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RESEARCH ARTICLE

Organ Index, Toxicological, and Bioaccumulation Study of Sub chronic Intraperitoneal Injected Silver Nanoparticles in Albino Mice Males

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Abstract

To investigate the sub chronic toxicity effects of intraperitoneal injected silver nanoparticles (Ag NPs) (24.52 nm), Albino mice males repeatedly exposed to three different doses (12.5, 25, and 50 mg/kg) of Ag NPs for 4 weeks. The accumulations of injected doses in different organs were evaluated. The results showed a significant changes in organs indexes with a good relations to the histopathological effects were noted which ranges between infiltration of inflammatory cells to coagulative necrosis in liver, multiple hemorrhage and cast forming in kidney, and amyloid deposition in spleen, thickening of intestinal villi, and sever interstitial broncho pneumonia as adverse effects in studied organs with dose related manner. Significant changes in hematological and biochemical parameters were detected combined with a considerable increasing in antioxidant enzymes in a dose dependent manner. The reduction in thyroid hormones levels T3, T4, and thyroid stimulating hormone (TSH). The highest accumulated Ag means values were recorded in liver, spleen, lung, and kidney respectively followed by brain, heart, small intestine, and testes in the order with dose depended manner which express the toxicological effects were recorded.

Keywords: *Ag NPs, Accumulation, Organ index, Biochemical parameters, Histopathology.*

Introduction and Literatures Review

Silver nanoparticles (AgNPs) was the most often incorporated in nano functionalized consumer products and applications in various fields over the world [1], although the highest volume of production related to SiO2 NPs, silver nanoparticles (Ag NPs) with mean annual production estimated with 55 tons, individually was the highest used in many consumer products with the widely expansion in NPs commerce which are used as antimicrobial agent in different products ranging from clothing, cosmetics, shoes, respirators surface coating, detergents, water filters and house purification systems, to laptops, and phones [2].

Recently, Ag NPs have gained increased attention because of their therapeutic [3], imaging, and diagnostics of cancer and other diseases by leading therapeutic or diagnostic target material in the area of interest, e.g., a tumors [4, 5].Although the growing AgNPs applications in many fields, apprehensions about their health and environmental effects combined with increasing in the opportunities of potential releasing to the environment and transporting to the ecosystems to make severe impacts on different types of biota remain unsolved. The net result of all uptake and loss processes of nanoparticles described by bioaccumulation process which describes the accumulation and enrichment of contaminants in organisms relative to that in the environment that leads to toxic effects in the organism and alteration of the animal's normal physiology and ecology [6].

Liver is the most common accumulation organs regardless of exposure routes [7], animal models, and physicochemical properties of NPs used, as their ability to form ions which enter the blood circulation and accumulate in several tissues, including the liver [8]. Ag NPs toxicity has been studied in different biological systems, such as bacteria [9], mammalian cells, and in vivo models [10, 12].

In this study, we extended previous studies to evaluate the toxicity of intraperitoneal injected Ag NPs and their possible effects on organ index, different hematological and biochemical parameters. Accumulation of injected doses in different organs were studied and their impacts in histopathological changes resulting from AgNPs exposure which have also been examined. This study noted some biochemical changes and histological effects which not recorded as an effect of Ag NPs exposure in pervious studies of animal models over the world. Also this study used the organ index side by side with biochemical biomarkers as a good tool to explain the histopathological effects.

Materials and Methods

Nanoparticles

Ag NPs about (20 nm) were purchased as grey to black nano powder from NANOSHEL company-USA, with purity of 99 %, 10.5 g/cm3 density, and spherical morphology. Size average and surface morphology were characterized using scanning probe microscope (SPM) and atomic force microscope (AFM) from FILIPS- Germany.

Animal Housing

Healthy adult male (8-10 week aged) Swiss albino mice with average weight 25±2 gm were purchased from the national center for drug control and research-Ministry of Health. All mice were housed in polypropylene cages under controlled conditions of temperature 25 ± 5°C, humidity of 50-60 %, and 12 ± 2 hours light/dark cycles. Standard diet pellet and water ad libitum were used for feeding.

The animals were kept for7 days before starting the experiments for acclimatization to laboratory conditions. All animals were dealt in accordance to the guidelines of the Care and Use of Laboratory Animals-National Research Council and in accordance with the guidelines of the international guidelines for animal experimentation.

Ag NPs Suspension Preparation

The concentrations of Ag NPs suspension were prepared with deionized distilled water and homogenized by vortex for 20 sec., then exposed to probe ultrasound sonication from (Soniprep 150 MES-UK) in pulsed mode in ice bath for 60 min [13].

The prepared suspensions were immediately exposed to mice with intraperitoneal injection.

Acute toxicity Assay of Ag NPs and Mortality Percentage

The experiment was conducted using nine experimental groups and one as a control (six animals per each group).

Nine series of concentrations were used in acute toxicity test (50, 100, 150,200, 250,300, 350, 400, 450, and 500 mg/kg) intraperitoneally administrated as a single dose, the experiment carried out in two replicate per concentration. Live/dead animals were counted after 48 of administration. The probit analysis was carried out using by Finney's [14] method, and the LD50 was derived from the best-fit line obtained. Mortality rate were calculated by the ratio between the total numbers of animals at the start to dead number at the end of experiment.

Sub Chronic Toxicity Test for Ag NPs

One control and three treated groups of mice males (10 animals per each group) were used in this experiment. Treated groups were intraperitoneal injected with different concentrations of Ag NPs (12.5 mg/kg, 25 mg/kg, and 50 mg/kg respectively) three times a week for four weeks. These three concentrations for each group were calculated by approaching to 1/5th, 1/10th and 1/20th of the calculated LD 50.

After 24 of the last dosing, the mice anaesthetized using 0.1ml of (0.4 ml XYL-M2 and 0.6 ml Ketamine 10%). Blood collected to EDTA tube for hematological tests by heart puncher. Another blood samples used for serum separation using eppendorf centrifuge from (Eppendrof 5417R-Germany) at 3000RPM for 10min at 30 ºC and used for biochemical parameters tests. Organs (liver, spleen, kidney, brain, small intestine, testes, lung , and heart) were collected and divided to two groups, one group were kept (without washing) in -20 C until using in further bioaccumulation study, the second one were directed for organ index and histopathological examination.

Body Weight and Organ Index

Initial and weekly body weights of animals were recorded for all groups and

subsequently, body weight gain (gm/ week) and organ index was calculated. For organ index, After blood samples collected, the animal scarified, organs (liver, spleen, kidney, small intestine, brain, testis, lung, and heart) were collected and washed using solution of chilled saline, all adhering connective tissues and fat were removed, then weighed after dried using tissue papers, Organ index calculate using the equation describe below:

$$\text{Organ Index} = \frac{\text{Weight of organ (mg)}}{\text{Weight of the body (gm)}} \times 100$$

Hematological Parameters

Hematology analyzer from (Nihon Kohden Celltac ES-Indian) was used to measure the following hematological parameters in collected blood samples: white blood cells (WBCs), red blood cells (RBCs), hemoglobin (Hb), Lymphocyte, and monocyte counts according to the manufacturing instructions and using Quality Control Reagent to assess the validity of the assays.

Biochemical Parameters

Separated serum were used to determine total cholesterol (CHO), triglyceride (TG), low density lipoproteins (LDL), and high density lipoproteins (HDL), total protein (TP), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), albumin (ALB), bilirubin (BIL), Urea (UR) and creatinine (CR) were measured using kits from (SPINREACT, Spain).

Anti oxidant factors, Super oxide dismutase (SOD) measured using kit from (Cell Technology, USA), Glutathion peroxidase (GPx) using kit from (Abcam, UK). Thyroid hormones (T3), (T4), and thyroid stimulating hormone (TSH) measured using ELISA kit from (Abnova-USA, and Antibodies-Germany) the validity of these kits was assayed using Quality Control Reagent.

Bioaccumulation Study

Sub samples about 0.25-0.5 gm of organs tissue (liver, spleen, kidney, small intestine, brain, testis, lung, heart) were obtained from control and the three experimental groups and digest individually using microwave digestion system from Milestone-Italy as described in using 9 ml of 68-72% nitric acid HNO3 from Merk-UK, 1ml of 37% hydrochloric acid HCl from Panreac- Spain, and 2 ml of 30% hydrogen peroxide H2O2 from Panreac-Spain, the digested samples diluted to 25 ml using distilled water. The concentration of silver detect in each sample using flameless atomic absorption spectrophotometer (AAS) from Shimadzu-Japan, the concentrations expressed in µg of Ag/gm of sample.

Histopathological Study

At the end of experiment of toxicological study, anesthetized mice were sacrificed. liver, kidneys, spleen, small intestine were collected from three animals for each experimental and control groups, washed with normal saline solution and kept in 10% formalin solution provided by CHEM-LAB-Beigium for fixation , then dehydrated in serial concentration of ethanol 60, 70, 80, 90, and 100% and xylene (Sigma-USA).

The tissues were embedded in paraffin, and then sectioned at 5µm thicknesses with microtome provided by MICROM-Germany for staining by Haematoxylin and Eosin (H&E) stains from Merk-UK. The tissues were further examined using optical compound microscope from Olympus- Japan on 40x, 100x and 400x to detect any abnormal changes in the organs tissues.

Statistical Analysis

The results are presented as the mean ± standard error of means (SE). Analysis of variance (ANOVA) and least significant difference (LSD) were used to explain the differences between means of parameters in treated and control groups ($p \leq 0.05$) [15].

Results and Discussion

Characterization of Ag and NPs

According to the granularity distribution chart, the average diameter for Ag NPs sample were 24.52 nm with spherical shape determined using AFM (Fig.1).

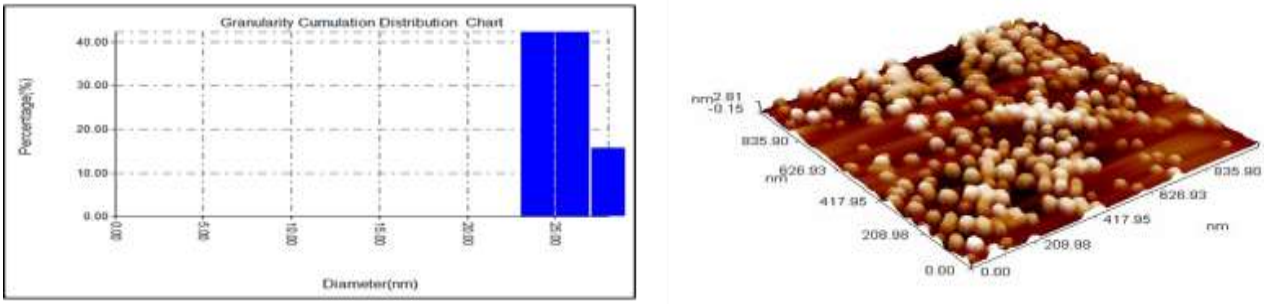

Figure 1: Characteristics of Ag, granularity distribution chart (left) and, spherical shape using AFM (right)

Acute Toxicity Assay of Ag NPs

Acute toxicity study is better described as LD50, which is defined as the dose which kills 50% of animals in a dose group [16]. LD50 were evaluated for intraperitoneal injected AgNPs with 251.47 mg/kg (Table 1; Fig 2). The calculated value of LD50 in current study were higher than those recorded by Elkhawass et al [17].In BALB/c mice using two methods of toxicological evaluation with169 and 213.8 mg/kg using Dixon's up-and-down method and AOT425statPgm methods respectively.

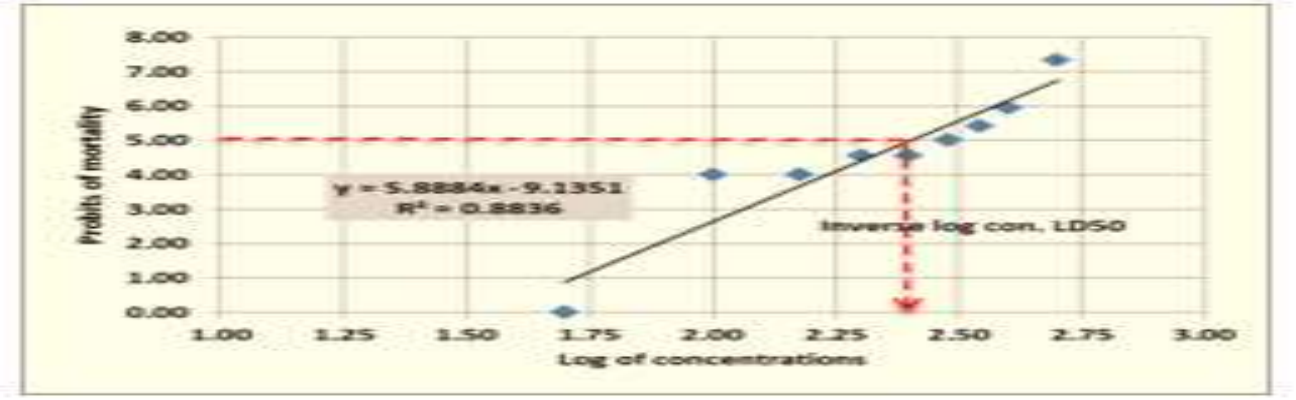

Figure 2: Toxicity curve of AgNPs after 48 h of intraperitoneal injection

Table 1: Median lethal dose LD50 after 48 h of intraperitoneal injection with Ag NPs using probit analysis method

Con. (mg/kg)	log of Con.	Mortality (%)	Probits	LD50 (mg/kg)
50	1.70	0	0.00	251.47
100	2.00	16	4.01	
150	2.18	16	4.01	
200	2.30	33	4.56	
250	2.40	33	4.56	
300	2.48	50	5.00	
350	2.54	66	5.41	
400	2.60	83	5.95	
500	2.70	100	7.33	

Sub Chronic Toxicity

Body Weight and Organ Index

Non significant (p>0.05) decreasing in body weight were noted in all exposed groups over weeks of injection compared with control group except G3 which exposed some increasing in weight specially after 1and 2 weeks which may because of some reduction in T3 and T4 hormones that detected in this study (Table 2 A) before dwindling to records their higher weight loosing with-3.26 ±1.27 gm after 4 weeks of injection with the higher dose in G3. Injurious effects of Ag NPs in mice may be associated closely with the weight leakage and death [18], crossing of blood brain barrier (BBB) by metal NPs have a direct injurious effect on the central nervous system which probably directing to anorexia then weight loos[19].These results come to an agreement with Al-Bairuty and Taha [20] study after orally administration of 200 mg/kg Ag NPs for 5, 10, and 15 days which supposed these results to some

injurious effects in digestive system which will illustrated in histopathological study. In this study, the organ index of kidney, spleen, heart, lung, and brain, increased significantly ($p \leq 0.05$) after 2 and 4 weeks of injection with 12.5, 25, and 50 mg/kg Ag NPs (Table 3-). This increasing may referred to the accumulation of Ag NPs in organs as in brain, heart and spleen, also some inflammation impacts or edema in organs as response to the Ag NPs exposure which confirmed in this study by histopathological examination like thickening of the intestinal tissue in lung, cast formation and glomerular swelling also were companion with kidney index increasing in dose dependent manner. A significant decreasing in liver index was noted in G3 and testes index were noted in this study which related to tissue necrosis with dose dependent severity in testes. This result agreed with Taha and Ali [21] when noted significant decreasing in weights of testes tunica albuginea after 5, 10, and 15 days of orally administration of 200 mg/kg Ag NPs, these results combined with decreasing in sperms concentration and vitality.

Table 2: Weekly change in body weight (gm), weight gain (gm/week), and organ index after 4 weeks of intraperitoneal injection with different doses of AgNPs

Weekly body weight (gm) and weight gain (gm/week)

	Experimental groups							
	Control	weight gain (gm/week)	weight (gm) (G1) 12.5 mg/kg AgNPs	weight gain (gm/week)	weight (gm) (G2) 25 mg/kg AgNPs	weight gain (gm/week)	weight (gm) (G3) 50 mg/kg AgNPs	weight gain (gm/week)
Time zero	29.69 ± 0.39 a		26.80 ± 1.19 a		26.83 ± 0.82 a		26.67 ± 0.88 ab	
1st. week	27.79 ± 0.87 a	1.10 ± 0.81 a	28.88 ± 1.62 a	-4.88 ± 1.62 a	24.78 ± 1.04 a	-1.17 ± 0.88 a	28.87 ± 1.25 c	8.87 ± 1.82 b
2nd. week	28.38 ± 1.18 a	1.70 ± 0.81 a	21.88 ± 2.08 a	-4.18 ± 2.08 a	22.84 ± 1.28 a	-2.88 ± 1.14 a	27.14 ± 1.40 bc	1.64 ± 1.40 b
3 rd. week	29.84 ± 1.37 a	8.10 ± 1.01 a	24.24 ± 2.78 a	-4.88 ± 2.20 a	28.82 ± 1.68 a	-2.28 ± 1.08 a	26.18 ± 1.84 a	-0.41 ± 1.84 a
4 th week	30.74 ± 1.18 b	4.00 ± 0.80 b	23.64 ± 2.80 a	-2.28 ± 2.21 a	22.78 ± 2.07 a	-3.14 ± 1.81 a	22.34 ± 1.81 a	-3.28 ± 1.27 a

All data expressed with mean ± standard error of means (SEM), similar small letters means no significant difference between means values on $p<0.05$

Organ index

Organs	Experimental groups				
	Control	G1 12.5 mg/kg AgNPs	G2 25 mg/kg AgNPs	G3 50 mg/kg AgNPs	LSD
Liver	6.42 ± 0.15 b	6.57 ± 0.31 b	6.67 ± 0.00 b	5.11 ± 0.12 a	1.16
Spleen	0.61 ± 0.04 a	0.89 ± 0.06 ab	1.08 ± 0.26 ab	1.23 ± 0.20 b	0.50
Kidney	1.36 ± 0.03 a	1.79 ± 0.06 b	1.80 ± 0.03 b	2.08 ± 0.08 c	0.17
Small intestine	12.43 ± 0.05 ab	11.28 ± 0.74 a	12.43 ± 0.36 ab	12.75 ± 0.39 b	1.42
Testes	1.21 ± 0.01 c	0.82 ± 0.08 b	0.66 ± 0.05 a	0.61 ± 0.07 a	0.16
Brain	1.44 ± 0.01 a	1.59 ± 0.03 b	1.64 ± 0.06 bc	1.76 ± 0.07 c	0.14
Lung	0.82 ± 0.02 a	1.04 ± 0.06 b	1.02 ± 0.03 b	1.18 ± 0.09 b	0.18
Heart	0.51 ± 0.00 a	0.66 ± 0.04 b	0.63 ± 0.03 b	0.72 ± 0.04 b	0.12

All data expressed with mean ± standard error of means (SEM), similar small letters means no significant difference between means values on $p<0.05$

Hematological Parameters

According to Sarhan and Hussein [11] the injected Ag NPs interact with blood and all their components causing wide variety of immunogenic responses, like inflammation, hematological parameters alternations, including red and white blood cells, these changes may be due to the increasing in immunogenic response [22], or disorder in signaling pathway and cell maturation disturbance that affects on the RBC s division and other cells development [23]. All Ag NPs treated groups exhibited announced decreasing in red blood cells (RBC) and hemoglobin (Hb) values ($p<0.05$) comparison with control group the lowest means recorded in G2 which may related to the hemorrhage effects in G1and G2 kidney as showed by histopathological examination (Table

3).These results agreed with those of Tiwari et al. [24].

In Wistar rats after intravenous administration of 4, 10, 20, and 40 mg/kg, they concluded the toxic effects of doses > 20 mg/kg. White blood cells (WBC) considered the active functional cells of specific and non specific immune system, their count reflect a whole picture of immune system function [25]. Reduction in WBC means in treated groups compared with control one indicates the retraction in immune system that makes the animal susceptible to any external dangerous agent [26].

Lymphocytes and monocytes values recorded means lower than those in control group with 28.70±6.70×10^3/ul and 1.10±0.12×10^3/ul in G3for lymphocytes and monocytes respectively, which may be referred to the noted infiltration of lymphocytes and monocytes in studded organs tissue which illustrated in histopathological study.

Biochemical Parameters

A significant decreasing (P≤0.05) CHO, TG, LDL, and HDL with injected dose increasing manner compared with control group (Table 3). Lowest means 62.67±1.76 mg/dL, 58.00±1.73 mg/dL, 38.73±1.05 mg/dL, and 11.00± 0.58 mg/dL for CHO, TG, LDL, and HDL respectively recorded with the highest concentration 50 mg/kg Ag NPs in G3. These declined concentrations due to decreasing of cholesterol that carried by LDL and HDL, and may be combined with the lipid peroxidation and free radical releasing in the body by oxidative stress effect [27].

Also the cholesterol rich environment improved the Ag NPs uptake by macrophage within 24 h of exposure, which cause increasing in cholesterol uptake by role of Ag NPs as a carrier for cholesterol in to the cell [28]. The decreasing in HDL and LDL means as the effect of Ag NPs exposure recorded in the current study were not reported before in animal model, while decreasing in TG levels were reported beforein previous studies [29, 30].

A respectable growing (p≤0.05) in means of liver enzymes AST, ALT, and ALP were clarified in experimental groups by the increasing doses of Ag NPs in contrasting with control one to record their highest means in G3 with 167±2.65 U/L, 49.33±1.76 U/L, and 181.67±1.33 U/L respectively which gave an indicator that the liver tissue were damaged, this illustration were proven then by histopathological test. On the other hand TP and ALB showed a concidrable decreasing compared with control group (p≤0.050) with 4.10±0.06 gm/dL, and 2.13±0.09 gm/dL respectively which may relate to the free radicals liberated from Ag NPs attacked the hepatocytes that caused ALT releasing and entering to the blood [12].

Also the liver cells degenerations noted in histopathological test which will be illustrated later may be responsible for the elevation in AST. According to Adeyemi and Adewumi [31] the adaptation mechanism by animals may be translated by the alterations in the enzymes levels to reduce stress decreed by Ag NPs exposure. The current study results agreed with those of Al Gurabi et al [32].

In notification a significant rising in hepatic damage symptoms such as altitude in levels of ALT, AST, and ALP enzymes, DNA damage and cell death of hepatocytes of albino mice that treated with 7.8 mg/ kg of Ag NPs. A significant increasing (p≤0.05) in urea and creatinine concentrations in experimental groups compared with control one with clear manner of increasing relative to the dose concentration which marked the severity of toxicological dysfunction effects of AgNPs on kidney using creatinine as a good index of renal function [33, 34].

The potential capacity of Ag NPs to motivate the oxidative stress was studied by evaluating serum SOD and GPx. Compared to control group, a statically significant increasing (p≤0.05) in serum SOD and GPx enzymes means with manner of dependent on dose concentration of Ag NPs with the higher means recorded in G3 with 19.97±0.07 U/L and 15.23±0.07 U/L respectively which may be related to the increasing the oxidative stress as mentioned in some previous studies [35, 36] which induced by the highly interaction between Ag NPs and proteins and different enzymes in tissue [37] and involve in reducing or preventing the mechanism of protection by the interaction with the antioxidant systems with dose depending [38] and leading to more production of (ROS) reactive oxygen species, which may start a response of inflammation [37, 39].

The present study reflects a considerable decreasing in means of T3, T4 concentrations in groups treated with the three different doses of Ag NPs ($p<0.05$), lowest means values noted in G3 with 0.67±0.01 ng/ml and 4.31±0.01 ng/ml respectively, this reduction may related to the increasing the oxidative stress [10] as the high levels of ROS generated by nanoparticles in addition to their extensive ability of tissue penetration that lead to inflammation or structural and functional disruption effects on thyroid gland [40, 41] or on the liberation of thyroid stimulating hormone (TSH) Sulaiman et al [42].Which clarified by the highly significant decreasing in TSH means levels in experimental groups injected with Ag NPs.

Table 3: Hematological and biochemical parameters means values in mice after 4 weeks of intraperitoneal injection with different doses of AgNPs 12.5 mg/kg (G1) , 25 mg/kg (G2) , and 50mg/kg (G3)

Parameters	Experimental groups			
		G1	G2	G3
	Control	12.5 mg/kg AgNPs	25 mg/kg AgNPs	50 mg/kg AgNPs	LSD
RBC (×10^6/ul)	8.37 ± 0.12 ^{c}	5.39 ± 0.24 ^{a}	5.35 ± 0.12 ^{a}	6.14 ± 0.03 ^{b}	0.47
Hb (g/l)	12.57 ± 0.31 ^{c}	8.40 ± 0.24 ^{b}	7.87 ± 0.17 ^{a}	8.60 ± 0.08 ^{b}	0.50
HCT (%)	38.17 ± 2.09 ^{c}	30.15 ± 0.04 ^{b}	28.10 ± 0.08 ^{ab}	26.05 ± 0.78 ^{a}	3.52
WBC (×10^3/ul)	9.00 ± 0.38 ^{d}	3.00 ± 0.00 ^{a}	4.90 ± 0.41 ^{b}	5.70 ± 0.00 ^{c}	0.78
lymphocyte (×10^3/ul)	60.83 ± 2.74 ^{c}	45.20 ± 0.82 ^{b}	40.80 ± 5.40 ^{b}	28.70 ± 6.70 ^{a}	9.72
Monocyte (×10^3/ul)	2.90 ± 0.37 ^{b}	1.80 ± 0.62 ^{a}	1.15 ± 0.18 ^{a}	1.10 ± 0.12 ^{a}	0.57

All data expressed with mean ± standard error of means (SEM), similar small letters means no significant difference between means values on $p<0.05$

Parameters	Experimental groups			
		G1	G2	G3
	Control	12.5 mg/kg AgNPs	25 mg/kg AgNPs	50 mg/kg AgNPs	LSD
CHO (mg/dL)	124.00 ± 1.53 ^{d}	91.33 ± 1.86 ^{c}	75.67 ± 0.88 ^{b}	62.67 ± 1.76 ^{a}	5.07
TG (mg/dL)	92.67 ± 1.76 ^{d}	82.00 ± 1.15 ^{c}	64.67 ± 1.76 ^{b}	58.00 ± 1.73 ^{a}	5.30
HDL (mg/dL)	28.33 ± 0.67 ^{d}	19.67 ± 0.33 ^{c}	13.67 ± 0.33 ^{b}	11.00 ± 0.58 ^{a}	1.63
LDL (mg/dL)	74.00 ± 2.08 ^{d}	55.93 ± 1.14 ^{c}	48.33 ± 1.07 ^{b}	38.73 ± 1.05 ^{a}	4.58
TP (gm/dL)	5.83 ± 0.03 ^{c}	5.06 ± 0.04 ^{b}	4.82 ± 0.06 ^{b}	4.10 ± 0.06 ^{a}	0.14
AST (U/L)	72.00 ± 1.15 ^{a}	148.33 ± 1.86 ^{b}	160.50 ± 3.67 ^{c}	167.00 ± 2.65 ^{c}	7.02
ALT (U/L)	12.33 ± 1.33 ^{a}	22.00 ± 2.08 ^{b}	43.00 ± 0.58 ^{c}	49.33 ± 1.76 ^{d}	3.69
ALP (U/L)	92.33 ± 0.33 ^{a}	127.33 ± 2.73 ^{b}	162.67 ± 2.33 ^{c}	181.67 ± 1.33 ^{d}	6.27
ALB (gm/dL)	3.30 ± 0.00 ^{d}	2.91 ± 0.04 ^{c}	2.58 ± 0.11 ^{b}	2.13 ± 0.09 ^{a}	0.19
BIL (mg/dL)	0.35 ± 0.01 ^{a}	0.51 ± 0.02 ^{b}	0.69 ± 0.01 ^{c}	0.80 ± 0.02 ^{d}	0.04
UR (mg/dL)	29.33 ± 0.67 ^{a}	40.00 ± 1.15 ^{b}	54.00 ± 1.00 ^{c}	56.67 ± 1.20 ^{c}	3.35
CR (mg/dL)	0.34 ± 0.03 ^{a}	0.65 ± 0.02 ^{b}	0.89 ± 0.02 ^{c}	0.89 ± 0.02 ^{c}	0.07
SOD (U/L)	10.00 ± 1.01 ^{a}	17.20 ± 0.82 ^{b}	19.17 ± 0.09 ^{bc}	19.97 ± 0.07 ^{c}	2.12
GPx (U/L)	6.87 ± 0.07 ^{a}	11.13 ± 0.82 ^{b}	12.47 ± 0.09 ^{c}	15.23 ± 0.07 ^{d}	1.18
T3 (ng/ml)	0.88 ± 0.01 ^{c}	0.79 ± 0.015 ^{b}	0.76 ± 0.02 ^{b}	0.67 ± 0.01 ^{a}	0.05
T4 (ng/ml)	4.83 ± 0.01 ^{d}	4.57 ± 0.01 ^{c}	4.43 ± 0.05 ^{b}	4.31 ± 0.01 ^{a}	0.07
TSH (ng/ml)	0.21 ± 0.01 ^{c}	0.20 ± 0.01 ^{bc}	0.18 ± 0.01 ^{b}	0.14 ± 0.00 ^{a}	0.03

All data expressed with mean ± standard error of means (SEM), similar small letters means no significant difference between means values on $p<0.05$

red blood cells (RBC) , hemoglobin (Hb) , hematocrit (HCT), white blood cells (WBC) , Lymphocytes , monocytes ,total cholesterol (CHO) ,triglyceride (TG) , high density lipoprotein (HDL) , light density lipoprotein (LDL) , total protein (TP) , aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) , albumin (ALB), bilirubin (BIL) , blood urea (UR) , creatinine (CR) , Super oxide dismutase (SOD), Glutathion peroxidase (GPx) , thyroid hormones (T3) , (T4), and thyroid stimulating hormone (TSH)

Bioaccumulation Study

As their unique properties, nanoparticles have a tendency to aggregate. Furthermore, some metal containing nanoparticles have the ability of forming and releasing ions that easily enter the blood stream and reach the vital organs. The over load of metal ions May leads to serious tissue damage [43].Statically, high significant difference ($p\leq0.05$) were cleared among concentrations means in different organs treated with AgNPs with growing manner associated with increasing dose concentration (Table 4).Liver, the major reticuloendothelial a phagocytic system recorded an important levels of Ag specially in G3 with 3.02±0.12 µg/gm as their highly capturing and retaining capacity [44, 45] by Kupffer cells mainly and secondary by hepatocytes and endothelial cells [46, 47].An important concentrations level of Ag were observed in spleen 4 weeks of injection in all

groups with (1.24±0.03 µg/gm-2.24±0.19 µg/gm).

These values were explained by the massive filtering activity by non specific scavenging properties of the red pulp macrophages and marginal zone to nanoparticle uptake [48].Roughly all nanoparticles types, especially in small dimensions of size have a high probability of clearance through kidneys. Thus, they have a high chance of accumulation and causing some adverse effects [8]. An important means of Ag were observed in kidney in range of (0.98±0.04 µg/gm-1.35 ± 0.04 µg/gm) with pattern depended on dose concentration, these considerable levels may indicate the deposition of un cleared portion of Ag in the basement membrane of renal glomerulus units [49].

Means with range of (0.72±0.07µg/gm-0.99±0.00 µg/gm) of Ag were noted in small intestines which still lower than those were recorded in liver which may indicate the lower excretion ability of intraperitoneal injected AgNPs by intestine in to feces. Regarding on the deposition and translocation of nanoparticles, their ability of crossing the biological barriers of BBB and the BTB was very important [49].However, physicochemical properties of nanoparticles play an important role in passing through these barriers [50], the mechanism were remained unclear [51]. Highest mean value recorded 0.92±0.12 µg/gm and 1.67±0.00 µg/gm in G3 after 4 weeks of repeated injection noted in testes and brain respectively. These results identified the ability of Ag NPs to cross blood- testes barrier BTB and blood brain barrier BBB [52, 53].

Explained by numbers of researchers were related to increase the deposition percentage in the sub epithelial zone of basement membrane [54]. Other studies indicate the role of macrophage in Ag accumulation in alveolar phagolysosomes [55]. In heart, the highest mean levels of Ag , 1.19 ±0.04 µg/gm were observed in G3 of after 4 weeks of repeated injection with high significant difference ($p<0.05$). Whereas non significant concentrations levels of Ag were detected in heart in compared with other studded organs were observed by Tang et al [56].After intravenous injection with 62.8 mg/kg in rat for 2, 4, 8, 12, 18, 24 weeks.

Table 4: Silver concentrations means in different organs after 4 weeks of intraperitoneal injection with different doses of Ag NPs, G1:12.5mg/kg, G2: 25 mg/kg, and G3: 50 mg/kg

Organs	Experimental groups			
	Control	G1 12.5 mg/kg AgNPs	G2 25 mg/kg AgNPs	G3 50 mg/kg AgNPs	LSD
Liver	0.00 ± 0.00 ^{a}	1.56 ± 0.06 ^{b}	2.42 ± 0.19 ^{c}	3.02 ± 0.12 ^{d}	0.38
Spleen	0.00 ± 0.00 ^{a}	1.24 ± 0.03 ^{b}	1.67 ± 0.07 ^{c}	2.24 ± 0.19 ^{d}	0.32
Kidney	0.00 ± 0.00 ^{a}	0.98 ± 0.04 ^{b}	1.21 ± 0.05 ^{c}	1.35 ± 0.04 ^{d}	0.11
small intestine	0.00 ± 0.00 ^{a}	0.72 ± 0.07 ^{b}	0.94 ± 0.06 ^{b}	0.99 ± 0.00 ^{b}	0.15
Testes	0.00 ± 0.00 ^{a}	0.67 ± 0.12 ^{b}	0.76 ± 0.05 ^{b}	0.92 ± 0.12 ^{b}	0.29
Brain	0.00 ± 0.00 ^{a}	0.80 ± 0.00 ^{b}	0.96 ± 0.00 ^{c}	1.16 ± 0.00 ^{d}	0.01
Lung	0.00 ± 0.00 ^{a}	0.71 ± 0.05 ^{b}	1.34 ± 0.01 ^{c}	1.76 ± 0.21 ^{d}	0.36
Heart	0.00 ± 0.00 ^{a}	0.63 ± 0.03 ^{b}	1.02 ± 0.06 ^{c}	1.19 ± 0.04 ^{d}	0.12

All data expressed with mean ± standard error of means (SEM), simillar small letters means no significant difference between means values on $p<0.05$

Histopathological Study

The histopathological effects were clarified in G2. General mild hepatitis which characterized by marked infiltration of mono nuclear leukocytes mainly lymphocytes and monocytes within sinusoids were observed (Figure 3-C).These effects were developed in G3 to generalize coagulative necrosis (Figure 3-D).These results correlated with increasing in levels of ALT, AST in blood which can used as a good indicators for the hepatic alternations on cellular level.

These findings were diagnosed by previous studies of Kermanizadeh et al [57]. The sections of the renal cortex and medulla showed multiple focal hemorrhage in G1 and G2 groups (Figure 3-F, G) with mild vacular degeneration and cast formation in G1and G3 (Figure 3-F, H), and cloudy swelling in G2 and G3 (Figure 3-G and H).

These results indicated by the elevation in urea and creatinine mean levels and increasing the organ index of kidney that

marked possible renal damages which approved by histopathological examination .The multiple hemorrhage and cast forming has not been reported previously as one of histopathological effects of Ag NPs exposure in animal models . present study indicate the adverse inflammatory effects of Ag NPs in spleen which may resulted by silver ions releasing and the accumulation of Ag NPs. Deposition of pinkish a cellular and amorphous material in marginal zone of white pulp, splenic sinusoids and in surrounding connective tissue causing moderate atrophy of white pulp in G2 and amyloid deposition in G3 (Figure 3-K, L) which is the first recorded as one of the adverse effects of Ag NPs in animal model.

These effects indicated early by increasing spleen organ index with means considered high as amyloid deposition indicated with histopathological examination which characterized by inflammatory- inducing deposition of extracellular material of amorphous protein which correlated with serum amyloid A [58, 59, 60, 61].The sections of intestine showed mild enteritis which characterized by thickening of intestinal villi in all groups (G1, G2, and G3) and their lamina propria showed infiltration of lymphocytes with marked epithelial sloughing in G1 (Figure 3-N).

Mild bronchitis characterized by marked peri bronchial thickening associated with infiltrations of mono nuclear lymphocytes were observed in lung , with mild interstitial pneumonia and mild thickening of inter alveolar tissue in G1(Figure 3-R) which progressed to sever interstitial broncho pneumonia in G3 (Figure 3-T) as oxidative stress resulting by Ag NPs distribution to different organs, and it is the first recording of this type of Ag NPs histopathological effects in lung followed intraperitoneal injection which recorded before by Amin et al.,2015 in Albino rat after orally administration of 26.878 and 425.990 mg/kg for 28 days.

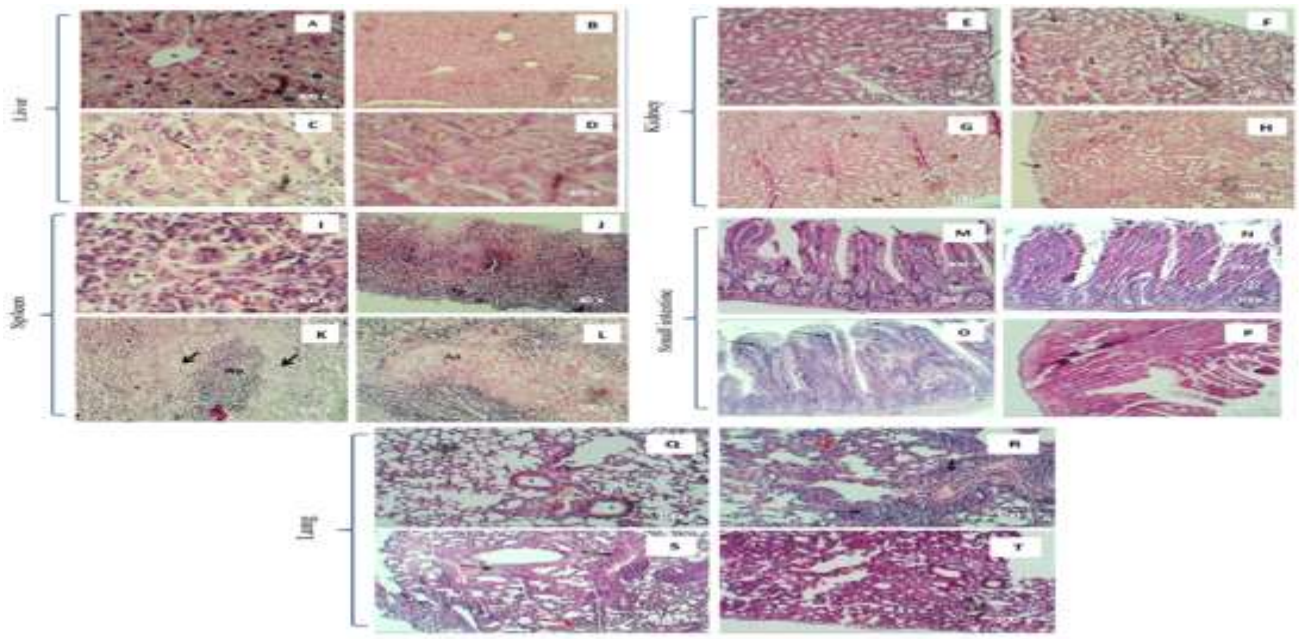

Figure 3 : Histopathological effects in control and G1, G2, and G3 groups after 4 weeks of intraperitoneal injection with 12.5 , 25 , and 50 mg/kg respectively , sections in liver (A-D): control group (A) showes normal structure of liver contain capsule , central vein (Cv) , Hepatocytes cords, and sinusoid , (B) normal appearance of liver G2 , (C) mild hepatitis with infiltrations mono nuclear leukocytes (Black arrows) , (D) marked hepatic coagulative necrosis of hepatocytes in G3 ; sections in kidney (E-H) : control group (E) section in renal cortex shows capsule (arrow), glomerulus and normal renal tubules (Rt) , multiple focal hemorrhage (H) in G1(F) and G2(G) groups , mild vacular degeneration (black row) and cast formation (c) in G1(F)and G3(H), cloudy swelling (Cs) in G2 (G) and G3(H) ; sections in spleen (I-L): control group (I) magnified section shows megakaryocytes (Mg), lymphocytes (black arrow), macrophages (red arrow), blood vessel (Bv) & septum (se), normal appearance of splenic parenchyma in G1(J) , amyloid deposition (arrows) in G2(K) , huge amyloid deposit (Ad) in G3(L) under 100x ; sections in small intestine (M-P): control group (M) shows villi (arrows) , lamina propria (P), epithelium (E), crypt of Lieberkühn (Cl) and tunica muscularis (Tm), for G1(N) epithelial sloughing (Black arrows) and infiltration of mono nuclear leukocytes (lymphocytes) (Red arrows), thickening of villi associated with infiltration of mono nuclear leukocytes (lymphocytes) (arrows) in G2(O), thickening of intestinal wall in G3 (P) ; sections in lung (Q-T): section of lung in control group (Q) shows: bronchioles (B), alveolar sac (As), alveolar duct (Ad), alveolus (A) & blood vessel (Bv) , in G1 (R) peri bronchial and perivascular lymphocytic infiltrations (Black arrows) and mild thickening of interstitial tissue (red arrow) , for G2 (S) moderate peri bronchial and perivascular lymphocytic infiltrations (Black arrows) and moderate thickening of interstitial tissue (red arrow) , for G3 (T) sever interstitial broncho pneumonia , (H&E) stain , 40x,100x, and 400x

Conclusions

An important effect on body weight, organ index, hematological, biochemical parameters were noted combined with severe histopathological effects with necrosis in liver, multiple hemorrhage and cast formation in kidney, moderate atrophy and amyloid deposition in spleen, thickening of intestinal villi of small intestine, and sever interstitial broncho pneumonia in lung. Liver, spleen, lung, and kidney respectively are the major organs were silver accumulated after repeatedly injection with the three studied doses.

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