Toxicological assessment of multi-walled carbon nanotubes combined with nonylphenol in male mice

Hao Fang¹, Yibin Cui²*, Zhuang Wang¹, Se Wang¹

¹ Collaborative Innovation Center of Atmospheric Environment and Equipment Technology (AEET), School of Environmental Science and Engineering, Nanjing University of Information Science and Technology, Nanjing, China, ² Nanjing Institute of Environmental Sciences, Ministry of Environmental Protection, Nanjing, China

* cyb@nies.org

Abstract

Carbon nanotubes have attracted increasing attention attributable to their widespread application. To evaluate the joint toxicity of multi-walled carbon nanotubes (MWCNTs) and nonylphenol (NP), we investigated the toxicological effects of NP, pristine MWCNTs, and MWCNTs combined with NP in male mice. After exposing male mice by gavage for 5 days, intracellular superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity, as well as malondialdehyde (MDA) and glutathione (GSH) levels in tissues were determined to evaluate in vivo oxidative stress. In addition, genotoxicity was assessed by examining DNA damage in mouse liver and sperm via the comet assay, and transmission electron microscopy (TEM) was used for direct visual observations of mitochondrial damage in the liver. Results from the oxidative damage and DNA damage experiments indicate that after adsorbing NP, MWCNTs at a high dose induce oxidative lesions in the liver and cause DNA damage in mouse sperm; these data offer new insights regarding the toxicological assessment of MWCNTs.

Introduction

There has been a rapid growth in the application of nanoscale materials, attributable to the development of nanotechnologies [1]. Carbon nanotubes (CNTs), which were discovered in 1991 [2], have attracted a great deal of attention owing to their unique structural, electrical, and mechanical properties, which make them potentially useful in extremely small-scale biological, electrical, and mechanical applications. Evidence suggests that CNTs adsorb organic pollutants effectively. For instance, CNTs adsorb environmental endocrine disruptors (EEDs), neutral dissolved organic matter, and trihalomethanes more extensively than activated carbon materials do [3–5]. Therefore, CNTs are potential adsorbents of these organic pollutants.

Nonylphenol (NP) is a xenobiotic compound used globally to manufacture nonylphenol ethoxylate surfactants and is widely known as a type of EED [6]. Because of its low solubility and high hydrophobicity, NP accumulates under certain environmental conditions, such as in
wastewater treatment plants, river water, sediments, and soils. For example, NP can be detected in the surface water of the Daliao river estuary of China, with concentrations ranging from 83.6 to 777 ng/L [7]. It has also been reported that NP exists in pork, chicken, and beef, with concentrations ranging from 0.50 to 0.67 mg/kg [8, 9]. Previous studies have confirmed that NP may interfere with animals and various types of cells more than other EEDs via several mechanisms, including increased proliferation of mammary gland cells [10], production of telemetric associations and chromosomal aberrations [11], irreversibly influencing the reception of fear-provoking stimuli in male rats at a low dosage of 0.1 mg/kg/day [12], inhibiting the growth and differentiation of murine neural stem cells and inducing apoptosis [13], and inducing oxidative stress [14] and reproductive toxicity in Muridae animals [15]. In particular, much attention was paid to NP-induced disruption of male reproductive toxicity, such as reduced testis and epididymis weights as well as decreased sperm count and motility [15, 16].

Although studies have shown that electrochemical-assisted adsorption on multi-walled CNTs (MWCNTs) removes 4-nonylphenol (4-NP) efficiently [17], there is growing concern regarding the safety of CNTs [18–21]. Intense investigations of the adverse health effects have focused on CNT toxicity both in vivo and in vitro. To date, several in vivo studies have shown that CNTs may induce various toxicities, including an increase in the inflammatory response, oxidative stress, granuloma formation, and fibrosis [22–25]. In vitro investigations have confirmed these physiological and biochemical responses and provide further support to explain the increased incidence of oxidative stress in cells after exposure to CNTs [26–30]. In addition, NP adsorption on MWCNTs facilitates its bioavailability in the earthworm (Eisenia fetida) and increases ecological risks [31]. A recent study indicates that MWCNTs cause toxicity to the invertebrate, Daphnia magna, in water [32]. However, to date little is known about the environmental health risks resulting from NP adsorption on MWCNTs.

In this study, CD-1 (ICR) mice were exposed to 4-NP, MWCNTs, or 4-NP adsorbed on MWCNTs (MWCNTs+NP) by gavage; in vivo oxidative effects and genotoxic responses to stress, including intracellular superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity, as well as malondialdehyde (MDA) and glutathione (GSH) levels were evaluated. We assessed the genotoxic response by measuring DNA damage in mouse liver and sperm via the comet assay. Further, transmission electron microscopy (TEM) was used for direct visual evidence of mitochondrial damage in liver cells. The objective of this study was to evaluate oxidative and genotoxic effects of NP adsorbed on MWCNTs in mice, along with potential mechanisms.

**Materials and methods**

**Materials**

MWCNTs made via chemical vaporization deposition (CVD) were obtained from Shenzhen Nanotech Port Co. Ltd. (Shenzhen, China). Morphology and specific surface area of the MWCNTs were determined via TEM (H-7500, Hitachi, Japan) and multipoint Brunauer-Emmett-Teller (BET) analysis, respectively. 4-NP was purchased from Acros Organics Co. Ltd. (New Jersey, USA), and purity was greater than 99%.

**Animals and exposure procedure**

CD-1 (ICR) male mice (18 ± 2 g, 4 weeks) were purchased from Beijing Vitalriver Experimental Animal Technology Co. Ltd. (Beijing, China). The animals were housed and maintained on a commercial pellet diet, given distilled water ad libitum, and kept in plastic cages in a ventilated animal room. Room temperature was controlled at 22 ± 1 °C and relative humidity was maintained at 60 ± 10%, with a 12 h light/dark cycle. The mice were acclimated to this

**Competing interests:** The authors have declared that no competing interests exist.
environment for 1 week. All experimental animal procedures were approved by the Ethics Committee of Nanjing University of Information Science and Technology. Animal management was performed strictly in accordance with the standards of the Animal Ethics Committee of Nanjing University of Information Science and Technology. All sections of this report adhere to the ARRIVE Guidelines for reporting animal research [33]. A completed ARRIVE Guidelines Checklist is included in S1 Checklist.

The animals were randomly divided into five groups of five animals each as follows: (1) normal saline control group (CK group), (2) dimethyl sulfoxide (DMSO) vehicle control group (DMSO group), (3) 4-NP treatment group administered 5 mg/kg body weight (NP group), (4) MWCNTs treatment group administered 100 mg/kg body weight (MWCNTs group), and (5) 100 mg/kg MWCNTs+NP treatment group administered 5 mg/kg (MWCNTs+NP group). In addition, 100 μmol/L hydrogen peroxide (H₂O₂) was used as a positive control to ensure that the comet assay was functioning properly. For the NP group, 50 mg 4-NP was dissolved in 100 ml DMSO. Since DMSO has been reported to have low toxicity in mice [34], it is necessary to set a DMSO vehicle control group. Materials in the MWCNTs+NP group were pretreated as previously described, with some modifications [31]. Briefly, 20 mg 4-NP was dissolved in 100 ml DMSO; 200 mg MWCNTs was then added to the solution. The mixture was placed into a water-bathing constant temperature vibrator (THZ-82, Changzhou, China) and vibrated at 150 rpm for 24 h. After standing for 24 h, the mixture was centrifuged at 21000 × g for 10 min and the concentration of 4-NP was measured in the supernatant using high performance liquid chromatography (HPLC, Alliance 2695, Waters, USA) [35]. Results indicate that 50 mg/g 4-NP adsorbed on the MWCNTs. The MWCNTs+NP were collected (100 mg), dried, and administered to the MWCNTs+NP group. Prior to animal treatment, the MWCNTs and MWCNTs+NP were suspended in normal saline and dispersed by ultrasonic vibration for 15 min. Suspensions were subjected to dynamic light scattering (DLS) spectroscopy (ZEN 3600, Malvern, UK) for the characterization of size and dispersity. Particle size distributions were determined on the basis of number, volume, and scattering intensity [36]. All animals were treated by gavage of a 0.2 ml suspension once per day for 5 days.

After 5 days, no adverse event was found in any of the experimental groups. The mice were sacrificed and the liver, kidneys, heart, spleen, and lungs from each group were collected to evaluate intracellular oxidative damage. The liver and sperm were also collected to assess the genotoxic response.

**Analytical procedure**

SOD and GSH-Px activity, as well as MDA and GSH levels were measured in each group to determine oxidative damage to the liver, kidneys, heart, spleen, and lungs. Before experimental analysis, each tissue (5 per group) was cut into pieces and mixed with ice-cold 0.86% NaCl to form 10% tissue homogenate. The mixture was then homogenized with an ultrasonic processor (JY-250, Zhejiang, China) and centrifuged at 600 × g (4˚C) for 15 min. The supernatants were used in the enzymatic assays. The activities of SOD, GSH, GSH-Px and the levels of MDA were determined using commercial assay kits purchased from Nanjing Jiancheng Biotechnology Institute (Catalog number: A001-1, A006-1, A005, A003-1, China). SOD activity was determined using a xanthine-xanthine oxidase and nitro blue tetrazolium (NBT) system. The endpoint of SOD activity was detected based on the presence of red substances in the reaction system by absorbance at 550 nm after 40 min of reaction time at 37˚C. One unit of SOD was defined as the amount of protein that inhibited NBT reduction by 50% [37]. MDA levels were assessed using the thiobarbituric acid (TBA) assay [38]. The absorbance of red TBA-MDA complex was determined at 532 nm. GSH reacts with 5, 5’-dithiobis (2-nitrobenzoic acid)
(DTNB) to produce stable yellow substances and the absorbance was detected at 420 nm. The GSH-Px activities were also measured using the assay kit based on the principle that oxidation of GSH and H$_2$O$_2$ could be catalyzed by GSH-Px to produce oxidized glutathione (GSSG) and H$_2$O. The decrease in GSH at 412 nm during the 5 min of reaction time at 37˚C indicates GSH-Px activity in tissues. The MDA and GSH content as well as the GSH-Px activity were calculated per the detailed instructions on the assay kits. All the enzymes and MDA contents were detected using a spectrophotometer (UV1102Ⅱ, Techcomp, Shanghai, China).

The comet assay was performed according to the method described by Singh et al., with some modifications [39]. After the mice were sacrificed, the livers and testicles were obtained in 35-mm glass plates and washed twice with phosphate-buffered saline (PBS). These two tissues were transformed into a 10-ml beaker and cut into small pieces; then, the liver and sperm cells were collected through a 150-μm mesh. Cell suspensions were centrifuged at 860 × g for 3 min and cells were re-suspended in PBS. Prior to the comet assay, a trypan blue dye-exclusion staining assay was used to ensure that cell viability was greater than 95%. Electrophoresis was conducted at 4˚C for 20 min at 25 V and 100 mA in the dark. Slides were then stained with ethidium bromide (EB) and scored using a fluorescent microscope (BX41, Olympus, Japan). Fifty images were randomly selected for each group and analyzed with CASP software per the method described by Collins et al. [40].

Liver samples were fixed in 2.5% glutaraldehyde and embedded as sections following routine techniques for TEM observations (H-7650, Hitachi, Japan) [41].

**Statistics**

The data are presented as means ± SD (standard deviation). Statistically significant differences among treatment groups were determined using one-way analysis of variance (ANOVA), followed by Tukey’s honest significant difference (HSD) post hoc test (equal variances) or Dunnett’s T3 post hoc test (unequal variances). All the above tests were performed using SPSS 16.0 software. Differences were considered statistically significant when the $p$-value was less than 0.05.

**Results**

**Characteristics of MWCNTs**

The MWCNTs were 10 μm (Fig 1) in length, with a 10–20 nm outer diameter and purity greater than 99%. The special surface area of the MWCNTs was 500 m$^2$/g according to BET analysis.

**Characterization of MWCNTs and MWCNTs+NP in dispersion**

DLS measurements of MWCNTs and MWCNTs+NP suspensions showed that their size distribution ranged from 37 nm to 550 nm, with an average hydrodynamic size of 125 nm and 137 nm, respectively (S1 Fig).

**Oxidative damage in the liver, kidneys, lungs, heart, and spleen of male mice**

To investigate the intracellular response to MWCNTs+NP, several anti-oxidative enzymes and antioxidants were evaluated. SOD and GSH-Px activity, as well as GSH and MDA content in the liver, kidneys, lungs, heart, and spleen of mice are shown in Figs 2–5. No significant differences were observed in antioxidative enzyme activity or antioxidant levels in the five organs among the treated and control groups, except for that in the liver. SOD activity in the liver
significantly decreased ($p < 0.05$) after administration of MWCNTs; however, this effect was attenuated in the MWCNTs+NP group ($p < 0.01$) (Fig 2).

GSH-Px enzymes can protect organs from oxidative damage by consuming $H_2O_2$ *in vivo* and can be used as an indicator of intracellular oxidative stress. Liver GSH-Px activity in the MWCNTs+NP group was lower ($p < 0.05$) than that in the CK group (Fig 3). In addition, GSH depletion in the liver was significantly different ($p < 0.01$) from the CK group after exposure to MWCNTs+NP (Fig 4).

We measured MDA content in the organs to determine the extent of lipid peroxidation. MDA content was higher in the livers from the MWCNTs group ($p < 0.05$) than that in the livers from the CK group and relatively higher ($p < 0.01$) than that in the livers from the MWCNTs+NP group (Fig 5).

**DNA damage in mouse liver and sperm**

To evaluate DNA damage in the liver and sperm, tail DNA and olive tail moment (OTM) were determined via the comet assay; the results are shown in Figs 6 and 7. DNA damage in mouse liver after exposure to MWCNTs and MWCNTs+NP was significantly different from that in mouse liver from the CK group (Fig 6, $p < 0.05$), while DNA damage in mouse sperm was higher after exposure to MWCNTs and MWCNTs+NP (Fig 7, $p < 0.01$, $p < 0.001$, respectively).
respectively). No significant difference in DNA damage in the liver and sperm was observed in the DMSO and NP groups.

**Liver mitochondrial damage in mice**

We used TEM to investigate the comparative effects of MWCNTs and MWCNTs+NP treatment on cellular structures and organelles in mouse livers. Five days after exposure, 100 mitochondria were randomly selected to check the morphology in each animal and the results showed that mitochondrial abnormalities, including reduction, disorganization, and fractures (Fig 8B and 8C), were significantly greater in the MWCNTs and MWCNTs+NP groups than in the CK group (Fig 8A).

**Discussion**

In this study, we compared the anti-oxidative damages of NP, pristine MWCNTs, and MWCNTs+NP in mice. In addition, we evaluated genotoxic effects using the comet assay...
during acute toxicity tests. Although no differences in body weight and organ coefficients were observed in all five groups (data not shown), the enzyme and genotoxic results indicate that MWCNTs+NP exposure for 5 days in male mice results in greater toxicity than exposure to NP or MWCNTs alone. As a common mechanism for intracellular damage, oxidative stress has been clearly implicated in the induction of inflammation in many studies examining CNTs both in vivo and in vitro [1, 27, 42]. CNTs stimulate the generation of reactive oxygen species (ROS), which can damage lipids, carbohydrates, proteins, and DNA [43, 44]. ROS-mediated toxicity has also been observed in vitro for single-walled CNTs with a diameter of 8 nm and length of < 5 μm [30]. Normally, antioxidant enzymes such as SOD and GSH-Px reduce H$_2$O$_2$ and superoxide radicals, protecting polyunsaturated fatty acids from lipid peroxidation and further preserving the structure of the cell membrane. However, excess ROS production destroys the natural antioxidant defense system and leads to several sub-cellular injuries, including protein denaturation, membrane damage, and DNA damage [1]. In the present study, we investigated changes in SOD and GSH-Px activity, as well as GSH levels to compare the toxicity of NP, MWCNTs, and MWCNTs+NP. Our results suggest that MWCNTs+NP induce significant changes in GSH-Px activity and deplete GSH in the liver.

**Fig 3. Glutathione peroxidase (GSH-Px) activity in the organs of mice.** Results are expressed as the means ± SD (n = 5). Significant differences from the control (CK) group are denoted by *p < 0.05.

https://doi.org/10.1371/journal.pone.0200238.g003
although evidence suggests that pristine CNTs are not toxic or little toxic to animals when administered by gavage (50 mg/kg) or by the intraperitoneal route (250 mg/kg) [45, 46]. In this study, decreased SOD levels in the liver were observed in both the MWCNTs and MWCNTs+NP groups. This is likely attributable to the high concentration of MWCNTs (100 mg/kg) used in this study. It has been reported that the oral no-observed-adverse-effect-level value of NP seems to range from approximately 50 to 100 mg/kg [47]. In this study, we set a high concentration of MWCNTs (100 mg/kg) and a relatively low dose of NP (5 mg/kg). This is mainly due to the limitation of adsorbing capacity of NP on MWCNTs in our pilot study, which indicated that the largest extent of NP adsorption on MWCNTs was approximately 58 mg/g. In the meanwhile, although previous study showed animals were successfully administered via the intraperitoneal route by using an extremely high dose (250 mg/kg) [46], in this study we still chose the oral route for the sake of safety. The oxidative damage appeared to be higher in the 100 mg/kg MWCNTs+ 5 mg/kg NP group than in the other treatment groups. The adsorptive properties of MWCNTs may explain this phenomenon. The addition of NP to the MWCNTs may have exacerbated the induction of intracellular ROS generation by simultaneously exerting adverse effects on the antioxidant defense system. Although studies have suggested that NP is an environmental contaminant that results in adverse environmental health effects attributable to oxidative stress both in vivo and in vitro [14, 48–50], in our study, there

---

**Fig 4. Glutathione (GSH) levels in the organs of mice.** Results are expressed as the means ± SD (n = 5). Significant differences from the control (CK) group are denoted by **p < 0.01.**

https://doi.org/10.1371/journal.pone.0200238.g004
were no significant differences in toxicity between the NP and CK groups. We inferred from these data that the NP exposure dose and time used in this study were not sufficient to stimulate ROS generation and induce oxidative damage; however, when NP adsorbs to MWCNTs, it remains in the tissues for a longer duration.

MDA is a major peroxidation product that is formed under conditions of oxidative stress and can be used as an indicator of lipid peroxidation [51]. Instability of the plasma membrane results from active oxygen atoms generated by peroxide. It has been reported that most MWCNTs are excreted in the feces when administered to mice by gavage [52], and an in vitro study suggested that MWCNTs are not taken up by enterocytes [53]; however, in this experiment, significant increases in liver MDA levels in the MWCNTs and MWCNTs+NP groups were observed. In addition, MDA content in the MWCNTs+NP group was higher than that in the MWCNTs group. In combination with the observed changes in antioxidant enzyme activity, the MDA results suggest that most of the oxidative stress occurred in the liver of MWCNTs+NP-treated mice. We inferred from these data that some MWCNTs enter the circulatory system via the gastrointestinal tract, resulting in liver damage.

The comet assay is a useful tool for studying the genotoxic effects of CNTs [29, 54]. Currently, the genotoxic potential of CNTs is not clear, attributable to differences in experimental

---

**Fig 5. Malondialdehyde (MDA) content in the organs of mice.** Results are expressed as the means ± SD (n = 5). Significant differences from the control (CK) group are denoted as *p < 0.05 and **p < 0.01.

https://doi.org/10.1371/journal.pone.0200238.g005
design among studies, including the various models used, exposure routes, type of CNTs examined, administered concentrations, and assessed endpoints. Genotoxic responses may transpire via direct mechanical injury or as a secondary result of CNT-mediated ROS generation and oxidative stress [55]. The results of the comet assay in the present study clearly show DNA damage were observed in the liver and sperm from mice administered MWCNTs. It was reported that repeated intravenous injections of water-soluble MWCNTs to male mice (5 mg/
kg) can cause reversible testis damage without affecting fertility [56]. Further, DNA damage were highest in the liver and sperm of mice administered MWCNTs+NP. Although the investigation revealed that adsorption of another endocrine disruptor, bisphenol A (BPA), to a CNT (with the highest dose of 2.4 mg/kg BPA and 65 mg/kg carboxylated MWCNTs) reduced its endocrine disrupting effect in mice male offspring [57], we consider that the properties of MWCNTs+NP may differ from that of MWCNTs+BPA after adsorption. Another possible explanation is that MWCNTs+NP accumulate in mouse liver and sperm mitochondria [58].

Previous studies have already demonstrated that NP could lead to reproductive toxicity in Muridae animals such as rats [59] and mice [15, 60], and cause abnormal conditions including decreased testis weights and sperm motility, reduced SOD and GSH levels as well as increased MDA contents in the reproductive organs. As there was no significant difference in the DNA damage between the NP group and the CK group, we inferred that it is due to a short exposure time and low dose (5 mg/kg) used in this study. On the contrary, MWCNTs act as a carrier for NP, which then persists in the liver and sperm, causing additional DNA damage.

It was reported that NP placed in direct contact with the liver could lead to more gene activation than that caused by estradiol, indicating that tissue-specific effects should also be considered [61]. Since the liver suffered more oxidative damage from MWCNTs+NP and MWCNTs than other organs, TEM was used to directly observe mitochondrial damage in the liver. Disorganization and fractures in mitochondria with broken cristae and membrane were observed in the liver tissues treated with MWCNTs (Fig 8B), while these lesions were more severe in the liver tissues treated with MWCNTs+NP (Fig 8C), indicating that both exposure induce hepatic mitochondrial damage. This result provides direct evidence that mitochondria are candidate organelles for studying the toxicity of MWCNTs and MWCNTs+NP administered at high doses.

**Conclusions**

To investigate the toxicological effects induced by NP, MWCNTs, and MWCNTs+NP in mice, several anti-oxidative defense system parameters were examined, with the comet assay used specifically to study genotoxicity. No obvious acute toxicity was observed 5 days after exposure to NP at a dose of 5 mg/kg in mice. In addition, high doses of MWCNTs+NP induced more
oxidative lesions in the liver and caused more DNA damage in the sperm than pristine MWCNTs, as shown by measuring changes in markers of oxidative damage and via the comet assay.

Supporting information

S1 Checklist. Completed “The ARRIVE Guidelines Checklist” for reporting animal research in this manuscript.

(PDF)

S1 Fig. Dynamic light scattering (DLS) spectra of MWCNTs and MWCNTs+NP suspensions.

(TIF)

Acknowledgments

This project was supported by Startup Foundation for Introducing Talent (2014r020) of Nanjing University of Information Science and Technology, The Natural Science Foundation of Jiangsu Province (BK20170948), The Natural Science Foundation of Jiangsu Higher Education Institutions of China (17KJB610007), Top-notch Academic Programs Project of Jiangsu Higher Education Institutions (PPZY2015C222) and National Natural Science Foundation of China (31572623), China.

Author Contributions

Conceptualization: Hao Fang, Yibin Cui.

Data curation: Hao Fang, Yibin Cui.

Formal analysis: Hao Fang, Zhuang Wang.

Funding acquisition: Hao Fang, Yibin Cui.

Investigation: Hao Fang, Se Wang.

Methodology: Hao Fang, Yibin Cui.

Project administration: Hao Fang, Yibin Cui.

Resources: Hao Fang, Yibin Cui.

Software: Hao Fang, Zhuang Wang.

Supervision: Hao Fang, Yibin Cui.

Validation: Hao Fang.

Visualization: Hao Fang, Se Wang.

Writing – original draft: Hao Fang, Yibin Cui.

Writing – review & editing: Hao Fang, Yibin Cui.

References

1. Nel A, Xia T, Madler L, Li N. Toxic potential of materials at the nanolevel. Science. 2006; 311: 622–627. https://doi.org/10.1126/science.1114397 PMID: 16456071

2. Iijima S. Helical microtubules of graphitic carbon. Nature. 1991; 354: 56–58.

3. Pan B, Lin DH, Mashayekhi H, Xing BS. Adsorption and hysteresis of bisphenol A and 17 alpha-ethinyl estradiol on carbon nanomaterials. Environ Sci Technol. 2008; 42: 5480–5485. PMID: 18754464
4. Pan B, Xing BS. Adsorption mechanisms of organic chemicals on carbon nanotubes. Environ Sci Technol. 2008; 42: 9005–9013. PMID: 19174865

5. Yang K, Xing BS. Adsorption of fulvic acid by carbon nanotubes from water. Environ Pollut. 2009; 157: 1095–1100. https://doi.org/10.1016/j.envpol.2008.11.007 PMID: 19084305

6. Soares A, Gueyssse B, Jefferson B, Cartmell E, Lester JN. Nonylphenol in the environment: A critical review on occurrence, fate, toxicity and treatment in wastewaters. Environ Int. 2008; 34: 1033–1049. https://doi.org/10.1016/j.envint.2008.01.004 PMID: 18282600

7. Li ZY, Gibson M, Liu C, Hu H. Seasonal variation of nonylphenol concentrations and fluxes with influence of flooding in the Daliao River Estuary, China. Environ Monit Assess. 2013; 185: 5221–5230. https://doi.org/10.1007/s10661-012-2938-9 PMID: 23064854

8. Ramarathnam N, Rubin LJ, Diosady LL. Studies on meat flavor. 3. A novel method for trapping volatile components from uncured and cured pork. J Agric Food Chem. 1993; 41: 933–938.

9. Ramarathnam N, Rubin LJ, Diosady LL. Studies on meat flavor. 4. Fractionation, characterization, and quantitation of volatiles from uncured and cured beef and chicken. J Agric Food Chem. 1993; 41: 939–945.

10. Colerangle JB, Roy D. Exposure of environmental estrogenic compound nonylphenol to 20 noble rats alters cell-cycle kinetics in the mammary gland. Endocrine. 1996; 4: 115–122. https://doi.org/10.1007/BF02782756 PMID: 21153266

11. Roy D, Colerangle JB, Singh KP. Is exposure to environmental or industrial endocrine disrupting estrogen-like chemicals able to cause genomic instability? Front Biosci. 1998; 3: d913–921. PMID: 9696883

12. Takayuki N, Katsuyoshi K, Shingo S, Haruna M, Yoshiyuki I, Shigeru K, et al. Behavioral Alterations in Response to Fear-Provoking Stimuli and Tranylcypromine Induced by Perinatal Exposure to Bisphenol A and Nonylphenol in Male Rats. Environ Health Persp. 2004; 112: 1159–1164.

13. Kudo C, Wada K, Masuda T, Yonemura T, Shibuya A, Fujimoto Y, et al. Nonylphenol induces the death of neural stem cells due to activation of the caspase cascade and regulation of the cell cycle. J Neurochem. 2004; 88: 1416–1123. PMID: 15009642

14. Chitra KC, Latchoumy candane C, Mathur PP. Effect of nonylphenol on the antioxidant system in epididymal sperm of rats. Arch Toxicol. 2002; 76: 545–551. https://doi.org/10.1007/s00204-002-0372-4 PMID: 12242613

15. El-Dakdoky MH, Helal MAM. Reproductive toxicity of male mice after exposure to nonylphenol. Bull Environ Contam Toxicol. 2007; 79: 188–191. https://doi.org/10.1007/s00128-007-9158-y PMID: 17701091

16. Aly HA, Domènèch Ò, Banjar ZM. Effect of nonylphenol on male reproduction: Analysis of rat epididymal biochemical markers and antioxidant defense enzymes. Toxicol Appl Pharm. 2012; 261: 134–141.

17. Li XN, Chen S, Li LY, Quan X, Zhao HM. Electrochemically enhanced adsorption of nonylphenol on carbon nanotubes: Kinetics and isotherms study. J Colloid Interface Sci. 2014; 415: 159–164. https://doi.org/10.1016/j.jcis.2013.10.021 PMID: 24267343

18. Warheit DB, Laurence BR, Reed KL, Roach DH, Reynolds GA, Webb TR. Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats. Toxicol Sci. 2004; 77: 117–125. https://doi.org/10.1093/toxsci/fkg228 PMID: 14514968

19. Lam CW, James JT, McCluskey R, Hunter RL. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. Toxicol Sci. 2004; 77: 126–134. https://doi.org/10.1093/toxsci/fkg243 PMID: 14514958

20. Shvedova AA, Kisin ER, Mercer R, Murray AR, Johnson VJ, Potapovich AI, et al. Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. Am J Physiol Lung Cell Mol Physiol. 2005; 289: L698–708. https://doi.org/10.1152/ajplung.00084.2005 PMID: 15951334

21. Singh R, Pantarotto D, Lacerda L, Pastorin G, Klumpp C, Prato M, et al. Tissue biodistribution and blood clearance rates of intravenously administered carbon nanotubes radiotracers. P Natl Acad Sci USA. 2006; 103: 3357–3362.

22. Muller J, Huaux F, Moreau N, Misson P, Heiller JF, Delos M, et al. Respiratory toxicity of multi-wall carbon nanotubes. Toxicol Appl Pharmacol. 2005; 207: 221–231. https://doi.org/10.1016/j.taap.2005.01.008 PMID: 16129115

23. Shvedova AA, Kisin E, Murray AR, Johnson VJ, Gorelik O, Arepalli S, et al. Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: Inflammation, fibrosis, oxidative stress, and mutagenesis. Am J Physiol Lung Cell Mol Physiol. 2008; 295: L552–565. https://doi.org/10.1152/ajplung.90287.2008 PMID: 18658273

24. Li Z, Hulderman T, Salmen R, Chapman R, Leonard SS, Young SH, et al. Cardiovascular effects of pulmonary exposure to single-wall carbon nanotubes. Environ Health Persp. 2007; 115: 377–382.
25. Poland CA, Duffin R, Kinloch IA, Maynard A, Wallace WAH, Seaton A, et al. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat Nanotechnol. 2008; 3: 423–428. https://doi.org/10.1038/nnano.2008.111 PMID: 18654567

26. Shvedova AA, Castranova V, Kisin ER, Schweger-Berry D, Murray AR, Gandelsman VZ, et al. Exposure to carbon nanotube material: Assessment of nanotubes cytotoxicity using human keratinocyte cells. J Toxicol Environ Health A. 2003; 66: 1909–1926. https://doi.org/10.1080/713853956 PMID: 14514433

27. Manna SK, Sarkar S, Barr J, Wise K, Barrera EV, Jejelowo O, et al. Single-walled carbon nanotube induces oxidative stress and activates nuclear transcription factor-kappaB in human keratinocytes. Nano Lett. 2005; 5: 1676–1684. https://doi.org/10.1021/nl0507966 PMID: 16159204

28. Brown DM, Kinloch IA, Bangert U, Windle AH, Walter DM, Walker GS, et al. An in vitro study of the potential of carbon nanotubes and nanofibres to induce inflammation mediators and frustrated phagocytosis. Carbon. 2007; 45: 1743–1756.

29. Yang H, Liu C, Yang D, Zhang H, Xi Z. Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by four typical nanomaterials: The role of particle size, shape and composition. J Appl Toxicol. 2009; 29: 69–78. https://doi.org/10.1002/jat.1385 PMID: 18756589

30. Ye SF, Wu YH, Hou ZQ, Zhang QQ. ROS and NF-kappaB are involved in upregulation of IL-8 in A549 cells exposed to multi-walled carbon nanotubes. Biochem Biophys Res Comm. 2009; 379: 643–648. https://doi.org/10.1016/j.bbrc.2008.12.137 PMID: 19121628

31. Hu CW, Cai Y, Wang WL, Cui YB, Li M. Toxicological effects of multi-walled carbon nanotubes absorbed with nonylphenol on earthworm Eisenia fetida. Environ Sci Proc Impacts. 2013; 15: 2125–2130.

32. Sanchis J, Olmos M, Vincent P, Farré M, Barceló D. New insights on the influence of organic co-contaminants on the aquatic toxicology of carbon nanomaterials. Environ Sci Technol. 2016; 50: 961–969. https://doi.org/10.1021/acs.est.5b03966 PMID: 26694946

33. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 2010; 8: e1000412. https://doi.org/10.1371/journal.pbio.1000412 PMID: 20613859

34. David NA. The pharmacology of dimethyl sulfoxide. Ann Rev Pharm. 1972; 12: 353–374.

35. Gundersen JL. Separation of isomers of nonylphenol and select nonylphenol polyethoxylates by high-performance liquid chromatography on a graphitic carbon column. J Chromatogr A. 2001; 914: 161–166. PMID: 11358209

36. Ghosh M, Chakraborty A, Bandyopadhyay M, Mukherjee A. Multi-walled carbon nanotubes (MWCNT) : Induction of DNA damage in plant and mammalian cells. J Hazard Mater. 2001; 914: 161–166. PMID: 11358209

37. Sun Y, Oberley LW, Li Y. A simple method for clinical assay of superoxide dismutase. Clin Chem. 1988; 34: 497–500. PMID: 3349599

38. Okhawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979; 95: 351–358. PMID: 36810

39. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988; 175: 184–191. PMID: 3345800

40. Collins AK, Ma AG, Duthie SJ. The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells. Mutat Res. 1995; 336: 69–77. PMID: 7528897

41. Horiuchi K, Naito I, Nakano K, Nakatani S, Nishida K, Taguchi T, et al. Three-dimensional ultrastructure of the brush border glycoalyx in the mouse small intestine: a high resolution scanning electron microscopic study. Arch Histol Cytol. 2005; 68: 51–56. PMID: 15827378

42. Reddy AR, Rao MV, Krishna DR, Himabindu V, Reddy YN. Evaluation of oxidative stress and anti-oxidant status in rat serum following exposure of carbon nanotubes. Regul Toxicol Pharmacol. 2011; 59: 251–257. https://doi.org/10.1016/j.yrtph.2010.10.007 PMID: 20955749

43. Kelly SA, Havilla CM, Brady TC, Abramo KH, Levin ED. Oxidative stress in toxicology: established mammalian and emerging piscine model systems. Environ Health Persp. 1998; 106: 375–384.

44. Shvedova AA, Pietroiusti A, Fadeel B, Kagan VE. Mechanisms of carbon nanotubes-induced toxicity: focus on oxidative stress. Toxicol Appl Pharmacol. 2012; 261: 121–133. https://doi.org/10.1016/j.taap.2012.03.023 PMID: 22513272

45. Matsumoto M, Serizawa H, Sunaga M, Kato H, Takahashi M, Hirata-Koizumi M, et al. No toxicological effects on acute and repeated oral gavage doses of single-wall or multi-wall carbon nanotubes material. J Toxicol Sci. 2012; 37: 463–474. PMID: 22687986
46. Liang GY, Yin LH, Zhang J, Liu R, Zhang T, Ye B, et al. Effects of subchronic exposure to multi-walled carbon nanotubes on mice. J Toxicol Environ Health A. 2010; 73: 463–470. https://doi.org/10.1080/15287980903523378 PMID: 20391125

47. Vazquez-Duhalt R, Marquez-Rocha F, Ponce E, Licea AF, Viana MT. Nonylphenol, an integrated vision of a pollutant. Scientific review. Appl Ecol Environ Res. 2005; 4: 1–25.

48. Gong Y, Han XD. Nonylphenol-induced oxidative stress and cytotoxicity in testicular Sertoli cells. Reprod Toxicol. 2006; 22: 623–630. https://doi.org/10.1016/j.reprotox.2006.04.019 PMID: 16777376

49. Aydogan M, Korkmaz A, Barlas N, Kolankaya D. The effect of vitamin C on bisphenol A, nonylphenol and octylphenol induced brain damages of male rats. Toxicology. 2008; 249: 35–39. https://doi.org/10.1016/j.toxic.2008.04.002 PMID: 18508178

50. Lee S, Cha M, Kang C, Sohn ET, Lee H, Munawir A, et al. Mutual synergistic toxicity between environmental toxicants: A study of mercury chloride and 4-nonylphenol. Environ Toxicol Phar. 2009; 27: 90–95.

51. Wang JX, Chen CY, Li B, Yu HW, Zhao YL, Sun J, et al. Antioxidative function and biodistribution of [Gd@C82(OH)22]n nanoparticles in tumor-bearing mice. Biochem Pharmacol. 2006; 71: 872–881. https://doi.org/10.1016/j.bcp.2005.12.001 PMID: 16436273

52. Deng X, Jia G, Wang H, Sun H, Wang X, Yang S, et al. Translocation and fate of multi-walled carbon nanotubes in vivo. Carbon. 2007; 45: 1419–1424.

53. Clark KA, O’Driscoll C, Cooke CA, Smith BA, Wepasnick K, Fairbrother DH, et al. Evaluation of the interactions between multiwalled carbon nanotubes and Caco-2 cells. J Toxicol Environ Health A. 2012; 75: 25–35. https://doi.org/10.1080/15287394.2011.5989105 PMID: 22047161

54. Kisin ER, Murray AR, Keane MJ, Shi XC, Schwegler-Berry D, Gorelik O, et al. Single-walled carbon nanotubes: Geno and cytotoxic effects in lung fibroblast V79 cells. J Toxicol Environ Health A. 2007; 70: 2071–2079. https://doi.org/10.1080/15287390701601251 PMID: 18049996

55. Johnston HJ, Hutchison GR, Christensen FM, Peters S, Hankin S, Aschberger K, et al. A critical review of the biological mechanisms underlying the in vivo and in vitro toxicity of carbon nanotubes: The contribution of physico-chemical characteristics. Nanotoxicology. 2010; 4: 207–246. https://doi.org/10.3109/17435390903569639 PMID: 20758587

56. Bai YH, Zhang Y, Zhang JP, Mu QX, Zhang WD, Butch ER, et al. Repeated administrations of carbon nanotubes in male mice cause reversible testis damage without affecting fertility. Nat Nanotechnol. 2010; 5: 683–689. https://doi.org/10.1038/nnano.2010.153 PMID: 20693989

57. Wang WW, Jiang CJ, Zhu LD, Liang NN, Liu XJ, Jia JB, et al. Adsorption of bisphenol A to a carbon nanotube reduced its endocrine disrupting effect in mice male offspring. Int J Mol Sci. 2014; 15: 15981–15993. https://doi.org/10.3390/ijms150915983 PMID: 25210847

58. Xu C, Liu Q, Liu H, Zhang CL, Shao WT, Gu AH. Toxicological assessment of multi-walled carbon nanotubes in vitro: potential mitochondria effects on male reproductive cells. Oncotarget. 2016; 7: 39270–39278. https://doi.org/10.18632/oncotarget.9689 PMID: 27248475

59. Lee PC. Disruption of male reproductive tract development by administration of the xenoestrogen, nonylphenol, to male newborn rats. Endocrine. 1998; 9: 105–111. https://doi.org/10.1385/ENDO:9:1:105 PMID: 9798737

60. Kyselova V, Peknicova J, Buckiova D, Boubelik M. Effects of p-nonylphenol and resveratrol on body and organ weight and in vivo fertility of outbred CD-1 mice. Reprod Biol Endocrin. 2003; 1: 30.

61. Watanabe H, Suzuki A, Goto M, Lubahn DB, Handa H, Iguchi T. Tissue-specific estrogenic and non-estrogenic effects of a xenoestrogen, nonylphenol. J Mol Endocrinol. 2004; 33: 243–252. PMID: 15291756