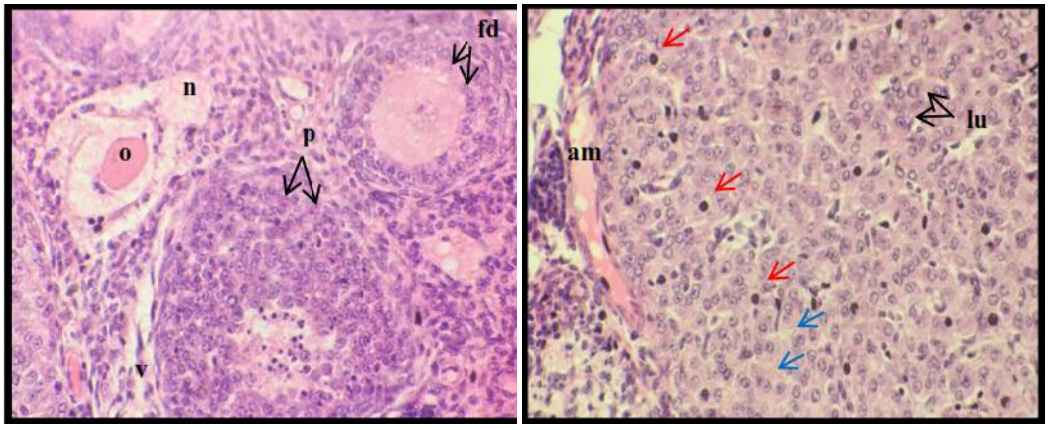


FIGURE: 3 & 4

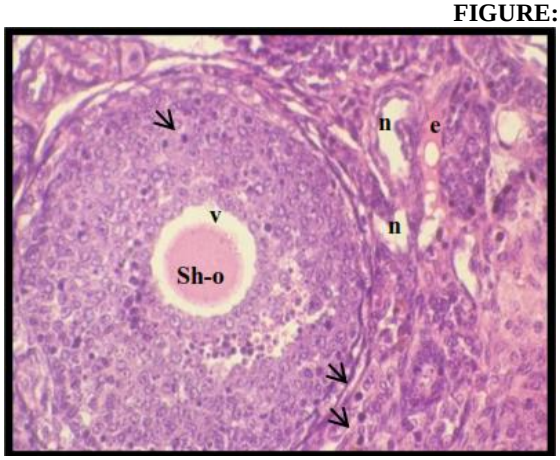


FIGURE: 5

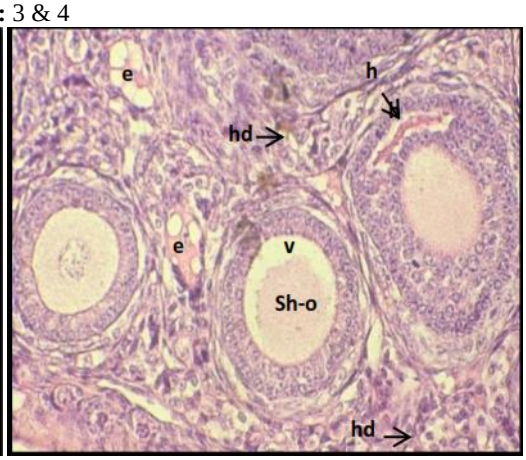
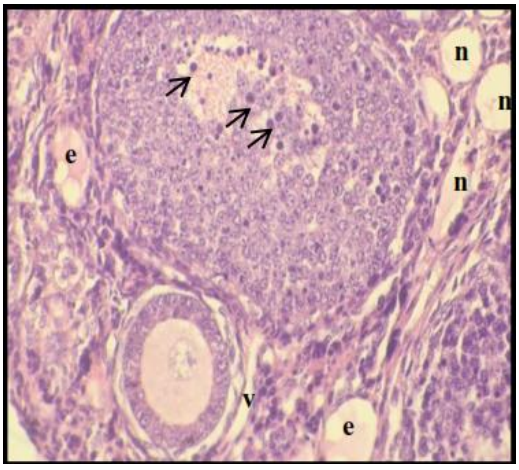


FIGURE: 6

Whereas, prolonged treatment (45days) of the PCO-induced mice with (0.5ppm) of silver NPs exhibited different pathological changes in the ovary. The oocyte of the antral follicle seemed shrunk and vacuolation appeared in the ooplasm in addition to some cells of the theca layer, silver NPs were deposited on granulosa layer, edema (e) was seen in the stroma in addition to some necrotic areas, as in figure (5) Hyalinazation is found in the granulosa layer of the late secondary follicle, some interstitial cells demonstrate hydropic degeneration (hd), edema has also be seen in the stroma, as in the figure (7). The same pathological changes were seen in figure (8) with deposition of silver NPs on granulosa cells of the growing follicle shown in black arrow.



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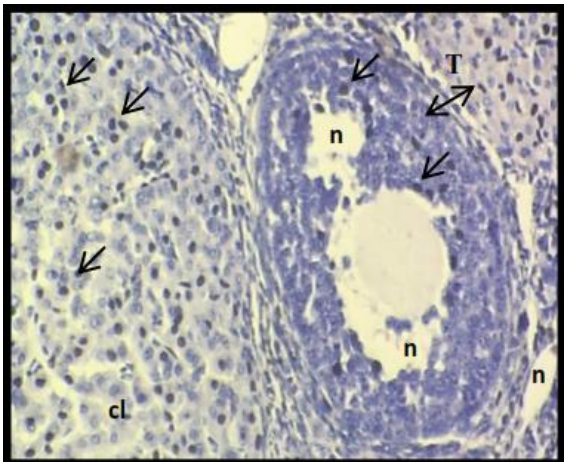

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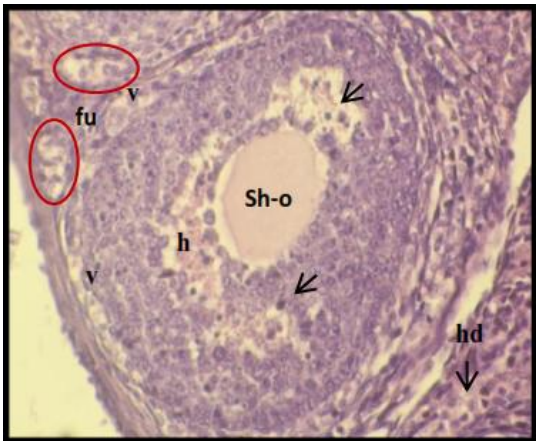

FIGURE: 9

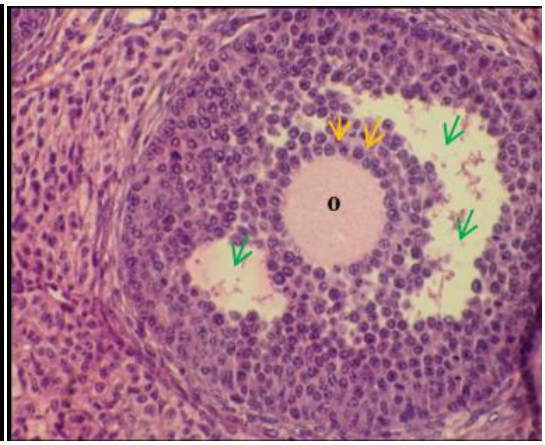

FIGURE: 10

The next (figure 8) represents section in an ovary of PCO-induced mouse treated with (1ppm) of silver NPs for 30 days. The oocyte of the antral follicle seemed shrunk and vacuolated, in addition to hyalinazation in the granulosa layer with vacuolation and hydropic degeneration in the granulosa and stroma cells, necrosis also was obvious in

the stroma. While figure (9) represents section in an ovary of a PCO-induced mouse treated with (1ppm) of silver NPs for 45 days. a growing follicle with shrunk oocyte appears in this section. Hyalinazation also can be seen in the growing follicle with deposition of silver NPs on the granulosa cells. Hydropic degeneration is obvious in the stroma with fusion (fu) of some stroma cells and necrosis. While, unremarkable histopathological changes have been seen in sections of the ovaries from the PCO- induced groups treated with metformin for 30 and 45 days, figure (10).

DISCUSSION

In this study, hCG hormone was applied to induce ovarian polycyst since both LH and hCG hormones share similar molecular structures and receptor (Choi and Smitz, 2014). Besides, low doses of hCG can induce follicular cysts (Srivatava and Krishna, 2006). Edema represents an accumulation of fluid in the extravascular spaces and it is a typical sign of inflammation (Kumar *et al*., 2007). It could be recognized in some sections of the PCO-induced mice treated with (1ppm) of silver NPs. This result came in line with Kang *et al*. (2012) who reported that the toxic effect of silver NPs was triggered by inflammation resulted from oxidative stress. Besides, nanoparticles mediate ROS generation and initiate a sequence of pathological events, including inflammation, fibrosis, genotoxicity, and carcinogenesis (Abdal Dayem *et al*., 2017). ROS production is generated during crucial processes of oxygen (O_2) consumption and certain amount of ROS is needed for the progression of normal cell function (Agarwal *et al*., 1998). Another histopathological change is vacuolation which appeared in theca cells and in the oocyte for the same treated groups. This type of degeneration is observed in mammalian cells after exposure to bacterial or viral pathogens as well as to various natural and artificial low-molecular-weight compounds (Shubin *et al*., 2016). These results were in consistence with results of Al-Gurabi *et al*. (2015) who observed a vacuole- related swelling in the cytoplasm of liver cells from mice exposed to silver NPs in addition to hydropic degeneration. Similar changes were observed in cells of liver and kidney in the groups of rats treated orally with different concentrations of silver NPs (Salman, 2014).In addition, mice treated with silver NPs exhibited a destruction in the blood brain barrier (BBB) in addition to swelling and neuronal degeneration (Park et al, 2010). The cytotoxic of effects the silver NPs may be due to their ability to attach to cell membrane, changing its permeability and leading to intracellular ROS accumulation and oxidative stress (El-Nouri *et al*., 2013). *In vitro* study on mammalian cells revealed that, silver NPs could interact with these cells and cause membrane damage and altering membrane permeability (AshaRani *et al*., 2009; McShan *et al*., 2014). Hydropic degeneration on the other hand, appeared due to disturbed in ions and fluid homeostasis that leaded to increase of intracellular water (Abdelhalim and Bashir, 2011). It is known that silver NPs induce the permeability of cell membrane to potassium and sodium and disturb the activity of Na-K-alpase and mitochondria (Sardari *et al*., 2012). This type of reversible cellular injury is caused by a failure of energy-dependent ion pumps in the plasma membrane leading to an inhibitory to maintain ionic and fluid homeostasis (Kumar *et al*., 2007).

Hyalinization, which is a type of degeneration was found in the stroma and the granulosa layer of some sections and this result was in agreement with El-Nouri *et al*. (2013) who observed excess of collagen deposition in the ovaries of rats treated orally with two doses (30 and 300 mg/Kg) of silver NPs once a day for two weeks. Collagen fibers that found in the fibrous connective tissue become abundant and blend together as a result, a dense a vascular tissue with uniform, structure less, glassy appearance under light microscope would form (Kumar, 2005). Results of an *in vitro* effect of silver NPs on porcine ovarian cells revealed that silver NPs directly affected cell proliferation and apoptosis through influence on expression of growth factor IGH-1, cyclin B1 and apoptosis (Kolesarova *et al*., 2011). So we assumed that silver NPs stimulated the proliferation of the cells that produce collagen fibers and cause the production of hyaline deposits. Amyloid, which is another type of degeneration was deposited in blood vessels of the PCO-induced ovary treated with (1ppm) of silver NPs and it was more obvious in the 45 days experiment. This substance is an extracellular deposition of misfolded proteins that aggregate to form insoluble fibrils (Lee *et al*., 2012). Amyloid is found in at least three different states, one of these is associated with chronic inflammation (Kumar, 2005) which might explain the result obtained in this study.

Necrosis was more obvious in the group treated with (1ppm) of silver NPs. This result has come in consistencewith Sarhanand Hussien (2014) who observed necrosis in hepatocytes after i.p. injection with silver NPs and it was attributed to the inhibition of mitochondrial respiratory chain which caused a reduction in the available energy for cells (Sardari et al, 2012; Sarhan and Hussien, 2014). Exposure to gold NPs (AuNPs) which has the same effect as silver NPs, most likely cause oxidative stress that was amplified by mitochondrial damage and necrosis (Pan *et al*., 2009). Whereas, *in vitro* studies of manufactured silver NPs on reproductive and development revealed that these structures induced necrosis, apoptosis and mitochondrial dysfunction in mouse spermatogonia stem cells (Lankoff *et al*., 2012). Moreover, *in vitro* studies revealed that the distinct mechanisms associated with toxicity of silver NPs included ROS generation and oxidative stress, interaction with cellular proteins and enzymes by binding to free thiol groups, and mimicry of endogenous ions (*e.g*. calcium, sodium, or potassium) leading to disturbances in ion regulation. Such mechanisms lead to cytokine production, cellular damage, and eventually apoptosis or necrosis (Recardati *et al*., 2016). Reactive oxygen or nitrogen species (ROS/RNS) generated naturally, in the cell or in response to environmental stress have long been implicated in tissue injury in the context of a variety of disease states. ROS/RNS can cause cell death by non physiological (necrosis) or programmed pathways (apoptosis) (Ryter *et al*., 2007). Pyknosis of nuclei that appeared in some sections, is a pattern of nuclear changes associated with necrosis due to breakdown of DNA and it is characterized by shrinkage of the cell and increase basophilia, the DNA condense into a solid shrunken mass (Kumar *et al*., 2007).

On the other hand, some sections demonstrated shrunk oocyte which might be due to the necrosis in the granulosa cells (Figures 6 and12). Granulosa cells supply essential nutrients (L-alanine and L-histidine and products of glycolysis) for the growth and development of oocyte and regulate their transcriptional activity (Matzuk *et al*., 2002). Otherwise, this effect might be due to the excessive accumulation of silver NPs in ovarian tissue which might reduce superoxide dismutase (SOD) activity. This enzyme is one of the most important enzymes that act as cellular antioxidant (Sakhee *et al*., 2011). Although, necrosis appeared in some sections of ovary of PCO- induced treated with silver NPs (0.5ppm) but, the oocyte seemed unaffected. These results came in line with an *in vitro* study by Syrvatka *et al*. (2015) who studied the effect of two types of silver NPs capped with two materials polyvinyl pyrrolidon (PVP) and bovine serum albumin (BSA) and found that, silver NPs at a concentration of 10µg/ mL inhibited the growth of granulosa cells but did not affect the maturation of oocytes to metaphase-2. It seemed that the zona pellucida layer protected the oocyte from the cytotoxic effect of silver NPs. Deposition of silver NPs and its toxicity were found in numerous sections of the ovaries in both concentrations and durations. In general, deposition of these particles were observed in different sizes and organs (Sarhan *et al*., 2014; Lee *et al*., 2013; Boudreau *et al*., 2016; Braakhius *et al*., 2014). It is known that nanoparticles penetrate cells without specific receptors on their outer surfaces by passive uptake or adhesive interaction besides, these particles are not necessarily located within a phagosome which, protects the rest of the cellular organelles from the chemical interaction with them (Buzea *et al*., 2007). Whereas, uptake of silver NPs occurs in most cells via endocytosis which, depends on time, dose and energy (Zhang *et al*., 2015). The particular size of silver NPs applied in this study was (50-100nm) in addition these particles were used without capping agents. Taking into consideration that in various environmental conditions silver can undergo spontaneous transformation leading either to creation of nanoparticles from silver ions, or agglomeration of silver NPs into greater particles(Likus *et al*., 2013).` In this study we tried to investigate the therapeutic property of silver NPs at a low and high concentrations and for two durations of treatments since Silver NPs possess many characteristics that make them attractive as medical devices, especially in therapeutic agents and drug delivery systems (Yoisungnern *et al*., 2015). But, the cytotoxic effect of these particles seemed more pronounced the benefits because they were used solyly without capping agents.

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