DOSE AND TIME DEPENDENT EFFECTS OF SILVER NANOPARTICLES (AGNPs) ON OVARIAN HISTOLOGY AND SERUM LEVELS OF SEX HORMONES IN FEMALE RATS

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ABSTRACT

Background: Silver nanoparticles (AgNPs) are the most commonly nanoparticles used in various areas of research, because of their characteristic physical properties as; optical, magnetic, mechanical properties, catalytic performance as well as the antimicrobial effects so that exposing human to increased levels of nanoparticles. However, not enough information’s are accessible about their potential effect on endocrine physiology. Therefore, this study aimed to investigate the time and dose dependent effects of AgNPs in ovaries function, sex hormones and histology in female rats.

Method: Sixty adult female spargue-dawely rats were divided into Three main groups each of (20) animals treated for (10, 20, and 30) days duration. within each treatment duration, animals were assigned into 4 subgroups each of 5 rats as follows; control treated with vehicle and three experimental subgroups treated with sequential doses 12.5, 25 and 50mg/kg of AgNPs 20-30nm by intra-peritonal injection. At the end of each treatment duration, animals were sacrificed, and blood samples were collected and analyzed for serum levels of LH, FSH, Estrogen and Progesterone. Ovary was removed and kept in buffered formalin for microscopic examination.

Result: Serum levels of Progesterone and FSH non-significantly altered by AgNPs in all treatment groups, while serum level Estrogen showed significant increase in short duration (10 days) with all treatment doses of 12.5, 25 and 50mg/kg but in long duration (30 days) showed significant decrease in serum level Estrogen in all treatment doses of 12.5, 25 and 50mg/kg, furthermore, Treatment for (20 day), with 12.5 mg/kg showed significant elevated in the level of Estrogen. While, 25 and 50mg/kg caused highly significant decrease in Estrogen levels for same duration (20 day) when compared to control groups. This result was confirmed by histological examination of ovary tissues. As well as, the study of ovary weight showed the long-term exposure 30 day to 50 mg/kg of AgNPs caused a highly significant increase in weights of ovaries.

Key Words: Silver Nanoparticles, Ovary, LH, FSH.

1. INTRODUCTION

Nanoparticles (NPs) are small atoms with size ranging between 1-100nm where the name ‘nano’ indicates one billionth or 109 units (Kholoud et al., 2010). The metallic NPs display different properties than they found at larger bulk size. These properties are accredited to their small size and larger surface area to volume ratio (Yu et al., 2007). The NPs with smaller sizethan (50nm) are able of entering the cells and move out of the blood vessels if their size is less than 20nm (Yihand Wei, 2005).

It is well known that silver ions are highly toxic on the mammaliancells. Therefore, this AgNPs can affect organs such as liver, kidney, heart, lung, thyroid, Brain and others. to date, little is understood concerning the distribution, accumulation, and target organ of AgNPs in organisms. Recently there has been a concern of the wide use of nanoparticles and its potential hazards. But regulatory control over the use or disposal of such products is lagging due to insufficient assessment of the toxicology of silver nanoparticles (Stensberg et al., 2011). Therefore, this study investigated the dose and time dependent effect of exposure to AgNPs on Ovarian histology and function exemplified by measurement of serum levels of sex hormones in female rats.

2. MATERIALS AND METHODS

2.1. Preparation of Silver Nanoparticle (AgNPs) Solution

- The AgNPs used in this study was obtained from skyspringnanomaterials.
- The product of AgNPs has the following characteristic properties:
  • Gray powder of with 99.95% purity
  • particle size of 20-30nm diameter, specific surface area ~20 m2/g, morphology (spherical) and have 10.5 g/cm3 density.
- Concerning the preparation of injected suspension of AgNPs; Initially stock suspension of (40mg/ml) concentration was freshly prepared by dispersing the weighed amount of AgNPs in deionized water, and then mixed by vortex for 10 min. From this stock suspension two additional diluted AgNPs suspensions of (10 and 20) mg/ml final concentrations by diluting the calculated volume of stock suspension with deionized water to achieve the final required
concentration. The prepared dilutions then mixed, and the required volume calculated and injected to animals by intraperitonal route.

2.2. Animals: Sixty female Sprague-dawley rats were purchased from National Center for Drug Control and Research (NCDCR)/ ministry of health, Baghdad, Iraq. Females ratshaving body weight range between 225-250gm were selected as the animal model for the study of AgNPs effects. The animals were maintained at controlled laboratory conditions of temperature (25 ±20 C) and humidity, with 12 hours light-dark cycle along the time of study. Animals were allowed to feed standard rat pellet ad libitum with free access to tap water. After the end of experimental treatment all animals were performed in compliance with ethics committee.

2.3. Experimental design and treatment: Animals were randomly divided into 12 groups, each of 5 rats. Three control groups, and nine treatment groups.
- Group (1, 2 and 3) control
- Group (4, 5 and 6) treated with low dose (12.5ppm) of AgNPs intraperitoneally for 10, 20 and 30day respectively.
- Group (7, 8 and 9) treated with middle dose (25ppm) of AgNPs intraperitoneally for 10, 20 and 30day respectively
- Group (10, 11 and 12) treated with high dose (50ppm) of AgNPs intraperitoneally for 10, 20 and 30day respectively.

The rats were anesthetized by ether before getting sacrificed, and blood samples were taken directly from the heart. Ovary was removed, and weight then kept with formalin 10% be used for histological examination.

2.4. Hormonal examination: Blood samples were centrifuged at (3000) rpm for (10 min). Then each sample was collected and frozen at -20C until analysis. Serum levels of LH, FSH, Estrogen and Progesterone were determined using ELISA technique for the quantitative determination of concentrations of female sex hormones LH according to Kosasa (1981), FSH according to Odell (1981), Estrogen and Progesterone according to Abraham (1981).

2.5. Histological examination: The procedure described by Adeyemi and Akanji (2010) were used. in Briefly, ovary was fixed in 10% formalin, dehydrated through ascending grades of ethanol (70%, 90% and 95%), cleaned in xylene and embe- ded in paraffin wax (melting point 56°C) and stained with hematoxylin and eosin. The photo- micrographs were captured at X100 using the software Presto Image Folio package.

2.6. Statistical analysis: The obtained data were analyzed statistically using unpaired t-test in comparison with control and ANOVA analysis for group comparison. P values less than 0.05 were considered as significant.

3. RESULTS AND DISCUSSION

Effect on Ovarian Hormones: The statistical analysis of serum progesterone levels was demonstrated in Figure 1, which showed that intraperitoneal injection of different doses 12.5, 25 and 50 of AgNPs for different durations 10, 20 and 30day, non-significant increase (P≥0.05) serum progesterone levels as compared with control groups, as well as, non-significant results were found among all groups included in this study.

Concerning estrogen hormone Figure 2 displayed the statistical analysis of serum estrogen levels (E2), which very highly significant increased (P<0.001) after 10 days of treatment with all doses used in this study 12.5, 25 and 50mg/kg in comparison to control groups. While, prolongation of treatment duration to 30days showed high significant (P<0.01) decrease in E2 levels was observed with all doses 12.5, 25 and 50.

Treatment for intermediate duration 20days, only the small dose 12.5mg/kg very highly significant elevated (P<0.001) the level of E2. While, both medium and high doses 25 and 50 mg/kg caused highly significant decrease (P<0.001) in E2 levels for same duration 20days when compared to control groups.

However, a dose and time dependent differences was observed among different treatment groups. Where, prolongation of treatment duration to 20 and 30days, considerably reduced the levels of E2 relative to those observed with short duration 10 days. In addition, increasing the dose up to 50 mg/Kg give rise to significant decrease in E2 in comparison to low dose 12.5mg/Kg within each of treatment duration.
Figure 1: Progesterone levels in response to different doses of AgNPs and different treatment duration. Data expressed as mean ± SD. ANOVA (P≥0.05) among all groups.

Figure 2: Estrogen levels in response to different doses of AgNPs and different treatment duration. Data expressed as mean ± SD. (***) Very highly significant increase (P<0.001) relative to control group. (***) Highly significant increase (P<0.01) relative to control group. (a,b,c) represent the significant difference between groups.

Normally, the ovarian hormones E2 and P4, secreted in response to other female sexual hormones of anterior pituitary to regulate reproduction (Christensen et al., 2012). A process that mediated by hypothalamus-pituitary-gonadal axis (HPG). This axis controlled by positive and negative feedback mechanism (Plant, 2015).

In ovaries, E2 production occur in granulosa follicle cells (Nelson and Bulun, 2001). Therefore, several evidences postulated a detrimental effect of environmental nanomaterials on performance of mammalian female reproductive health, by impairment of normal gonadal process, fertility and hormone production (Abdulsattar, 2015). However, few studies evaluating the toxic effect of AgNPs on reproduction and development. The finding of this study showed different effects of AgNPs on E2 levels. A significant estrogenic effect was found with different doses of AgNPs that used for (10 days) duration. This increase suggested to result from possible activation of cytochrome P450 aromatase enzyme, which involved in metabolic conversion of testosterone to E2 (Zhao et al., 2013). This consistent with previous study to Gao et al. (2012) which indicated to a significant increase in E2 levels with all doses (2.5, 5 and 10) mg/kg of titanium oxide nanoparticles (Tio2-NPs) in female mice after oral administration. It is
satisfactorily that NPs can increased membrane permeability of biomembranes that may intensify cellular efflux of E2 from granulose cell, and the lipid storage sites. Findings of this study disagreed with other study reported by Esmaeillou et al., (2013), which demonstrated non-significant change in serum level of E2 of female Wister rat after administration of oral daily dose (333.33 mg/Kg) of ZnO-NPs 20-30nm for five consecutive days. Also, this study revealed that long term exposure to high doses of AgNPs result in significant reduction of serum E2 level. This result could be attributed to possible disruption of functional integrity of mitochondrial within ovarian cells that consequently reduce conversion of cholesterol to pregnenolone resulting in reduced levels of E2 produced by these cells. These results comparable to that reported by others, studied the effect of AuNPs on granulosa cells (Stelzer and Hutz, 2009). Where they postulated that toxicity of NPs may in part mediated by increasing the level of ROS, which can affect cell signaling and ATP synthesis that subsequently altered expression of genes encoding of proteins involved in synthesis and/or release of ovarian hormones.

Regarding progesterone levels, which non-constantly secreted along and during estrous cycle in non-pregnant animals, therefore, the obtained data showed no significant changes (P≥0.05) for all doses and durations used in this study. As, there are no studies about the effect of AuNPs on granulosa cells (Stelzer and Hutz, 2009). However, studies of NPs effect on female reproductive hormones using pregnant animals showed variable data, where reduced levels of P4 was observed in response to nanoparticle-rich diesel exhaust (NRDE-NPs) administrated to pregnant rats by inhalation exposure for 5hr daily (Li et al., 2013). While, Karimpour et al., (2016) injected pregnant Wister rat intraperitonially from 7 to 18th day of pregnancy with different doses of AgNPs 250, 500 and 1000ppm, found a significant increase of P4 level associated and decrease of E2 level at dose 250ppm.

**Effect in Gonadotropin hormones (LH and FSH):** Statistical analysis of data related to luteinizing hormone (LH) was plotted in Figure 3. The results revealed no significant suppression (P≥0.05) in serum levels of LH in animals treated with different doses 12.5, 25 and 50gm/kg of AgNPs for both short and intermediate durations 10 and 20 days respectively in comparison to control groups. While, high significant decrease (P<0.01) in serum levels of LH were observed with long term treatment (30 day) with each of the three doses levels used in this study 12.5, 25 and 50mg/Kg as compared with control groups.

Investigation of differences among all different treated groups used in this study demonstrated that groups treated with different doses for short and intermediate duration have a comparable result with both the control and those exposed to different doses for long term duration 30 days. In this study analysis data related to serum FSH levels presented in Figure 4, which showed that all experimental doses used in this study 12.5, 25 and 50mg/kg non-significantly affect serum FSH levels for all treatment durations 10, 20 and 30 days as compared to control groups, as well as, non-significant differences(P≥0.05) were observed among all treated groups.

![Figure 3: LH levels in response to different doses of AgNPs and different treatment duration. Data expressed as mean ± SD. ( **)high significant difference P< 0.01 in comparison to control. (a,b) represent the significant difference between groups.](image-url)
Number of later studies suggested that exposure to some NPs may pose a threat to both male and female reproductive health, by disturbing the functional and/or structural integrity of reproductive glands causing alteration in sex hormone levels (Sycheva et al., 2011). Results of previous in vitro and in vivo studies demonstrated that NPs are able to affect testosterone production in Leydig cells by maintaining the conversion of transported cholesterol to pregnenolone in Leydig cells (Payne, 1990). Whereas, this effect did not observe with increased concentration of NPs.

The results of this study showed a time dependent decrease in serum levels of LH, which found to be significant after 30 days of exposure to AgNPs. While, no significant decrease (P≥0.05) in FSH levels was observed at all treatment durations of this study. These results were found to be in the line of other studies reported by Rezaei-Zarchi et al., (2013) who found non-significant decrease (P≥0.05) in FSH levels in male rats treated with different oral doses 25, 50, 100 and 200mg/kg of AgNPs, with significant reduction in serum levels of LH. This disruption of normal sex hormone levels induced by NPs may attributed to their possible effect on mitochondria of functional cells that lead to reduce their secretory activity. Furthermore, AgNPs known to induce oxidative stress and increase the releasing of ROS which enhance the oxidation of cellular macromolecules such as proteins (Carlson, et al., 2008).

However, physico-chemical properties as charge, reactivity and functionalization and the degree of agglomeration in biological fluids may in part involved in hormonal disrupting effect of NPs (Gatoo, et al., 2014).

Other study showed that the levels of Plasma (LH and FSH) non-significantly changed in animals treated with AuNPs suggesting the direct impact of NPs on testicular hormonal synthesis and release. Since, the levels of LH and FSH released from pituitary remained unmodified (Li et al., 2013). In the same direction, previous experimental study showed a dose-dependent effect of metal NPs on ovarian cell viability following both acute and sub-acute exposure (Di Virgilio et al., 2010; Wang et al., 2011; Hussain, 2017). Also, others showed non-significant alteration in serum LH and FSH of female rat treated with zinc oxide NPs (ZnO-NPs) (Esmaeillou et al., 2013). On contrast, others established a significant reduction in levels of LH and FSH with sub chronic exposure to Titanium oxide NPs (Mahdieh et al., 2015). A study reported by Omidi et al., (2015) also approved that treatment of female rat with high dose 400mg/Kg of Zirconium oxide NPs induced a significant decrease (P<0.05) in levels of LH and FSH which inconsistent with results of this study. Other study showed that treatment of Wister rats with high dose (40mg/kg) of ZnO-NPs intra-peritoneally induced significant decrease (P<0.05) in FSH while no change observed in LH serum level also reported that its possible NPs inhibit function of endocrine system by blocking of pituitary-hypothalamus axis and it is may be because of reduction in GnRH level (Reza et al., 2013), An
Opposing results was observed by Behnammorshed et al., (2015), explained that animals treated with AuNPs 25, 50 and 100mg/kg significantly increased LH and FSH levels. Other conflicting results for the alterations in LH and FSH levels induced by metal-based NPs were documented by Baki et al., (2014), who found that treatment of male rats orally with different doses 25, 50, 100 and 200mg/kg of AuNPs showed non-significant reduction in FSH (P≥0.05), while, LH levels significantly increased (P<0.05). As many studies demonstrated that the small size of NPs facilitated their penetration across variety of bio membrane including blood brain barrier, so that they have tendency to alter reproductive and development by their central effect on hypothalamic-pituitary-axis (McAuliffe and Perry, 2007). Accordingly, in this study the observed effect of AuNPs on LH and FSH may in part mediated by central effect of AuNPs.

**Effect on Ovarian Histology:** The microscopic examination of ovarian sections of all control groups appeared with normal morphology, they contained numerous follicles at different development stages. In addition, number of primary graffian follicles with normal corpus luteum was observed. The follicles appeared with distinct zonal granulosa encircled the oocyte, with compressed theca cells, and some primoidal follicles Figure 5.

Ovaries of animals treated with different doses of 12.5, 25 and 50mg/kg of AuNPs showed dose and time dependent increase in congestion of blood vessels in area of stroma relative to control groups. Moreover, short term exposure 10days to AuNPs revealed an increment in the number of secondary graffian follicles, and this result is consistent with hormonal increase in the level of E2 in response to treatment with different doses of AuNPs for 10 days duration. These results agreed with previously reported data suggested that exposure to AgNPs enhanced E2 effect on reproduction in concentration, and suppress follicular atresia of secondary and tertiary follicles (Asarea et al., 2012). On contrast, ovaries of animal’s exposed to intermediate and high dose 25 and 50mg/ kg for 20 and 30 days durations displayed several histological changes when to control groups, where some vacuoles and congested blood vessels found in corpus luteum with clear atretic follicles. The stromal connective tissue appeared with vacuolated interstitial cells. Moreover, an increment in the number of corpus luteum and mild to moderate deposition of collagen fibers in stroma that increased with dose and duration of exposure to AgNPs (Figures 6, 7, and 8). Accordingly, these results agreed with those of previous study reported that chronic exposure to different doses of AgNPs induced histological changes in ovary cells of experimental animals (El-Nouri et al., 2013).

Other in vitro studies suggested that the toxicity of AgNPs mediated by induction of either apoptosis or necrosis of different targets is concentration and time dependent (Ahmadi, 2009; Braydich-Stolle, 2005). However, Tiwari and colleague (2011) documented safe exposure to AgNPs at doses below 10mg/kg, while, doses above 20mg/kg considered to be toxic with repeated exposure. In same manner, AgNPs exhibits toxicity to different biological targets as reported to be mediated by the effect of AgNPs various bio membranes affecting their permeability either by release of silver ion or generation of reactive oxidant producing cellular oxidative changes (Kim et al., 2008). Accumulation of these changes increased with time to overwhelm the cellular defense capacity, which subsequently result in disruption of mitochondrial function and impairment of cellular respiration and cell death (Carlson et al., 2008)

![Figure 5: Section of ovary from rat control groups showing consist numerous numbers of primary follicles and primary graffian follicles with corpus luteum. (H and E) 100x.](Image)
Dose and time dependent effects ...

In this study results of microscopic examination of ovarian tissue were in agreement with hormonal results, where there are no changes in values of Progesterone and FSH in different doses for all treatment durations with AgNPs compared to control groups. While, a significant (P<0.05) decrease in serum levels LH was observed with long term 30 days of treatment with 25 and 50mg/kg of AgNPs. As well as, Estrogen levels showed significant increase in short term duration 10 days and
significant decrease in the chronic duration (30 day) in comparison to control groups.

**Effect on Ovary Weight:** In present study analysis of data presented in table 1, and Figure 9 showed; non-significantly (P≥0.05) increase in weights of ovaries of animals treated with different doses 12.5, 25 and 50mg/Kg of AgNPs after both, short 10 days, and intermediate 20days time in comparison to control. Moreover, non-significant (P≥0.05) results were obtained when comparison done among experimental groups with different doses for each duration 10 and 20day. While, with long term of treatment (30 day), only animals injected with low dose 12.5mg/Kg of AgNPs for 30 days showed non-significant increase in weights of ovaries in comparison to control. The long-term exposure 30 day to high dose 50 mg/kg of AgNPs produces a highly significant increase (P<0.001) in weights of ovaries relative to control and other treatment groups, with exception to those treated with 25mg/Kg for 30 days, which revealed a comparable non-significant(P≥0.05) result to control and all treatment groups.

Large number of evidences indicated that metal NPs including AgNPs have reproductive toxicity by affecting both reproductive and somatic cells (Taylor et al., 2012). As well as, it was previously reported that changes in organ weights and body weight may give an indication about the potential toxicity of chemical compound (Kim et al., 2007; Hamza, and Rashid, 2017). In this respect, the result of this study showed that animal groups exposed to increased doses of AgNPs have a significant increase in body weight and ovary weight after (30 day) of treatment. These results found to be agreed with observed histological changes in ovarian tissue such as vascular congestion, increased number of secondary graffian follicles and corpus lutium.

These findings were supporting by previous work Gao et al., (2012) who reported that long term exposure to (TiO2NPs) produced ovarian dysfunction and altered cell histology, and the expression of gene encoding cell proliferation and inflammatory response in ovaries.

**Table 1:** The mean values of ovaries weight (gm) of animals treated with different doses of AgNPs for different durations.

<table>
<thead>
<tr>
<th>Treatment Durations (day)</th>
<th>Weight of Ovaries (gm) in different Treatment groups</th>
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<tr>
<td></td>
<td>Control N=5</td>
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<tr>
<td></td>
<td>12.5 mg/Kg N=5</td>
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<tr>
<td></td>
<td>25mg/Kg N=5</td>
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<tr>
<td></td>
<td>50 mg/Kg N=5</td>
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<td>154±0.02607 a</td>
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<td>0.157±0.03094 a</td>
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<td></td>
<td>0.161±0.02881 a</td>
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<tr>
<td></td>
<td>0.177±0.069336 a</td>
</tr>
<tr>
<td>20</td>
<td>0.144±0.00185 a</td>
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<tr>
<td></td>
<td>0.163±0.05585 a</td>
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<tr>
<td></td>
<td>0.17±0.060415 a</td>
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<tr>
<td></td>
<td>0.218±0.058907 a</td>
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<tr>
<td>30</td>
<td>0.159±0.00134 a</td>
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<td></td>
<td>0.181±0.04581 a</td>
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<td>0.240±0.03886 a</td>
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All data expressed as mean ± SD. (***) Highly significant differences (P<0.01) compared to control group -
- (****) Very highly significant differences (P<0.001) compared to control group -
- Values with non-identical superscript are significant among all groups.

**Figure 9:** Weight of ovary in response to different doses of AgNPs and different treatment duration. Data expressed as mean±SD. (****) Very highly significant increase (P<0.001) relative to control group. (a,b) represent the significant difference between groups.
Conclusion: In summary, we observed that ovaries are affected by chronic exposure to high dose of AgNPs which may serve as an indication of AgNPs toxicity. Further studies are needed to explore such finding.

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REFERENCES


