THE TOXIC EFFECT OF MULTI WALL CARBON NANOTUBES ON SWISS ALBINO RAT

Zoology Department, Faculty of Science, Cairo University.
*National Center for Radiation Research and Technology-Egyptian Atomic Authority.
** Pathology Department, Faculty of Veterinary Medicine, Cairo University.

ABSTRACT
Production and usage of multi-walled carbon nanotubes (MWCNTs) have widely increased over the last years. Nanoparticles with their sizes below 100 nm are able to enter and be stored in organs (such as liver, lung, testes and brain) and caused toxic effects. The aim of the present study is to investigate the MWCNTs toxicity, if any, on liver tissue. A total number of fifteen male albino rats were used in the present study; five rats as control and ten rats were intravenous injected with a single dose of MWCNTs (30 mg/ kg body weight, 20-50 nm in diameter and 1 µm in length). Histopathological, histochemical, apoptotic, necrotic and ultra-structure studies were carried out in the present work. The results showed different histopathological changes on liver tissues of MWCNTs treated rats, such as focal hepatic necrosis with inflammatory cells infiltration, hydropic degeneration of hepatocytes, and some increases in collagen fibers deposition in the portal area after 28 days of MWCNTs injection. High appearance of necrotic and apoptotic cells was also noticed. Ultrastructure study of liver tissue of rats treated with MWCNTs showed abnormal hepatocytes with irregular nuclear envelope and nuclear chromatin material, swollen and fused mitochondria with destructed cristae, lysis of some cytoplasmic organelles and rupture of the endoplasmic reticulum. Shrunken nucleus and abundance of vacuoles and lysosome were also observed in the hepatocytes. It could be concluded that MWCNTs have a toxic effect on liver tissue and hepatocytes of rats.

Keywords: Carbon nanotubes; Nanoparticles; Toxicity; Liver tissue.

INTRODUCTION
Nanoparticles (NPs) can be used in medicine for cancer treatment, infectious diseases, and diagnostic procedures with new imaging sensors and agent (Kagan et al., 2005; Shvedova et al., 2009). In general, the NPs have usually been made from transition metals, silicon, metal oxides, and different forms of carbon (carbon nanotubes and fullerenes). Inorganic NPs do not include carbon atom while carbon nanotubes and carbon fullerenes are titled as organic NPs (Ferreira et al., 2013).
Carbon nanotubes (CNTs) have attracted much more industrial interest because of their unique properties (Kaiser et al., 2011). They could be manufactured as single- or multi-walled tubes. The majority of diameters of single-walled carbon nanotubes (SWCNTs) are less than 10 nm, while those of multi-walled carbon nanotubes (MWCNTs) are generally above 20 nm up to 150 nm (Hou et al., 2003; Donaldson et al., 2006).
CNTs have been used in biomedical engineering, tissue engineering, drug delivery, gene therapy, biosensors, cancer therapy, vaccine delivery, imaging and diagnostics (Spana et al., 2012). There are increasing probabilities for humans to contact CNTs as a result of their wide applications in the biomedical and material science field. Production and usage of MWCNTs have widely increased over the last years and nanoparticles with their sizes below 100 nm are able to enter and be stored in organs such as liver, lung, testes, and brain, causing toxic influences (Dan and Wan, 2016).
The aim of the present study is to investigate the toxicity of MWCNTs on liver of male albino rats as being a vital organ as there is a scarcity of researches on this subject.
MATERIAL AND METHODS

Animals
Fifteen (15) healthy male albino rats (*Rattus norvegicus*) weighing 100-120 g, purchased from the Egyptian Holding Company for Biological Products and Vaccines (Cairo, Egypt), were used in this study.

The animals were housed in specially designed individual plastic cages, five per cage, under standard conditions of 12-h light /12-h dark cycle, normal temperature, good ventilation and humidity range. The animals were provided with a pellet concentrated diet containing all the necessary nutritive elements and tap water *ad libitum*. Animals were acclimatized to laboratory conditions before starting, for fourteen (14) days, the experiment. They were maintained according to the Ethics Committee of the National Research Center and in accordance to the "Guide for the Care and use of Laboratory Animals " published by the US National Institutes of Health (NIH publication No.85-23, 1996).

Characteristics and Preparation of MWCNTs
MWCNTs were obtained from the Egyptian Petroleum Research Institute (EPRI). It was synthesized by using a chemical vapour deposition (CVD) method. According to the product specification, MWCNTs are 1 µm in length and 20 – 50 nm in diameter, their purity is > 93% and has impurities less than 7 %. The impurities are cobalt oxide, magnesium oxide and amorphous carbon. MWCNTs were suspended in saline using 0.1 % Tween 80 and then sonicated for about 1 hour using ultra-sonic bath (Branson 1510, England).

Experimental Design
Fifteen (15) healthy male albino rats were used in the whole study. They were divided into two groups,
Group 1: sham untreated control rats (n=5).
Group 2: rats intravenous injected through the caudal vein by a single dose of 30 mg/kg bw MWCNTs (n=10).
All the animals were euthanized after 7 and 28 days of MWCNTs injection.

Liver tissues were collected for histopathological, apoptotic and necrotic and ultra-structure studies. Sections (4 – 5 µm) for histopathological study were stained with haematoxylin and eosin stain (Bancroft and Gamble, 2002). Microscopic examined and a number of photomicrographs were taken at known magnification using a light microscope (OLYMPUS BX50). For histochemical study, the obtained tissue sections were stained by Masson's trichome for the detection of collagen fibers deposition (Carson, 1990).

Apoptotic and necrotic detection were evaluated according to the method of Coligan *et al.* (1995) and Ribble *et al.* (2005) using propidium iodide and acridine orange (PI/AO) stains, tissue sections were examined and photographed with a fluorescent microscope. Electron microscopic sections were prepared according to the method of Bancroft and Gamble (2002) and examined and photographed using JEOL JEM.1010 transmission electron microscope (TEM).

RESULTS

Histological and histopathological study
The liver of control group of male albino rat showed the normal histological structure presented previously elsewhere (Fig. 1, A). Liver of rats treated with 20 – 50 nm MWCNTs (30 mg/kg bw) showed hydropic degeneration of hepatocytes, focal hepatic necrosis associated with inflammatory cells infiltration and abundance of Küpffer cells(Fig. 1, B and C) after 7 days of MWCNTs injection. Same changes were observed when the rats were treated with the same dose and studied after 28 days (Fig. 1, E). Cytoplasmic vacuolation of centro lobular hepatocytes (Fig. 1, D), dilatation and congestion of hepatic sinusoids (Fig. 1, F) and a massive infiltration of portal area with inflammatory cells (Fig. 1, G) were also detected.
Collagen fiber deposition in the liver tissue

Light microscopic examination of liver sections of the control rats stained with Masson's trichrome showed normal distribution of the collagen fibers supporting the walls of the blood vessel, and around the hepatocytes (Fig. 2, A). Liver of rat treated with 20 – 50 nm MWCNTs (30 mg/kg bw) exhibited some increase in the collagen fibers deposition of the portal area (stained blue) (Fig. 2, B) on day 7 after MWCNTs injection. Normal collagen fibers deposition surrounding the hepatocytes was also observed, while after 28 days of treatment, some increase was noticed in the central vein wall (Fig. 2, C) and in the portal area (Fig. 2, D).

Apoptosis and necrosis detection

Apoptotic and necrotic changes were stained by PI/AO Compartments are represented in the liver tissue of normal untreated rat by a vital green fluorescent tissue cells (Fig. 3, A). Liver sections of rats treated with 20 – 50 nm MWCNTs (30 mg/kg bw) and examined after 7 days of injection showed high appearance of necrotic (orange cells) and apoptotic (bright yellow cells) hepatocytes (Fig. 3, B). The aforementioned high apoptotic appearance of hepatocytes was also noticed after 28 days of treatment (Fig. 3, C).

Ultra structure study of the liver tissue

Electron micrographs of liver sections of control rat showed normal hepatocytes, as well as nucleus, mitochondria, lysosomes and rough endoplasmic reticulum (Fig. 4, A). While those treated with 30 mg/kg bw MWCNTs after 7 days displayed rupture in some areas of the endoplasmic reticulum, swollen electron dense and fused mitochondria with ruptured membranes and degenerated cristae. Also, abnormal nucleus with irregular nuclear envelope and nuclear chromatin condensation was observed. Vacuoles and lysis of some cytoplasmic organelles were also apparent (Fig. 4, B & C). After 28 days of the same treatment, shrunken nuclei, red blood cells, ill-defined mitochondria with ruptured membranes and deteriorated cristae, abundant lysosomes and vacuoles were also observed in the hepatocytes (Fig. 4, D & E).

DISCUSSION

There are increasing possibilities for humans to contact CNTs because of their extensive applications in the field of biomedical and material science. MWCNTs have potential applications in many diverse commercial processes, and thus human exposures are possible.

The histopathological alterations observed in the liver of rats treated with 30 mg/kg bw MWCNTs, in the present study, were summarized as cytoplasmic vacuolation of hepatocytes, focal hepatic necrosis associated with inflammatory cells infiltration as well as dilatation and congestion of hepatic sinusoids, abundance of Küpffer cells, in addition to portal infiltration with inflammatory cells, compared to control liver.

Liver sections of rats stained with Masson's trichrome showed also increased collagen fiber deposition in the portal area, while, normal collagen fiber deposition were supporting walls of the blood vessels, central veins and around the hepatocytes. On the other hand, examination of liver tissues under fluorescent microscope showed a high appearance of apoptotic and necrotic hepatocytes.

The ultrastructure examination of liver hepatocytes treated with 30 mg/kg bw MWCNTs showed swollen and fused mitochondria with rupture of their cristae, rupture and lysis of the endoplasmic reticulum cisternae as well as abnormal nucleus with irregular nuclear envelope and abnormal nuclear chromatin material were noticed after 7 days of the MWCNTs injection. In addition to the stated observations, ill-defined mitochondria, dilated blood sinusoid with red blood cell, shrunken nucleus, abundance of vacuolation and lysosomes were also detected after 28 days of the MWCNTs injection compared to control group.
These results are in accordance with Zhang et al. (2018), who studied the effects of MWCNTs on the liver of adult mice and their offspring. They observed histopathological changes in the liver tissues of the mice offspring. The hepatocytes of female treated mice were loose and highly vacuolated, while their offspring liver showed irregular arrangement of liver cells, hepatic sinus dilation, unclear lobular structure, significant inflammatory cell infiltration, and some lipid droplets.

These results were also in agreement with Zhang et al. (2017), who demonstrated that pristine multi-walled carbon nanotubes (P-MWCNTs) induced high level of inflammation. They also summarized that P-MWCNTs could cause different degrees of pathological injury in the liver of mice such as, structural disorder of liver lobules and loss of some sinusoidal space, mild sinusoidal congestion and mild vacuolar degeneration of hepatocytes.

In addition, Hougaard et al. (2013) showed microfoci of necrosis, enlargement of single hepatocytes, increased number of Kupffer cells and binucleated hepatocytes in experimental animals exposed to MWCNTs compared to the control liver. Ji et al. (2009) showed severe inflammatory cell infiltration in the portal region, cellular and focal necrosis at a dose of 60 mg/kg MWCNTs dispersed in Tween-80 (T-MWCNT). Moreover, Awasthi et al. (2013), demonstrated the histopathological changes of the liver of Swiss albino mice exposed to MWCNTs showing remarkable morphological alterations such as individual cell necrosis indicated by karyorrhexis as well as sinusoid dilation, hepatocyte disruption, vacuolation, swelling, fatty changes, hemorrhagic clots and necrotic changes when compared to controls. Many studies showed also that MWCNTs can induce inflammation, fibrosis and angiogenesis (Boyles et al., 2015).

It is well known that the liver is the first organ to encounter ingested nutrients, vitamins, drugs, metals and environmental toxicants that enter portal blood. The functions of liver can be destructively altered by liver injury resulting from chronic or acute exposure to toxicants (Klaassen and Watkins, 2015). Because the liver is a reticuloendothelial system organ, nanoparticles entering circulation mostly accumulate in the liver cells and are primarily taken up by macrophages of the liver, where they activate an inflammatory response through an oxidative stress mediated mechanism (Ahmad et al., 2012). These cells participate in acute and chronic responses of liver to toxic compounds (Roberts et al., 2007). The later authors added that activation of Kupffer cells by toxic agents both directly and indirectly resulted in releasing an array of inflammatory responses. It should be taken into consideration that CNTs may damage or interfere with DNA (Lam et al., 2006; Kolonsiaj et al., 2007) and this could explain the observed damage in the liver tissue, high appearance of apoptotic and necrotic hepatocytes, due to the treatment with MWCNTs. Apoptotic bodies derived from the damaged hepatocytes can activate quiescent stellate and Kupffer cells, and these activated cell populations can in turn promote inflammatory and fibrogenic responses (Lee et al., 2011).

It has been found that CNTs entered the liver via the circulation and were taken up by Kupffer cells being a part of the mononuclear phagocyte system. Kupffer cells are the resident macrophages of liver and play an important role in its normal physiology and homeostasis. The activation appears to control the acute hepatocyte injury along with chronic liver responses (Roberts et al., 2007).

On the other hand, many previous studies have pointed out that reactive oxygen species (ROS) is a key player, which mediates many of the effects of nanomaterials. In vitro study also showed that the toxicity of MWCNTs to the hepatocyte cell line was at least in part mediated by reactive oxygen species/oxidative stress (Kermanizadeh et al., 2012). Both in vitro and in vivo studies have shown that nanoparticles are strongly associated with toxicity by increasing the level of pro-inflammatory mediators and/or intracellular ROS levels. Nanoparticles are also known to up-regulate the transcription of various pro-inflammatory genes, including tumor necrosis factor-α (TNFα) and IL (interleukins)-1, IL-6 and IL-8, by activating nuclear factor-kappa B (NF κB) signaling. These sequential molecular and cellular events are known to cause oxidative stress, followed by severe cellular genotoxicity and then programmed cell death (Khanna et al., 2015). This finding supports
and explain the observation of apoptotic and necrotic cells in the liver of MWCNTs of the studied groups.

Kam and Dai, (2005) reported that CNTs can be internalized by clathrin mediated endocytosis. Clathrin-coated pits are formed under the plasma membrane when CNTs come in contact with cells, then each pit forms a vesicle inside the cell. The lysosomes act as the waste removal system of the cell by digesting undesirable materials in the cytoplasm, from outside and inside the cell. Material from outside the cell is taken-up through endocytosis (Mindell, 2012). This finding supports the abundance of lysosomes in liver hepatocytes observed by TEM in the present study.

Xu et al. (2016) imaged the uptake of MWCNTs was imaged by TEM in vitro and showed that MWCNTs accumulated in the mitochondria of GC-2pd (the mice spermatocyte cell line) caused potential mitochondrial DNA damage, and decreased oxygen consumption rate of the cells compared to control group. They also found that the ATP content detected in GC-2pd cells treated with MWCNTs was lower than control cells in addition to Jiang et al. (2013) found that functionalized MWCNTs via carboxylation (MWCNTs-COOH) have a cytotoxic effect and induced apoptotic cell death of macrophages in vitro. They showed that MWCNTs-COOH induced the release of cytochrome C from mitochondria to the cytosol, increased the expression of pro-apoptosis Bcl-2 proteins and suppressed the expression of pro-survival Bcl-2 proteins.

Fang et al. (2018), studied also the in vivo effect of MWCNTs on liver hepatocytes in male mice using TEM and found that mitochondrial abnormalities, included reduction, disorganization, and fractures. They found also that MWCNTs have oxidative stress by producing ROS and genotoxic effect. Excess production of ROS destroyed the antioxidant defence system and resulted in many sub-cellular injuries, including membrane damage, protein denaturation and DNA damage (Nel et al., 2006). This finding supports the ultrastructure results observed in hepatocytes of the present study.

This study may conclude that MWCNTs intravenous injected in male albino rats showed some histopathological changes to the liver tissue, increased collagen fiber deposition in portal area and may also cause apoptosis of liver cells. MWCNTs have also a toxic effect on mitochondria of hepatic cells studied by TEM.

REFERENCES


EXPLANATION OF FIGURES

**Fig. 1:** (A): Light micrographs of liver sections of control rats showing normal hepatocytes (arrow), centrilobular vein (CV), blood sinusoids and Kupffer cells (I). (B) & (C): Liver of rat treated with 30 mg/kg bw MWCNTs, after 7 days of MWCNTs injection showing hydropic degeneration of hepatocytes (⃣), abundant Kupffer cells (I) and focal hepatic necrosis associated with inflammatory cells infiltration (★). (D, E, F & G): Liver of rat treated with 30 mg/kg bw MWCNTs, after 28 days of MWCNTs injection (D): showing cytoplasmic vacuolation of centrolobular hepatocytes (curved arrow). (E): showing hydropic degeneration of hepatocytes (⃣), abundant Kupffer cells (I) and focal hepatic necrosis associated with inflammatory cells infiltration (★). (F): showing dilatation and congested of hepatic sinusoids ( ). (G): showing massive infiltration of portal area with inflammatory cells (★). (H&E X 400).

**Fig. 2:** Photomicrographs of liver sections of rats. (A): Control liver section showing normal distribution of collagen fibers around the central vein and hepatocytes. (B), (C) & (D): Liver sections of rats treated with 20 – 50 nm MWCNTs (30 mg/kg bw) after 7 days of MWCNTs injection (B): showing some increase in collagen fibers deposition in the portal area ( ). After 28 days of MWCNTs injection (C): showing normal distribution of collagen fibers surrounding the hepatocyte ( ) (D): showing some increase in collagen fiber deposition around central vein and bile duct ( ). (Masson trichrome stain, X 400 for all micrographs).

**Fig. 3:** Fluorescent photomicrograph of liver of rat showing (A): Control liver with viable green cells in liver tissue. (B) & (C): Liver tissue treated with 30 mg/kg bw MWCNTs (B): High appearance of ) after ( ) and apoptotic hepatocytes (bright yellow color)★ (necrotic ) hepatocytes at 28 days (7 days of MWCNTs injection. (C): Apoptotic after treatment. (PI/AO stain, X250).

**Fig. 4:** Electron micrographs of hepatocytes of rat liver showing (A): normal hepatocytes, nucleus (N), nucleolus (Nu), mitochondria (M), lysosomes (Ly) and rough endoplasmic reticulum (rER). (B), (C), (D) & (E) Liver of rat treated with 30mg/kg bw MWCNTs, after 7 days of MWCNTs injection (B): Ruptured endoplasmic reticulum (rER), swollen electron dense and fused mitochondria with rupture of some mitochondrial membranes and cristae (M), abnormal nuclear structure (N) with irregular nuclear envelope and nuclear chromatin material. (C): Mitochondria with some ruptured cristae (M), vacuoles (V) and lysis of ), after 28 days of MWCNTs injection★ (some cytoplasmic organells (D): Dilated blood sinusoid (BS) with presence of red blood cell (RBC), ill-defined mitochondria (M) and vacuoles (V) & (E): Shrunken nucleus (N), ill-defined mitochondria (M), lysosomes (Ly) , and vacuoles (V).
Fig. 1
Carbon nanotubes toxicity in albino rats

**Fig. 2**

**Fig. 3**
Fig. 4
التأثير السمي لأنابيب الكربون النانونية متعددة الجدران على كبد الجرذان السويسري البيضاء

صفاء غريب السيد – نعمة حنفي أحمد* - كوكب عبدالعزيز أحمد* - علياء محمود عيسى**
قسم علم الحيوان – كلية العلوم جامعة القاهرة *مركز القومي للبحوث والتقنية=lية العلوم - كلية الطب البيطرية – جامعة القاهرة 

ازداد إنتاج واستخدام أنابيب الكربون النانونية متعددة الجدران على نطاق واسع خلال السنوات الأخيرة والمواد النانونية ذات الأحجام الأقل من 111 نانومتر لها القدرة ان تدخل وتختزن بعض الأعضاء مثل الكبد والرئة والخصيتين والدماغ وتشير تأثير سام على هذه الأعضاء. لذلك تهدف هذه الدراسة إلى بحث مدى سمية أنابيب الكربون النانونية متعددة الجدران – إن وجدت – على أنسجة وخلايا الكبد في ذكور الجرذان السويسري البيضاء.

في هذه الدراسة، تم استخدام عدد خمسة عشر (15) من ذكور الجرذان البيضاء بخمسة (5) كمجمعة ضابطة ومثل حجرة وحيدة من أنابيب الكربون (31) ملم جرام لكل كيلو جرام من وزن الجسم لكل جرذ، حيث كان قطر هذه الأنابيب 21-نانومتر وطولها 1 ميكرومتر. واستخدمت الدراسات البيولوجية والكيميائية، والعصبية، والأنسجة، وفحص التركيب الدقيق لخلايا الكبد باستخدام المجهر الإلكتروني النافذ.

و بعد 7 و 28 يومًا من حقن أنابيب الكربون النانونية متعددة الجدران، وجد أن لها تأثيرات نسيجية مختلفة على أنسجة كبد الجرذان، مثل النخر الكبدى البورى مع ارتشاح الخلايا الالتهابية، التنكس المائي لخلايا الكبد، وانتشار الزهاد في نسيج الكبد. كما أظهرت الظاهرة عن طريق الفحص باستخدام المجهر الإلكتروني النافذ لخلايا الكبد وجود خلايا كبدية غير طبيعية، وخصوصًا في خلايا الكبد، من بعض مظاهر هذا الخلل وجود عديدات متكونة من الخلايا المتجلبة، وظهور نواة منكسة، ونواة أخرى ذات نسيج غير منظم ومواد نوية غير طبيعية. كما لوحظ أيضاً وفراة من الفجوات والليوسومات.

من هذه الدراسة يستنتج أن أنابيب الكربون النانونية متعددة الجدران ذات تأثير سام على أنسجة الكبد والخلايا الكبدية في ذكور الجرذان السويسري البيضاء.