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TOXICITY STUDY OF GOLD AND SILVER NANOPARTICLES ON EXPERIMENTAL ANIMALS

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ABSTRACT

Both Silver (AgNPs) and gold (AuNPs) nanoparticles are increased being utilized broadly in many industries and biomedical products to enhance their performance. However, humans are increasingly being exposed to the two metal-NPs, which also been shown to be highly potential toxic to mammals. Meta-NPs has been demonstrated to have the capability to cross biological barriers cell membranes and subsequently interact with intracellular structures. The present in vivo study assessed the toxicological potential of AgNPs and AuNPs, in 30 mature male albino rats, assigned to three groups, to receive intraperitoneal injection of 0.25 mg/kg b.w of AgNPs (G1) or AuNPs (G2) or vehicle only (G3) daily for 21 days. Liver function makers, thyroid function hormones, testosterone hormone, inflammatory biomarkers and plasma proteins as well as histological characteristics were tested. The results revealed increased liver enzymes indicating liver toxicity and injury. Liver inflammation was manifested by elevated inflammatory cytokines interleukin-6 (IL-6) and tumour-necrosis factor alpha (TNF-α). Thyroid hormones were elevated indicating hyperthyroidism, while testosterone hormone was diminished indicating their potential to cause infertility in males. However, the low-dose of AgNPs and AuNPs has no noticeable changes in histomorphologic picture of liver. In conclusion, both metal-NPs are potentially toxic, with AgNPs exhibiting a greater toxicity effect than AuNPs. Toxicity mechanisms include direct cellular injury (lysis) and induction of oxidative stress.

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Introduction

Nanoscience and nanotechnology, which entail manipulation of matter on subatomic scale, has rapidly produced a wide range of nanomaterials containing nano-scale particles of less than 100 nanometres. Rapid characterisation of nanomaterials over the last two decades, has led to the identification of novel nanoparticles with altered or enhanced properties suitable for materials science and biology including nanomedicine [1]. Biological studies have shown than nanoparticles (NPs) have the capability to not only cross cell membranes and biological barriers, but also initiate chemical interactions with intracellular structures [2]. Intracellular NPs exhibit unusual reactivity, catalytic, and chelation potential due to their nano-scale, large surface area to volume ratios, electronic characters, and aggregation behaviour. Thus, their interactions with intracellular structures and chemical elements potentiate dysfunctions at cellular and molecular levels [3]. These dysfunctions imply that NPs have the potential to cause cytotoxicity, genotoxicity, and carcinogenicity to both humans and animals [4].

Humans are increasingly being subjected to NPs via occupational inhalation and skin exposures, non-dietary ingestion exposures, and via some injectable drugs [1]. The application of NPs in the production of antimicrobial agents, therapeutics, fluorescent cell labels, and as gene-delivery vectors, has put humans at risk of occupational exposure to NPs. This is likely to occur at any point during the manufacturing cycle of NP-based products, especially during disposal stages [5]. By this account, a thorough knowledge on how engineered NPs interact with cells, tissues, and organisms has become increasingly essential; especially, in regard to their severe hazards to public health [1].

Metal-based NPs, particularly from transition metals, have attracted much interest due to their extensive application in medical and industrial fields [3]. Metal NPs, especially silver (AgNPs) and gold (AuNPs) have revolutionized the fields of

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engineering and material science, especially photonics, pharmaceutics, optics, biotechnology, catalysis, and medical science [6]. Products infused with AgNPs are extensively being utilized broadly in some products, especially for sanitation and hygiene (washing powders, deodorants, cosmetics, room sprays, water disinfectants, sports and other textiles), food handling (food supplements), and medical devices [2]. AgNPs are preferably used over other metal-based NPs, because they exhibit reliable and broad-spectrum antibacterial activity [6].AuNPs, on the hand, have large reactive surfaces, remarkably high electron conductivity, and unique optical properties and can be stabilized in suspension formulations [7]. Although AuNPs had a lower application than AgNPs, due to their relatively lower reactivity, the discovery of wet chemical synthesis coupled with surface functionalization of AuNPs has in the last decade inspired their wide application in biotechnology and biomedical fields [8]. Their application includes targeted drug delivery systems, bio-imaging, and bio-sensing devices, chemotherapy agents including photothermal therapy [9].

While the bulk forms of some metals, namely Ag, Au, and Cu, are relatively inert, they exhibit increased toxicity potential as their particle sizes decrease to nano-scale [3]. Recently, the increased exposures to AgNPs and AuNPs have been associated with the quick development of nanotechnology and the utilization of these metal-based NPs in biomedical applications [10]. This has warranted studying the potential toxicity of the two metal-based NPs to humans in order to develop biocompatible alternatives and possible prophylactic measures.

Bioaccumulation and toxicity of AgNPs has been shown in many publications [2]. Several studies,reviewed by [8], have demonstrated cellular uptake, biodistribution, and toxicity potential of AuNPs. Both AgNPs and AuNPs can interfere with a number of biochemical processes by interacting withcellular components [3]. However, there is paucity of toxicology data on the impact of AgNPs and AuNPs onbiochemistryat the cellular level.

The aim of this study was to examine the toxicological effect of AgNPs and AuNPs on liver function, hormone function, and thyroid function as well as inflammatory markers in male albino rats injected intraperitoneally with AgNPs or AuNPs.

Materials and Methods

Animals

30 males Sprague-Dawley albino rats (120 - 130 g) were bought from Animal House Colony of King Fahd Medical Research Center (KFMRC), Jeddah, KSA. Rats were left for 7days for acclimatization to the research conditions. The rats were kept in a controlled room (12h light/dark cycles), with a maintained room temperature (24-25 ^{o}C). The rats were given standard food pellets and water *ad libitum*. The experiment was carried out according to the Ethical Committee of King Fahad Medical Research Center. Jeddah, KSA.

Metal nanoparticles

Both AgNPs and AuNPs were bought from Sigma-Aldrich (St Louis, MO, USA). The stabilized suspension of AgNPs and AuNPs were provided in sodium citrate solution (0.02 mg/mL) and in 0.1 mM phosphate-buffered saline, respectively. The suspensions of NPs were kept at 4 °C until use. The characteristics of individual metal-NPs are summarized in Table 1.

Table 1: Characteristics of AgNPs and AuNPs

Metal-NPs	Colour	shape	specific surface area (m^2/g)	Density (g/cm^3)	Transmission Electron microscopy TEM (nm)	X-ray diffraction (nm)	Purity (%)
AgNPs	Brown	Spherical	5	10.49	< 100	21.8	99.5
AuNPs	Brown	Spherical	3.3	19.32	< 100	24.6	99.9

Study groups

Rats were assigned into three groups (ten rats in each group). Rats in group1 were given intraperitoneal injection of AgNPs (0.25 mg/kg body weight) daily for 21 days [11]. Rats in group 2 were injected intraperitoneal with AuNPs (0.25 mg/kg body weight) daily for 21 days [8]. Rats in the control group were injected intraperitoneal with PBS vehicle only. After 21 days, the animals were starved overnight and euthanized by ether anaesthesia and fasting-blood samples drawn from their hepatic portal veins. The blood samples were then centrifuged ready for biochemical analysis.

Biochemical investigations

Biochemical investigations were performed using the Enzyme-linked immunosorbent assay (ELISA) technique. Serum biochemical analysis included quantification of liver enzymes, serum proteins, hormones and Inflammation biomarkers. Quantification of liver enzymes; alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was performed according to methods described originally by [12], and alkaline phosphatase (ALP) was quantified according to methods described originally by [13]. Serum proteins (serum total protein and total bilirubin) were quantified according to methods described originally by [14 and 15] respectively. Serum levels of TSH, total T3, and T4 hormones were quantified using

commercial kits according to methods described previously by [16, 17 and 18], respectively. Serum testosterone hormone was quantified using ELISA technique according to methods described previously by [19]. Serum levels ofIL-6 , IL-10 and TNF-α were quantified using commercial R&D Systems Quantikine ELISA Kits (R&D systems, inc. USA). Serum C-reactive protein (CRP) was quantified according to methods described previously by [20].

Histopathologic examinations

Liver autopsy samples were harvested from 3 rats randomly selected from each of the 3 study groups. The liver specimens were fixed in 10% neutral buffered formalin (NBF) at 4° C for 24 hours. After fixation, the fixed liver tissues were rinsed with tap water followed by several changes of PBS. The rinsed liver specimens were subjected to ethanol dehydration, followed by xylene and finally embedded in a paraffin wax. The liverspecimens were then sectioned at thickness of 4 microns using a sledge microtome. Liver sections were then stained with hematoxylin and eosin (H & E) [21].

Statistical analysis

Statistical analysis of the obtained experimental data was analysed using SPSS software package (Version 20) for windows. Data were calculated as mean ± standard deviation (SD) of ten rats. One-way analysis of variance (ANOVA) test was carried out to estimate whether the individual serum biochemical tests differed across the three study groups. Student's t-test was utilized to determine the significant differences between the treatment groups and the control one. The differences among data were significant atp ≤0.05.

RESULTS

Liver function

The toxic impacts of AgNPs and AuNPs on hepatic function are summarised in Table 2. Serum liver function makers (liver enzyme activity) were altered. Activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were pronouncedly elevated in AgNPs and AuNPs injected groups (G1 and G2) than in control group (G3). Total protein and albumin were markedly reduced in the treated groups than in control group, while total and direct bilirubin were dramatically increased in the treated group than the control group. In summary, the toxicity effect of AgNPs on liver function makers were more pronounced than that exhibited by AuNPs.

Table 2: Serum liver function markers in treated versus control groups.

Parameters	G1(AgNPs)	G2(AuNPs)	G3 (control)
ALT (U/L)	56.50±3.87[a]	47.50±2.08[a]*	19.25±2.22
AST(U/L)	48.25±2.06[a]	47.25±2.6[a]	21.00±2.5
ALP (U/L)	260.30±5.85[a]	189.00±7.70[a**]	78.25±10.31
Total protein (g/dl)	6.07±0.17[b]	5.97±0.17[a]	6.85±0.28
Albumin (g/dl)	3.65±0.13[a]	3.43±0.09[a]	4.40±0.18
Total bilirubin (mg/dl)	1.00±0.089[a]	0.87±0.015[b***]	0.63±0.08
Direct bilirubin (mg/dl)	0.17±0.008[a]	0.16±0.012[a]	0.12±0.009

Results are calculated as mean ± S.D. (n=10), [a]P≤ 0.001, [b]P ≤ 0.01 versus the controlrats. $^{*}P \leq 0.001$, $^{**}P \leq 0.01$, $^{***}P \leq 0.05$ compared with AgNPs group.

Hormone function

The toxic effects of AgNPs and AuNPs on hormone function are summarised in Table 3.The two treated groups, exhibited significant reductions in testosterone hormone levels with respect to the control group. thyroid hormones: T3, T4 and TSH were significantly elevated in both treated groups than control, indicating their toxic effects on thyroid gland.

Inflammation markers

The toxic effects of AgNPs and AuNPs on serum inflammation makers are summarised in Table 4. C-reactive protein (CRP) concentration was dramatically elevated in the metal-NPsinjected groups than the control. Interleukin-6 (IL-6) and TNF-α were significantly elevated in the both treated groups than the control, while IL-10 levels markedly diminished in AgNPs and AuNPs injected groups than the control group. The overall toxic effects were more in AgNPs-treated rats than the AuNPs-treated ones.

Table 3: Hormone levels in in treated versus control groups.

Parameters	G 1 (AgNPs)	G 2(AuNPs)	G3 (control)
Testosterone (ng/ml)	1.2±0.04[a]	0.26±0.003 [a*]	4.74±0.16

Triiodothyronine—T3 (ng/ml)	1.25±0.04 [a]	1.16±0.04[a**]	0.86±0.02
Thyroxine—T4 (µg/ml)	6.97±0.15 [a]	5.64±0.23 [a*]	4.84±0.043
Thyroid-stimulating hormone—TSH (µU/ml)	1.87±0.008 [a]	3.50±0.013[a**]	0.22±0.022

Values are calculated as mean ± S.D.(n=10), [a]P≤ 0.001, [b]P ≤ 0.01 versus the control group. $^{*}P \leq 0.001$, $^{**}P \leq 0.01$ compared with AgNPs group.

Table 4: Levels of serum inflammation markers in treated versus control groups.

Parameters	G1 (AgNPs)	G2(AuNPs)	G3(control)
CRP (mg/L)	0.42±0.067 [a]	0.475±0.021 [a]	0.21±0.032
IL-6 (pg/ml)	17.85±0.76 [a]	16.19±0.49 [a**]	10.35±0.63
TNF-α (pg/ml)	36.07±1.16 [a]	25.13±2.65 [a*]	9.62±0.78
IL-10 (pg/ml)	20.08±1.6 [a]	23.10±1.75 [a***]	37.85±1.53

Results are represented as mean ± S.D. (n=10), [a]$P \leq 0.001$ compared with the control group. $^{*}P \leq 0.001$, $^{**}P \leq 0.01$ compared with AgNPs group.

Histopathologic examination

The histopathologic characteristics of liver tissues of nanoparticle-injected rat groups with respect to the control are shown in Figure 1. Both treatment groups exhibited normal histological structure of the liver tissue as the control.

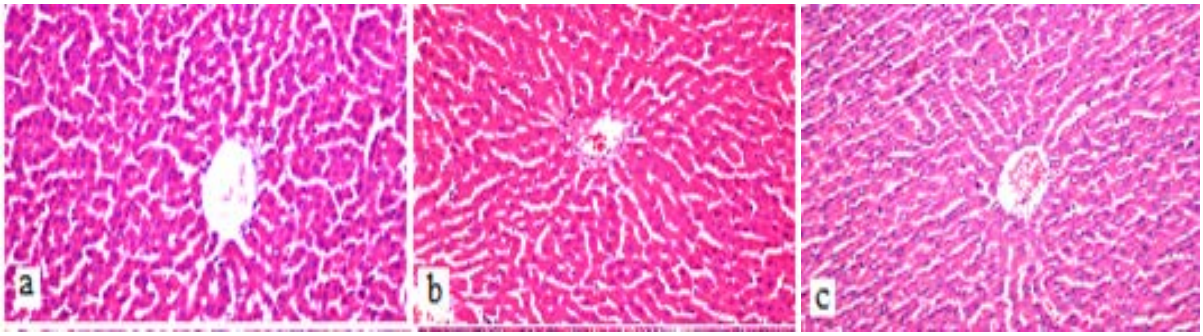

Figure 1: Light micrographs showing liver histomorphological characteristics of the treated (G1 and G2) versus the control group (G3); a) Light micrographs of a liver section of the control group showing normal histological structure of the central vein and the surrounding hepatocytes. b) and c) show that both G1 and G3 groups have normal histological structure.

Discussion

Some studies have made good attempt to explain possible mechanisms under, which metal-NPs, induce toxic effect on humans and animals as well as microorganisms. The toxic effect of inorganic nanoparticles on microorganisms is quite understood. A rent study demonstrated that AgNPs induces its toxic effect in bacteria and aquatic microalgae via particle-specific mechanisms. While the mechanisms are not fully understood, it was observed to be due to ionic toxicity and oxidative stress [22]. The mechanisms of nanoparticle-induced cellular toxicity are largely due to several physical and physical factors, especially: 1) size of particle mobility across cell membranes [23]; (2) high solubility that is different from that of its surface coating and particle composition; (3) higher aggregation rate to enhancecellular bioaccumulation; (4) interaction with biomolecules to form nanoparticle-protein complexes with cellular proteins/enzymes and DNA to altercellular, physiological, and biochemical processes; and; (5) ability to generate copious amounts of reactive oxygen species (ROS), which subsequently promote cellular injury by oxidative stress.

Some studies regarding toxicity of metal-NPs are conflicting; *in vivo* studies often showing positive toxicity. While uptake ofmetal-NPs via skin is possible, it appears that oral route is the most efficient for nanoparticle-induced toxicity. Following oral administration of 20 nm noncoated or <15 nm PVP-coated AgNPs, significant amounts of AgNPsalong with silver ionswere detected in severalrat tissues and organs, especially liver and biliary system[24], heart muscle[25], including thyroid and parathyroid glands [24]. The present *in vivo* study evaluated the toxicity of AuNPs and AgNPs via intraperitoneal route in rats.In line with [24], findings in the present study demonstrated toxicity of both AuNPs and AgNPs in rat affecting the liver and thyroid glands. Both metal-NPs significantly affected liver function, hormonal function and inflammation makers, but not histomorphological characteristics of liver tissue. Liver biochemical makers indicated elevated hepatic enzymes, particularly, alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase. This indicated hepatic toxicity induced by both metal-NPs. Nanoparticle-induced hepatic injury was also manifested by elevated bilirubin

due to increased breakdown of red blood cells in the liver (haemolysis) coupled with impaired protein synthesis, marked by diminished levels of total protein and albumin. Our result is coped with a previous *in vivo* study [25], that also demonstrated that AuNPs induced injury to cardiac myocytes. However, while the present study demonstrated no histological alterations of the liver tissue by both the 24.6 nm AuNPs and 21.8 nm AgNPs, [25] demonstrated altered histological alterations of cardiac muscle tissue following treatment with 10 nm AuNPs. This supports that small nanoparticle size significantly increases the toxicity of metal-NPs. In the *in vivo* study, [25] demonstrated that AuNPs toxicity was influenced by not only particles size, but also dosage. This was demonstrated in a recent *in vivo* study, where a dose-dependent build-up of AgNPs and silver ions was observed in all the tissues examined, though this did not significantly result in changes in liver or body weight [26]. Similarly, in an *in vivo* investigation by [27] revealed that injected BALB/C mice with AuNPs (8 mg/kg/week) of different sizes (3, 5, 50, and 100 nm) showed no adverse impacts. However, AuNPs of 8-37 nm in size caused severe illness in mice, with increase in liver Kupffer cells.

While small size is a critical factor in the toxicity of metal-NPs, higher doses can increase toxicity of otherwise larger-sized NPs. According to [1], larger size NPs can still cause toxicity by principally increasing dose. For instance, 42nm AgNPs were observed to increase levels of alanine aminotransferase and alkaline phosphatase enzymes, when rats were administered orally with high doses of 1 mg/kg / body weight/day. This was also reported in a recent study by (28], which demonstrated severe liver toxicity following administration of high doses of AgNPs. However, the size of nanoparticles should be <100nm, to cause any toxic effect. This was demonstrated in previous study by [29], where rats exposed orally to 183 nm silver particles administered in significantly higher doses of more than 1000 mg/kg of bw/day for 2 weeks, did not significantly induce toxicity. The activity of sensitive liver enzymes, especially, alkaline phosphatase, alanine aminotransferase and cytochrome P450 enzymes was not increased.

In the present in vivo study, a slight reduction of total proteins and albumin observed in both AuNPs- and AgNPs-treated groups. While this can be attributed to liver injury, since albumin synthesis occurs in the liver, this could be contributed by the adsorption of the proteins on the surfaces the metal-NPs, a phenomenon called opsonisation [30]. This can be attributed to the ability of the two metal-NPs to bind to, and form meta-protein complexes with biomolecules, especially proteins. Thus, as the metal-NPs inter a mammalian blood-stream, they are immediately coated with proteins, due to their electrostatic properties that tend to attract other electronegative molecules to its high surface area to volume ratio. This coupled with its chelationproperty makes the metal-NPs bind to and form stable complexes with albumin and apolipoproteins [30]. By this account, the reduced serum total protein and albumin, following administration of the two metal-NPs could also be attributed to opsonisation and chelation of the proteins. Moreover, opsonisation and chelation of plasma proteins by the metal metal-NPS enhances their biological distribution and assimilation into various tissues [31].

Findings from a recent *in vitro* study suggest that AgNPs are capable of penetrating cell membranes and other biological barriers, aggregate, and subsequently bioaccumulate[3]. Surprisingly, it also appears that AgNPs can also passively penetrate into cell organelles, especially mitochondria, where they disrupt membrane potential, and therefore, induce the production of reactive oxygen species (ROS). Accumulation of ROS increases oxidative stress, which underpins cytotoxicity of nanoparticles [32]. Due to their high-ATP requirement, hepatocytes are the most vulnerable cells to nanoparticle-induced toxicity as observed in the present *in vivo* study. In a recent *in vitro* study, [33] demonstrated AgNPs induced liver toxicity by reducing ATP, reducing activity of the antioxidant enzymes, especially glutathione (GSH). Thus, AgNP-treated cells were abnormal in size and shape, displaying cellular shrinkage. However, in the present *in vivo* study, the histomorphologic characteristics of liver cells appeared normal. This finding is in agreement with a study by [26], which stated that low-dose AgNPs has no effects on the morphological structure of rat liver. In addition, this could also be explained by the presence of remedial mechanisms in *in vivo* systems, which counter NP-induced toxicity, unlike in *in vitro* systems. This was demonstrated in a previous *in vivo* study [11], where liver macrophages were observed to eliminate AgNPs by phagocytosis, though the process resulted in increased endogenous production ofROS. However, higher doses of AgNPs (2mg/kg bw) were shown to cause mild-to-severe, dose-dependent hepatic lesions. It induced aggregation of inflammatory cells, liver necrosis and fibroplasias—formation of fibrous (connective) tissue as observed during wound healing [6].

Thyroid and parathyroid glands are also susceptible to toxicity induced by AuNPs and AgNPs. Findings from the present *in vivo* study indicated toxicity of these glands, manifested by a significant increase in three thyroid hormones: triiodothyronine, thyroxine, and TSH; and significantly diminished levels of testosterone hormone. Thyrotoxicosis potentiates thyroid hyperthyroidism (overactive thyroid) and associated complications. On the hand, diminished testosterone hormone, which regulates gonadal morphology and spermatogenesis [34] and maturation of male germ cells increases risk of male infertility [35 and36]. AgNPs has been demonstrated to diminish the number of the testosterone-producing Leydig cells and alter sperm morphology as well as motility [37]. While the present study demonstrated the potential of AuNPs and AgNPs to cause infertility, a recent *in vivo* study by [38] demonstrated that sub-acute exposure to AgNPs, was not significantly toxic to reproduction of male mice. The treatment did not significantly decrease the weight of testis and sperm concentrations, indicating that AgNPs did not impair spermatogonial stem cells *in vivo*. However, while AuNPs and AgNPs may not significantly affect spermatogenesis, it is likely to affect sperm quality and therefore, infertility in mammalian males [37].

Metal-NPs can induce organ injury coupled with inflammation, which can negatively affect critical functions of organs, including organ failure. Findings in the present study demonstrated elevation of serum inflammation markers following treatment with AuNPs and AgNPs. The pro-inflammatory TNF-α, and IL-6 were significantly elevated while the anti-inflammatory IL-10, were significantly diminished consistent with findings from another *in vivo* studies [39 and 40]. CRP was also acutely elevated and is usually expressed under the stimulation of TNF-a, IL-1 and IL-6.An acute phase of induction of proinflammatory cytokines was observed in liver and kidneys of rats treated with AuNPs [6 and 41]. The present *in vivo* study demonstrated a significantly higher pro-inflammatory induction by AgNPs than AuNPs. A previous *in vitro* study by [42] demonstrated that, while cellular uptake of 60nm AuNPs was successful and aggregated within intracellular vacuoles murine macrophages, they were however, not cytotoxic, as they failed to induce proinflammatory responses (IL-6, TNF-α). Furthermore, use of smaller sizes of AuNPs, such as 2-4 nm, 5-7 nm and 20-40 nm, were still non-toxic to Human Fetal Lung Fibroblast Cells (MRC-5). However, the toxicity of AuNPs appears to be highly dose-dependent, as AuNPs of concentration ≥ 10 ppm, successfully induced cellular apoptosis [43], up-regulation of IL-1, IL-6 and (TNF-α) in the MRC-5 cells [39]. Increased inflammation inductions coupled with impaired anti-oxidative mechanism increases the risk of extensive damage to tissues leading to failure of critical organs, especially liver, kidney and heart [11).

A previous *in vivo* study by [44] demonstrated no evidence of dose-dependent effects onblood biochemistry and tissue histologyin mice treated with different intraperitoneal doses (40, 200, and 400 microg/kg/day) of 12.5 nm AuNPsfor 8 days. Despite, the lower toxicity potential of AuNPs compared to AgNPs, AuNPs exhibits significantly rapid uptake intoKupffer cells of the spleen and liver as well as mesenteric lymph nodes with less elimination phase. Kupffer cells have been shown to have a spectacular uptake capacity, taking up larger NPs of up to several hundreds of nanometers [45]. Clearance of NPs is believed to be via lysosomal exocytosis, emptying into the biliary canaliculus, where there are excreted by biliary system depending on particle sizes [46]. However, hepatobiliary clearance of metal-NPs may not be sufficient. Therefore, long-term bioaccumulation of metal-NPscan result in necrosis of liver and spleen tissues [47 and 48].Necrotizing effect of AuNPs has been shown to be largely due to their solubility in the intracellular spaces and bioaccumulation in the extracellular spaces [49].

Although AgNPs are potentially toxic, a previous study demonstrated some therapeutic effect of AgNPs in mice model of peritonitis. The mice treated with AgNPs, exhibited reduced accumulation of inflammatory cells and production of inflammatory cytokines. Via an upregulated expression of TGF-β1, AgNPs was observed to stimulate proliferation of skin keratinocyte and the production of VEGF and IL-10 [50]. Furthermore, it was demonstrated that AgNPs promoted local formation of new blood vessels (angiogenesis) and therefore, facilitated healing in a mouse model of burn wounds [51]. Therefore, although, AgNPs has therapeutic benefits, its toxicity and therapeutic benefits should be weighed before a clinical decision to use them is reached.

Conclusion

Although both AgNPs and AuNPs, have therapeutic benefits, findings from the present *in vivo* stud, as supported by the available literature, demonstrated their high toxicity potential to mammals including humans. The toxicity of two metal-NPs has the potential to disrupt thyroid function and liver function, cause liver injury and inflammatory, affect male fertility by suppressing testosterone hormone.

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