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Gold Nanoparticle: Synthesis, Characterization, Clinico-Pathological, Pathological, and Bio-Distribution Studies in Rabbits

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***Abstract*—**This study evaluated the acute toxicity and tissue distribution of intravenously administered gold nanoparticles (AuNPs) in male rabbits. Rabbits were exposed to single dose of AuNPs (300 µg/ kg). Toxic effects were assessed via general behavior, hematological parameters, serum biochemical parameters, and histopathological examination of various rabbits' organs. Inductively coupled plasma–mass spectrometry (ICP-MS) was used to determine gold concentrations in tissue samples collected at predetermined time intervals. After one week, AuNPs exerted no obvious acute toxicity in rabbits. However, inflammatory reactions were observed in liver, lungs and kidneys accompanied with mild absolute neutrophilia and significant monocytosis. The highest gold levels were found in the spleen and liver followed by lungs, and kidneys. These results indicated that AuNPs could be distributed extensively to various tissues in the body, but primarily in the spleen and liver.

***Keywords*—**Gold nanoparticles, toxicity, pathology, hematology, liver function, kidney function.

I. Introduction

GOLD in its bulk form has long been considered an inert, nontoxic, bio-compatible, noble metal with some therapeutic (and even medicinal) properties. However, when the size of the typical objects decreases into nanoscopic dimensions (nanometer dimensions), gold behaves very differently than in bulk and its safety, as far as a promising material for biomedical applications is concerned, is no more unquestionable and many important concerns in the risk assessment for humans have been raised. The a priori assumption that gold nanoparticles are intrinsically bio-compatible must be rejected. On the other hand, the term itself of toxicity is rather vague. From a theoretical point of view, toxicology is related to the adverse effects that a generic substance exerts on living organisms. In this context, it must be recognized that, if exposure occurs in sufficient quantities, all materials are toxic (this basic principle of toxicology was expressed by Paracelsus more than five centuries ago, i.e., many drugs that are beneficial at low doses are toxic at high doses) [1]. Hence, a very important aspect is the identification of the most relevant dosimetry for particle toxicity [2]. The basic question to be addressed is: how toxic are gold nanoparticles at the potential concentrations at which they might be used for therapeutics? At present, even if a considerable number of reports have appeared, the problem remains basically unsolved. Gold nanoparticles [AuNPs] have been widely used in current medical and biological research, including targeted delivery of drugs [3], imaging and diagnosis of many diseases [4], intravenous contrast agent for imaging and noninvasive detection of lung cancer and many other topics [5]. Many labs have tried to investigate, from different points of view, the safety of using gold nanoparticles. Along this line, much experimental work has been done, which confirms the non-toxicity of gold NPs [6]-[8]. However, on the contrary, as much conflicting researches are also present, which revealed the toxicity of gold NPs [9]-[11].

The aim of the study is to investigate does the per se reduction of the size makes GNPs toxic to rabbits.

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II. Materials and Methods

A. Synthesis of Gold Nanoparticles

Colloidal gold nanoparticles were synthesized by the optimization of classical Citrate reduction method [12].

B. Surface Modification of Gold Nanoparticles

Surface modification of gold nanoparticles was necessary to increase its colloidal stability in physiological fluid by adding poly –ethylene glycol (PEG) according to method described by [13].

C. Characterization of Gold Nanoparticles

The prepared gold nanoparticles were characterized by using UV-Vis Spectrophotometer (PERKIN-ELEMER Lambda 40 B double beam), Zeta sizer (Malvern, UK, and Model: Zeta sizer nano series (Nano ZS), Size range (nm):0.6:6000 nm.) and High Resolution Transmission Electron Microscopy (JEOL, JEM-1400) which was used for determination of GNPs shape and size [14].

D. Animals and Experimental Protocol

Thirty six- day old New Zealand white male rabbits, around 1 kg weight were purchased from El Hady farm (a certified breeder) were used for this experiment. The rabbits were housed individually in cages, received food and water *ad libitum* and a daily illumination of 16 hours of light. All experiments using animals were performed according to the protocol approved by the Institutional Animal Care and Use

Committee at Cairo University. The rabbits were acclimatized for one week in the laboratory conditions prior to being randomly divided into two groups of fifteen animals each. Group (1) served as control and *i.v* injected with 1 ml saline. While group 2; rabbits were *i.v* injected with single dose of 300ug/kg b. wt. of the prepared gold nanoparticles according to [15]. All rabbits were kept under observation along the experimental period for their behavior, fur changes, or any abnormalities.

On 3rd, 5th, and 7th day post inoculation five animals from each group were euthanized prior to which, three blood samples were collected from the ear vein of each animal. One on EDTA and was used for routine hemogram picture. The second blood sample was taken in a test tube with double oxalate as anticoagulant and the third was taken in vacutainer tube without anticoagulant and allowed to stand for 30 minutes at room temperature. The second and third samples were centrifuged at 1500 r.p.m. for 20 minutes. The clear supernatant plasma or serum was decanted into clean and dry tubes and was used for glucose and other biochemical analysis respectively. Immediately after euthanization, liver, kidneys, spleen, and lungs were dissected out of the body, wiped off blood, weighted. Part of each organ was stored at -20°c till use. The other part was kept in buffered neutral formalin 10% for histopathological studies.

E. Hematological Studies

Full hemogram picture was evaluated for all animals including RBCs count, packed cell volume (PCV), hemoglobin concentration (Hb), total leukocyte count (TLC) and differential leukocytic count (DLC) [16].

F. Serum Biochemical Analyses

Separated serum samples were used for determination of total proteins [17] and albumin [18]. Globulins were assayed by subtracting value of serum albumin from the value of serum total proteins. Albumin/Globulin ratio was obtained by subdividing the values of serum albumin by those of serum globulins. Blood urea [19], creatinine [20], the activities of ALT and AST [21] and glucose concentration [22] were also assayed. Serum biochemical parameters were assayed using reagent kits supplied by Stan Bio-Laboratories incorporation, USA.

G. Determination of Gold Distribution in Tissue

The stored tissue specimens of liver, kidneys, spleen, and lungs at -20C° were prepared for evaluation of their gold content using Inductively Coupled Plasma–Mass Spectrometry (ICP-MS) according to the method described by [23] where, a known amount of tissue was weighed and digested by a mixture of perchloric and nitric acids.

H. Histopathological Examination

Formalin fixed tissue specimens of liver, kidneys, lungs, and spleen were routinely dehydrated in ascending grade of alcohol, then cleared with xylene, embedded in paraffin, and sectioned at 5 µm thickness. The obtained sections were stained with H & E and examined microscopically [24].

I. Statistical Analysis

Data were presented as Mean ± SD. Variables were statistically analyzed by student's t test and two-way ANOVA, using software SPSS (version 16). When differences were significant, LSD (Least Significant Difference) was performed to find the individual differences between groups. Statistical analyses were performed according to [25].

III. Results

A. Synthesis and Characterization of AuNPs

In the present work, tri sodium citrate served the dual role of both a reducing agent and a stabilizer. Tri sodium citrate initially acted as a reducing agent to reduce the Au (III) ions to Au (0) then acted as a stabilizing agent by forming a layer of citrate ions over the AuNPs surface.

1. UV-Visible Absorption Spectrophotometry

The absorption spectrum for the prepared solution of citrated AuNPs is characterized by the excitation of the Plasmon resonance in the neighborhood at 518 nm (Fig. 1).

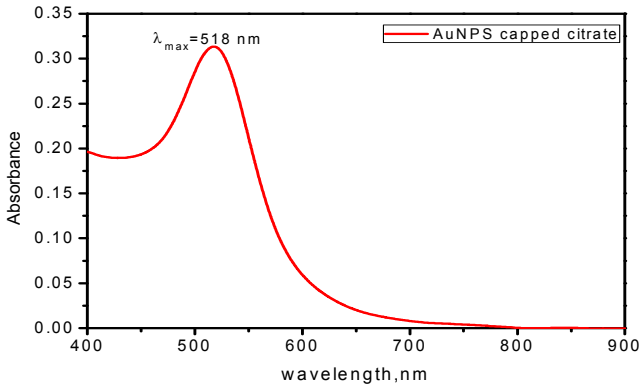

Fig. 1 Spectrophotometer results of AuNPs characterization showing peak absorption (0.33) at wave length 518nm

2. Transmission Electron Microscopy (TEM) imaging

The size, shape and size distribution of the prepared gold nanoparticles have been measured by TEM imaging. A representative TEM image of these particles showed that; they were mostly spherical, Fig. 2. From the sizes of a great number of particles measured on the TEM images, an average size (diameter) of the synthesized AuNPs capped citrate was 7± 2 nm, Fig. 3.

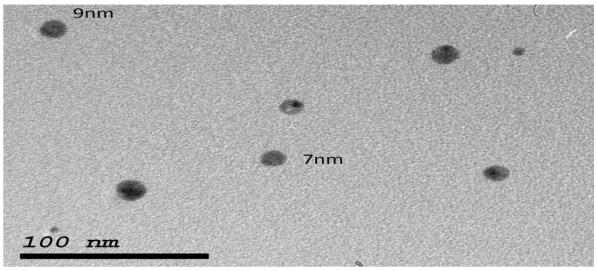

Fig. 2 Transmission electron microscope image showing spherical shape of the prepared AuNPS with different sizes

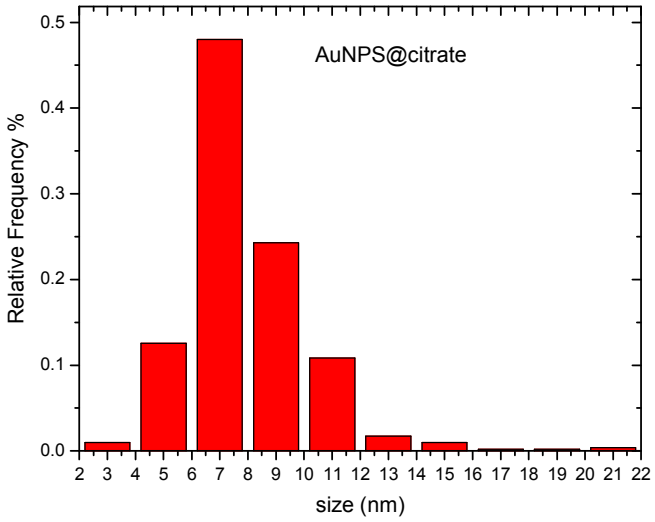

Fig. 3 Histogram chart provides the size distribution of the synthesized AuNPs capped citrate on the TEM images. It shows that an average size (diameter) of the prepared AuNPs is around 7± 2 nm

3. Zeta Potential

The zeta potential of the prepared citrated AuNPs was found to be around -12.3 mV, verifying that gold nanoparticles were charged and stable (Fig. 4).

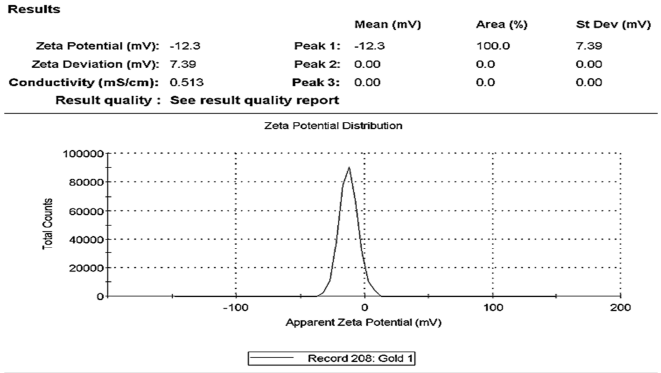

Fig. 4 The zeta potential of citrated AuNPS was found to be around -12.3 mV

B. Results of the Erythrogram

Mean values of erythrocyte count (RBCs), packed cell volume (PCV), hemoglobin concentration (Hb), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) of both groups are presented in (Table I). It was observed that GNPs administration to rabbits did not show any significant difference in all of the examined blood parameters compared to those of the control group at different time intervals 3rd, 5th and 7th day.

TABLE I
THE ERYTHROGRAM PICTURE IN BOTH CONTROL AND GNPS TREATED GROUPS AT DIFFERENT TIME INTERVALS

Parameter / Group	RBCs (x10^6 cell/µ/l)			Hb (g/dl)			PCV (%)			MCV(fl)			MCHC (%)		
	3rd	5th	7th	3rd	5th	7th	3rd	5th	7th	3rd	5th	7th	3rd	5th	7th
control	6.43± 0.15	6.43± 0.15	6.43± 0.15	11.50 ±1.00	11.50 ±1.00	11.50 ±1.00	40.67 ±2.52	40.67 ±2.52	40.67 ±2.52	63.30 ±5.38	63.30 ±5.38	63.30 ±5.38a	28.25 ±0.74a	28.25 ±0.74a	28.25 ±0.74a
GNPs	6.17 ±0.15	6.17 ±0.21	6.33 ±0.21	10.93 ±0.12	10.83 ±0.29	10.97 ±0.15	39.00 ±1.00	38.67 ±1.15	39.67 ±1.53	63.25 ±1.31	62.71 ±0.55	62.63 ±1.30	28.04 ±0.48abA	28.03 ±0.80aA	27.66 ±0.69abA

Means with different **small letters** (a, b, c, d) within the same **column** are significantly different at P value ≤ 0.05.
Means with different **CAPITAL letters** (A, B, C, D) within the same **row** for each **parameter** are significantly different at P value ≤ 0.05.

C. Results of Leukogram

GNPs administrated group showed mild increase in total leukocytic count associated with significant mild absolute neutrophila at 3rd day and significant monocytosis at 3rd, 5th and 7th day post administration in comparison with control set (Table II).

D. Results of Biochemical Analyses

1. Proteinogram

It was noticed that single administration of GNPs to rabbits did not significantly influence the levels of total protein, albumin, globulin and A/G ratio when compare them with those of control group (Table III).

2. ALT, AST, BUN, Creatinine and Glucose Conc.

Rabbits of GNPs administrated group showed non-significant difference in their levels of ALT, AST, glucose, BUN and creatinine in comparison with those of control group as presented in Tables IV and V.

TABLE II
RESULTS OF LEUKOGRAM IN CONTROL AND GNPS TREATED GROUPS AT DIFFERENT TIME INTERVALS

Parameter / Group	WBC (x10^3 cell/µl)			Neutrophils (x10^3 cell/µl)			Lymphocytes (x10^3 cell/µl)			Monocytes (x10^3 cell/µl)			Esinophils (x10^3 cell/µl)		
	3rd	5th	7th	3rd	5th	7th	3rd	5th	7th	3rd	5th	7th	3rd	5th	7th
Control	9.1 ±0.6ab	9.1 ±0.6ab	9.1 ±0.6ab	3.7 ±0.3c	3.7 ±0.3b	3.7 ±0.3b	4.4 ±0.4a	4.4 ±0.4a	4.4 ±0.4a	0.79 ±0.1c	0.79 ±0.1c	0.79 ±0.1b	0.18 ±0.1a	0.18 ±0.19a	0.18 ±0.19a
GNPs	10.46 1.80aA	9.53 0.41aA	9.40 1.01aA	5.02 0.93aA	3.43 0.32 bB	3.75 0.31 bB	3.92 0.78 abA	4.38 0.14 aA	4.07 0.58 abA	1.38 0.20 aA	1.71 0.12 aA	1.32 0.38 aA	0.13 0.11 aA	0.67 0.11 aA	0.25 0.22 aA

Means with different **small letters** (a, b, c, d) within the same **column** are significantly different at P value ≤ 0.05.
Means with different **CAPITAL letters** (A, B, C, D) within the same **row** for each **parameter** are significantly different at P value ≤ 0.05.

TABLE III
PROTEINOGRAM IN CONTROL AND GNPS TREATED GROUPS OF RABBITS AT DIFFERENT TIME INTERVALS

Parameter / Group	Total protein (g/dl)			Albumin (g/dl)			Globulin			A/G		
	3rd	5th	7th	3rd	5th	7th	3rd	5th	7th	3rd	5th	7th
Control	5.17± 0.18a	5.17± 0.18b	5.17± 0.18b	2.5± 0.12a	2.5± 0.12a	2.5± 0.12b	2.65± 0.21b	2.65± 0.21b	2.65± 0.21b	0.96± 0.10a	0.96± 0.10a	0.96± 0.10a
GNPs	5.16± 0.22aA	5.25 0.07bA	5.16 0.13bA	2.18 0.24aB	2.28 0.07aA B	2.51 0.05bc A	2.88 0.03ab B	2.97 0.01bA	2.86 0.08bB	0.75 0.19ab B	0.76 0.12bB	0.89 0.05ab AB

Means with different **small letters** (a, b, c, d) within the same **column** are significantly different at P value ≤ 0.05.
Means with different **CAPITAL letters** (A, B, C, D) within the same **row** for each **parameter** are significantly different at P value ≤ 0.05.

TABLE IV
RESULTS OF ALT, AST ENZYME VALUES AND GLUCOSE CONC.IN DIFFERENT GROUPS OF RABBITS AT DIFFERENT TIME INTERVAL

Parameter / Group	ALT(U/L)			AST(U/L)			Glucose (mg/dl)		
	3rd	5th	7th	3rd	5th	7th	3rd	5th	7th
Control	14.00 ±0.00 b	14.00 ±0.00 b	14.00 ±0.00 b	15.00 ±0.00 b	15.00 ±0.00 b	15.00 ±0.00 b	101.38 ±2.47 b	101.38 ±2.47 c	101.38 ±2.47 c
GNPs	14.00 ±0.00 bA	14.00 ±0.00 bA	14.00 ±0.00 bA	17.00 ±6.88 aA	15.00 ±0.00 bA	15.00 ±0.00 aA	102.16 ±1.28 abA	102 ±2.61 cA	100.98 ±1.59 cA

Means with different **small letters** (a, b, c, d) within the same **column** are significantly different at P value ≤ 0.05.
Means with different **CAPITAL letters** (A, B, C, D) within the same **row** for each **parameter** are significantly different at P value ≤ 0.05.

TABLE V
RESULTS OF BUN AND CREATININE LEVELS IN CONTROL AND GNPS TREATED GROUPS AT DIFFERENT TIME INTERVALS

Parameter / Group	BUN (mg/dl)			Creatinine (mg/dl)		
	3rd	5th	7th	3rd	5th	7th
Control	31.59 ±2.07a	31.59 ±2.07c	31.59 ±2.07b	0.51 0.06a	0.51 0.06c	0.51 0.06b
GNPs	33.68 ±2.54aA	31.90 ±0.70cA	31.63 ±1.57bA	0.44 ±0.05aB	0.55 ±0.05cA	0.47 ±0.06bAB

Means with different **small letters** (a, b, c, d) within the same **column** are significantly different at P value ≤ 0.05.
Means with different **CAPITAL letters** (A, B, C, D) within the same **row** for each **parameter** are significantly different at P value ≤ 0.05.

TABLE VI
GOLD TISSUE DISTRIBUTION IN LIVER, SPLEEN, KIDNEYS, LUNG AT DIFFERENT TIME INTERVALS IN GNPS GROUP

Organ	3rd day	5th day	7th day
Liver	7.24 ± 4.25 ab	10.05 ± 2.85a	5.66 ± 5.25 a
Spleen	8.57 ± 1.33 a	6.90 ± 0.20 b	6.20 ± 2.36 a
Kidney	0.56 ± 0.45 c	0.43 ± 0.05 c	0.93 ± 0.81 a
Lung	3.33 ± 2.69 bc	1.80 ± 0.50 c	1.90 ± 0.66 a

Means with different letter (a) within the same row are statistically the same at P value ≤ 0.05.

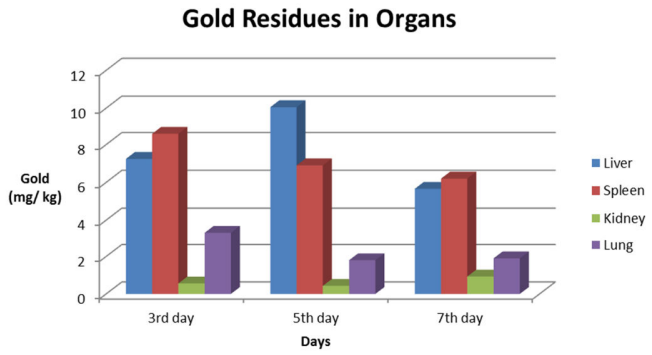

Fig. 5 Gold tissue concentrations in liver, spleen, kidneys, lung at different time intervals in GNPs group

E. Results of Tissue Distribution of GNPs

Results illustrated in Table VI and presented in Fig. 5 demonstrated the mean GNPs concentration at different time intervals 3rd, 5th and 7th day post single dose of GNPs administration. The obtained data revealed that GNPs were accumulated mainly in liver and spleen and to less extent in kidney and lung.

F. Results of Histopathological Examinations

Microscopical examination of tissue sections from different organs of control group revealed normal histological picture. GNPs administrated rabbits revealed mild to moderate tissue alterations started at day 3 and decreased in intensity at day 7. Examination of liver sections of this group revealed congestion of the hepatic blood vessels, increased number of binucleated cells and kupffer cells activation, Fig. 6 (a). Swelling of the hepatic cells was evident with mild degree of granular and vacuolar degeneration as well as scattered necrotic cells, Fig 6 (b). Kidney showed marked swelling of the tubular epithelial linings with variable degrees of granular degeneration and few scattered necrotic cells, Fig. 6 (c). Mild congestion of the interstitial and glomerular blood capillaries, Fig. 6 (d) was also evident. Lung showed marked congestion of the peribronchial and perialveolar blood capillaries, Fig. 6 (e) with slight thickening of the interalveolar walls. The later accompanied with mild mononuclear inflammatory cells infiltration especially in the peribronchial areas, Fig. 6 (f). The examined sections of the spleen of rabbits of this group revealed no obvious changes.

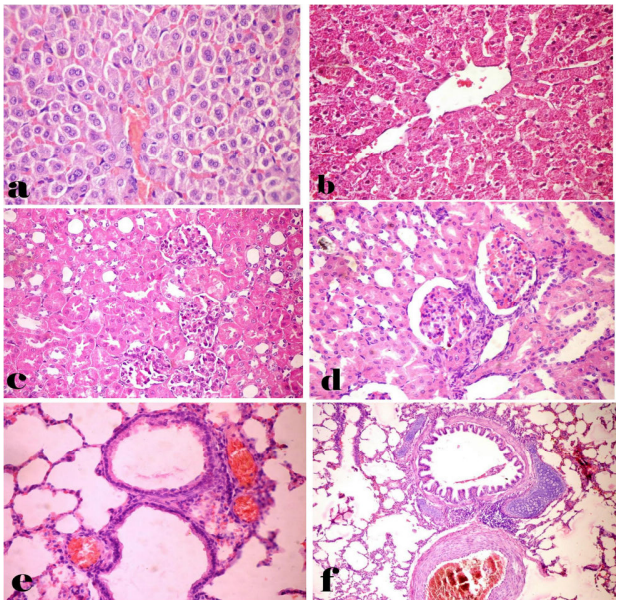

Fig. 6 (a) and (b): Liver of nanoparticle treated rabbit showing (a) marked congestion of central vein and hepatic sinusoids, increased number of binucleated cells and Kupffer cells activation. (b) granular degeneration of hepatocytes and scattered necrotic cell (arrow). (c) and (d): Kidney of nanoparticles treated rabbit showing (c) swelling of the tubular epithelial linings with variable degrees of granular degeneration and scattered necrotic cells. (d) congestion of the glomerular blood vessels with swelling of the tubular lining epithelium (e) and (f): lung of nanoparticles treated rabbit showing (e) congestion of the perialveolar and peribronchial blood capillaries (f) mild inflammatory cells infiltrates in the peribronchilal area (H&E X 400)

IV. Discussion

The unique physiochemical properties of the nanoparticles combined with the growth inhibitory capacity against microbes has led to the upsurge in the research on nanoparticles and their potential application as antimicrobials. It is quite evident that some of metallic compounds possess antimicrobial properties. Recently, the confluence of nanotechnology and biology has brought to fore metals in the form of nanoparticles as potential antimicrobial agents that may be due to large surface area to volume ratio [26]. Nanoparticles have unique and well defined physical and chemical properties which can be manipulated suitably for desired applications.

A primary goal of gold nanoparticles synthesis for practical applications is to produce mono-dispersed nanoparticles with a well-defined shape. Therefore, careful selection of the reducing agent and stabilizer are critical steps which can be more easily controlled when the nanoparticles are synthesized. Hence, we were able to successfully synthesize water-soluble, highly mono-dispersed, spherical AuNPS with a known chemical composition. For these experiments, tri sodium citrate served the dual role of both a reducing agent and a stabilizer. Trisodium citrate initially acts as a reducing agent to reduce the Au (III) ions to Au (0) and then acts as the stabilizing agent by forming a layer of citrate ions over the AuNPs surface, inducing enough electrostatic repulsion between individual particles to keep them well dispersed in the medium and prevents the aggregation or further growth of the particles [14].

The colloidal gold solution proved to be very stable in time; no spectral change has been observed a yearlong after preparation. This indicates electrostatic stabilization via citrate anions bonded on the AuNPs surface. Therefore, AuNPs capped with citrate are negatively charged composites [27].

The overall charge that the particle acquires in a particular medium can be determined by measuring the zeta potential of the suspension. The resulting repulsive force can be used to predict the colloidal stability and agglomeration state of nanoparticles [28]. The zeta potential of the citrated AuNPS was found to be around -12.3 mV, verifying it was charged and stable.

Erthrogram (RBCs count, Hb conc, PCV, MCV and MCHC) values of GNPs administrated group were almost the same and did not show any significant difference in comparison with control group. This finding is consistent with previous study of [15], while disagree with [29]. These variations may be related to size and /or dose difference.

Leukogram results of GNPs group showed normal leukocyte count in comparison with that of control group which agreed with [11], [29], [30].

Regarding neutrophil count of GNPs group, it was significantly higher at 3rd day than that of control group which is in agreement with [29], [31]. The elevation in neutrophil count could be attributed to enhanced release of different cytokines including both pro-inflammatory and inflammatory cytokines caused by the nanoparticles [32]. That elevated neutrophil count is in disagreement with [11], [30] which may be size and/or dose related.

The observed tremendous monocytosis in GNPs administrated group is mainly due to the role of monocyte in clearance of the circulation from foreign bodies [33].

Proteinogram of GNPs administrated group showed insignificant difference from control group which agree with many authors as [11], [34], [35]. On the other hand, the levels of ALT, AST and glucose of GNPs administrated group did not show any significant changes in comparison with those levels of the control group. These findings agree with [35], [36]. İn addition, BUN and creatinine results of GNPs group did not show any significant difference in comparison with control group. These findings are in agreement with those of [31], [34], [36].

Concerning tissue distribution results, GNPs were accumulated first in significant amount in liver and spleen and to less degree in lungs and kidneys at 3rd and 5th day then at 7th day post injection GNPs were accumulated at almost similar amount in all organs. These results agree partially with [37], [38]. The later partial disagreement is mainly related to difference in timing of sample collection and their experimental design.

Histopathological examination of GNPs administrated group revealed mild to moderate tissue alterations starting at day 3 and were significantly decreased in intensity at day7.

Examination of liver sections of this group revealed congestion of the hepatic blood vessels, with mild degree of hepatocellular granular and vacuolar degeneration, increased number of binucleated cells and kupffer cells activation. These findings are in agreement with those of [31], [39]-[41]. The later changes may be due to enhanced defense mechanism against foreign bodies [30], intoxication, or hemodynamic changes [42] or alteration in inflammation and apoptosis related gene [41].

Kidney showed congestion of the interstitial and glomerular blood capillaries with variable degrees of degenerative and necrotic changes of the renal tubular epithelial linings. These findings are consistent with previous studies of [30], [42], [43] and could be attributed to the toxic effect of GNPs during their clearance by renal tissue.

Lungs showed marked congestion of peribronchial and perialveolar blood capillaries with slight thickening of the interstitial tissue. The later accompanied with mild mononuclear inflammatory cells infiltration especially in the peribronchial areas. These findings agree with [44] while disagree with [35], [36], this disagreement may be dose, route and/or size related.

Regarding the examined sections of the spleen of rabbits of this group, no obvious lesions could be observed which agree with [45] while disagree with [42] which may be related to size difference or the use of silica as coating agent in the auther experiment.

In conclusion, the results of the present study revealed mild to moderate changes in the hematological and serum biochemical parameters as well as histological picture of the examined organs caused by the used size of GNPs. Despite these preliminary studies, more accurate studies need to be taken for designing GNPs with less immunogenicity and cytotoxicity to be used *in vivo*.

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