Silver Nanoparticles Cause Some Physiological Changes in Ovaries of Mice Treated with Human Chorionic Gonadotropin (hCG)

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Abstract

Silver nanoparticles is widely applied in recent years. These particles have minimal effects on FSH yet, they raise levels of LH hormone. Human chorionic gonadotropin hCG has a similar structure and function as LH. This hormone is used in ovulation induction in human and animals but, it may involve in the formation of cystic follicle. **The aim of this study is to examine** the effect of silver NPs on some physiological changes in ovaries of female mice treated previously with hCG. Mature female mice (32) in number were divided into the following groups (n=8) for each: G control which was excluded from treatment, G (hCG) treated by intraperitoneal injection with hCG solution(10 I.U) three times a week for two weeks, then they were sacrificed and ovaries were isolated and preserved for histological study. The rest(16), were divided into two groups according to the concentration of silver NPs solution(0.5 and 1ppm) which was administrated by (i.p) injection twice a week for 45 days. At the end of this period, isolated ovaries were sectioned and stained with routine stains haematoxylin and eosin. A significant increase ($P \leq 0.05$) in diameters of ovaries for both groups treated with hCG then with (0.5 and 1ppm) of silver NPs. Number of pre-antral follicles were significantly high($P \leq 0.05$) in the group treated with hCG+ 0.5 ppm of silver NPs. Number of antral was significantly high in the group treated with hCG. Whereas, number of corpora lutea in the group treated with hGC+ 1ppm silver NPs was significantly higher ($P \leq 0.05$). All the histopathological changes in the treatments were due to silver NPs.

Keywords: hCG, Cystic Follicle, Silver NPs, Corpus Luteum Cyst.

Introduction

Nanotechnology is progressively developing due to the production of nanoparticles (NPs) & Nano-products that comprise of fresh & size specified physiochemical properties which evidently vary from the bigger material (Tran *et al*., 2013). Silver is the single most used material in all of the nanotechnology (Biswas and Dey, 2015). Silver nanoparticles (NPs) is widely recognized for its activity to kill bacteria, the lack of cytotoxicity of silver NPsto mammalian cells has resulted in its use as antibacterial and antibiotic treatment for a long time (Seo *et al.*, 2014). Recently, many studies took up the effect of silverNPs and other nanoparticles on both male and female reproductive systems. The effect of oral administration of silver NPs (60nm in dimension) at different concentrations on the histological structure of testes and levels of sex hormones and gonadotropin in rats was studied by Baki *et al*, (2014). Although, these particles revealed minimal effects on FSH levels, they raise LH levels. This study was also assured by Attia, (2014) but in male mice. On the other hand, Luaibi, and Qassim (2017) documented that, an alteration in serum levels of LH arouse in rats treated orally with silver NPs for long term. The effect of silver NPs on the histological structure of ovaries obtained from lactating mice also was studied by (Mehdi and Al-naqeeb, 2018) and different types of degeneration and necrosis appeared in the architecture of these ovaries.

Ovarian follicular cysts are progressed in other mammals & humans as well due to the disruption in hormonal interactions that are mandatory for the development of normal follicles, normal follicular atresia and ovulation (Bogovich, 1992).In a study achieved by Manjarin *et al*, (2015) on giltsdata revealed that, treatment with human Chorionic Gonadotropin (hCG) followed by treatment with equine chorionic gonadotrophin (eCG) induces a higher number of luteal bodies compared to injection of eCG /hCG alone, thoughits usage is related to the development of cyst by means of dose dependence. In current research, female mature mice were treated with the hormone named as human Chorionic Gonadotropin (hCG). The resemblance of gonadotropin activity to the LH assist in binding to the receptors of LH on the small luteal cell membrane for the second messenger activation to enhance the

synthesis of progesterone (Santos *et al*, 2001; Bryn, 2015). Even though, the hCG and LH share a mutual receptor, however, each hormone activates an exclusive cascade of events subsequent to the binding of receptors (Choiand Smitz, 2014).Similarly, the hCG's low dosage can encourage the formation of ovarian follicular cysts in immature rats which have synchronized progesterone. Furthermore, persistent stimulus given by LH than that of LH serum elevation might be a stern factor for ovarian cyst development (Bogovich,1991).Further researches commended that the growth of ovarian cysts is related to the hypothalamo-hypophysealgonaldal axis' imbalance (Nora *et al*,(2018).

Areduction in number of graffian follicles (Ghorbanzadeh *et al*, 2011) and secondary follicles (Ghorbanzadeh *et al*., 2012) in ovaries of rats treated by intraperitoneal injection of silver NPs was observed. Additionally, the silver NPs' histopathological impacts on the rats' ovaries (El-nouri *et al*., 2013) and ovaries of polycystic induced female mice (Al-naqeeb *et al*., 2017) were also studied. Whereas, a significant alteration in the levels of E2, FSH and LH serum was perceived in the female mice induced with polycystic (PCO) serum while being treated with various silver NPs' concentrations by intraperitoneal injection (Fakhrildin *et al*, 2017) or oral administration (Luaibi and Qassim, 2017) at different durations.

So, current study was directed to examine the silver NPs' effect on the diameter of corpora lutea, ovaries, (pre-antral, antral) follicles' number and corpora lutea number in mice treated with hCG hormone. A histopathological study was also included.

Materials and Method

Animals

A number of mature female mice (28), weighting about(20-25) gm were purchased from the High Institute of Infertility Diagnosis & Assisted Reproductive Technology/ Al-Nahrain University's animal house. Moreover, animals were kept in the animal house of same institute, under standard conditions of temperature (25-28)°C and 12 hours light dark cycle throughout the period of experiments (45days) .

Treatment with hCG Hormone

Female mice (n=32)were injected intraperitoneally (i.p) with the hCG (human Chorionic Gonadotropin/ Choriomon) while giving a (10 I.U) dose between one day and another fortwo weeks, (Bogovich, 1991).

Only one group of mice were excluded from hCG injection and considered as control group (Gc). The rest, (n=24) were divided into the following subgroups after hCG injection:

G_{hCG} (n=8) = were sacrificed immediately after two weeks of treatment with hCG hormone.

G1 (n=8) = were treated with silver NPs(0.5ppm) by intraperitoneal injection, twice a week for 45 days.

G2 (n=8) = were treated with silver NPs(1ppm) by intraperitoneal injection, twice a week for two weeks.

At the end of experimental time, mice were sacrificed, ovaries were isolated from the surrounding tissues. Weights of these ovaries for each mouse were measured by sensitive balance, and then the isolated organs were preserved in Boun′s solution for (24) hours which was then replaced by ethanol(70%)for histological preparation.

Solution of Silver Nanoparticles (silver NPs)

Solution of silver NPs was prepared by Iranian Nanoparspanda Company at a concentration of (4000) ppm and a size of about (50-100) nanometer.Using Scanning Probe Microscope (SPM), characteristics of this solution was confirmed at the Chemistry department, College of Science/ Baghdad University. Furthermore, the solution was activated with the usage of ultrasound sonicator at the Physics department of College of Science, University of Baghdad every two weeks. In this study, two concentrations (0.5 and 1 ppm) were prepared from the stock. According to Ghorbanzadeh *et al*(2012) and according to the following formula: $C_1 V_1 = C_2 V_2$ (Skoog *et al*., 2004).

Histological Preparation

The histological preparation was done according to (Bancroft and Stevens, 2010). Briefly, it includes the following steps: dehydration with the assistance of ascending ethanol's grades (70%, 90% & 95%), clarification was done in xylene and after that embedded in the paraffin wax at 56°C melting point. At the end segmenting and lastly staining was carried out with eosin and hematoxylin.

Measurement Diameters of the Ovaries and Corpora Lutea

In order to study the histological changes of the ovaries, pictures were captured by using a special microscope supplied with camera (*Leica*). The ocular lens (10X/20) and the objective lens (4X/0.10) were used. These pictures then were downloaded on the computer and by using Motic Image Analyzer Plus2.0 program, diameters of ovarieswere measured. Each diameter was taken by extracting the average of the longest diameters. Diameters of corpus luteum were also measured by the same way.

Studying the Histological Structure of the Ovary

The histological changes of ovaries were examined using Olympus (a light microscope) that had objective

lenses of 40X while 10X ocular lenses. These changes include counting different follicular development stages conferring to Myers *et al*, (2004) with some modifications:

- Pre-antral follicles which include primordial, primary and secondary follicles; the identification of primordial follicles was done through an oocyte encircled by a whole squamous follicular cells layer, however, primary follicles demonstrate a single cuboidal granulosa cell's layer. While, secondary follicle was recognized as an oocyte that had been surrounded by two or even more granulosa layers.
- Early late antral follicles: usuallyown one or two smaller follicular fluid (antrum) areas, though, the antral follicles have a single yet larger antral space.
- The corpus luteum number was calculated and structure's diameter was also measured.

Statistical Analysis

The program Statistical Package for the Social Science (SPSS)& one way ANOVA was utilized for the data analysis and to find out the significant differences. Results were obtained on the base of least significant difference (P ≤0.05).

Results

Ovaries' diameters of the treated groups are illustrated in figure (1). Non-significant differences were found between the cystic follicle groups(Gcf) when related to the control. Whereas, a significant increment (p ≤ 0.05)was obvious in diameters of ovaries for the cystic follicle group (Gcf) with given 0.5ppm silver NPs in comparison to the both control and the cystic follicle group (Gcf). Similarly, cystic follicle group (Gcf) treated with (1ppm) of silver NPs revealed a significant increase (p≤0.05) as compared to the control and to the cystic follicle group(Gcf). In contrast, a significant reduction (p ≤0.05) in diameters of the ovaries were seen in the cystic follicle group (Gcf) treated with (1ppm) of silver NPs as compared to the cystic follicle group (Gcf) treated with (0.5ppm) of silver NPs.

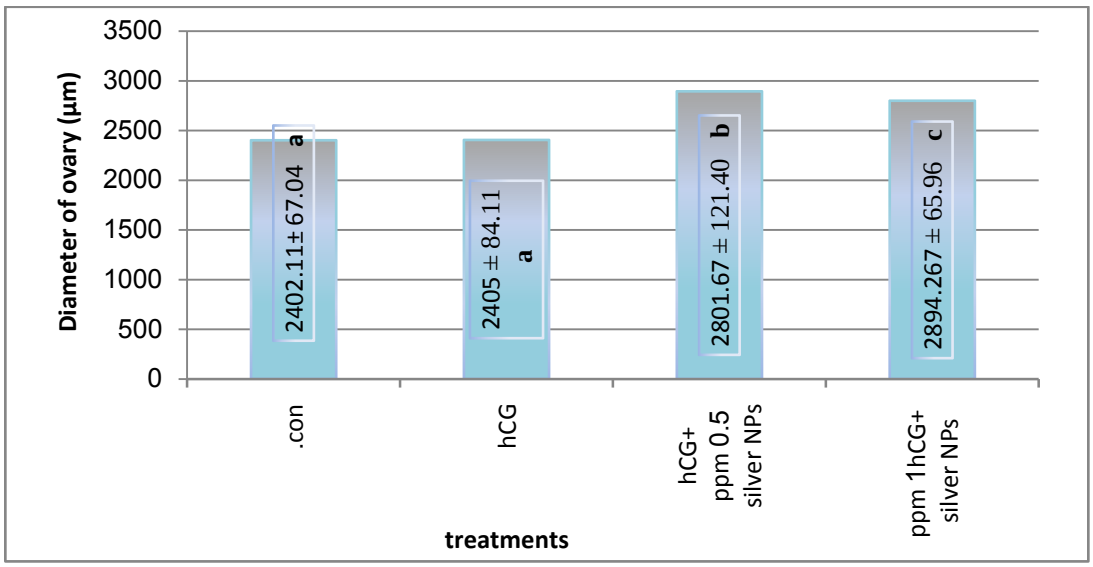

Figure-1: Effect of silver NPs (0.5 & 1ppm) on diameters of the ovaries of mice treated with hCG hormone. Means with similar letters are non significantly different, while means with different letters are significantly different (p≤ 0.05).

Figure (2) shows diameters of the corpus luteum for the control and the treated groups. A significant reduction (P≤0.05)was seen in diameters of corpus lutium for the group treated with hCG as compared to the control. But, non significant difference was seen in diametrs of corpus lutium in the group treated with hCG hormone + 0.5ppm of silver

NPs when compared to the group treated with hCG hormone. Similarly, a substantial decrease (P≤0.05) was found in diameters of corpus lutium for the group treated with hCG and silver NPs of 0.5ppm in comparison to the control. While, the group treated with hCG hormone + 1ppm of silver NPs revealed an important decline (P≤0.05) in diameters of corpus lutium as compared to the control. Whereas, the same group revealed a significant elevation (P≤0.05) in corpus lutium diameters than that of the both hCG treated group and the group treated with hCG+ 0.5 ppm of silver NPs.

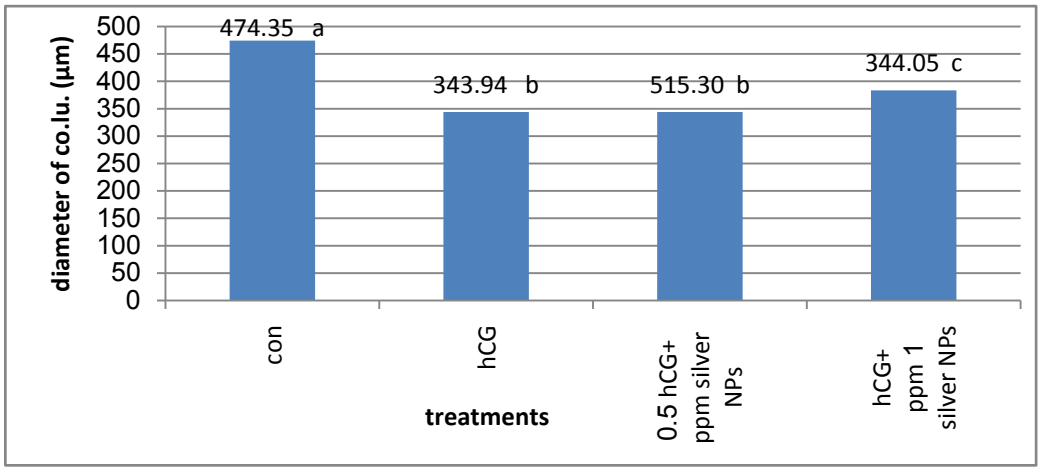

Figure-2: Effect of silver NPs (0.5 & 1ppm) on diameters of the corpus luteum in ovaries of hCGtreated mice. Means with similar letters are insignificantlydifferent, while means with different letters are significantly different (P ≤0.05)

Number of pre-antral (primordial, secondary & primary) follicles of control group and the treatments is illustrated in figure (3).Only one group of the treatments revealed a significant elevation (P ≤.0.05) in pre-antral follicle's number. It was the group treated with hCG and silver NPs (0.5ppm) in relation to the hCG treated group, control group and group treated with hCG as well as (1ppm) of silver NPs.

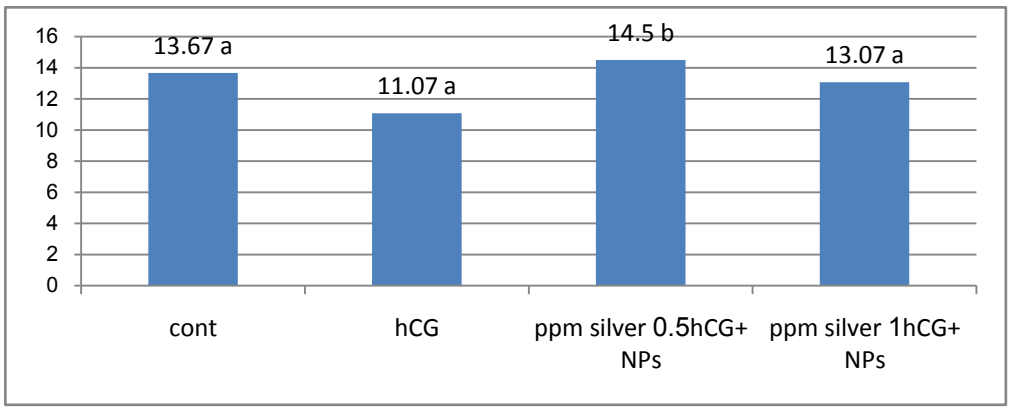

Figure-3: Effect of silver NPs (0.5 & 1ppm) on number of pre-antral (primordial, primary and secondary follicles) in ovaries of hCG treated mice.Means with similar letters are nonsignificantlydifferent, while means with different letters are significantly different (P ≤0.05)

Number of antral follicles for the control and the treatments are illustrated in figure (4). A significant increase (P ≤0.05) was found in the antral follicles' number in hCG hormone treated group in relation to the control. However, treatment of another group with hCG+ (0.5ppm) of silver NPs and the group treated with hCG+ (1ppm) of silver NPs.

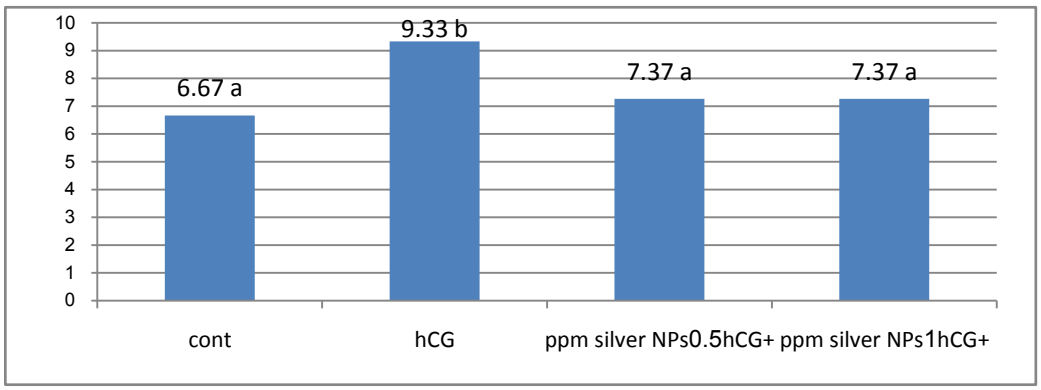

Figure-4: Effect of silver NPs (0.5 & 1ppm) on number of antralfollicles in ovaries of hCGtreated mice.Means with similar letters are insignificantlydifferent, while means with different letters are significantly different (P ≤0.05)

Number of corpus lutium for the control and the treatments is illustrated in figure (5). A substantial increase of about (P≤ 0.05) in the corpus lutium number was evident in the group that had been treated with hCG+ (0.05ppm) of silver NPs in comparison to the control group, further, the group treated with hCG+ (0.05)ppm and the group treated with hCG +(1ppm) of silver NPs.

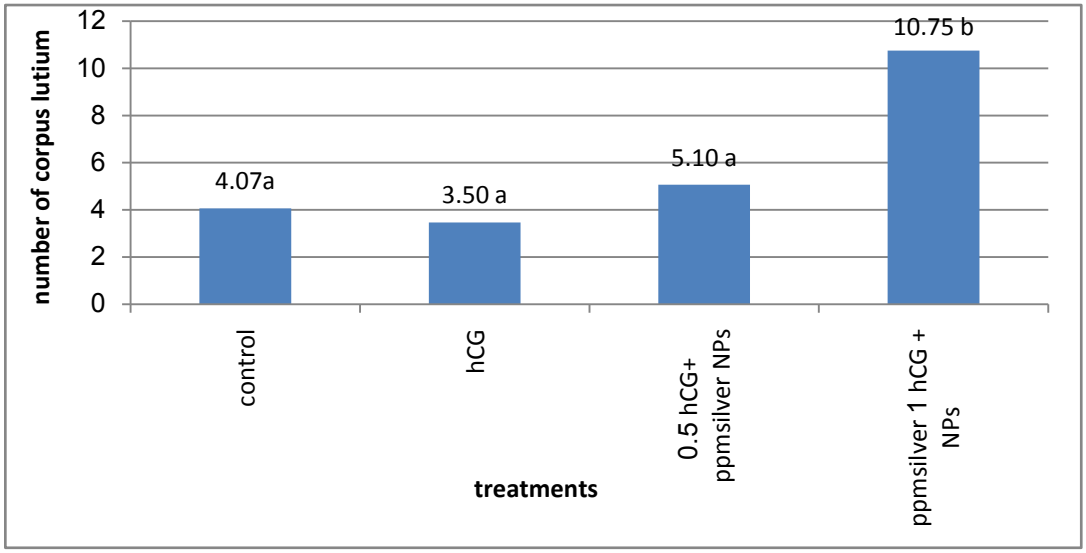

Figure-5: Effect of silver NPs (0.5 & 1ppm) on number of corpora lutea in ovaries control and the group treated with hCG hormone. Means with similar letters are non significantly different, while means with different letters are significantly different (P ≤0.05)

Histopathological Study

A section in an ovary of a mouse from control group is represented in Fig.6.

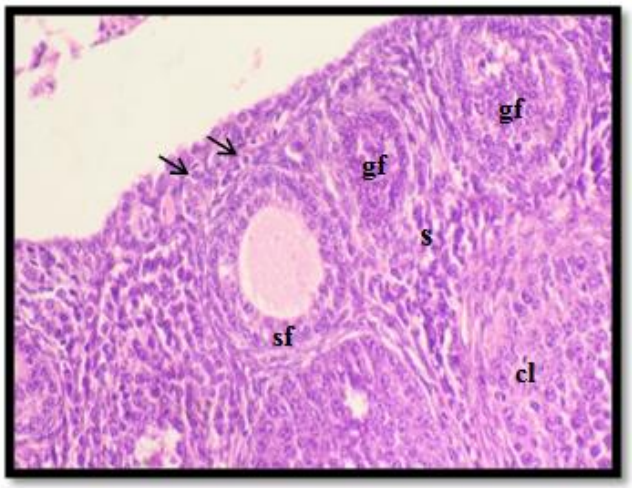

Fig.6: Section in an ovary from the control group showing: germinal epithelium(arrows), secondary follicle with two rows of granulosa cells (sf), growing follicles at different stages (gf), corpus lutium (cl) and stroma.H&E400X

A section in an ovary of a mouse treated with hCG hormone group is illustrated in figure (7) **A**. a number of atretic small antral follicles (red arrows), increase in size of stroma (double head arrow). **B**. a large cystic follicle with highly stimulated theca layer and remnant of granulosa cells(red arrows) with no obvious oocyte.

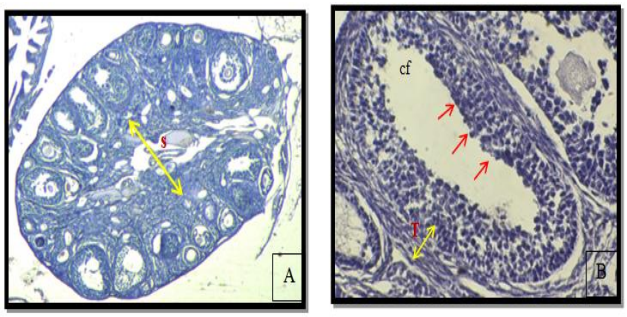

Fig.7: A.Section in an ovary of a mouse treated with hCG hormone.(100X)B. Large cystic follicle (cf), thick theca layer(T), residual of granulosa cells (red arrows). H&E.400X

The next figure (8) represents section in an ovary from the group treated with hCG and (0.5ppm) silver NPs. Pre-antral follicle with shrunk oocyte(sh-o) with vacoulation (v), hydropic degeneration (h) in granulosa, stroma cells and theca shell , pyknosis (p) in some granulosa cells, edema (e)and necrosis (n) in the stroma. In another section. Fig(9) from the same treated group, two pre-antral follicles appeared with hydropic degeneration in the stroma cells, theca shell can hardly be distinguished some with hydropic degeneration, pyknosis was obvious in some granulosa cells.

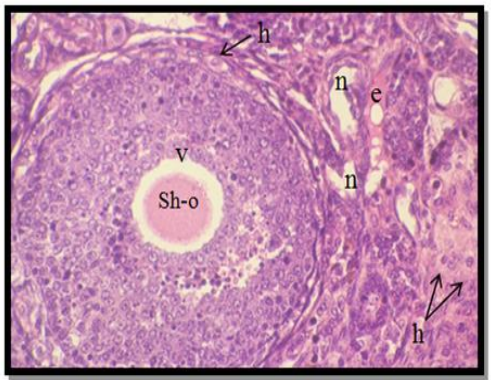

Fig.8: Section in an ovary from the group treated with hCG+ (0.5ppm) silver NPs. Pre-antral follicle with shrunk oocyte (sh-o) and vacoulation (v), hydropic degeneration (h) in granulosa, theca cells and stroa, edema (e) & necrosis (n) in the stroma.H&E.400X

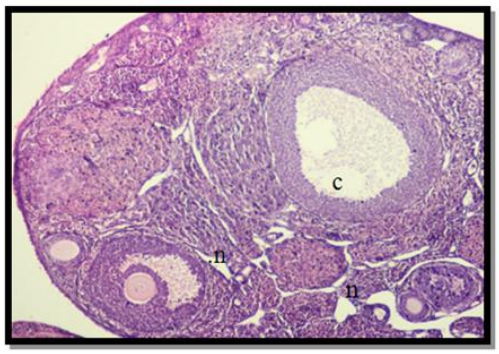

Fig.9: Section in an ovary of group treated with hCG+ (1ppm) silver NPs. Corpus lutem cyst (c), necrosis (n) in the stroma.H&E.100X

Figure (9) represent sections in an ovary from the group treated with hCG+ 1ppm of silver NPs. Large cystic follicle, degeneration appeared in most stroma cellsin addition to necrosis (n).The next figure (10), represent section in an ovary from the group treated with hCG + (1ppm) silver NPs. A growing follicle with sever damage, degeneration in addition to pyknosis (p) in most granulosa cells, vocoulation (v) and fragmentation of the oocyte. Undistinguishable theca sheet. Degeneration and necrosis (n) in the stroma cells.

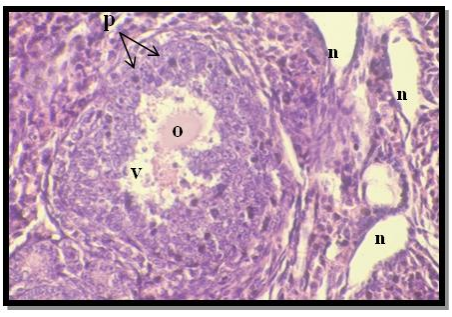

Fig.10: Section in an ovary of group treated with hCG+ (1ppm) silver NPs.growing follicle with vacoulation (v) of the oocyte, pyknosis (p) in some granulosa cells, undistinguished theca shell, necrosis (n) in the stroma.H&E.100X

Discussion

This article took up the effect of silver NPs on some physiological parameters of mice's ovaries which were previously treated with hCG hormone. On regard diameters of the ovaries, an increment was obvious in both groups treated previously with hCG hormone then with (0.5 and 1ppm) of silver NPs.

No data were obtained on effect of hCG hormone on diameters of ovaries. So these changes may be attributed to silver NPs. The significant increase in diameters of the ovaries in both groups treated previously with hCG then with silver NPs(0.5 and 1ppm). run in parallel to results of Luibi and Qassim(2017) who observed a significant increase in ovarian weights for rats treated with 50 mg/kg of silver NPs for 30 days. These results can be attributed to inflammation which, is defined as the basic process whereby tissues of the body respond to injury(Stankov, 2012).Since silver NPs is known to induce inflammation (Abdal Dayem *et al*., 2017).

In this study, a relative elevation was seen in number of pre-antral follicles for both treatments (0.5 and 1ppm) figure (8).These results are incompatible to results of (Ghorbanzadeh *et al*, 2012) who observed a reduction in number of secondary follicles in ovaries of rats treated by intraperitoneal injection (i.p) of silver NPs (1and 10ppm) for 30 days. In addition, Lytvynen *et al*., (2017) noticed a decrease in number of living follicular cells surrounding the oocyte by either necrosis or apoptosis as a result of intravenous injection of silver NPs(30nm) at two dose (2mglkg and 4mg/ kg) at a frequency of ten times a day.

This result may be correlated to the elevated levels of FSH caused by treatment with silver NPs. Since, the development of follicles to maturity is FSH dependent (Mason *et al*., 1996). Besides granulosa cells of primary and secondary follicles have FSH receptors (Fox, 2016). According to Fakhredin *et al*,(2017) an increment in serum levels of FSH was seen in polycystic induced (PCO) female mice treated with (0.5ppm) of silver NPs by i.p injection for 45 days. An explanation of that is nanoparticles affect hormone secretion by the ovary and the hypothalamic pituitary gonadal axis (HPOA) through passing the blood brain barriers (BBB) into the hypothalamus and secretory cells of pituitary and change the secretion of GnRH, LH and FSH (Hou and Zho,2017). Whereas, our explanation is correlated to the degenerative changes appeared in granulosa cells in the group treated with hCG+ 0.5ppm of silver NPs, Fig (10).Granulosa cells secret a protein known as inhibin. It acts in an endocrine manner to suppress FSH

secretion and locally to enhance follicles development (Welt *et al*., 2012). So, we assumed that secretion of inhibin by granulosa cells and consequently the negative feedback mechanism between FSH and inhibin was disturbed which, caused an elevation in FSH levels.

The mice estrus cycle is distributed into four stages as pro-estrus, estrus, di-estrus and met-estrus while having a time period of four to five days except the pregnancy interruption or even anestrus or pseudo-pregnancy (Byers *et al*, 2012). In our recent research, the female mice were subjected to the hCG hormone which is used for ovulation induction. It was reported by Santos *et al,* (2001) that, cows treated with hCG revealed an increases with the incidence of three-wave follicular cycles, where, the appearance of 3rd wave dominant follicle is deferred. This reflects the increment of the antral follicles' number in the group that had been treated with hCG hormone as shown in figure (9).

While, the reduction in number of antral follicles caused by treatment with (0.5 or 1ppm) of silver NPs can be explained by the ability of these particles to accumulate in theca and granulosa cells and affect steroidogenesis (Hou and Zhu, 2017).Similar results was shown by Ghobanzadeh *et al*(2011) on regard number of graffian follicles. But, these results were not compatible to results of (Elaheh *et al*., 2017) who observed a substantial upsurge in number of graffian & primoidal follicles in ovaries of rats treated orally with silver NPs and isoniazid drug.

A significant elevation in corpora lutea number was observed in the ovaries of mice that were treated with 1ppm silver NPs and hCG. The outcomes appeared to be related to the (Syrvatka *et al*.,2014) that observed a significant elevation in the concentration of progesterone secreted by corpus luteum in the group of female rabbits treated with subcutaneous injection (sc.i) of silver NPs solution at the gestational day 1. For our understanding, the main source of P_4 is corpus luteum which is a prerequisite for pregnancy establishment as well as its maintenance and regulates various reproductive functions (Khanghah and Kor, 2013). Moreover, Hou and Zhu,(2017) reported that, the *in vitro* application of metal nanoparticles (TiO_2 and AgNps) as an inducers for the detection of nanoparticles' effects on the secretion of steroid hormone through porcine granulosa cells instigated the alterations in steroid hormone secretion, progesterone P_4 and estradiol E_2.

Previously in this article, we have noticed that hCG gonadotop in has identical activity to the LH as it binds to the receptors of LH on smaller luteal cell membranes for the activation of 2nd messenger that further increases the synthesis of progesterone(Santos *et al*, 2001; Bryn, 2015). The most extensively acknowledged hypothesis explains the ovarian cyst formation is due to the variations in the release of LH from the pituitary hypothalamus (Vanholder *et al*., 2006). Besides, reduced activity of steroidogenic active regulatory (StAR) protein by some nanoparticles would cause a decrease in levels of E_2 but, levels of LH will elevate (Baki *et al*., 2014) . This explain the presence of large cyst (which may be luteal cyst) as shown in figure (9). Otherwise, these cysts may result in prolonged, noncyclic progesterone stimulation (National Toxicology Program) as a result of hCG injection and treatment with silver NPs (Hou and Zho, 2017) as shown in figure (2-B).

Edema as shown in figure (8) represents an accumulation of fluid in the extravascular spaces and it is a typical sign of inflammation (Kumar *et al*., 2007). As explained by Kang *et al*. (2012), the toxic effect of silver NPs is triggered by inflammation and resulted from oxidative stress. Besides, the cytotoxic effects of silver NPs may be due to theirability to attach to cell membrane, changing its permeability and leading to intracellular reactive oxygen species (ROS)accumulation and oxidative stress (El-Nouri *et al*., 2013). As a result of ROS generation, a sequence of pathological events (inflammation, fibrosis, genotoxicity, and carcinogenesis will appear.

Vacuolation appeared in oocyte of the group treated with (0.5ppm) and in granulosa cells of the group treated with (1ppm) of silver NPs. This type of degeneration is observed in mammalian cells after exposure theo several artificial or natural compounds of low molecular weights (Shubin *et al*., 2016). These results were similar to results of Al-Gurabi *et al*. (2015) who perceived a swelling associated to a vacuole in hepatic cell cytoplasm of mice that had been treated with silver NPs in addition to hydropic degeneration. Similar changes were seen in cells of liver and kidney in the group of rats which took different concentrations of silver NPs by oral administration (Salman, 2014).Moreover, hydropic degeneration appeared due to disturbed ions & fluid homeostasis which led to the intracellular water escalation (Abdelhalim and Jarrar, 2011). Silver NPs induce the cell membrane permeability to the sodium & potassium while disturbing the Na-K pump's activity and mitochondrial cell membrane's activity as well (Sardari *et al*., 2012). This typeof reversible cellular injury is caused by failed energy relied ion pumps of plasma membrane while instigating the inhibition to sustain the ionic & fluid homeostasis (Kumar *et al*., 2007).

Necrosis was more obvious in group that was subjected to the silver NPs of 1ppm. This result has come in consistence with Sarhan and 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 existing energy of cells (Sardari *et al*, 2012; Sarhan and Hussien, 2014).On the other hand, *in vitro* studies exhibited that the distinct mechanisms associated with toxic effects of silver NPs include ROS generation and oxidative stress. Moreover, also with the interactions of enzymes and proteins of cells while binding to the thiol groups for free while imitating the endogenous ions for example, potassium, calcium or sodium that led to the ion's uneven distribution. Thus, these mechanisms instigate the cytokine synthesis, cellular damage, and ultimately, necrosis or apoptosis (Recardati *et al.*, 2016).

As it is well recognized that reactive nitrogen or oxygen species (RNS/ROS) rise naturally in cells or due to the environmental stress reaction. Moreover, these compounds have extensively been related to the injury of tissue in disease state perspective. These RNS/ROS can be a reason of cell decease through non-physiological (necrosis) or programmed pathways (apoptosis) (Ryter *et al*., 2007). Besides, pyknosis of nuclei that was seen in some sections, is a pattern of nuclear changes associated with necrosis due to breakdown of DNA and it is further described by cell shrinkage and increase basophilia, the DNA condense into a solid shrunken mass (Kumar *et al*., 2007).

Conclusion

The increase in number of corpora lutea caused by silver NPs reflects the stimulatory effect of these particles to induce ovulation induction and increase progesterone secretion which, is approved by other authors. Nevertheless, silver NPs still have deterioration effects (degenerations and necrosis) on ovarian tissue.

Recommendation

Additional immunohistochemical study on receptors of sex hormones in reproductive system should be achieved to ensure the ability of silver NPs to induce ovulation. Taking in consideration the size of particles, mode and duration of administration and the dose to eliminate the deterioration effects of these particles.

Acknowledgment

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