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Low doses of multi-walled carbon nanotubes elicit hepatotoxicity in rats with markers of oxidative stress and induction of pro-inflammatory cytokines

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ABSTRACT

The investigation into the potential health risks associated with the use of engineered nanoparticles is a major scientific interest in recent years. The present study elucidated the involvement of pro-inflammatory cytokines, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in carboxylated multi-walled carbon nanotubes (MWCNTs)-induced hepatotoxicity. Pubertal rats were exposed to purified MWCNTs at 0, 0.25, 0.50, 0.75 and 1.0 mg/kg for 5 consecutive days. Results indicated that exposure to MWCNTs caused liver damage evidenced by significant elevation in serum activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) when compared with control. Moreover, MWCNTs significantly decreased superoxide dismutase (SOD) and glutathione S-transferase (GST) activities as well as glutathione level whereas it significantly increased catalase (CAT) and glutathione peroxidase (GPx) activities in liver of the treated rats. Moreover, the dose-dependent increase in hepatic hydrogen peroxide (H_2O_2) and lipid peroxidation levels were accompanied by marked increase in micronucleated polychromatic erythrocytes (MNPCE) in the MWCNTs-treated rats. Administration of MWCNTs significantly increased serum concentrations of pro-inflammatory cytokines namely interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) in the treated rats. Immunohistochemical analysis showed significantly increased COX-2 and iNOS protein expressions in the liver of MWCNTs-treated rats. In conclusion, carboxylated MWCNTs induces hepatic damage *via* disruption of antioxidant defense systems, promotion of pro-inflammatory cytokines generation and expression of COX-2 and i-NOS in rats.

1. Introduction

The exceptional properties of carbon nanotubes have motivated their incorporation into innovative products globally. Carbon nanotubes can exist as single-walled (SWCNTs) which consists of a single sheet of graphene rolled to form a cylinder or multi-walled (MWCNTs) which consist of several concentric graphene tubes with diameters of up to 100 nm [1]. Both forms of carbon nanotubes are widely used in medicine, nanoelectronics, engineering, agriculture and daily consumable products [2,3]. There is an increasing research attention on these nanoparticles in recent years because excessive exposure to them has been demonstrated to pose great health risks to both animals and humans [4,5]. Indeed, exposure to MWCNTs is a global concern due to their potential similarities to hazardous asbestos fibers [6,7].

Previous studies have independently demonstrated several toxicological effects of MWCNTs in different experimental models. Moreover, research into the hepatotoxicity induced by MWCNTs is important because the liver is the major site of xenobiotic metabolism. Earlier studies on MWCNTs-induced hepatotoxicity indicated that oral administration of single dose of MWCNTs at 60 and

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100 mg/kg to Swiss mice decreased superoxide dismutase (SOD) and catalase (CAT) activities and caused histopathological changes in the liver of the treated rats [8]. Intravenous injection of MWCNTs at 10 and 60 mg/kg to Kunming mice for 15 and 60 days induced hepatotoxicity by altering the expression of cytochrome P450, a gene involved in drug metabolism [9]. Moreover, single intravenous injection of MWCNTs functionalized with single strand DNA (ss-DNA-MWCNTs) at 270 mg/kg produced transient oxidative stress and inflammation in Wistar rats [10].

The modern trend in toxicology involves investigating the cellular responses to toxic chemicals at environmentally relevant concentrations, which is the condition usually encountered by the resident population. Hence, in contrast to earlier studies where high doses of MWCNTs were used, we previously demonstrated that low doses (i.e. 0.25, 0.5 and 0.75 mg/kg) of carboxylated MWCNTs induced hepatotoxicity in mice and reproductive toxicity in rats *via* induction of oxidative stress [11,12]. The mechanism underlying the hepatotoxicity resulting from carboxylated MWCNTs exposure is not fully known. It remains to be investigated if alterations in inflammatory mediators including cytokines, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) contribute to previously reported hepatotoxicity elicited by low doses of carboxylated MWCNTs in rats. Given these gaps in knowledge, the present study investigated the involvement of glutathione-dependent enzymes, pro-inflammatory cytokines, COX-2 and iNOS in hepatotoxicity induced by repeated administration of carboxylated MWCNTs in rats.

2. Materials and methods

2.1. Chemicals

Carboxylated multi-walled carbon nanotubes (MWCNTs) from NanoLab Inc. (Newton, MA) were received from Professor Anita K. Patlolla of the Molecular Toxicology Research Laboratory, NIH-RCMI Center for Environmental Health, CSET, Jackson State University, Jackson, Mississippi, USA. 1-chloro-2, 4-dinitrobenzene (CDNB), 5′, 5′-dithiobis-2-nitrobenzoic acid (DTNB), epinephrine, hydrogen peroxide, glutathione (GSH), trichloroacetic acid (TCA) and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals and reagents were of analytical grade and were procured from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Physicochemical characterization of functionalized multi-walled carbon nanotubes

Multi-walled carbon nanotubes (MWCNTs) (outer diameter of 15–30 nm, lengths of 15–20 μm, purity > 95%) were synthesized by NanoLab Inc. (Newton MA, USA) using catalytic chemical vapor deposition technique. The MWCNTs were subsequently heated under argon (2 l/min) to 2000 °C at the rate of 10 °C/min to remove iron-impurities. Purified MWCNTs [purity > 95% by Thermogravimetry analysis (TGA)] were then subjected to a reflux process in sulfuric/nitric acid (3:1) to functionalize their surfaces leading to a large concentration of carboxyl (COOH) groups on the nanotube surfaces. The resulting functionalized carboxylated nanotubes have 2–7% COOH by weight. The morphology and size of these pure MWCNTs were read by Transmission Electron Microscope (TEM). Prior to visualizing the samples with TEM, MWCNTs were directly placed on a TEM grid and allowed to dry. Further, the surface areas were assessed by the isothermal gas adsorption BET method [13] using a Micromeritics Flowsorb 2300 (Norcross, USA). To characterize our system, MWCNTs were dispersed in 1% tween-80 and sterile saline using physical mixing and ultrasonication. Eventually, the purified long MWCNTs had a diameter of 11.5 nm, length of 12 μm and specific surface area of 42 m^2/g, respectively.

2.3. Animal model and care

Fifty pubertal male Wistar rats (8 weeks old) weighing between 160 and 180 g obtained from the Department of Biochemistry, University of Ibadan, Ibadan were used for this investigation. The rats were housed in plastic cages placed in a well-ventilated vivarium, under standard laboratory conditions of a 12 h/12 h light/dark cycle and provided rat chow and water *ad libitum*. Animal care and experimental etiquettes were executed according to the approved guidelines set by the University of Ibadan Ethical Committee, which is in accordance with the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Science (NAS) and published by the National Institute of Health.

2.4. Experimental design

Following one week of acclimatization, the rats were assigned into five groups of ten rats each. Pure carboxylated MWCNTs suspension was administered intraperitoneally to the rats at the doses of 0.25, 0.5, 0.75 and 1.0 mg/kg for 5 consecutive days. Control rats were administered saline plus 1% tween-80 in the same manner as in the treatment groups. The doses of carboxylated MWCNTs used in the present study were chosen based on the previously published data from our laboratories [11,12]. Twenty-four hours following the final treatment, the blood was collected from retro-orbital venous plexus into plain tubes before the rats were sacrificed by cervical dislocation. Subsequently, serum samples were obtained by centrifugation of the clotted blood at 3000 g for 10 min and were stored frozen at −20 °C until the liver function indices were analyzed.

2.5. Assessment of oxidative stress biomarkers

Liver samples from control and MWCNTs-treated rats were homogenized in 50 mM Tris–HCl buffer (pH 7.4) containing 1.15% potassium chloride. The homogenate was subsequently centrifuged at 12,000 g for 15 min at 4 °C and the supernatant used for biochemical assays. Hepatic protein concentration was determined according to the method described by Bradford [14] using bovine serum albumin as standard. Superoxide dismutase (SOD) activity was assayed according to the method described by Misra and Fridovich [15]. Catalase (CAT) activity was assayed using hydrogen peroxide as a substrate according to the method described by Clairborne [16]. Level of reduced glutathione (GSH) was assayed at 412 nm according to the method described by Jollow et al. [17]. Glutathione peroxidase (GPx) activity was assayed according to the method described by Rotruck et al. [18]. Glutathione-S-transferase (GST) activity was assayed according on the method described by Habig et al. [19]. Hydrogen peroxide level was assayed according to the method described by Wolff [20]. Malondialdehyde (MDA) level, an index of lipid peroxidation (LPO), was assayed according to the method described by Farombi et al. [21] with slight modification. All assays with the exception of SOD and CAT activities were analyzed using a SpectraMax plate reader (Molecular Devices, CA, USA).

2.6. Measurement of liver function and pro-inflammatory biomarkers

Serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) were all were analyzed using available

commercially available kits from Randox Laboratories Limited (UK). The serum concentrations of pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) were evaluated using commercially available ELISA kits (Abcam Plc, UK).

2.7. In vivo micronucleus (genotoxicity) assay

Genotoxicity assay was performed according to established procedure [22]. Briefly, a small opening to the marrow was created at the proximal ends of the femurs with a pair of scissors. Subsequently, the femur was immersed in fetal calf serum and the bone marrow carefully released by aspiration and flushing on glass slides. The bone marrow suspension was then smeared on the slide, fixed in methanol for 3–5 min and allowed to dry for 24 h. The slides were thereafter stained with May-Gruenwald followed by 5% diluted Giemsa solution for 30 min. Further, the slides were rinsed in phosphate buffer followed by distilled water and air dried. Subsequently, the dried stained slides were mounted in DPX with coverslips and examined under the microscope at 100× magnification using oil immersion. The incidence of micronucleated polychromatic erythrocytes (MNPCE) was recorded using a tally counter.

2.8. Immunohistochemical staining of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) protein expression

The level of hepatic COX-2 and iNOS expression was assessed in a 5 μm thick sections of formalin fixed liver embedded in paraffin. Following deparaffinization of the sections in xylene and rehydration with graded alcohol, antigen retrieval was performed by immersing the slides in 10 mM citrate buffer at 95–100 °C for 25 min and the peroxidase activity quenched in 3% H_2O_2/methanol solution. The liver sections were subsequently blocked in goat serum followed by an overnight incubation at 4 °C in the goat anti-COX-2 and rabbit anti-iNOS primary antibodies. The slides were subsequently washed with tris buffer saline and then incubated with horse-radish peroxidase labeled anti-rabbit monoclonal secondary antibodies (Dako, Agilent Technologies, US). Immune complexes were visualized using 0.05% 3, 3-diaminobenzidene and the slides examined under light microscope (Leica DM 500, Germany). The images were captured using a digital camera (Leica Biosystems, UK) attached to the microscope. The quantitative assessment of COX-2 and iNOS protein expression was performed by counting 10 non-continuous sections per eye and a total of 20 eyes per group by investigators who were blinded to the study. The levels of expression of the proteins were expressed as percentage of the total cells counted.

2.9. Statistical analyses

Statistical analyses were done using one-way analysis of variance (ANOVA) to compare the experimental groups followed by Bonferroni's post-hoc test using GRAPHPAD PRISM 5 software (Version 4; GraphPad Software, La Jolla, California, USA). Values of $P < 0.05$ were considered significant.

3. Results

3.1. Carboxylated MWCNTs induce hepatic lipid peroxidation by disrupting antioxidant defense system

To test the impact of low doses of MWCNTs, we administered MWCNTs *via* i.p. injection into rats and monitored the hepatic redox status. Fig. 1 depicts the effects of carboxylated MWCNTs on hepatic redox status in rats. Administration of carboxylated MWCNTs caused a dose-dependent significant decrease in the SOD and GST activities as well as GSH level when compared to the control. The percentage decrease in SOD activity was 20%, 48%, 69% and 97%; GST activity decreased by 25%, 43%, 81% and 96% whereas GSH level decreased by 29%, 41%, 74% and 84% at 0.25, 0.5, 0.75 and 1.0 mg/kg respectively, when compared with the control. Conversely, MWCNTs treatment significantly increased hepatic CAT and GPx activities in the treated rats when compared to the control. The percentage increase in CAT activity was 31%, 37%, 46% and 49% whereas GPx activity increased by 9%, 22%, 29% and 31% at 0.25, 0.5, 0.75 and 1.0 mg/kg respectively, when compared with the control. Further, there was a dose-dependent significant elevation in the hepatic level of MDA, a biomarker of lipid peroxidation in the experimental rats. The percentage increase in H_2O_2 level was 31%, 36%, 39% and 44% whereas lipid peroxidation level was increased by 32%, 51%, 64% and 66% at 0.25, 0.5, 0.75 and 1.0 mg/kg respectively, when compared with the control.

3.2. Carboxylated MWCNTs elicit dose-dependent increase in biomarkers of hepatic damage, pro-inflammatory cytokines and genotoxicity

Next, we studied the effects of the MWCNTs on biomarkers of hepatic damage, pro-inflammatory cytokines and genotoxicity. Fig. 2 represents the effects of carboxylated MWCNTs treatment on markers of liver toxicity, pro-inflammatory cytokines and genotoxicity. The serum activities of AST, ALT, ALP and GGT were dose-dependently increased following repeated exposure of rats to MWCNTs. AST activity significantly ($P < 0.05$) increased by 35%, 53%, 56% and 58% whereas ALT activity significantly increased by 24%, 31%, 37% and 38% at 0.25, 0.5, 0.75 and 1.0 mg/kg respectively, compared with the control. ALP activity was significantly increased by 13%, 24%, 32% and 35% at 0.25, 0.5, 0.75 and 1.0 mg/kg respectively whereas GGT activity was increased by 17%, 32%, 41% and 49% at 0.25, 0.5, 0.75 and 1.0 mg/kg respectively, when compared with the control group.

Moreover, there was a significant, dose-dependent increase in serum levels of pro-inflammatory cytokines namely IL-1β, IL-6 and TNF-α in rats treated with at 0.25, 0.5, 0.75 and 1.0 mg/kg when compared with control. The serum level of IL-1β increased by 28%, 36%, 40% and 46%; IL-6 increased by 24%, 29%, 30% and 38% whereas TNF-α level increased by 17%, 34%, 42% and 47% at 0.25, 0.5, 0.75 and 1.0 mg/kg respectively, compared with the control. In addition, administration of carboxylated MWCNTs significantly increased the frequency of micronucleated polychromatic erythrocytes (MNPCE) in a dose-dependent manner in the treated rats. The percentage increase in the frequency of MNPCE in rats administered with MWCNTs at 0.25, 0.5, 0.75 and 1.0 mg/kg were 60%, 80%, 83% and 87% respectively, when compared with the control.

3.3. Carboxylated MWCNTs induce dose-dependent increase in the expression of pro-inflammatory enzymes

Further, investigation into the influence of carboxylated MWCNTs on pro-inflammatory enzymes was performed by analyzing the immunohistochemical expression of COX-2 and iNOS in liver of the treated rats. As shown in Figs. 3 and 4, repeated administration of carboxylated MWCNTs resulted in a dose-dependent increase in the intensity of COX-2 and iNOS expression in the liver of the treated rats when compared to control. The percentage increase in the intensity of hepatic COX-2 expression in rats administered MWCNTs at 0.25, 0.5, 0.75 and 1.0 mg/kg were 29%, 60%, 71% and 75% whereas hepatic iNOS expression increased by 19%, 63%, 75% and 80% respectively, when compared with the

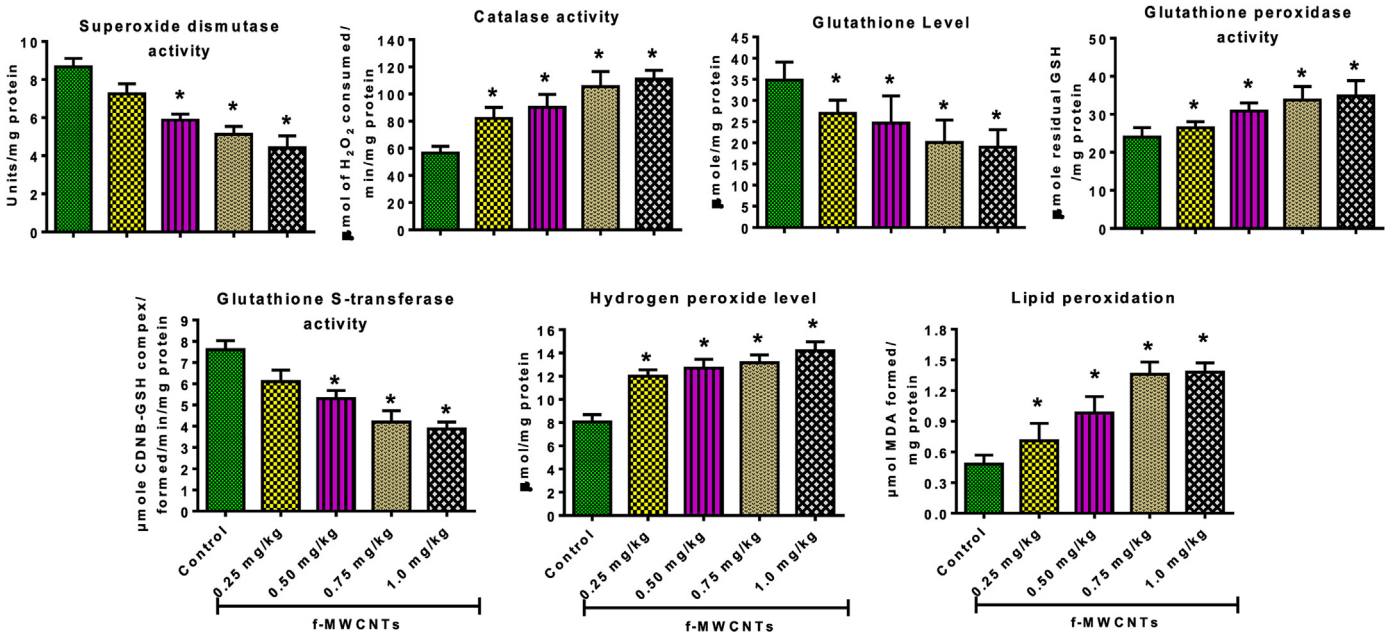

Fig. 1. Hepatic antioxidant enzyme activities and levels of glutathione and lipid peroxidation following administration of carboxylated MWCNTs to rats for 5 consecutive days. Each bar represents mean ± SD of ten rats. *: Values differ significantly from control ($p < 0.05$).

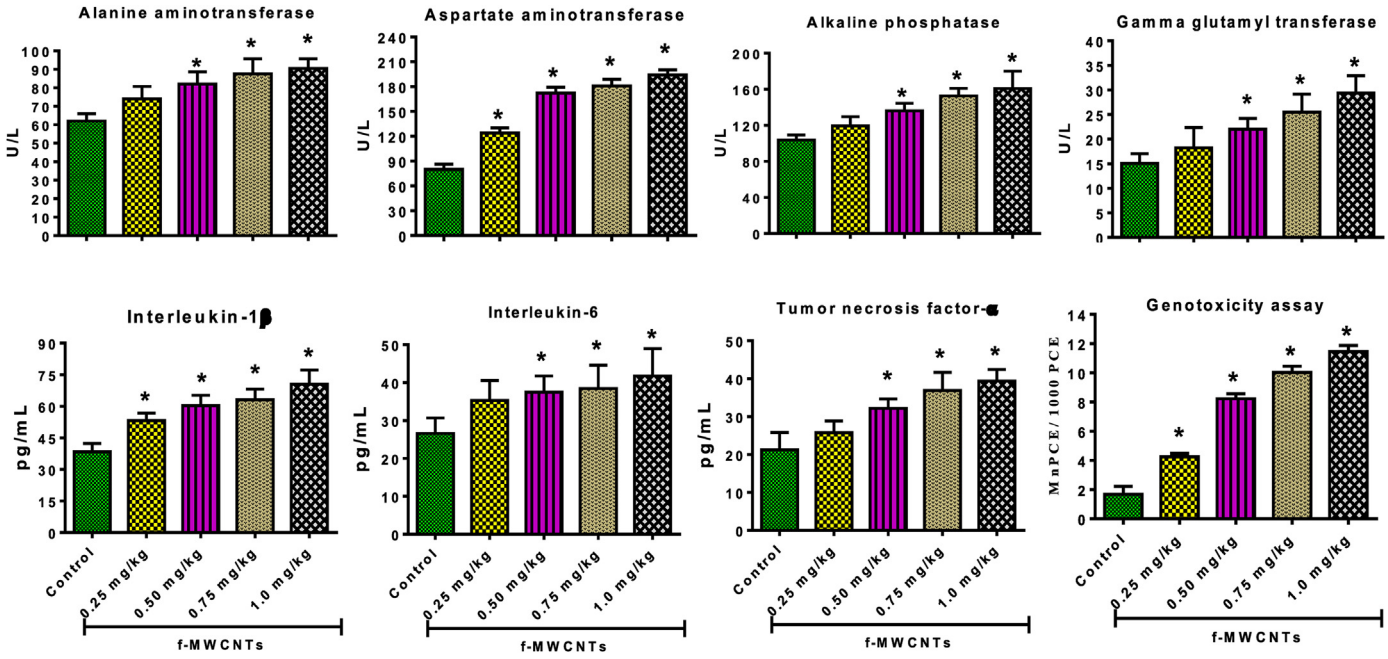

Fig. 2. Biomarkers of hepatic damage, inflammation and genotoxicity following administration of carboxylated MWCNTs to rats for 5 consecutive days. Each bar represents mean ± SD of ten rats. *: Values differ significantly from control ($p < 0.05$).

control.

4. Discussion

Nanotechnologies generate several kinds of engineered nanomaterials which have promising application benefits. Although the potential environmental and human health risk assessment of nanomaterials is still ongoing, the cellular and molecular effects of these nanotubes is yet an enigma and not fully understood. The present investigation demonstrated, for the first time, that pubertal exposure of rats to low doses of purified, long carboxylated MWCNTs elicited hepatotoxicity *via* disruption of antioxidant defense systems, increase in pro-inflammatory cytokines and expression of COX-2 and i-NOS in rats.

The natural antioxidant defense mechanisms against free radicals and ROS mediated tissue damage consists of enzymatic and non-enzymatic antioxidants. The first line of cellular defense against oxidative insult is executed largely by the mutual action between SOD, which accelerates the conversion of endogenous cytotoxic superoxide radicals to H_2O_2, and CAT which converts

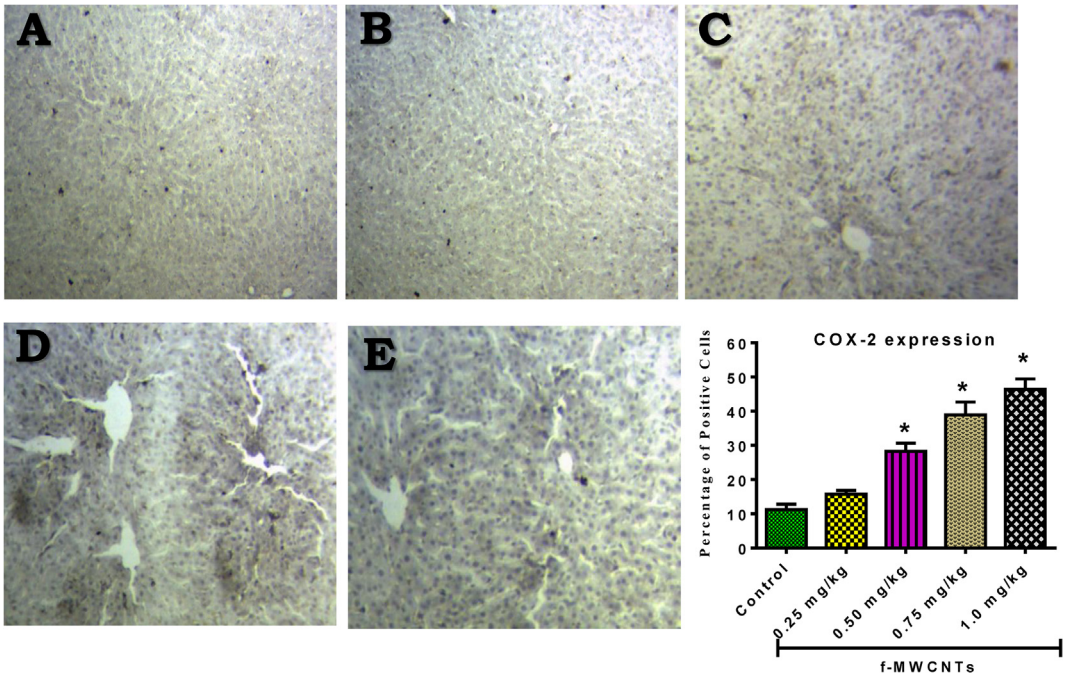

Fig. 3. Hepatic histopathology guide showing the influence of carboxylated MWCNTs on COX-2 expression in control and treated rats. Percentage of positive cells are presented in the graph. Each bar represents mean ± SD of ten rats per group. *: Values differ significantly from control ($p < 0.05$).

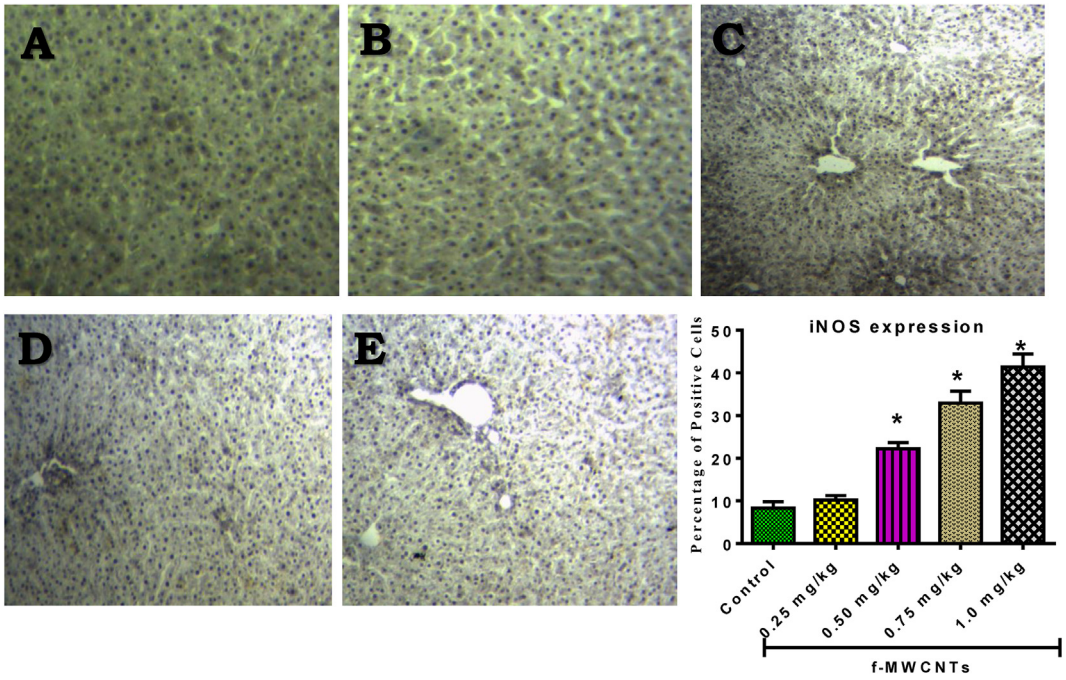

Fig. 4. Hepatic histopathology guide showing the influence of carboxylated MWCNTs on iNOS expression in control and treated rats. Percentage of positive cells are presented in the graph. Each bar represents mean ± SD of ten rats per group. *: Values differ significantly from control ($p < 0.05$).

deleterious peroxide radicals into water and oxygen [23]. The dose-dependent decrease in the hepatic SOD activity in MWCNTs-treated rats indicates enzyme inhibition which may lead to hepatic accumulation of the cytotoxic superoxide radicals in the rats. The increase in the CAT activity possibly suggest adaptive response of the cell to combat induction of cellular ROS.

Moreover, the harmful effects of H_2O_2 molecules is related to the direct activity emanating from their oxidizing properties and the indirect activity in which they produce a more toxic species namely as hydroxyl radicals and hypochlorous acid [24]. Glutathione is a non-enzymatic endogenous antioxidant known to provide secondary line of defense against intracellular generation of free radicals and peroxides following exposure to xenobiotics [25]. GPx protects cellular macromolecules against oxidative damage by catalyzing the GSH-dependent elimination of H_2O_2 and lipid hydroperoxides in cells [25,26] whereas GST, a phase-II metabolizing

enzyme, catalyzes biochemical conjugation of toxic electrophilic molecules with GSH [27,28]. The diminution in the hepatic GSH level and GST activity indicates overutilization of GSH in the scavenging of free radicals and inhibition of GST-mediated detoxification process in the MWCNTs-treated rats. Moreover, the marked increase in the hepatic GPx activity and the elevated H_2O_2 and LPO observed in MWCNTs-treated rats indicate failure of the cellular adaptive GPx response of mitigate oxidative damage in the liver of MWCNTs-treated rats.

Aminotransferases namely AST and ALT are localized in peripotal hepatocytes where they play a pivotal role in transamination reactions during amino acid metabolism. They are recognized biomarker enzymes of early acute hepatic damage because their serum or plasma activities increase following loss of hepatocyte structural integrity and leakage [29]. Moreover, serum ALP activity is a measure of the integrity of hepatobiliary system and the movement of bile into the small intestine. The present investigation demonstrated that short-term exposure to MWCNTs treatment elicited hepatic damage evidenced by the dose-dependent, significant increase in serum AST, ALT, ALP and GGT activities in the treated rats. The increase in serum ALP activity in MWCNTs-treated rats indicates an obstructive event or cholestatic effect which was corroborated by the similar elevation in serum GGT activity in the treated rats.

Further, the present study demonstrated that administration of purified MWNCNTs to rats caused a dose-dependent, significant increase in the serum levels of the pro-inflammatory cytokines namely IL-1β, IL-6 and TNF-α when compared with the control. TNF-α is well-known to play an essential role in recruiting immune cells at the sites of injured tissues. TNF-α is recognized as a "master-regulator" of cytokine production during an inflammatory response [30]. Thus, the increase in the serum levels of IL-1β, IL-6 and TNF-α in MWCNTs-treated rats clearly indicates induction of inflammation in the treated rats. The dose-dependent increase in the frequency of micronucleated polychromatic erythrocytes observed in the present study indicates the mutagenic potential of MWCNTs in the treated rats.

Besides, elevation in the TNF-α level reportedly up-regulate iNOS to increase NO production [31] which consequently aggravates cellular damage when it reacts with superoxide anion to form peroxynitrite, a reactive nitrogen species [32]. Cyclooxygenase-2, a rate limiting enzyme in the biosynthesis of prostaglandin, is well established to be largely inducible in response to pro-inflammatory stimuli [33]. In the present study, immunohistochemical analysis demonstrated that MWCNTs treatment resulted in an increased expression of COX-2 and iNOS in liver of the treated rats, thus corroborating the serum biochemistry data on pro-inflammatory cytokines. The increase in the expression of iNOS and COX-2 in liver of rats treated with MWCNTs in the present investigation could contribute to hepatic damage *via* induction of inflammation and nitrosative stress in the treated rats.

In conclusion, repeated short-term exposure to purified carboxylated MWCNTs elicits hepatic oxidative damage in pubertal rat *via* disruption of antioxidant defense systems, promotion of pro-inflammatory cytokines generation and expression of COX-2 and i-NOS. Moreover, MWCNTs exposure may pose genotoxic threat to exposed individual.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Transparency document

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