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Selenium nanoparticles effects of in reducing hepatotoxicity brought on by cisplatin

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ABSTRACT

Objective: The objective of this study is to investigate the protective role of Selenium Nanoparticles (SeNPs) on hepatotoxicity induced by cisplatin. This aim can be achieved by assessment of oxidative stress and antioxidants markers in liver homogenate [malondialdehyde (MDA), Superoxide dismutase (SOD), Glutathione (GSH), Glutathione peroxidase (GSH~PX) and Catalase (CAT)], serum liver function [(Alanine amino transferase (ALT), Aspartate amino transferase (AST), Total Protein (TP) and Albumin(Alb)], Complete blood count (CBC) and Histopathological examination of liver tissues. ***Material & Methods:*** Thirty adult male rats weighing 250±20g divided equally and randomly into four groups: Group I (negative control group), Group II (Cisplatin group), Group III (Selenium Nanoparticles group) and Group IV (Selenium Nanoparticles + Cisplatin). The rats of Group II were be injected intraperitoneally as a single dose by cisplatin (10mg/kg), meanwhile selenium nanoparticles (2mg/kg/day) were administrated alone (Group III) or in combination with cisplatin (Group IV). ***Results:*** Cisplatin treatment caused a significant decrease in antioxidant enzymes activities (SOD, GSH, GSH~PX and CAT), total protein and albumin levels and count of red blood cells, white blood cells and platelets while it caused elevation in MDA levels, ALT and AST activities. These biochemical parameters were confirmed by histopathological study as selenium nanoparticles improved the hepatotoxicity induced by cisplatin. ***Conclusion:*** Selenium Nanoparticles reduced cisplatin-induced hepatotoxicity, improved liver function and protected rats from liver injury.

I. Introduction

Cisplatin is the most effective chemotherapy used universally as cytotoxic agent for the treatment of many types of cancer including sarcomas, testicular, ovarian, breast, lung, bladder and lymphomas but it causes many side effects as hepatotoxicity [1]. The mechanism of action of cisplatin as anticancer agent is that it

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targets DNA of the cancer cells and interacts with its purine bases to prevent its replication and stop biological functions; so prevent the malignant cells proliferation [2] by impairing the normal repair mechanisms of its DNA which causes cell-cycle arrest and death [3].

Unfortunately, cisplatin induces oxidative stress by releasing reactive oxygen species that attack liver, also cisplatin increases cytochrome P450 level causing liver damage [4]. Selenium compounds have been found to attenuate cisplatin-induced toxicity in both target and non-target organs without affecting its antitumor activity [5].

Selenium nanoparticles have been produced to induce selenoproteins effectively with lower toxicity and acceptable bioavailability that confirmed by all of the comparative toxicity and efficacy studies [6].

The aim of this study is to investigate the protective role of Selenium Nanoparticles on hepatotoxicity induced by cisplatin in male rats.

II. Materials and Methods

II.1. Material:

II.1.1. Chemicals:

Cisplatin (diamminedichloridoplatinum (II))**:** Chemical formula: (cis-(Pt Cl_2 $(NH_3)_2$)), it was manufactured by Hikma Specialized Pharmaceuticals Company (Badr city, Cairo, A.R.E) in the form of Unistin Vial 50ml/50mg.

Selenium Nanoparticles**:** All chemicals for preparation were obtained from Sigma-Aldrich and used without further purification.

Biochemical Kits: Sandwich ELISA assay kits of rat glutathione peroxidase (GSH-PX), rat glutathione reduced (GSH), Malondialdehyde (MDA), Super Oxide Dismutase (SOD) and Catalase (CAT) were purchased from (Biodiagnostic, Dokki, Giza, Egypt).

Animals**:** Thirty adult male Swiss albino rats weighing 250±20g were purchased from Faculty of Veterinary Medicine, Zagazig University, Egypt. They were housed in cages free from any source of crucial contamination under controlled laboratory conditions with an ambient temperature degree 25±2ºC and the light/dark cycle was 12 hours at the animal house Faculty of Science, Zagazig University.

II.1.2. Ethical approve:

The experimental animals have been handled under the standards and guidelines of the Zagazig University Center Ethics Committee published by (ZU-IACUC/1/F/34/2023).

II.1.3. Experimental design:

After two weeks acclimatization period on a normal diet and *add libitum*, rats were weighed again divided equally and randomly into four groups each one contained six rats and each rat weighed 250 ±20 g as following:

Group I (negative control group): received a single dose intraperitoneally injection of normal sterile saline solution (0.9% NaCl).

Group II (**C**isplatin group): received a single dose intraperitoneally injection of Cisplatin (10mg/kg) [7].

Group III (Selenium Nanoparticles group): received intraperitoneally injection of Selenium nanoparticles (2 mg/kg/day) for 10 sequential days [8].

Group IV (Selenium Nanoparticles + Cisplatin): received intraperitoneally injection of Selenium nanoparticles (2 mg/kg/day) for 10 sequential days [8], then received a single dose intraperitoneally injection of Cisplatin (10 mg/kg) [7].

II.1.4. Sampling:

At the end of the study all animals were fasted over night; the blood and tissue samples were collected as following:

*II.1.4.1. Blood sample collection***:**

Venous blood samples were collected from the retro-orbital plexus of the animals by capillary glass tubes using light ether anesthesia according to procedure described

by Joslin [9]. The blood collected into a clean tube containing Ethylene diamine tetra acetic acid (EDTA) to prepare plasma (stored at -20ºC) for Complete Blood Count (CBC) analysis, and into a clean centrifuge tube to prepare serum by incubation at 37ºC and centrifugation for 15 minutes at 3000 r.p.m for estimating biochemical tests.

***II.1.4.2. Liver tissue samples collection*:**

Liver tissue were excised from each rat and divided into 2 parts*:*

(a) Frist Part one was collected and stored at -20ºC for preparing the homogenate:

Preparation of liver homogenate:

0.1 g tissue + 1 ml phosphate (KH_2PO_4) buffer solution (pH7) were homogenized for 5 minutes using a glass porcelain homogenizer and then centrifuge at 7000 x g for 15 minutes, all processes are carried out at 4ºC. [10] and used for estimating the following parameters (MDA, SOD, GSH, GSH~PX and CAT).

(b) Second part was collected from rats and preserved in 10% neutral buffered formalin, processed and stained with haematoxylin and eosin (H&E) dyes for histopathological studies using a light microscope.

II.2. Methods:

II.2.1. Synthesis of Selenium nanoparticles:

The wet chemical reduction method was modified to synthesis SeNPs. In this method, 5 mg of Polyvinylpyrrolidone (PVP) and 2.5mL of 0.2M of cetyltrimethylammonium ammonium chloride (CTAC) solution was added to a solution of 0.07 M ascorbic acid (40mL) under stirring. Then, after 5 min of stirring, 1mL of 0.25M of sodium selenite was added drop-wise till the solution colour changed from colourless into orange colour [11].

II.2.2. Biochemical evaluation:

II.2.2.1. Oxidative stress and antioxidants markers: *Malondialdehyde (MDA)* as a product of lipid peroxidation was estimated according to Satoh method [12]. Superoxide dismutase (SOD) activity, Glutathione (GSH) reduced levels, Glutathione peroxidase (GSH~PX) and Catalase activities were measured according to methods described by Nishikimi et al., [13], Beutler et al., [14], Paglia and Valentine [15], and Aebi [16]; respectively.

*II.2.2.2. Assessment of Liver functions***:**

Alanine aminotransferase (ALT), and Aspartate aminotransferase (AST) activities were be estimated according to Schumann and Klauke method [17], and Karmen et al. method [18]; respectively. Additionally, Total protein (TP) and albumin (ALB) levels were carried in sera samples according to Gornall method [19], and Doumas et al. method [20]; respectively.

II. 2.2.3. CBC analysis:

CBC was Performed by Automatic **CBC analyzer (Sesmex Kx-21)** for all EDTA blood samples.

II.2.3. Histological examination for liver:

A few liver tissues were washed, neutral buffered formalin 10% dehydrated in increasing amounts of alcohol, and then fixed in paraffin. The sections were then cut at a thickness of 5 m, stained with H & E, and examined under a microscope for histological analysis in line with the Bancroft et al. protocol [21].

II.2.3.1. The semi-quantitative score system of H&E-stained liver sections

The microscope slides were seen with a light microscope (Olympus Soft Imaging Solutions GmbH, Munster, Germany). The Shackelford et al. (2002) [22] scale states that semi-quantitative analysis (counts and measures) can be performed manually or digitally using image analysis and stereological techniques to provide numerical values. The severity of lesions in the tissues is described as grade 0. Grade1: Minimal modification, Grade2: Slight (equivalent to mild), Grade3: Moderate, Grade4: Marked (equivalent to severe), and Grade5: Massive (equivalent to extremely severe). Normal structure (unharmed).

***II.4. Statistical Analysis*:**

Data was statistically analyzed with the SPSS software (SPSS Inc.) version 20.0. Variables were expressed as mean ± standard deviation (SD) or as median and interquartile range (1QR) for continuous non-normally distributed variables. A two-sided P value < 0.05 was an indication of to be statistical significance. The correlations among markers were analysed using bivariate test.

III. Results

III.1. Selenium nanoparticle synthesis

SeNPs have a spherical shape and an average size of 24.9 2.022 nm as seen in the TEM image. Selenium nanoparticles shows good stability with zeta potential 32.4 mv, as reported by Keshta et al., [23].

III.2. Oxidative stress and antioxidants markers:

Fig 1. showed the statistical difference of oxidant and antioxidant enzymes in liver homogenate in all groups. The mean value of MDA levels recorded (741.8±27.1 (nmol/g tissue) in GII that increased compared to their corresponding values (622.3±17.1 (nmol/g tissue) in GI. The mean levels of MDA reduced compared to their corresponding values (522±50.6 (nmol/g tissue) and 675.2±15.1 (nmol/g tissue) in GIII and GIV respectively (P< 0.0001).

The mean activities of SOD, GSH~PX, CAT and GSH levels, recorded (1466.6±96 (U/g tissue), 69.1±3.2 (U/g tissue), 170.9±45.9 (U/g tissue) and 19.9±5.2 (mg/g tissue) in GII respectively, these activities decreased compared to their corresponding values to (2405.5±198.8 (U/g tissue), 81.9±1.2 (U/g tissue), 275.6±7.3 (U/g tissue) and 66.7±5.4 (mg/g tissue) in GI respectively. These values increased to be (3216.3±67.2 (U/g tissue), 87.4±2.8 (U/g tissue), 293.2±7.3 (U/g tissue) and 101±19 (mg/g tissue) in GIII respectively, and (2021.6±102.7 (U/g tissue), 75.9±1.8 (U/g tissue), 246±18.1 (U/g tissue) and 46.9±8.1 (mg/g tissue) in GIV; respectively compared to their corresponding values in GII.

III.3. Estimation of liver function tests:

Table (1) illustrated the liver functions (ALT, AST, TP and Alb) in all the studied groups. ALT and AST activities in G2 (161.5±24.5 (U/L) and 403.5±59.1 (U/L)); respectively increased compared to their corresponding values in G1 (45.2±2.5 (U/L) and 245.4±9.5 (U/L)); respectively and decreased in G3 (33.2±1.3 (U/L)and 213.5±8.9 (U/L)); respectively and G4 (119.9±7.6 (U/L) and 285.4±15.4 (U/L)); respectively compared to their corresponding values in G2 (161.5±24.5 (U/L) and 403.5±59.1 (U/L)); respectively (P< 0.0001), while mean values of TP and Alb levels in G2 (6.9±0.32 (g/dl) and 3±0.11 (g/dl)); respectively decreased compared to their corresponding values in G1 (8.3±0.14 (g/dl) and 3.8±0.12 (g/dl)); respectively and increased in G3 (8.3±0.28 (g/dl) and 4.2±0.14 (g/dl)); respectively and G4 (8±0.4 (g/dl) and 3.8±0.21 (g/dl)); respectively compared to their corresponding values in G2 (P< 0.0001).

***III.3. Complete blood count (CBC) parameters*:**

Table 2 illustrated CBC parameters in different groups. The mean value of Hb in G2 (16.5±0.31 (g/dL)) increased compared to its corresponding value in G1 (13.4±0.36 (g/dL)), and decreased in G3 (13±0.14 (g/dL)) and G4 (14.6±0.25 (g/dL)) compared to their corresponding values in G2 (P<0.0001).

Meanwhile the mean values of RBC, WBC and Platelets in G2 (6.6±0.14 (M/µL), 2.8±0.18 (K/µL) and 491.8±2.3 (K/µL)); respectively decreased compared to their corresponding values in G1 (8.1±0.1 M/µL, 6.4±0.15 K/µL and 529.8±3.3 K/µL); respectively and increased in G3 (8.5±0.33 M/µL, 9.6±0.3 K/µL and 987±8.0 K/µL); respectively and G4 (7.5±0.26 M/µL, 7.0±0.25 K/µL and 790.8±45.8 K/µL); respectively compared to their corresponding values in G2 (6.6±0.14 M/µL, 2.8±0.18 K/µL and 491.8±2.3 K/µL); respectively (P<0.0001).

Meanwhile the mean value of HCT in G2 (49.2±0.28 %) was non-significantly decreased

compared to its corresponding value in G1 (50.0±0.14 %) (P>0.05), and decreased in G3 and G4 (45.7±0.88 % and 38.6±1.5 %); respectively compared G2 (P<0.0001). The mean value of MCV in G2 (55.7±0.4 (fL)) decreased compared to G1 (61.1±0.57 (fL)) (P<0.0001), and non-significantly decreased in G3 and G4 (55.3±0.81 (fL) and 54.9±0.55 (fL)); respectively compared to G2 (P>0.05). Furthermore, the study showed that the mean value of MCH in G2 (18.8±0.21 (pg)) was non-significantly increased compared to G1 (18.1±0.31 (pg)) (P>0.05), and were non-significantly decreased in G3 and G4 (18.1±0.31 (pg) and 18.7±0.93 (pg)) respectively, compared to G2 (P>0.05). The mean value of MCHC in G2 (33.5±0.1 (g/dL)) was non-significantly increased compared to G1 (33.5±0.14 (g/dL)) (P>0.05), and significantly decreased in G3 (32.3±0.67 (g/dL)) compared to its corresponding value in G2 (P=0.001), and was non-significantly increased in G4 (34.3±1.24 (g/dL)) compared to its corresponding value in G2 (P>0.05).

***III.4. Histopathology of liver sections of different groups*:**

Fig 2 A&B showed liver sections of G1 and G3 respectively, with normal hepatic parenchyma and normal central vein, hepatocytes, blood sinusoids, and portal tract. While liver section of G2 showed dilatation and congestion of hepatoportal blood vessel, hyperplasia in the bile duct and fibrous connective tissue proliferation in the portal tract (Fig 2 C). Liver section of G4 showed dilatation and congestion of hepatoportal blood vessel and hyperplasia in the bile duct and newly formed bile ductules (Fig 2 D).

III.4.1. The semi-quantitative score system of H&E-stained liver sections

The results of liver sections stained with H&E are displayed in table 3 in a semi-quantitative manner. The collagen fibres deposition, Bile duct proliferation, and blood vessels were massive marked as grade 5 in Cis group (GII) compared to GI" negative control group" while, it was recorded grade 0 and grade 1 in SeNPs group (GIII). Additionally, SeNPs+ Cis group (IV) recorded grade moderate and mild soring compared to Cis group. This affirmed SeNPs' ability to protect the liver from the hepatotoxicity that Cisplatin induces.

IV. Discussion

Cisplatin enters the cell via passive transport and reaching higher concentrations in the liver and undergoes hepatic metabolism by cytochrome P450 enzyme complex, due to cumulative effect of cisplatin in the liver, hepatotoxicity takes place even if the patients received it in low doses [2]. Selenium compounds have been found to attenuate cisplatin-induced toxicity in both target and non-target organs without affecting its antitumor activity [5]. The current work aimed to study the protective role of Selenium Nanoparticles on hepatotoxicity induced by cisplatin. The hepatotoxicity induced by cisplatin caused due to releasing of free radicals that caused a devastating effect on hepatocytes, In agreement with the experimental results of Korkmaz et al. the cellular toxicity occurred either through lipid and protein peroxidation or through DNA damage caused by a highly toxic and pro-apoptotic nitrosating agent called peroxynitrite (ONOO) which interacts covalently with most types of biomolecules leading to risk of oxidative/nitrosative stress even at low concentration [24], peroxynitrite promotes generation of hydroxyl (OH.) and nitrogen dioxide (NO2.) radicals as intracellular oxidant that inhibits SOD as well as other antioxidants [25].

The excess production of superoxide anion which detoxified by SOD leads to increase in H_2O_2 which in turn leads to suppressing CAT and GSH-px activities in coverting of H_2O_2 into H_2O this explains the decrease in SOD, GSH-px and CAT. Meanwhile the significant increase in MDA levels refers to the increase in lipid peroxidation occurred by free radicals generated by cisplatin [8]. In agreement with the experimental results of Wang et al. the significant increase in ALT and AST and the significant decrease in total protein and albumin occurred due to liver damage effect of cisplatin that caused as a result of the attack of free radicals released by cisplatin to the hepatocytes that led to increase in liver enzymes levels

released from the damaged hepatocytes and decrease in its production for functional proteins [26].

The increase in Hb% recorded in this study might be as a result of RBCs destruction that released Hb and the evidence was deficiency in RBCs count in cisplatin group compared to negative control group this is may be due to the attack of free radicals released by cisplatin to the RBCs that caused damaging in RBCs and releasing Hb contents. Selenium nanoparticles administration prevented this damage by trapping these free radicals, but these results conflicted with Nasr who stated that Hb% in the group received cisplatin declined compared to negative control group and did not illustrate the cause for this [27].

Regarding to RBC, the effect of cisplatin on it causing decline in its count might be due to destruction of bone marrow cells or increase osmotic fragility of RBCs so cisplatin might lead to anemia as a result of either impaired erythropoiesis or suppression the activity of hematopoietic tissue or accelerated RBCs breakdown because of increased RBCs mechanical fragility, altered RBCs membrane permeability and/or defective iron metabolism which confirmed by [27]; [28].

WBCs count decreased due to destruction of bone marrow cells which confirmed by Ohno et al. stated that 50% from patients received low dose of cisplatin-based chemo radiation therapy and 58% from patients received high dose recorded leukopenia grade 3 versus only 7% received radiation therapy alone [29]; but the results were not compatible with (Nasr, 2014) stated that administration of cisplatin increased WBCs count because of infection and inflammation caused by cisplatin treatment and its metabolism [27].

The effect of cisplatin on platelets caused a decline in its count was compatible with (Nasr, 2014 ; Sirag, 2009) and may be due to either through decreasing the production of platelets by inhibiting the activity of bone marrow or increasing its consumption or aggregation [27]; [30].

The histological examination of liver preparations in the current study were compatible with many previous reports that showed the normal architecture of liver in cisplatin group revealed dilatation and congestion of hepatoportal blood vessel, hyperplasia in the bile duct and fibrous connective tissue proliferation in the portal tract this was mainly formed by transaminases and bilirubin elevation in circulation [31], due to oxidative stress [32] that caused liver damage as a result of enhancing cytochrome P450 level [4], and cytochrome-P450-2E1 enzyme (a member of cytochrome P450) by cisplatin therapy [33].

Selenium is an essential micronutrient trace element has a potent detoxifying effect against heavy metals by acting as a cofactor of the anti-oxidant system which protects macromolecules and lipids in the cellular membranes against free radicals. The rats treated with selenium showed restoration of the hepatic cellular organization along with attenuation of the vascular congestion, histopathological changes, inflammation, and collagen deposition. The ameliorating effect of selenium on hepatic fibrosis was attributed to its effect on decreasing the stellate cell activity and their collagen degradative properties [34].

V. Conclusion

Exposure to cisplatin results in histological and functional changes in the liver. This hepatotoxicity may have been brought on by oxidative stress damage brought on by reactive oxygen species. The current study's findings support the value of delivering selenium nanoparticles as a defence against the toxicity caused by cisplatin by playing a crucial function in securing the free radicals generated.

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Table 1: Statistical comparison of Liver function parameters (alanine amino transferase (ALT), aspartate amino transferase (AST), total protein and albumin) in different studied groups of adult male rats:

Groups	ALT (U/L)	AST (U/L)	TP (g/dl)	Alb (g/dl)
Negative control (GII)	45.2±2.5	245.4±9.5	8.3±0.14	3.8±0.12
Positive control Cisplatin (GII)	161.5±24.5	403.5±59.1	6.9±0.32	3.0±0.11
Selenium Nanoparticles (GIII)	33.2±1.3	213.5±8.9	8.3±0.28	4.2±0.14
Selenium Nanoparticles + Cisplatin (GIV)	119.9±7.6	285.4±15.4	8.0±0.40	3.8±0.21
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
G2 vs others	a,b,c	a,b,c	a,b,c	a,b,c

a Represents comparing G2 group with G1 group, **b** Represents comparing G2 group with G3 group, **c** Represents comparing G2 group with G4 group. Selenium nanoparticles (SNP); Cisplatin (Cis), (P value < 0.0001) represents comparing G2 group with others for all parameters were extremely significant.

Table 2: Statistical comparison of complete blood count (CBC) parameters (Hemoglobin (Hb), Red blood cell (RBC), White blood cell (WBC), Platelet, Hematocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH) and Mean corpuscular hemoglobin (MCH) and Mean corpuscular hemoglobin concentration (MCHC)):

Groups	Hb (g/dL)	RBC (M/µL)	WBC (K/µL)	Platelet (k/µL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)
Negative control (G1)	13.4±0.36	8.1±0.10	6.4±0.15	529.8±3.3	50.0±0.14	61.1±0.57	18.1±0.31	33.5±0.14
Positive control Cisplatin (G2)	16.5±0.31	6.6±0.14	2.8±0.18	491.8±2.3	49.2±0.28	55.7±0.40	18.8±0.21	33.5±0.1
Selenium Nanoparticles (G3)	13.0±0.14	8.5±0.33	9.6±0.30	987.0±8.0	45.7±0.88	55.3±0.81	18.1±0.31	32.3±0.67
Selenium Nanoparticles + Cisplatin (G4)	14.6±0.25	7.5±0.26	7.0±0.25	790.8±45.8	38.6±1.5	54.9±0.55	18.7±0.93	34.3±1.24
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.43	0.001
G2 vs others	a,b,c	a,b,c	a,b,c	a,b,c	NS,b,c	a,ns,ns	Ns,ns,ns	Ns,b,ns

a Represents comparing G2 group with G1 group, **b** Represents comparing G2 group with G3 group, **c** Represents comparing G2 group with G4 group. Selenium nanoparticles (SNP); Cisplatin (Cis), (P value < 0.0001) represents comparing G2 group with others for some parameters were extremely significant; (P value = 0.43) represents comparing G2 group with others for MCV were non-significant (**ns**); (P value = 0.001) represents comparing G2 group with G3 for MCHC were very highly significant.

Table (3): The semi-quantitative score system of H&E-stained liver sections

	Negative control	CIS	SNP	SNP+CIS
Collagen fibers deposition	0(normal)	5(severe change)	1 Minimal	2 (mild)
Bile duct proliferation	0(normal)	5(severe change)	1 Minimal	3 Moderate
Blood vessels	0(normal)	5(severe change)	1 Minimal	3 Moderate

Grade1: Minimal change, Grade2: Slight (same as mild), Grade3: Moderate Grade4: Marked (same as severe), Grade5: Massive (same as very severe)

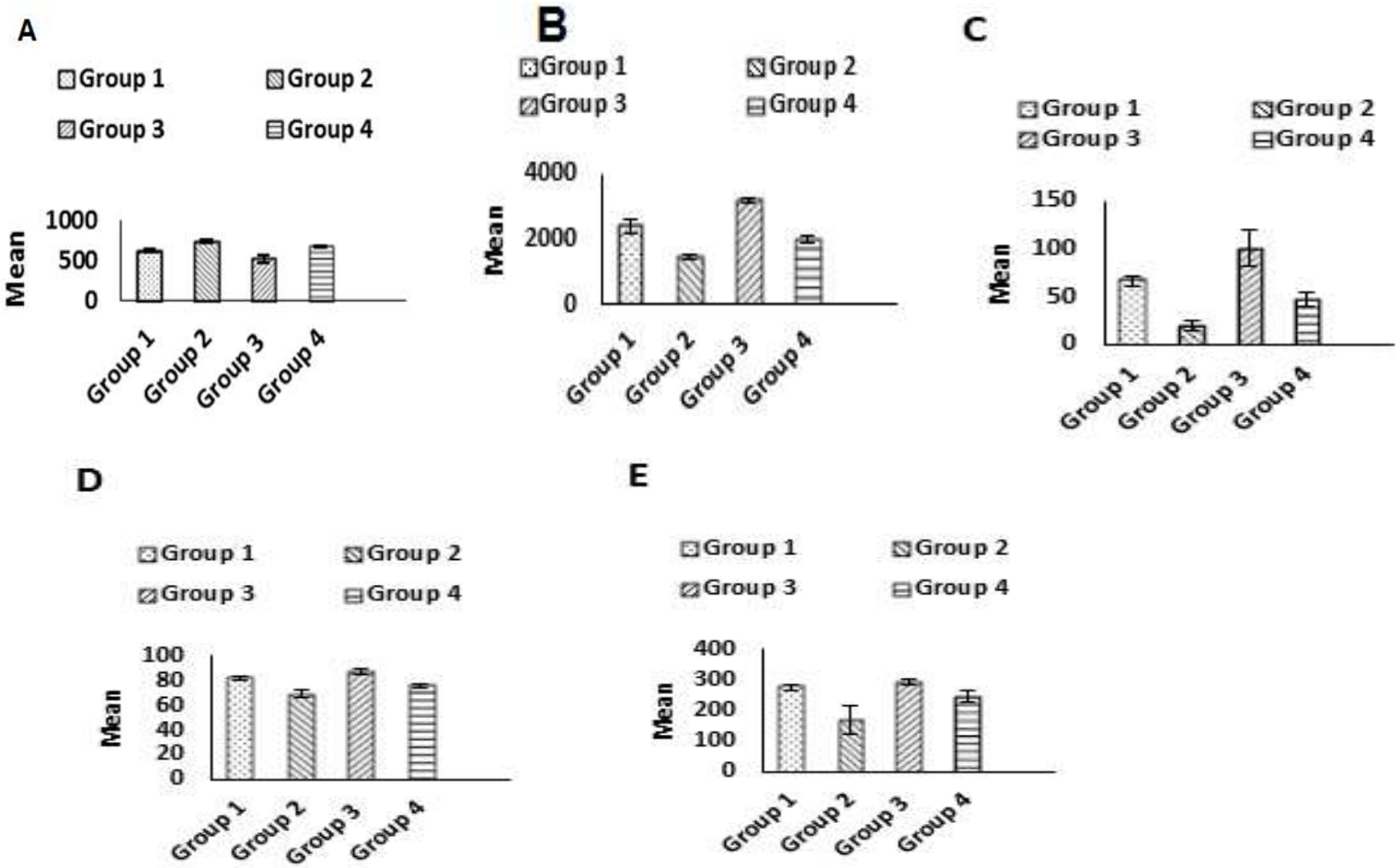

Figure 1: Statistical comparison of Oxidative stress markersand antioxidants in testis homogenate of different studied adult male rats groups. **A**: malonaldhyde (MDA), **B**: (superoxide dismutase (SOD), **C**: glutathione (GSH), **D**: glutathione peroxidase (GSH~PX) and **E**: catalase (CAT).

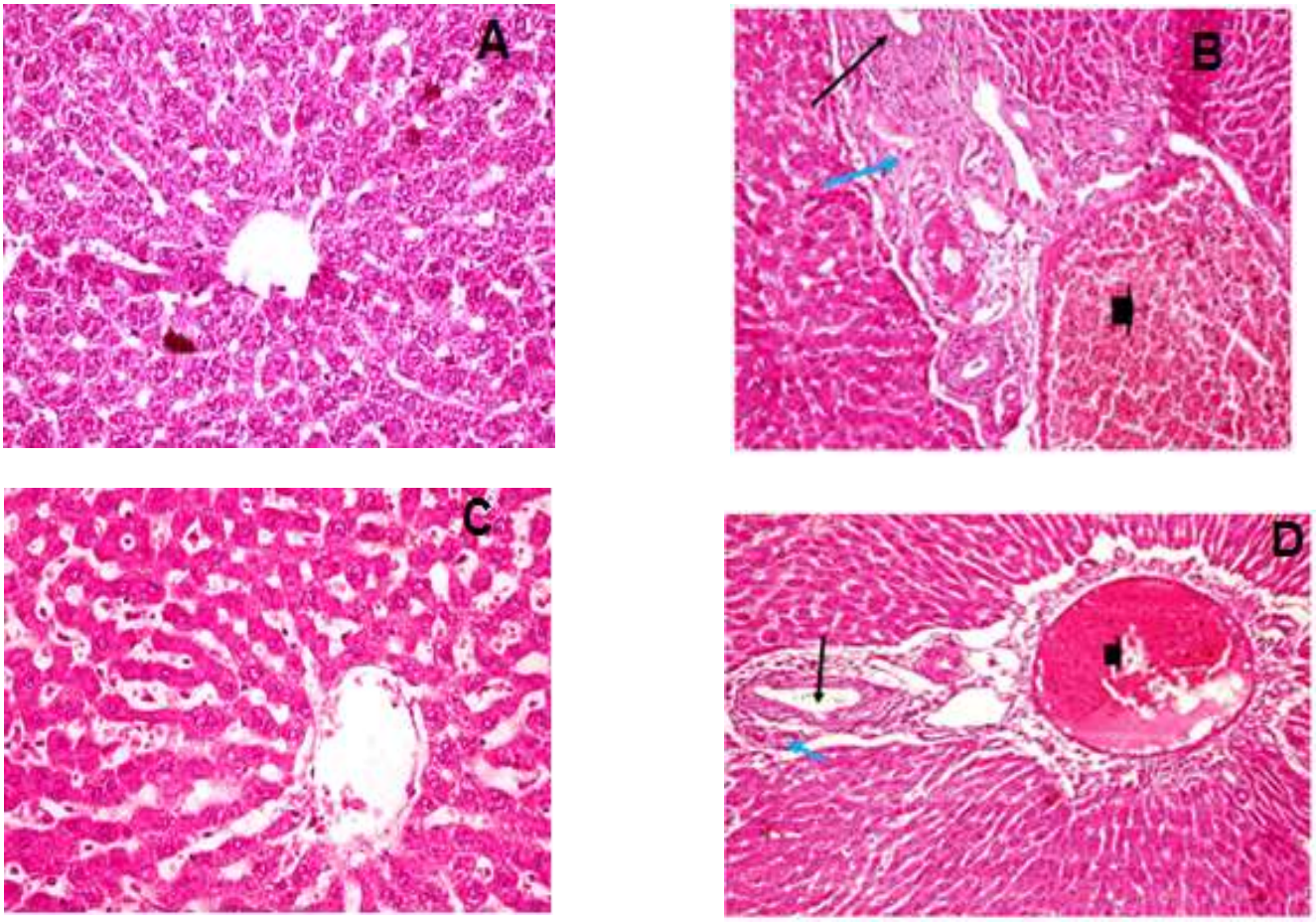

Figure 2: Histological changes of teists sections of different studied adult male rats groups.(A) **(Negative Control G1)**: liver showing normal hepatic parenchyma with normal central vein, hepatocytes, blood sinusoids, and portal tract, (H&E X 400). **(B) Positive control Cis (G2):** liver showing dilatation and congestion of hepatoportal blood vessel (arrow head), hyperplasia in the bile duct (black arrow) and fibrous connective tissue proliferation in the portal tract (blue arrow), (H&E X 400). **(C) SeNPs (G3):** liver showing normal hepatic parenchyma with normal central vein, hepatocytes, blood sinusoids, and portal tract, (H&E X 400). **(D) SeNPs+Cis (G4):** liver showing dilatation and congestion of hepatoportal blood vessel (arrow head) and hyperplasia in the bile duct (black arrow) and newly formed bile ductules (blue arrow), (H&E X 400).