INTRODUCTION

During the past decades, the field of nanotechnology was rapidly expanded, and metal nanoparticles (NPs) displayed wide applications in the industrial and medicinal fields. Accordingly, the increased human exposure to NPs may represent a threat to the human health. Due to their antimicrobial activity, the silver NPs (AgNPs) are the most widely used NPs in the nano-based products [1]. Previous data have shown that exposure to AgNPs resulted in a variety of toxicological effects. Although to date insufficient studies are available about the potential systemic toxicity of the AgNPs, they suggested that the nature of nanotoxicity is dose-dependent and explored their potential cytotoxicity explored at high concentrations [2]. On the other hand, the low concentration of AgNPs may be involved in the alteration of cellular signaling pathways [3,4]. The small particle size of AgNPs enables them to cross biomembranes and localize at any site within the target organs including the liver, spleen, lung, kidney, thyroid, brain, and gonads inducing toxicity [5,6]. The thyroid gland plays an important role in the regulation of several physiological functions such as metabolic processes, neuronal growth, bone remodeling, and cardiac functions [7,8]. Accordingly, changes in the levels of thyroid hormones may significantly affect human health. However, many studies have clarified the possible contribution of NPs in such changes. The endocrine system is highly sensitive to the environmental pollutants, and it has been reported that about 10% of the population suffer from thyroid gland disorders, which may be considered as a major public health problem. Therefore, previous data have focused on the possible negative impact of environmental NPs as endocrine disrupting materials [9]. Although, the mechanisms by which AgNPs induce toxicity are not well recognized. It has been suggested that free oxygen radical production may explain the toxicity of AgNPs, as they can induce a wide range of physiological and cellular effects such as inflammation, DNA damage, and apoptosis [10,11]. The endocrine system is highly sensitive to the environmental pollutants, and it has been reported that about 10% of population suffer from thyroid gland disorders, which may be considered as a major public health problem [9]. Therefore, the present study was designed to investigate the impact of exposure time and concentration of AgNPs on thyroid gland structure and function in female rats.

METHODS

Preparation of AgNPs solution

AgNPs powder (SkySpring Nanomaterials, Houston, USA) of 99.95% purity was used in the present study. The particles have a spherical shape (20–30 nm) with a specific surface area of 20 m²/g and 10.5 g/cm³ density. AgNPs stock suspension (40 mg/ml) was freshly prepared by dispersing the weighed amount of AgNPs in deionized water and mixed by vortex for 10 min. From this stock suspension, two additional diluted suspensions, the required dose volumes were calculated and injected intraperitoneally to the rats.

Animals

A total of 60 female Sprague-Dawley rats (225–250 g) were purchased from the National Center for Drug Control and Research/Ministry
of Health, Baghdad, Iraq. All animals were maintained at controlled laboratory conditions of temperature (25±2°C) and humidity with 12 h light-dark cycle along the time of the study. The animals were allowed to a standard rat pellet and water ad libitum. The research protocol was approved by the Animal Research Ethics Committee of Al-Mustansiriyah University in accordance with the globally adopted guidelines of the animal care and experiments.

Experimental design and treatment
The rats were randomly allocated into 12 groups (each of 5 rats). The groups (1, 2, and 3) were served as control and received the vehicle alone. The groups (4, 5, and 6) were exposed to 12.5 mg/kg of AgNPs suspension intraperitoneally (i.p) for 10, 20, and 30 days, respectively. The groups (7, 8, and 9) were exposed to 25 mg/kg AgNPs i.p for 10, 20, and 30 days, respectively. Finally, the groups (10, 11, and 12) were exposed to 50 mg/kg of AgNPs i.p for 10, 20, and 30 days, respectively. At the end of the exposure time, the animals were anesthetized by ether and blood samples were obtained directly from the heart. The animals were dissected, and the thyroid glands were removed, weighed and kept in 10% buffered formalin for the preparation of microscopic section. The blood was left to clot and centrifuged for 10 min at 3000 rpm. The resulted serum was utilized for the measurement of T3, T4, and thyroid-stimulating hormone (TSH) levels using ELISA technique according to standard procedures [12-14].

Histological examination
According to the procedure described by Adeyemi and Akanji [15], the thyroid tissue was fixed in 10% formalin, dehydrated with ascending grades of ethanol (70%, 90%, and 95%), then cleaned in xylene and embedded in paraffin wax (melting point 56°C). Tissue sections were prepared and stained with hematoxylin and eosin. The photomicrographs were captured at ×100 using the software Presto Image Folio package.

Statistical analysis
The data were presented as mean ± SD and analyzed, using GraphPad Prism 6.1 software, with unpaired t-test and ANOVA. p<0.05 was considered to be significant.

RESULTS
Thyroid weight
The exposure to a high-dose (50 mg/kg) of AgNPs for 20 and 30 days produced a significant (p<0.05) increase in the weight of thyroid gland compared with control groups, while nonsignificant changes were observed with the other doses. Moreover, short-term exposure (10 days) to any of three doses of AgNPs has no significant effect on the thyroid gland (Fig. 1).

Thyroid hormones
In the present study, the serum levels of T3, T4, and TSH displayed nonsignificant changes (p>0.05) among all groups (12.5, 25, and 50 mg/kg) at different time intervals (Figs. 2 and 3). Meanwhile, a highly significant reduction (p<0.01) in the mean values of the T4 achieved after the long and intermediate duration of exposure (30 days) to 50 mg/kg of AgNPs. The exposure to 25 mg/kg showed a comparable result only after 30-day duration compared with the control group and the other exposed groups (Fig. 4).

Histological study
In the control group, the thyroid tissue sections showed normal structure of variable sized follicles, which are filled with colloid materials. The lining epithelial cells appeared with low cuboidal morphology (Fig. 5). After 10 days of intraperitoneal injection of AgNPs doses (12.5, 25, and 50 mg/kg) did not impact the structural integrity of the thyroid tissue. Slight changes were observed when the exposure duration was extended to 20 and 30 days at low and intermediate doses. Only the groups of animals exposed to a high-dose (50 mg/kg) for 20 and 30 days showed distinct histological changes in the thyroid tissues, appeared as segregation of follicles and depletion of the colloid materials that associated with an increase in the spaces between the follicles (Figs. 6 and 7) compared with the control group. However, 20-day exposure shows a mild effect in term of structural changes of the follicles relative to those of 30-day exposure.

Fig. 1: Effect of different silver nanoparticles concentrations on the thyroid gland weight at different exposure periods. **Significantly different compared with the corresponding control group (p<0.01); *** very highly significant differences compared with the corresponding control group (p<0.001); values with different letters (a,b) are significantly different among different groups (p<0.05)

Fig. 2: Effect of rat exposure to different doses of silver nanoparticles on the serum T3 levels at different treatment duration. No significant changes were reported among the groups (p>0.05)

Fig. 3: Effect of rat exposure to different doses of silver nanoparticles on the serum T4 levels at different treatment duration; **significantly different compared with the corresponding control group (p<0.01); ***very highly significant differences compared with the corresponding control group (p<0.001); values with different letters (a,b) represent the among the different groups are significantly different (p<0.05)
DISCUSSION

The wide applications of AgNPs in nonmedical and pharmacological products directed the search toward safety guidelines and possible toxicity mediated by the release of biologically active Ag+ into the human body [16]. In this concern, the results of the present study agreed with the data reported by Soukup et al., where they found a significant increase of thyroid gland weight in animals with hypothyroidism due to thyroid gland hypertrophy [17]. The function of the thyroid gland is often maintained by a negative feedback mechanism, which involves the interplay between the hypothalamus (through the TRH release) and the TSH released from the pituitary gland. This regulatory effect adjusts the levels of the circulating thyroid hormones (T3 and T4) [18]. These thyroid hormones played critical roles in the metabolic and developmental functions of the body. Therefore, any alteration in the levels of these hormones will negatively affect the processes of development and differentiation [19]. Many chemicals were reported to disrupt thyroid function in laboratory animals and humans [20]. Since the small particle size of NPs including AgNPs contributes to extensive tissue penetration, it can be postulated that they may disrupt the structure and function of the thyroid gland. However, previous studies showed a reliable data in this respect [21], and this raises the need for more studies to assess the effect of exposure time and concentrations of AgNPs on the thyroid function.

In the present study, assessment of the thyroid status as hyperthyroid or hypothyroid was done by measuring the serum levels of T3, T4, and TSH. Even though TSH is considered as a biomarker for hypothyroidism, many xenobiotics can modify the circulating thyroid hormones levels without considerable changes in the TSH level [22]. Our data were in tune with the previous finding, as the present study reflects a significant reduction in the serum level of T4, with chronic exposure to increased concentrations of AgNPs, suggesting a time- and dose-dependent thyroid disrupting effect of AgNPs. However, no significant alterations (p>0.05) were found in the levels of T3 and TSH [18, 22]. Other previous study reported that chronic exposure to sub lethal dose of silver nanoparticles disrupts thyroid hormone signaling, which may explain the result of this study concerning thyroid function after long term exposure (30 days) to high dose of AgNPs (50 mg/Kg) [24]. Meanwhile, others suggested that the reduction of T4 due to the endocrine disruptor metals was attributed to the alteration in the expression of thyroid hormone synthesis gene in hypothalamic-pituitary-thyroid axis [25]. In general, most of the thyroid disruptor chemicals affect the thyroid status by interfering with the different steps involved in thyroid hormone production such as iodine trapping process, cellular uptake, and interconversion between thyroid hormones, disposition, and elimination of the hormone [26]. Since many properties of AgNPs, including particle size, shape, composition and surface chemistry, may facilitate cellular penetration of these particles and can induce irreversible...
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tievative damages to both cells and organelles specially the mitochondria. Accordingly, AgNPs may alter the mitochondrial function of the thyroid epithelial cells, which might reduce cellular ATP production required as an energy source for synthesis and release of thyroid hormone[25]. Accordingly, the low level ofT₄ may in part resulted from the effect of AgNPs on the iodine transport system, expression of thyroid binding protein, as well as enhancing the clearance of the hormone [27]. However, the absence of the significant response of TSH release to decreased levels of T₄ could be attributed to the possible interference of AgNPs with the central regulatory mechanism on the thyroid hormone production, as reported with other thyroid disruptor metals like cadmium [28]. Moreover, AgNPs may alter the mitochondrial function of the thyroid epithelial cells, which might reduce cellular ATP production required as an energy source for synthesis and release of thyroid hormone [27]. It has been reported that AgNPs induced mitochondrial dysfunction and activated the caspase-3 that may consequently directed the cell toward mitochondrial-dependent apoptosis [29]. However, other study disagreed with our finding, where they found that daily exposure of animals to different doses (20, 50 and 150 mg/kg) of iron oxide NPs for 15 consecutive days resulted in a significant increase in the serum levels of T₄, accompanied by significant reduction in TSH serum levels [30]. This can be explained by the ability of the absorbed NPs to circulate with blood and passed to accumulate within different organs including thyroid tissue as their distribution was known to be size dependent [31-33]. Other researchers reported that the possible mechanism by which AgNPs induce thyroid toxicity is by increasing the release of reactive oxidative species that induce cellular oxidative injury. They found that intraperitoneal administration AgNPs can induce oxidative injury in the hepatic and renal tissues through the inhibition of mitochondrial electron transport chain required to maintain cellular energy production[34-36]. However, the generation of reactive oxygen species may be associated with inflammatory reaction and genotoxic events [37]. Moreover, it has been found that smallest AgNPs can destruct small bio-membranes by suppression of cellular ATP level which followed by cell death [38,39].

Actually, the generation of Reactive oxygen species (ROS) has both beneficial effects as defense molecules against microorganisms, as well as; they may act as signaling molecules to develop cellular stress responses which directed the cell toward death. Therefore, with time increasing levels of ROS can adversely affect the structural and functional integrity of various biological organs [40,41]. In this study, we have suggested that hormonal and structural alteration of the thyroid gland could be one of the consequent events to oxidative injury induced by chronic exposure to AgNPs. The results of microscopic examination of the thyroid tissue were compatible with the results of thyroid hormone results, where a considerable histological alteration in the thyroid tissue was observed after exposure to a high-dose (50 mg/kg) of AgNPs during 20–30 days [Figs. 6 and 7], manifested by the depletion of follicular colloidal materials and necrotic injury of the follicular epithelial cells, which was found to be increased with longer duration.

CONCLUSION
According to the results, we can conclude that the thyroid gland was affected by chronic exposure to a high-dose of AgNPs, which may be considered as an indication of AgNPs toxicity. Further studies are needed to explore such a finding.

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AUTHORS’ CONTRIBUTION
All authors contribute equally in the collection of data and performing data analysis of the present study. In addition, the paper was written by the corresponding author.

CONFLICTS OF INTEREST
None declared.

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