



In vivo assessment of toxicological potential of graphene oxide nanosheets in Sprague Dawley rats

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Abstract

Graphene oxide nanosheets (GON) have encouraging applications in the field of biology, particularly in drugs delivery and therapeutics due to its distinctive properties. Despite of its greater applications *in vivo* studies but data of toxicity *in vivo* is scares. Therefore, a study was designed to assess the *in vivo* toxic potential of GON in Sprague Dawley rats by involving 25 rats distributed into 5 groups having 5 replicates. The groups were named as control (without any treatment), placebo (receiving deionized water intraperitoneally) and three treated groups (G₁-G₃) exposed with GON intraperitoneally @ 1.5 or 2.5 or 3.5mg/kg of bodyweight on alternate day for 30 days. After 30 days of exposure toxicity induced by GON was assessed. No death or change in body morphology and behavior were observed during the whole experiment. In present study, exposure of GON @ 2.5 and 3.5mg/kg BW induced toxicity, which was evident by the alteration in somatic index of liver, liver function enzymes concentrations (ALT, AST and ALP), markers of oxidative stress (MDA and LPO), enzymes of antioxidative system (CAT and GSH) and histopathology of liver of treated groups compared with control. Normal histology was observed in control and deionized treated rats while treated rats with GON showed dilation in central vein, pyknotic nuclei and degeneration of hepatocytes in a dose dependent manner. Therefore, much attention is required for the investigation of dose dependent toxicity of GON so that strictly monitored dose could be used *in vivo* applications.

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Introduction

The nanosized materials have attained great attention due to its unique properties like small size, variety in shape, good physicochemical and electrical properties, and bioapplications like delivery of drugs, proteins, nucleic acid, specific antibodies and in fighting against diseases (Bahader *et al.*, 2016; Priyadarsini *et al.*, 2019). The biological use of nanostructured materials in therapy and diagnosing of different diseases is mainly based on the large capacity for carrying drugs, emission of light and Raman properties than other nanoparticles (Park *et al.*, 2009). The fate of nanomaterials in *in vivo* models is influenced by various factors such as exposure route, chemistry of nanomaterials, physiology of the environment, shape, size, dose, purity, stability, bioconjugation and duration of exposure (Almeida *et al.*, 2011; Kenry and Lim, 2016).

Carbon nanomaterials have recently been extensively researched in biomedical applications owing to their immense properties like physical, chemical, electrical and mechanical properties due to different structures of carbon (Chong *et al.*, 2014). Carbon nanomaterials have wide applications in scientific community, but particularly in the delivery of drugs and capability of diagnosis by graphene and nanotubes have attained great attention recently (Kostarelos *et al.*, 2009; Liu *et al.*, 2009; Yang *et al.*, 2010; Shen *et al.*, 2012; Chung *et al.*, 2013; Mendes *et al.*, 2013).

Basically, graphene is derived from graphite having thick layer of single carbon sheets with two dimensions, sp^2 hybridized configuration and hexagonally arranged carbon atoms that make it honey comb like appearance. It provides building blocks for the formation of other molecules of graphite such as larger fullerenes and carbon nanotubes (Geim and Novoselov, 2007). Graphene has many derivatives such as graphene oxide, graphene oxide nanosheets and reduced graphene oxide, nanoribbons, quantum dots, aerogels, nanopores, three dimensional foams. Graphene has become a super star among other carbon nanoparticles in the field of nanotechnology by

having high surface area to volume, great electrical and thermal conductivity, and mechanically tough material (Geim and Novoselov, 2007; Li and Gilja, 2008). Due to maximum performance and optimizing characteristics, graphene and graphene based materials are produced on large scales and applied in various fields like formation of solar cells, sensing of biomolecules, diagnosing of different diseases and against bacteria, and viruses (Yan *et al.*, 2011; Akhavan *et al.*, 2012; Kostarelos and Novoselov, 2014; Wu *et al.*, 2014; Kumar *et al.*, 2016; Xu *et al.*, 2017). Graphene has played revolutionary role in nanoelectronics products and their composites, targeted drug and gene delivery, imaging of tumors in cells, tissue engineering and cancer photo thermal therapy (Dikin *et al.*, 2007; Zhang *et al.*, 2010; Li *et al.*, 2012; Yang *et al.*, 2012; Singh *et al.*, 2014). Graphene nanomaterials has ability to cross barrier between blood and brain therefore, used in carrying the anticancer drugs (Wan *et al.*, 2017). Graphene oxide nanosheets is a derivative of graphene, containing heavy groups that make them highly soluble in aqueous environment which increases their application in biomedical research and transport of the targeted drugs (Guo and Mei, 2014). Good heat conductivity, excellent strength, charge mobility and highly fixed surface area have made graphene based materials vast use in sensors formation (Allen *et al.*, 2009; Hadi and Mollaei, 2018; Ozdemir, 2018). Derivatives of graphene like quantum dots widely applied in imaging of diagnosis and in photothermal therapy as compared to conservative quantum dots, therapy of strokes by 3D graphene foam and its conjugates in medicine regeneration, sequencing of DNA and treatment of water by applying nanopores of graphene (Merchant *et al.*, 2010; Schneider *et al.*, 2010; Cohen and Grossman, 2012; Schneider and Dekker, 2012; Li *et al.*, 2013; Surwade *et al.*, 2015; Tabish *et al.*, 2018).

Graphene is versatile in functions due the presence of carbon, carbocyclic and hydroxyl group on the surface. The sheets of graphene have negative charge on dispersion in water, therefore, form a stable suspension in water (Park and Roof, 2009). The

presence of OH group on the surface of graphene gives hydrophilic nature and therefore, graphene is a precursor for the synthesis of various nanocomposites (Zhu *et al.*, 2010). Graphene has many reaction sites on surface due to presence of functional groups for peptides, many proteins, nucleic acids, metals and other nano-materials by covalent and non-covalent bondings, therefore, widely used in electrochemical biosensors, and conjugates of proteins with graphene also provide a platform for the synthesis of nanoparticles with unique properties that make these conjugates more biocompatible and biodegradable (Zhang *et al.*, 2013; Liu *et al.*, 2013). Recently, much attention is given in nanotechnology for the development of nanosized graphene sheets that solve the problem of entry in cells and crossing barriers like blood brain barrier to enhance therapeutic treatments. Nanoparticles with diameter 100nm can easily enter the cells, less than 40nm can reach the nuclei, while smaller than 35nm can reach the brain by crossing the blood brain barrier for therapeutic purposes (Augustine *et al.*, 2017; Habiba *et al.*, 2015; Guo *et al.*, 2017).

Graphene oxide has vast applications in biology and medicines and released into the environment through various sources and has become a potential risk for both environment and living things. Now recently, public concern to biosafety has become a major subject regarding the issues related to wide use and large production of graphene and graphene based nanomaterials (Patlola *et al.*, 2016; Zhang *et al.*, 2017; Pecoraro *et al.*, 2018). Previous studies in *in vitro* and in *in vivo* experiments have presented the picture of toxicity caused by graphene and its derivatives that mainly depends on size, morphology, dose and time of exposure. The mechanism involved in toxicity of graphene is by producing reactive oxygen species and its hydrophobic surface by interacting with biological tissues and lipid contents of cell membranes (Nel *et al.*, 2006; Chen *et al.*, 2012; Lammel *et al.*, 2013; Nikodinovska *et al.*, 2015). It is found from previous studies conducted on graphene biosafety, graphene nanoparticles enter blood and transport to other organs either injected intravenously, intraperitoneally

or through feeding, where they accumulate in liver, kidney, spleen, lungs and induce inflammation (Akhavan *et al.*, 2016; Lee *et al.*, 2011; Seabra *et al.*, 2014; Patlola *et al.*, 2016; Kovbasyuk and Mukhir, 2016). Cytotoxicity caused by carbon based nanomaterials including apoptosis, inflammation and mortality of living cells is also confirmed from the recent research (Sasidharan *et al.*, 2012; Singh *et al.*, 2012; Chowdhury *et al.*, 2013).

A toxicity of pure graphene and their derivatives has been described by many researchers in their recent research articles both in *in vivo* and *in vitro* biosystems but a debate is still present on the issue of biocompatibility of graphene and graphene based materials. There is inconsistency in the studies of toxicity of graphene due to diversity in single layer graphene, a few layer graphene, functionalized and nanocomposites (Wick *et al.*, 2014). The use of graphene and their derivatives is increasing in many fields especially in biomedical and in industry, therefore investigations on toxicity of graphene also has importance both in *in vitro* and *in vivo* conditions. The current study is planned to share the deep knowledge about the *in vivo* toxicity in model animal rat and the findings obtained from experiment will be helpful to assess the health risks linked to the application of graphene oxide nanosheets in living systems.

Materials and methods

Stock solution preparation of Graphene oxide Nano sheets

The unique nanoparticle, GONS was provided in pure powder form by the University of Exeter, U.K. The fresh stock solution was prepared by dispersing GONS in deionized water in sterilized 50ml falcon tubes at 1mg/ml and sonicated for 60 minutes at 65°C in ultrasonic bath (Universal Ultrasonic cleaner). Mechanically agitation was done at vortex (DLAB MX-S, China) for 1-2 minutes for further dispersion before injection to experimental animals (Fig. 1).

Animals

Healthy, free from ectoparasites, 25 Sprague Dawley Rats were purchased from the animal house,

Department of Physiology, Government College University, Faisalabad, Pakistan, kept in plastic basket and transferred in animal house of Department of Pharmacy, Government College University, Faisalabad, Pakistan for experiments. The weight of rats was 102 ± 6.25 g.

The rats were housed in standardized polypropylene cages. The animals were given commercial food and tap water freely during whole experimental periods. The temperature of animal house was maintained at 25 °C. The study protocol was approved by the local ethical committee on animal experimentation of Government College University, Faisalabad, Pakistan. Rats were acclimatized for two weeks and after that grouping was done by tagging on tails with by using permanent markers.

Study design and animal exposure

To assess the toxicity of unique group of Graphene, Graphene nanosheets, a dose-time response experiment was conducted in Sprague Dawley rats. Five groups of animals were made with 5 rats per group. One was control without any treatment, one placebo (injected with deionized water) and three treated groups exposed to graphene nanosheets.

Three doses of GONs nanoparticles were designated as low (group 1), medium (group 2) and high (group 3) with concentrations as 1.5, 2.5 and 3.5 mg/Kg body weight of rat for 30 days at an alternate day with total 15 doses (Table 1). All injections of nanoparticles suspension and deionized water were given intraperitoneally to the rats with 1ml BD insulin syringe in units. Variations in the weights of rats were recorded weekly with weight balance.

Bioassays

Sampling of rats was done at the end of experiment i.e., after 30 days for the collection of organs and tissues for analysis of biological parameters such as hematology, biochemical analysis of serum, pathology of tissues, and changes in the activities of oxidative stress enzymes as glutathione, catalase, and melondialdehyde and lipid hydroperoxides.

Hematology and serum biochemistry

After cervical dislocation, approximately 1ml blood was collected in EDTA coated tubes for the evaluation of hematological parameters as WBCs, RBCs, HB, HCT, MCV, MCH, MCHC and PLT and 1.5ml blood was poured into anticoagulant free tubes for the monitoring of changes in ALT, AST, ALP and A/G ratio that shows the health of liver. Auto Hematology analyzer (BC Mindray 3600, Shenzhen, China) was used for hematology and chemistry analyzer (Microlab: 300, ELI Tech group, USA) for biochemical analysis of serum.

Pathology of tissues

After blood collection, dissection of rats was done to obtain liver that was rinsed in chilled phosphate buffer solution to remove debris and blood, dried with filter paper and weighed on Sartorius balance. Some portion of tissues were quickly fixed in 10% neutral buffered formalin solution for the purpose of histology. Tissues were processed in ascending grades of alcohol as 70%, 80%, 100% and 100% for dehydration for different time periods. After that, dehydrated tissues were transferred into xylene for two times as clearing agent. Paraffin wax was used for embedding, infiltration and sectioning at 3-4 μ m at microtome (Histo-line, MR-2258, Italy). Sections of tissues were stained with hematoxylin and eosin, examined the prepared slides on biological microscope under 40x objective lens for pathology (Model: MT 4300H, Meiji Techno Co. LTD. Japan) and photographed (Naureen *et al.*, 2018). Remaining tissue pieces were stored in labeled polythene bags at -20 °C for further analysis.

Analysis of Antioxidant stress enzymes

Preparation of tissue homogenates

Recommended weight of stored tissues were obtained for the preparation of homogenates. Homogenization of liver was done in 0.1 M Tris-HCL with pH-7.4 in bullet blender (Bullet blender 5 eppendorf, Model-BBY5E-CE, USA) for 3-4 minutes. Homogenate was centrifuged at 10000rpm for 15 minutes at 4°C, then supernatant was collected carefully in 2ml eppendorf and stored at -20 °C for analysis of antioxidative

enzymes.

Reduced Glutathione (GSH)

Sedlak and Lindsay (1968) procedure was followed for the analysis of GSH contents in liver exposed to GONS by spectrophotometer (Model: U-2800 Hitachi, Germany) at 412nm and values expressed in $\mu\text{M/g}$ tissue. The precipitation of tissue homogenate was done with 50% trichloroacetic acid, centrifuged at 1000rpm for 5min. 0.5ml supernatant was mixed with 20ml Tris-EDTA buffer with 0.2M, 8.9 pH, 0.1ml of 0.01M DTNB and kept for 5mints at room temperature.

Catalase

The contents of catalase in liver was estimated by following the method of Aebi (1974). The mixture was prepared by adding 1.95ml phosphate buffer (50mM, pH 7), .05ml supernatant and 1ml hydrogen peroxide (30mM). The measurement of absorbance was taken at 240nm with 30 and 15 seconds intervals and expressed in Unit per ml of tissue homogenate.

Melondialdehyde (MDA)

The end product of melondialdehyde in tissue supernatant was calculated according to Okhawa method (1979). The reaction mixture consisted of 0.2ml supernatant, 8.1%SDS, 1.5ml 20% acetic acid (pH 3.5) and 1.5ml 0.8% thiobarbutaric acid and volume of mixture was raised to 4ml by adding

distilled water. Test tubes were heated at 95°C for 60 minutes in water bath. The mixture was cooled down at room temperature and mixed with n-butanol and pyridine in ratio (15: 1), respectively and strongly shaken at vortex mixture. The upper organic layer was carefully removed for absorbance at 532nm after centrifugation at 4000rpm for 10 minutes. $\mu\text{M/g}$ tissue units were used for expression of lipid peroxidation.

Lipid hydroperoxides

The method of Jiang (1992) was used for the analysis of lipid hydroperoxides in tissue homogenates. 0.9ml Fox reagent was mixed with 0.1 ml tissue homogenate and heated at 37°C for half an hour. After incubation, the absorbance was read at 560nm and values were shown in mM/g tissues.

Statistical analysis

The data were statistically analyzed by means of Minitab 17 software through ANOVA in general linear model to find out the effect of graphene on different parameter. Tukey's test was performed to compare the means of different groups at $p < 0.05$.

Results

General observation

In current study, all rats under investigation were found normal with no signs of abnormal behavior, weakness and illness i.e., no overall change in the general health condition.

Table 1. Grouping of Sprague Dawley rats and their treatment schedule.

Groups	Tested Materials	No. and Rout of Injections	Sampling interval
Control	No		after Days 30
Placebo	Deionized water	Intraperitoneal (15)	after Days 30
Group 1	GONS (1.5mg/kg BW)	Intraperitoneal (15)	after Days 30
Group 2	GONS (2.5mg/kg BW)	Intraperitoneal (15)	after Days 30
Group 3	GONS (3.5mg/kg BW)	Intraperitoneal (15)	after Days 30

Body weight and somatic index of organs

No significant difference was found in the body weight of rats in control and treated groups in first two weeks ($p < 0.05$). After two weeks, in week 3rd and 4th significant difference was observed in the body weight of rats in treated and control groups. A

significant reduction in body weight was observed in rats treated with high dose of GONS (Table 2).

There were significant changes in liver, kidney and spleen relative weights in treated against control and placebo groups (Table 3).

Hematological and serological analysis

In current study, data shows significant differences in values of red blood cells (RBCs), white blood cells, (WBCs), hemoglobin (HB), mean corpuscular hemoglobin concentration (MCHC) and platelets (PLT) by having greater values in treated groups than

control and placebo groups (Fig. 2). Hematocrit (HCT) and mean corpuscular hemoglobin in treated groups have no significant difference with untreated groups (Fig. 2) but all standard parameters of blood in current study are in normal ranges.

Table 2. Mean \pm SD of weekly body weight (g) of rats exposed to Graphene oxide nanosheets for 30 days with p values.

Groups Weeks	Control	Placebo	Group 1	Group 2	Group 3	p values
0 week	108.25 \pm 5.38 ^A	109.75 \pm 3.86 ^A	100.75 \pm 6.55 ^A	107.00 \pm 8.12 ^A	104.00 \pm 4.69 ^A	0.258
Week 1	108.75 \pm 7.27 ^A	112.50 \pm 3.11 ^A	101.00 \pm 4.08 ^A	105.25 \pm 7.41 ^A	103.25 \pm 4.03 ^A	0.067
Week 2	117.00 \pm 6.00 ^A	117.50 \pm 1.91 ^A	112.50 \pm 6.40 ^A	111.75 \pm 1.70 ^A	109.25 \pm 2.99 ^A	0.069
Week 3	123.00 \pm 5.94 ^A	121.25 \pm 2.99 ^{AB}	117.00 \pm 3.65 ^{AB}	119.25 \pm 0.95 ^{AB}	114.50 \pm 2.08 ^B	0.030
Week 4	124.25 \pm 8.10 ^A	128.25 \pm 2.75 ^{AB}	118.25 \pm 2.36 ^{AB}	120.00 \pm 1.63 ^B	115.00 \pm 3.92 ^B	0.006

Means that do not share a letter in rows are significantly different ($p < 0.05$, one-way ANOVA).

Table 3. Mean \pm SD of Somatic index of different organs (Mean \pm SD) of rats exposed to Graphene Nanosheets for 30 days with p values.

Groups Organs	Control	Placebo	Group 1	Group 2	Group 3	p values
Liver	5.21 \pm 0.12 ^{ABC}	4.75 \pm 0.35 ^C	4.90 \pm 0.10 ^{BC}	5.30 \pm 0.17 ^{AB}	5.53 \pm 0.07 ^A	0.004
Kidney	1.16 \pm 0.01 ^A	1.16 \pm 0.03 ^A	0.86 \pm 0.07 ^C	1.03 \pm 0.02 ^B	1.21 \pm 0.03 ^A	0.000
Spleen	0.47 \pm 0.04 ^{AB}	0.46 \pm 0.03 ^{AB}	0.37 \pm 0.04 ^B	0.50 \pm 0.01 ^A	0.54 \pm 0.05 ^A	0.005

Means that do not share a letter in rows are significantly different ($p < 0.05$, one-way ANOVA).

The health of liver in graphene nanosheets exposed and unexposed rats was investigated by analysis of ALT, AST and ALP enzymes in serum. The values of liver enzymes were found significantly higher in treated rats as compared to placebo and control animals (Fig. 3), ($p < 0.05$).

Oxidative stress biomarkers in liver

Catalase (CAT)

Catalase is an important antioxidant enzyme in animals produced in defensive mechanisms and convert the free radicals as H₂O₂ that produce stress condition into water and oxygen. Fig.4 shows dose dependent increase in concentration of catalase enzyme in treated rats than control and placebo groups.

Glutathione (GSH) activity

The liver of rats exposed to Graphene oxide

nanosheets showed significant increase in the contents of GSH as compared to control and placebo groups. The effects of graphene oxide nanosheets on glutathione contents was found dose dependent i.e., increases with dose increasing (Fig. 4).

Melondialdehyde (MDA)

Melondialdehyde is produced during the lipid peroxidation in animals. Fig. 4 showed significantly ($p < 0.05$) high contents of MDA in the liver of graphene nanosheets treated rats with untreated rats.

Lipid Hydroperoxides (LPO)

The assay of LPO was followed to determine the contents of lipid hydro peroxides (LPO) in the liver of rats. The data presented in Fig. 4 shows the trend of increase in the level of lipid hydro peroxides significantly in treated rats as compared to control and placebo groups.

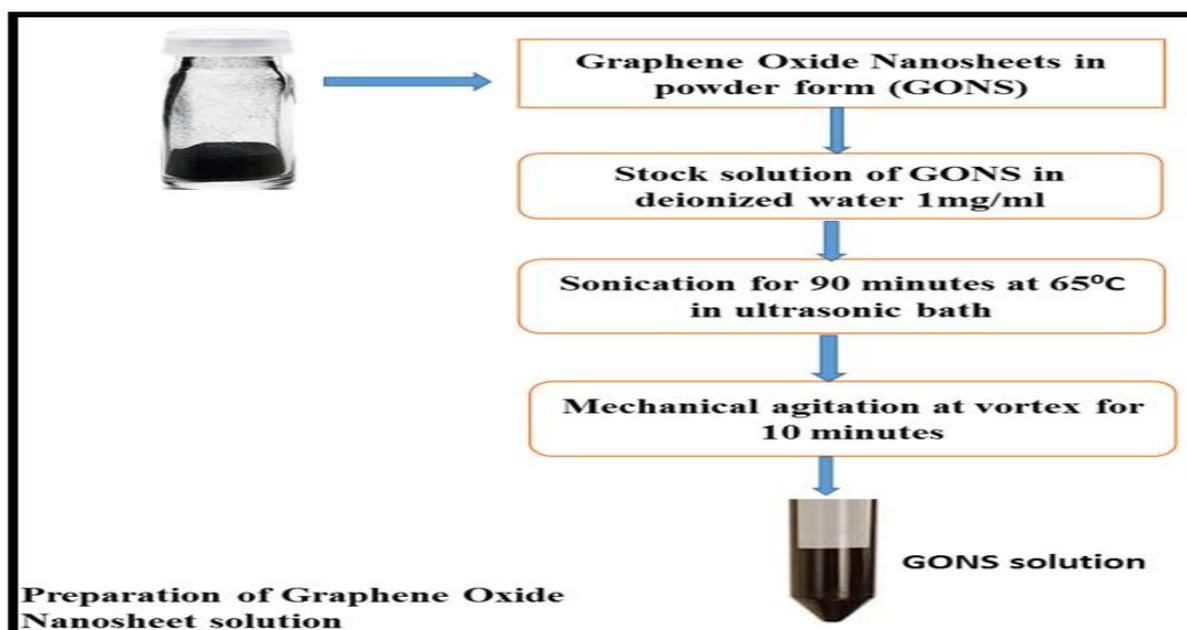


Fig. 1.

It showed the oxidative stress condition of tissue and consistently as the concentration of toxicant increases.

Distribution of graphene nanosheets

After the dissection of rats, gross morphology of organs and distribution of graphene oxide nanosheets were examined (Fig. 5A). There was found no change in the morphology of organs especially liver (Fig. 5, B, C photographs). Small aggregation of graphene nanosheets were found near the site of injection, in connective tissues of stomach, peritonium, lipid tissues of abdomen and in mesenteries at higher doses (3.5mg/kg BW rats) and less aggregation in low dose (1.5mg/kg) (Fig. 5, A). Small dots of graphene nanosheets were also noted in lipid tissues of liver. No distribution of nanosheets were examined in the control and placebo groups.

Pathology of liver

The liver of controlled and placebo rats was normal in histology with no lesions, normal central vein, and compact hepatocytes with sinusoids (Fig. 6). Microscopic observation of stained slices of liver in rats treated with low, medium and high concentration of graphene oxide nanosheets injected intraperitoneally showed dose dependent alterations in liver histology like focal area of necrosis in

hepatocytes, dilation of central vein, irregular shape of vein, vacuolization, pyknotic nuclei and binucleated cells were also found (Fig. 7).

Discussion

Overall, no significant change was found in the body weight of the rats but weight grew in last 4th week in control and treated groups. There was no clear difference in the appearance of selected organs but liver and spleen of treated groups with high dose showed slight dark in color as compared to control. This slight difference in color is due to the accumulation of graphene oxide nanosheets in organs as reported by Yang *et al.*, 2013. Weight of liver, spleen and kidney of the rats injected with GONS was higher than control due to slow excretion of nanosheets (Yang *et al.*, 2010). The liver is vital organ that detoxify broad range of particles by modifying, storing or breaking into another compounds not harmful for living systems through redox reactions. The imbalance in oxidation and reduction reactions may be responsible for initiation of oxidation that leads to liver diseases that may be metabolic or inflammatory (Cichoz-Lach and Michalak, 2014). Nanoparticles on accumulation may cause liver fibrosis (Li *et al.*, 2014) and therefore, liver is most vulnerable to damage caused by nanoparticles (Patlolla *et al.*, 2011).

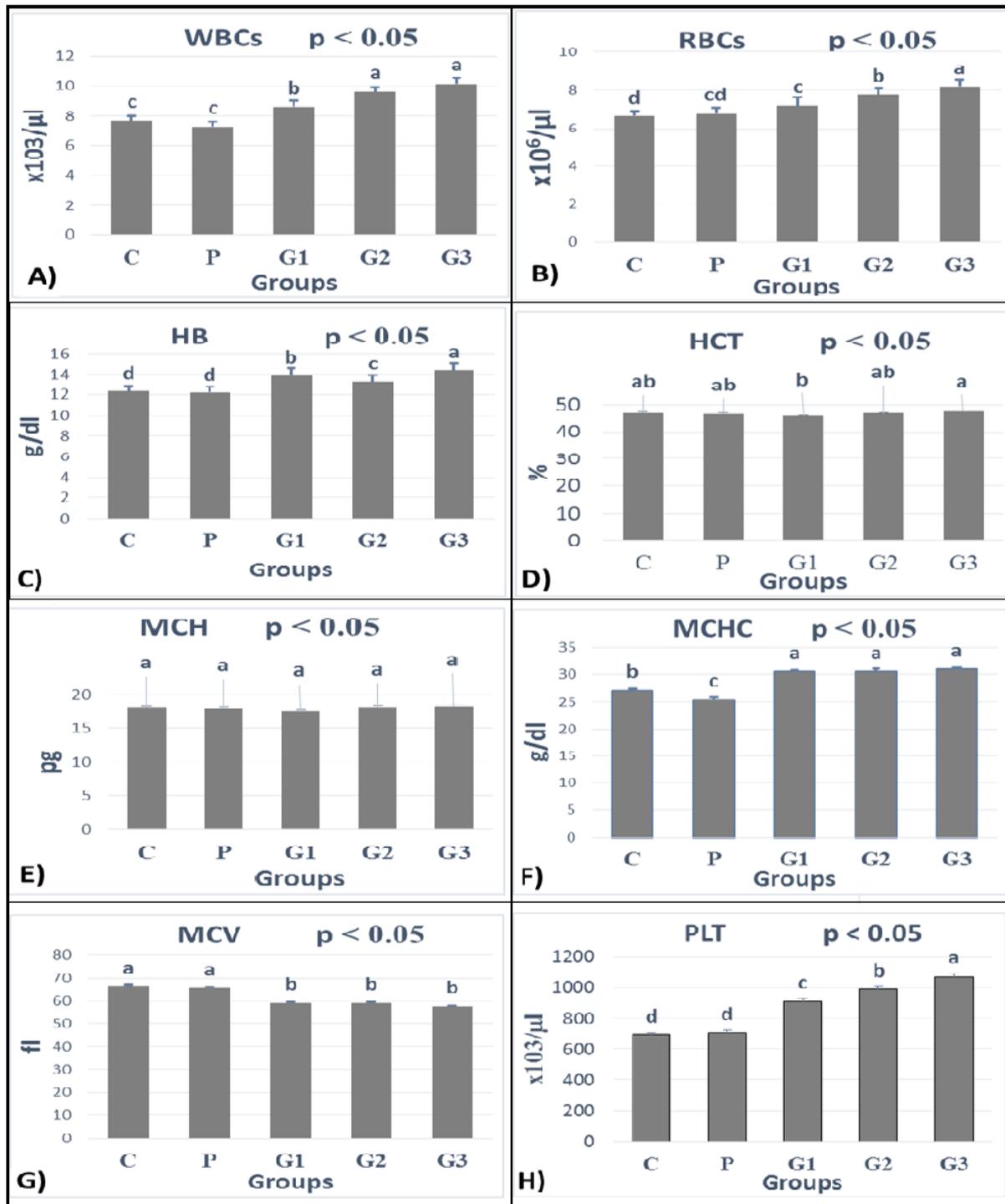


Fig. 2. Hematological profile of Sprague Dawley rats following intraperitoneal injection of GONS for 30 days. White blood cells A, number of red blood cells B, hemoglobin concentration C, hematocrit D, grams of mean corpuscular hemoglobin E, mean corpuscular hemoglobin concentration F, mean corpuscular volume G, platelets H. positive control and deionized treated placebo group means. Each bar denotes Mean \pm SD.

For investigation of toxicity caused by graphene oxide nanosheets injected intraperitoneally in rats, significant fluctuations were noted in values of WBCs, RBCs, hemoglobin, hematocrit, MCH, MCHC, platelets and MCV among the treated groups and with

control but their values remained in normal ranges (Chong *et al.*, 2014). We analyzed liver function enzymes as ALT, AST and ALP in serum that are indicators of toxicity caused by drugs in clinical and animal laboratories. Hepatocytes produce these

enzymes in greater amount and used in the formation of glutamate by transfer of amino group (Tarrant *et al.*, 2013). In our study, these enzymes showed significant changes in their level with reference to control and placebo (injected with deionized water) that indicated slight injury in liver. Damaged liver produces more ALT, AST and ALP in serum (Ramaiah *et al.*, 2007; Ozer *et al.*, 2008; Tabish *et al.*, 2018). In present work, significant increase in the concentration of ALT and AST were noted in the serum of rats at higher doses of graphene oxide nanosheets in response to liver damage that increase the permeability of cell membrane (Patlolla *et al.*,

2011). Alkaline phosphatase, a key enzyme of phosphatases family, fluctuation in ALP level considered as indicator of injury in hepatocytes (Murakami *et al.*, 2004). In present findings, graphene nanosheets elevated the level of ALP enzyme in the serum of rats as compared to control and placebo that have possibility of liver injury that agree with the findings of Patlolla *et al.*, 2017, who reported that increased level of ALP in serum produced by the cells of biliary ducts is the first sign of liver damage that involve the blockage of biliary drainage system.

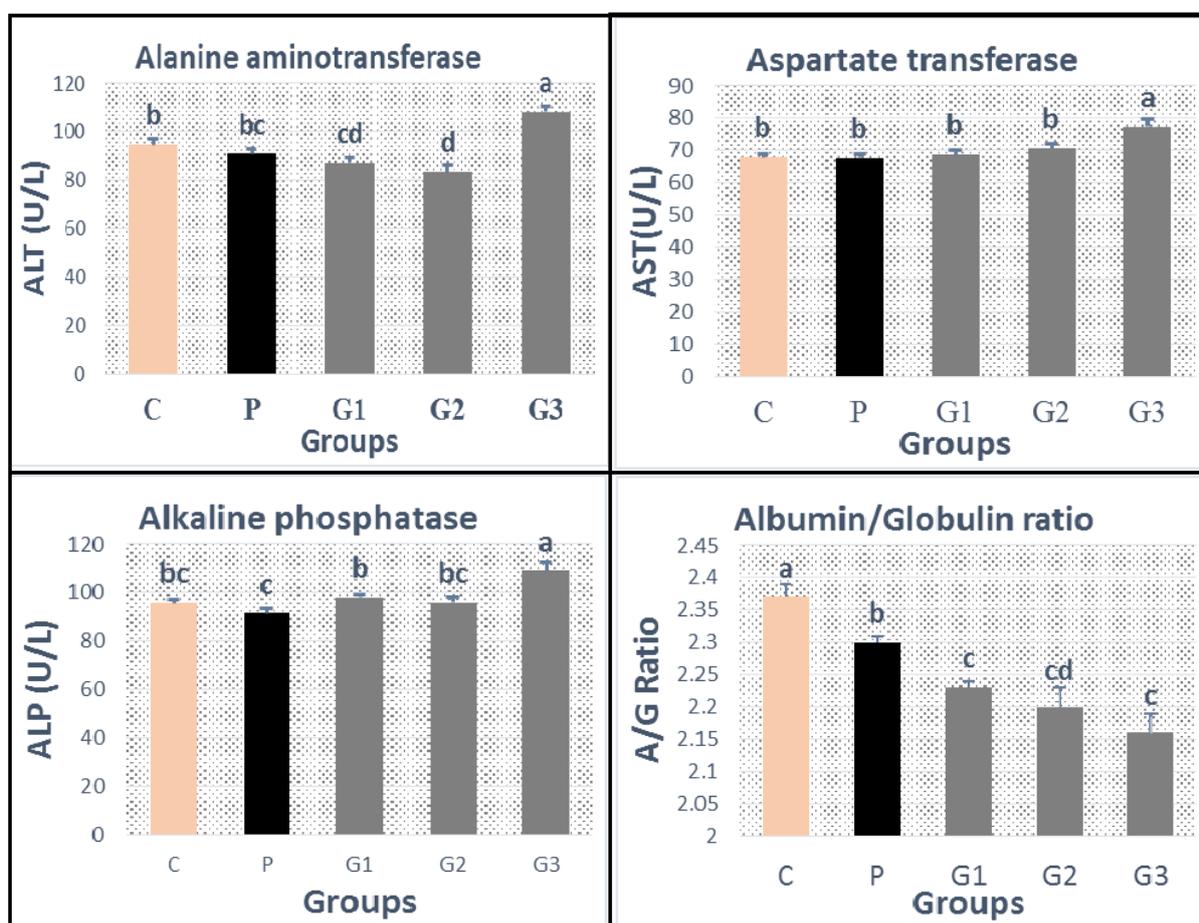


Fig. 3. Mean \pm SD of Liver function enzymes in Sprague Dawley rats among the control, placebo and treated groups. One way ANOVA, n = 3.

During the toxicological evaluation of graphene and their derivatives, well-known biomarkers, oxidative stress is widely used (Seabra *et al.*, 2014; Jarosz *et al.*, 2016). A balance is present between the generation of reactive oxygen species (ROS) and its removal from the cell but if the level of ROS severely increases,

responsible for the induction of apoptosis or necrosis that may lead to death of cells (Yang *et al.*, 2013; Khanna *et al.*, 2015). According to Zhang *et al.*, 2010, exposure to graphene and its family nanomaterials is responsible for the arising of antioxidant defense mechanism by producing reactive oxygen species.

Antioxidant system provides the protection against generation of free radicals produced during oxidation in stress conditions in vital organs by removing them into another products as water and oxygen (Patlolla *et al.*, 2016). During our present study, response of oxidative stress markers against application of graphene oxide nanosheets was evaluated by

measuring the activity of antioxidant enzymes in hepatocytes as Catalase, GSH, which involved in the detoxification of free radicals as H_2O_2 produced during oxidation process (Droge, 2002). Melondialdehyde (MDA) or TBARS is also evaluated which produced during the oxidation of lipid peroxides.

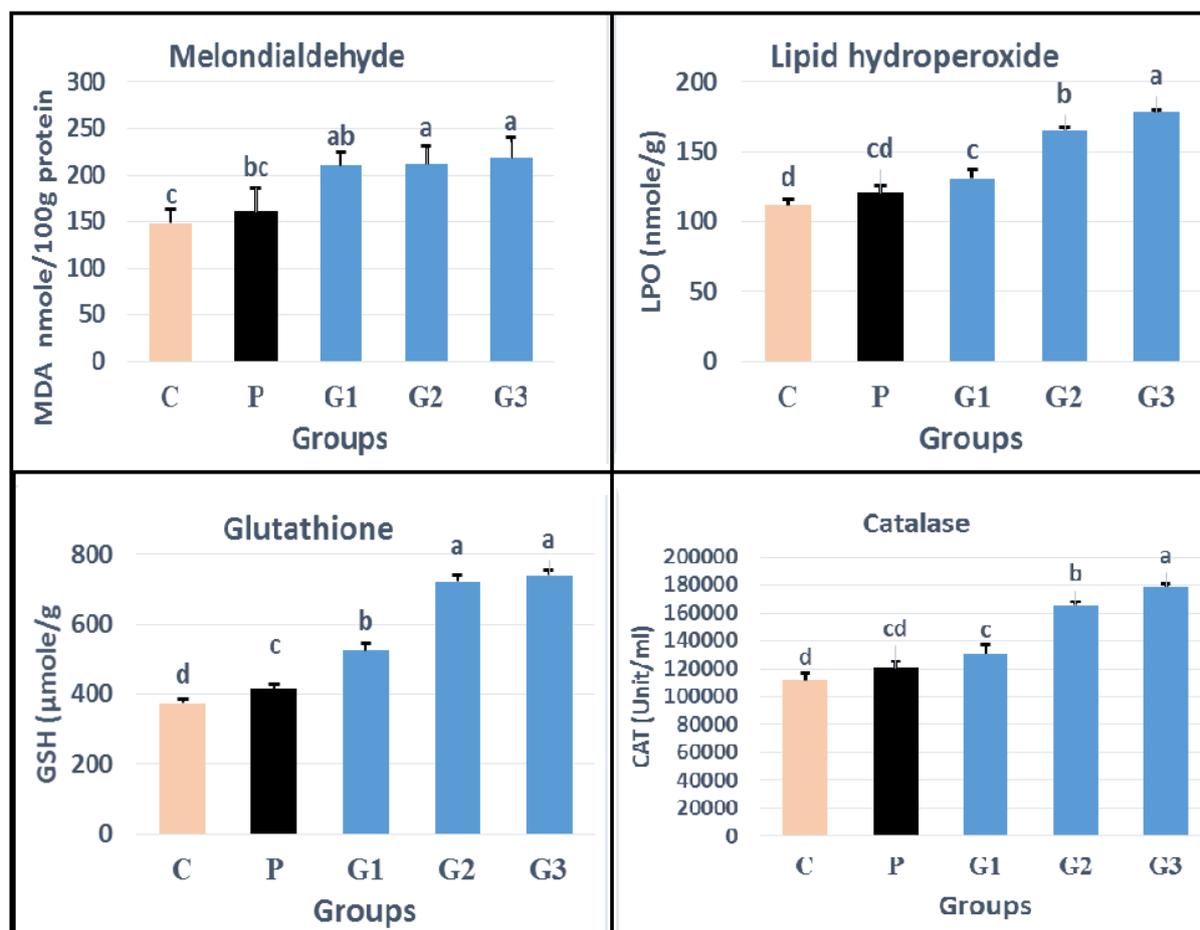


Fig. 4. Mean concentration of oxidative stress enzymes in the liver of Sprague dawley rats among control and treated groups.

The findings of present research showed a significant increase in antioxidant systems in the liver of rats exposed to graphene nanosheets intraperitoneally for 30 days as compared to control and placebo group. Our findings are in agree with Isalm *et al.*, 2014 and Patlolla *et al.*, 2016, who reported that increase in Catalase level may be a protective mechanism against the higher level of H_2O_2 in liver.

The increased level of MDA produced during oxidation of lipid peroxides agree with results of Chen *et al.*, 2016 who found that exposure of graphene

oxide nanomaterial responsible for oxidative stress in liver by increasing the amount of MDA in Zebra fish. The increase in the level of MDA during graphene oxide exposure is responsible to enhance the process of lipid peroxidation that may lead to destruction of cell membranes (Lin *et al.*, 2010).

The GSH is an important enzyme of defense system in liver cells that balance the free ion radicals that produce oxidative stress conditions (Ribas *et al.*, 2014) and liver is a key organ that maintain the homeostasis of GSH (Lu, 2013).

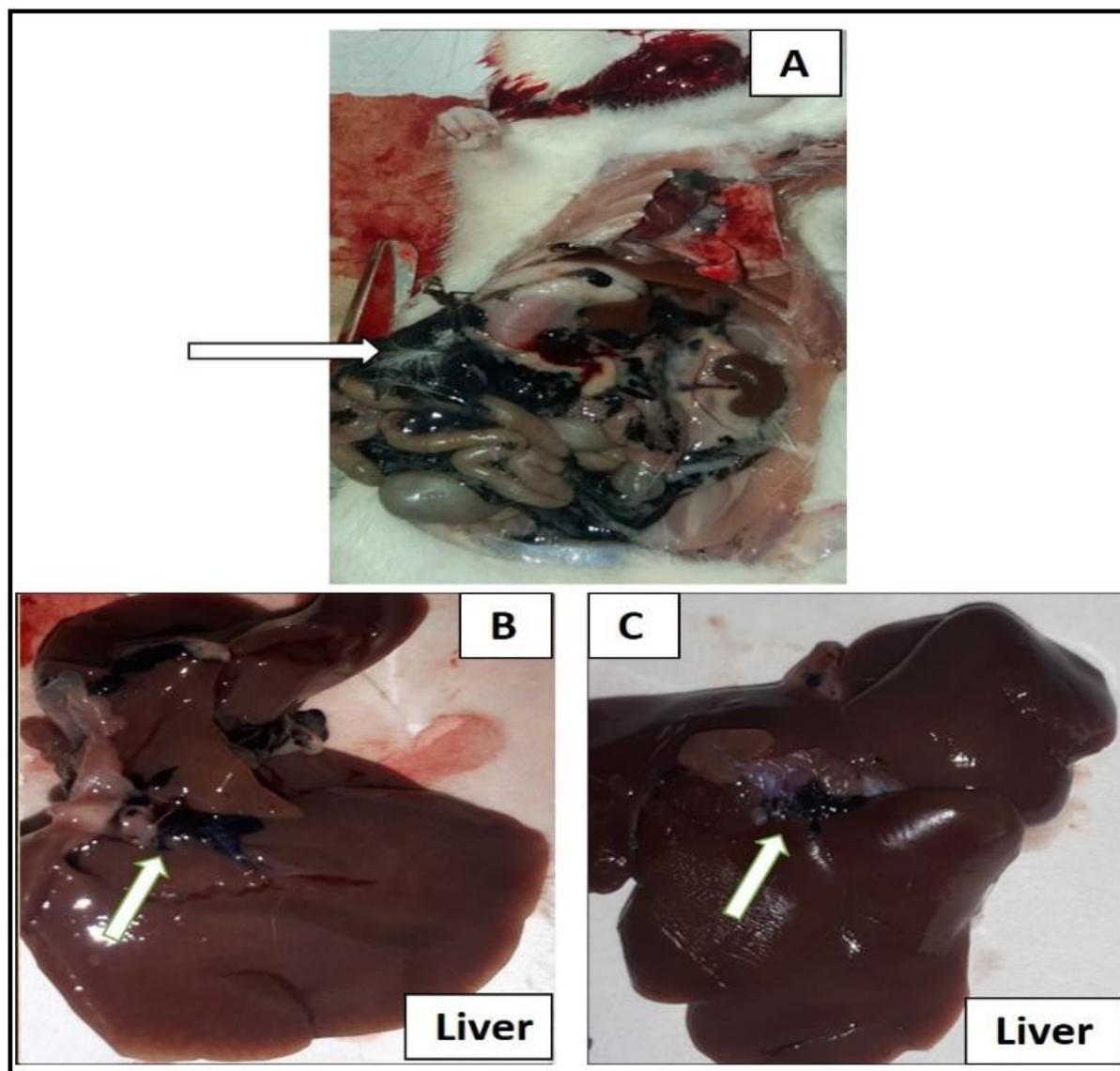


Fig. 5. Distribution of Graphene oxide nanosheets in rats. Black aggregates were seen in the region of injections and in mesenteries (White arrow, A). Gross anatomy and distribution of GONS in liver of rats at high dose (B) and low dose (C). No pathological features were seen in morphology of livers.

The findings of present work are in agree with Strojny *et al.*, 2015 who described the increased level of GSH in groups of rats exposed to graphene oxide nanoparticles. The elevated level of glutathione in the liver of GO exposed rats may be the result of structural similarity between graphene oxide and quinones, which have functional groups rich with oxygen molecules. Graphene oxide nanoparticles may also elevate the level of GSH in liver by producing the enzymes involved in the production of GSH (Forman *et al.*, 2009). According to Franco *et al.*, 2007 the de novo synthesis of GSH is compulsory in organs because of depletion in the level of GSH may cause

death of cells, toxicity in cells, production of radicals, oxidants. Thus it is hypothesized that most of pathology is related to low level of GSH not higher (Franco *et al.*, 2007).

Marked increase in the contents of Lipid hydroperoxides (LPO) were recorded in liver of rats treated with different doses of graphene nanosheets (GONS) for 30 days in present study. The oxidation of molecular oxygen that result in the production of superoxide radicals that increase the level of LPO in liver cells responsible for the loss of function and structure of membrane.

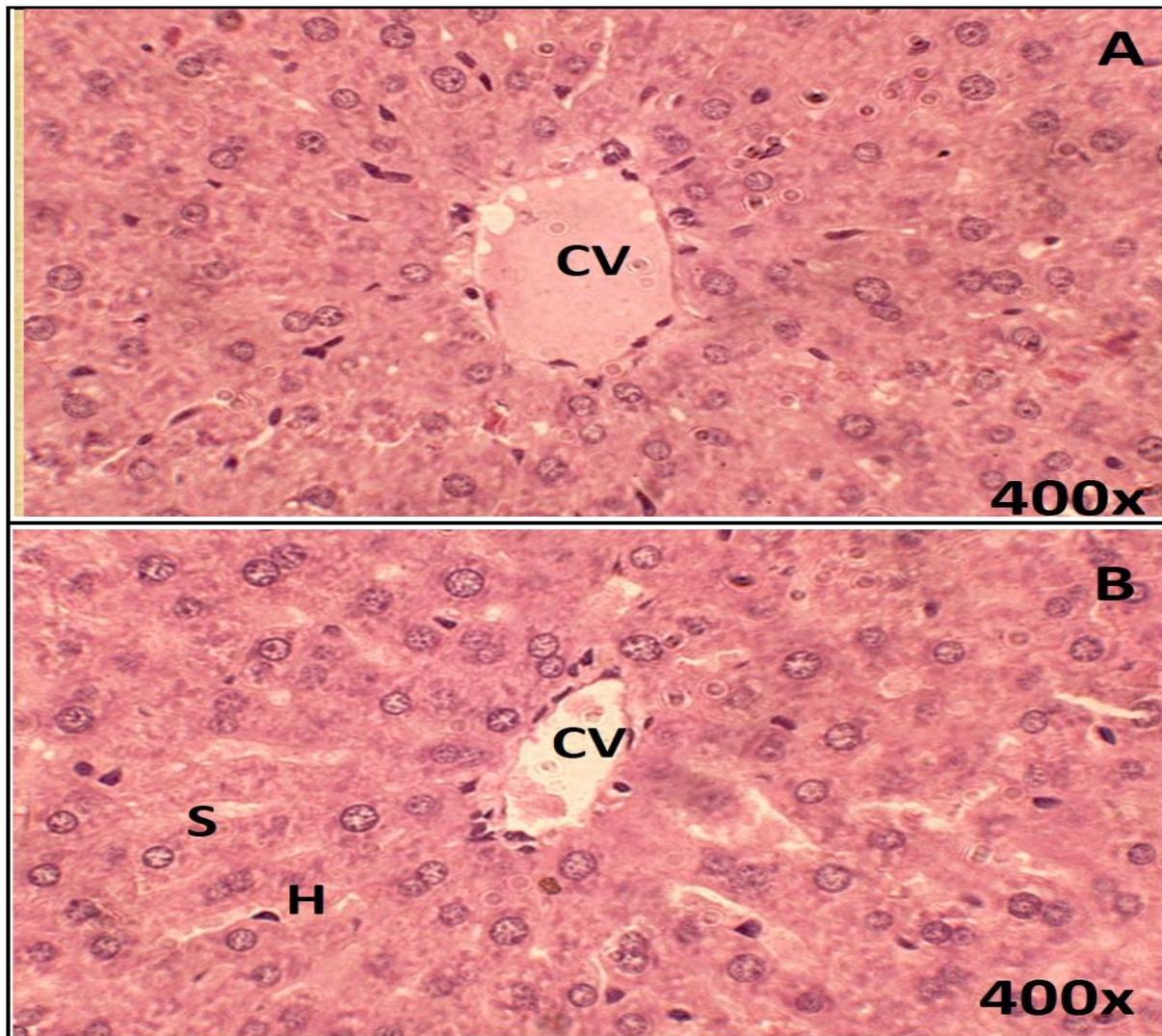


Fig. 6. Photomicrograph of liver tissues (H & E Staining) of control and placebo groups (A and B) of rats represented normal central vein (CV) surrounded with normal hepatocytes (H) having sinusoids.

During this reaction, MDA is also produced with stimulation of unsaturated fats peroxidation in cell membranes (Ray, 1991; Kale *et al.*, 1999; Sharma *et al.*, 2014).

The alterations in the structure of liver ranges from mild to severe after the exposure of graphene oxide and their family in model animal. In current study, prominent changes in liver of rats exposed to graphene oxide nanosheets were observed like vacuolization, damage of central vein, destruction of hepatocytes and dilation in sinusoids that depend on dose and time as compared to control and placebo group. The damage of liver may be due to the accumulation of graphene oxide nanosheets. The data of our present investigation is supported by previous

findings who mentioned the collection of graphene oxide nanosheets in lungs, kidney, spleen and liver administered either through intraperitoneal, oral, intravenous or through skin (Guo and Mei, 2007). Necrosis in hepatocytes at lower doses and severe with infiltration at higher doses may be attributed to the generation of reactive oxygen species in response to GO exposure resulting in cytotoxicity. The granuloma formation in liver in response to intravenous exposure of GO in rats reported by Yang *et al.*, 2011. It is reported from the previous investigations that graphene oxide induce pathology in liver by producing oxidative stress, directly attached with lipid bilayer of cell membrane or indirectly by combining with molecules. Hydrophobic nature of GO also support in the production cellular

toxicity (Zang *et al.*, 2010; Chen *et al.*, 2012; Lammel *et al.*, 2013). Enlargement of hepatic central vein and sinusoids reported by Li *et al.*, 2016 agree with our present data about the pathology of liver after intraperitoneal exposure of graphene oxide nanosheets. When any foreign material like graphene

oxide nanosheets enter the liver cells, immune cells recognize these foreign toxicant and activate the kupffer cells of liver which is defense system and uptake these nanomaterials by kupffer cells and cause the disturbance in the function of macrophages (Qu *et al.*, 2013; Yang *et al.*, 2013).

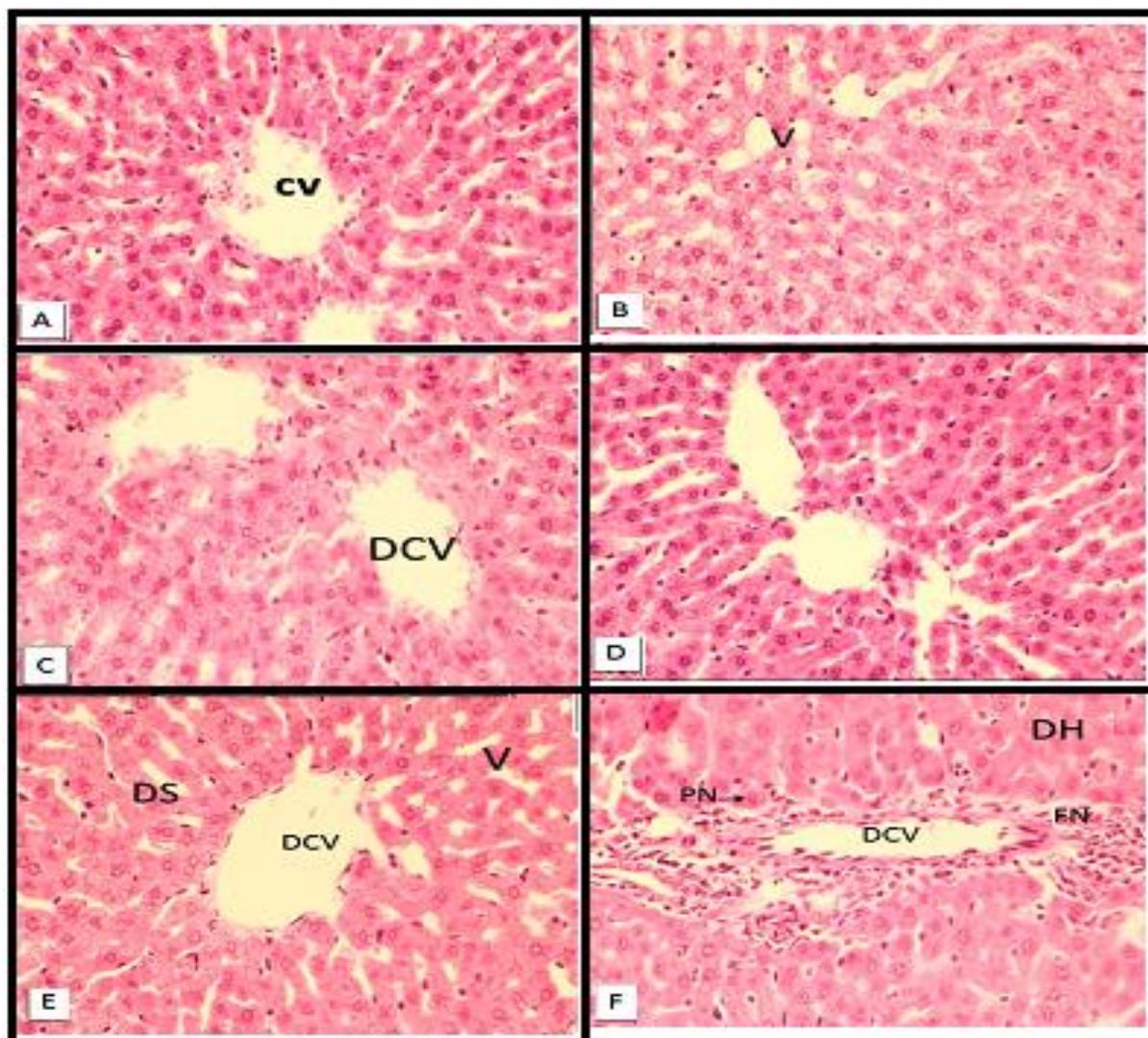


Fig. 7. Photomicrograph of liver tissues (400x H & E Staining) of Sprague dawley rats. **A-B** slides of rats treated with low dose of GONS showed irregularity in shape of central vein and vacuolation (V). Degeneration of central vein were observed in liver of medium dose treated rats (2.5mg/kg BW) **C-D**. Pyknotic nuclei (PN), focal area of necrosis (FN) and vacuolation (V), degeneration of vein (DCV), degeneration of hepatocytes (DH) dilation in sinusoids (DS) were seen after the administration of high dose of GONS (3.5mg/kg bw) in stained slides of liver (E-F).

Conclusion

It was concluded that graphene nanosheets induced oxidative stress mediated toxicity in the liver. Therefore, it should be used very carefully *in vivo* applications.

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