Recent Research in Genetics and Genomics 2019; 1(1): 8-28

Original article:

Potential protective effect of chlorophyllin against the genotoxicity and oxidative stress induced by nano-TiO_2 particles in mice

"Modulation of nano-TiO2 induced genotoxicity"

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Received: 05 October 2019; accepted: 23 October; published 01 November 2019

Abstract

Wide uses of titanium dioxide (TiO_2) nanoparticles in cosmetics, paints, toothpastes, sunscreens, food products, pharmaceuticals and nanomedical reagents increases human exposure and its risk. Thus this study was designed to investigate the possible protective effect of chlorophyllin (CHL) on nano-TiO_2 induced genotoxicity and oxidative stress in mice. Male mice were exposed to single i.p. injection with each of three dose levels of nano-TiO_2 (500, 1000 or 2000 mg/kg b.w) suspended in deionized water or CHL (200 mg/kg b.w.). Micronucleus and comet assays were carried out genotoxic endpoints. Moreover, oxidative stress was evaluated using five markers of oxidative damage to shed more light on its mechanism. A dose-dependent genotoxicity of nano TiO2 was evidenced by the observed significant elevations in micronuclei frequencies and DNA damage levels. The same end.-points indicated that TiO_2 genotoxicity was significantly decreased after CHL coadministration. Moreover, CHL significantly decreased malondialdehyde level, and significantly increased reduced glutathione level and superoxide dismutase, catalase and glutathione peroxidase activities, which were significantly disrupted by TiO_2 alone. In conclusion, the dose dependent nano-TiO_2 induced genotoxicity was diminished by CHL co-administration via its free radicals scavenger ability.

Keywords: chlorophyllin, TiO2 nanoparticles, genotoxicity, oxidative stress and mice

1. Introduction

Titanium dioxide (TiO_2) nanoparticles have been widely used in a wide variety of applications due to their high stability, excellent optical performance, high redox activity and electrical properties **(Park *et al.*, 2007)**. It is

used in a variety of consumer products (e.g., toothpastes, sunscreens, cosmetics, food products) **(Kaida *et al.*, 2004; Trouiller *et al.*, 2009)**, paints, pharmaceuticals and wastewater treatment **(Jeon *et al.*, 2010)**. Recently, TiO_2 was used as a photosensitizer for photodynamic therapy of endobronchial and esophageal cancers **(Ackroyd *et al.*, 2001)** and to kill viruses **(Zan *et al.*, 2007*)***.

Thus, increased human exposure to these nanoparticles. However, nano-sized TiO_2 has been shown to cause negative health effects, such as respiratory tract cancer **(Gurr *et al.*, 2005; Trouiller *et al.*, 2009)**, chronic pulmonary inflammation in rats **(Oberd¨orster *et al.*, 1992)** and proinflammatory effects in human endothelial cells **(Peters *et al.*, 2004)** in the absence of UV irradiation. In addition, nano-TiO_2 induced nephrotoxicity and neurotoxicity have also been proven **(Long *et al.*, 2007; Jeon *et al.*, 2010)**.

Moreover, its mutagenicity and genotoxicity have been evident. Nano-TiO_2 particles have been shown to be mutagenic using HPRT mutations assay **(Pan *et al.*, 2010)**. *In vitro*, nano-TiO_2 genotoxicity has been shown in different animal and human cell lines using different techniques: a dose-dependent induction of micronuclei and oxidative DNA damage (comet assay) has been evidenced in L5178Y mouse lymphoma cells **(Nakagawa *et al.*, 1997)**, Syrian hamster embryo cells **(Rahman *et al.*, 2002)**, human bronchial epithelial cell line **(Gurr *et al.*, 2005)**, cultured WIL2-NS human lymphoblastoid cells **(Wang *et al.*, 2007a)**, and fish cells **(Reeves *et al.*, 2008)**. Chromosomal aberration induction has also been observed in Chinese hamster lung CHL/IU cells **(Nakagawa *et al.*, 1997)**.

TiO_2 genotoxicity is poorly studied in vivo. It has been shown to induce micronucleus and DNA damage (comet assay) in mice **(Trouiller *et al.*, 2009)** and in rat (**Faddah *et al.*, 2013)**. Indeed, its in vivo genotoxicity was first reported by **Trouiller *et al.* (2009)** in mice. The authors indicated that nano TiO_2 (500 mg/kg) induced not only DNA single-strand breaks and double strand breaks but also chromosomal damage in bone marrow and/or peripheral blood. Moreover, maternal exposure to these nanoparticles during gestation results in significantly elevated frequencies of DNA deletions in offspring.

Nano-sized TiO_2 has been shown to induce oxidative DNA damage, lipid peroxidation, and increased hydrogen peroxide and nitric oxide production in the absence of photo-activation in many cell types both in vitro and in vivo **(Zhang and Sun, 2004; Trouiller *et al.* 2009; Saquib *et al.*, 2012)**.

The past years have seen rapid progress in the use of natural antioxidants as dietary supplements and in the prevention of various diseases such as cancer **(Bjelakovic et al., 2007)**. Chlorophyllin (CHL), a water-soluble sodium-copper derivative of chlorophyll, is a powerful antioxidant with a higher protective activity than chlorophyll itself **(Aung et al.,**

1989). Its antimutagenic, anticarcinogenic and radioprotective effects have been shown in many studies **(Abraham et al., 1994; Brenholt et al., 1995)**. It also presents an anti-clastogenic activity against various genetic substances in different experimental models **(Tet-al., 1997; Negrais et al., 2004)**.

Therefore, the current study was aimed to evaluate the genotoxic effects induced by nano TiO_2 <100 nm and the possible modulation of the potential toxicity of these nanoparticles by CHL in mice. Both of micronucleus and comet assays were used as cytogenetic and molecular end points in liver, brain and bone marrow cells. Additionally, levels of oxidative stress markers were biochemically evaluated by estimating hepatic malondialdehyde (MDA) and the reduced glutathione (GSH) levels as well as measurement of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidise (GPx) activities.

2. Materials and methods

2.1 Animals

Male Swiss Webster mice aged 10-12 weeks (25-28 gm) were obtained from the animal house of National Organization for Drug Control and Research **(NODCAR)**. Animals were supplied with standard diet pellets and water that were given ad Libitum, kept in plastic cages for 7 days to be accommodated with our laboratory conditions before being treated.

2.2 Chemicals

TiO_2 nanoparticles (mixtures of rutile and anatase) were purchased in the form of white powder (Sigma chemical Co., St. Louis, MO, USA, EC Number 236-675-5), its purity 99.5% based on trace metal basis analysis and with the size of <100 nm based on Brunauer-Emmett-Teller (BET) and < 50 nm based on X-ray diffraction (XRD). And chlorophyllin (CHL) that was purchased as green powder from Sigma chemical Co., St. Louis, MO, USA and was dissolved in sterile deionized water to prepare the desired dose (200 mg/kg b.w.).

2.3 Characterization of nano TiO_2 for injection

Characterization of TiO_2 nanoparticles were done in our previous study **(El ghor et al., 2014)** using X-ray diffraction (XRD) and transmission electron microscopy (TEM). The XRD was used for crystal phase identification and estimation of the average crystallite size. X-ray-diffraction (XRD) patterns of nano-TiO_2 particles were obtained at room temperature with a charge-coupled device (CCD) diffractometer (XPERT- PRO-PANalytical-Netherland) using Ni-filtered Cu Kα radiation.

The particle size and morphology of the powder were observed by transmission electron microscope (TEM). For TEM, TiO2 NPs were suspended in either Milli-Q water or CHL solution and sonicated at 40 W for 20 minutes. Samples for analysis by transmission electron microscopy (TEM) were prepared by drop coating a TiO2 nanoparticle solution on carbon-coated copper TEM grids. The films formed on the TEM grids were allowed to dry prior to measurement. TEM (a Tecnai G20, Super twin,

double tilt) operated at an accelerating voltage at 200 kV.

2.4 Doses of TiO_2 nanoparticles

In order to determine the lethal dose of TiO2 nanoparticles that causes the death of 50% of the animals (LD_{50}), mice were injected i.p. with a single dose of TiO_2 suspended in deionized water at the five dose levels 600, 1000, 3000, 5000 and 7500 mg/kg b.w. and observed for mortality, body weight effects, and the clinical signs for 14 days after dosing **(Warheit *et al.*, 2007)**.

No mortality or signs of toxicity were observed on mice injected i.p. with TiO2 nanoparticles at any of the dose levels used during the first 24 hours and until 14 days. Accordingly, the LD50 for nanosized TiO2 particles <100 nm was estimated to be greater than 7500 mg/kg for male mice. Based on these results and according to the guidelines recommended by **Tice *et al*. (1994)**, the highest dose used in the present study was 2000 mg/kg b.w. and the lowest dose was 500 mg/kg b.w. in addition to an intermediate dose of 1000 mg/kg b.w.

2.5 Treatment schedule

Animals were divided randomly into 11 groups of five animals each. Negative control group (group 1) and CHL control group (group 2) were injected i.p. with dist. H2O and CHL (200 mg/kg), respectively, and scarified 24 hour later. The other 9 groups were injected i.p. with each of three dose levels of nano-TiO_2 (500, 1000 and 2000 mg/kg b.w) suspensed in deionized water and scarified after 24 (groups 3, 5 and 7) or 48 (groups (4, 6 and 8) hour or in CHL solution and scarified after 24 hour (groups 9, 10 and 11).

2.6 Micronucleus assay

Bone marrow slides were prepared according to the method described by **Schmid (1975)**: bone marrow cells of one femur were flushed down and spread on clean slide. Air-dried, fixed and finally stained for 5 minutes in May-Grunwald – Giemsa stain mixture then mounted with DPX. 2000 polychromatic erythrocytes per animal were scored to determine the number of micronucleated polychromatic erythrocytes (MNPCEs). Also, polychromatic to normochromatic erythrocytes (PCEs/NCEs) ratio was determined per 1000 cell.

2.7 Comet assay

The alkaline (pH >13) comet assay was performed according to the method described by **(Tice *et al.*, 2000)**, with minor modifications, for bone marrow, liver and brain tissues. A femur was perfused with one ml of cold mincing solution (Hanks balanced Salt Solution (HBSS) Ca^{++} and Mg^{++} free with 20 mM EDTA, 10% DMSO) using needle into a micro-centrifuge tube, while a small piece of liver or brain was placed in 1 ml cold mincing solution and minced into fine pieces.

A 10 µl aliquot of cell suspension containing approximately 10000 cells (bone marrow, liver or brain cells) was mixed with 80 µl of 0.5% low melting point agarose (Sigma)

and spread on a fully frosted slide pre-dipped in normal melting agarose (1%). After solidification, the slides were placed in cold lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10) with freshly added 10% DMSO and 1% Triton X-100) for 24 hours at 4ºC in dark. Subsequently, the slides were incubated in fresh alkaline buffer (300 mM NaOH and 1 mM EDTA, pH>13) for 20 min.

The unwinding DNA was electrophoresed for 20 min. at 300 mA and 25 V (0.90 V⁄cm) and neutralized in 0.4 M Trizma base (pH 7.5) and finally, fixed in 100% cold ethanol, air dried and stored at room temperature until they were scored. The extent of DNA migration for each sample was determined by simultaneous image capture and scoring of 100 cells at 400 x magnification using Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK). The extent of DNA damage was evaluated according to the following endpoints measurements: **Tail length**: it is used to evaluate the extent of DNA damage away from the nucleus and expressed in µm, **% DNA in tail:** intensity of all tail pixels divided by the total intensity of all pixels in the Comet and **tail moment**: calculated as: tail moment= tail length × %DNA in tail/100.

2.8 Biochemical assays

Biochemical evaluation of hepatic MDA and GSH and SOD, CAT and GPx activities was done only in groups treated with 500 and 2000 mg/kg b.w. TiO2 alone or in combination with CHL at 24 hour sampling time only.

2.8.1 Malondialdehyde (MDA) level

Hepatic MDA was measured using method described by **Ohkawa *et al*. (1979)** that depends on thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) in acidic medium at temperature of 95°C for 30 min. to form thiobarbituric acid reactive product. The absorbance of the resultant pink product was measured spectrophotometrically at 534 nm. Results were expressed as nmol/g tissue used.

2.8.2 Reduced glutathione (GSH) level

Beutler *et al*. (1963) method was used to estimate hepatic GSH level and based on the reduction of Ellman's reagent [5, 5' dithio bis-(2-nitrbenzoic acid)] with glutathione (GSH) to form 1 mol of 2-nitro-5-mercaptobenzoic acid (yellow compound) per mol of GSH. The reduced chromogen is directly proportional to GSH concentration and its absorbance was determined at 405 nm. Results were expressed as mmol/g tissue used.

2.8.3 Superoxide dismutase (SOD) activity

SOD activity was measured using **Nishikimi *et al.* (1972)** method which based on its ability to inhibit the phenazine methosulphate_mediated reduction of nitroblue tetrazolium dye. Results were expressed as µ/g tissue used.

2.8.4 Catalase (CAT) activity

Hepatic catalase activity was measured using method described by **Aebi (1984)** that showed that CAT reacts with a known quantity

of H_2O_2. The reaction is stopped after exactly one minute with catalase inhibitor. In the presence of peroxidase, remaining H_2O_2 reacts with 3, 5-Dichloro-2-hydroxybenzene sulfonic acid and 4-aminophenazone to form a chromophore with colour intensity inversely proportional to the amount of catalase in the original sample. Results were expressed as µ/g tissue used.

2.8.5 Glutathione peroxidase (GPx) activity

Paglia and Valentine (1967) method was used to measure GPx activity depends on a reaction in which oxidized glutathione (GSSG), produced upon reduction of peroxides, and is recycled to its reduced state by the enzyme glutathione reductase. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm (A_{340}) providing a spectrophotometric means for monitoring GPx enzyme activity. The rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample. Results were expressed as µ/g tissue.

2.9 Statistical analysis

Results were expressed as mean ±S.D. Statistical analysis was performed using the T-test to test the significance level between groups. All statistics were carried out using Statistical Analysis Systems (**SAS) program (2005)** ®.

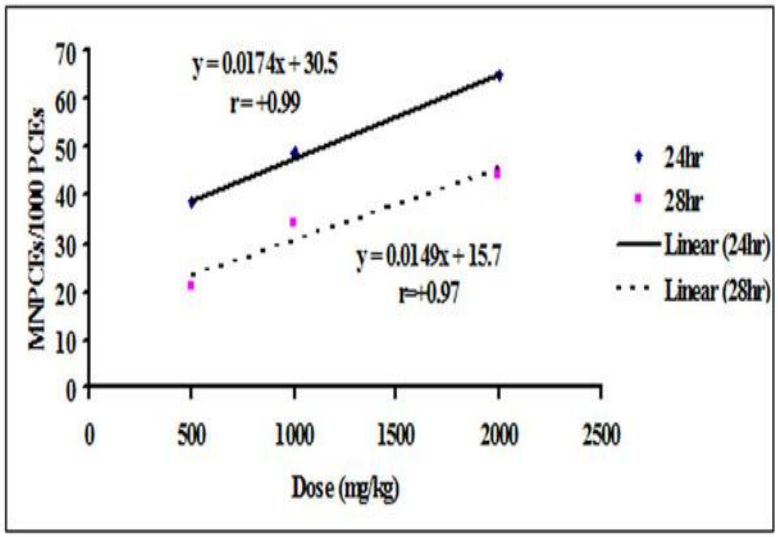

Fig. 1: Regression lines and correlation coefficients for micronuclei induced by the TiO2 nanoparticles tested doses after 24 and 48 hour sampling times.

3. Results

3.1 TiO_2 nanoparticles characterization

As shown in our previous study **El ghor et al., (2014)** the X-ray diffraction pattern of TiO_2-NPs confirmed the purchased form of rutile and anatase TiO_2 nanoparticles by the peaks that were corresponding with the tetragonal rutile and anatase structure of TiO_2 and the average crystallite size of these nanoparticles was found to be about 44 nm was calculated using Debye Scherrer's formula. Using TEM imaging the nano-TiO_2 particles

were found to be still in the nanoscale range in both water (45.63 ± 12.85 nm) and CHL (41.44 ± 15.1 nm) with polyhedral morphologies; however formed small agglomerates in aqueous solution thus samples were sonicated to disperse nanoparticles and prevent their aggregations.

3.2 Micronucleus assay

In comparison with the negative control values, CHL (200 mg/kg b.w.) alone did not induce any significant changes in both the number of micronucleated polychromatic erythrocytes (MNPCEs) and polychromatic to normochromatic erythrocytes (PCEs/NCEs) ratio (Table 1). On the contrary, nano TiO_2 at the three doses used (500, 1000 and 2000 mg/kg b.w.) significantly increased (at $p<0.001$) frequencies of MNPCEs and decreased (at $p<0.001$) PCEs/NCEs ratios at both 24 and 48 hour sampling times in a dose-dependent manner. Regression line analysis indicated a strong positive correlation ($r= +0.99$ and $+0.97$) between the increase in TiO_2 doses and the induction of micronuclei at sampling time 24 and 48 hour (Fig. 1).

Moreover, table 1 showed that coadministration of CHL and TiO_2 resulted in statistically significant decrease (at $p<0.001$) in MNPCEs when compared with that induced after administration of TiO_2 alone. However, this decreased frequencies of MNPCEs still significantly higher than the negative control level except in group 9 whose animals were coadministered CHL and the lowest dose used of TiO2 (500 mg/kg b.w.). The cytotoxic effect of TiO_2 manifested in the observed significant decrease in PCEs/NCEs ratios was significantly reduced when CHL was coadministered with TiO_2 and PCEs/NCEs ratios returned to the negative control level with the two lower doses of TiO_2 (500 and 1000 mg/kg b.w.).

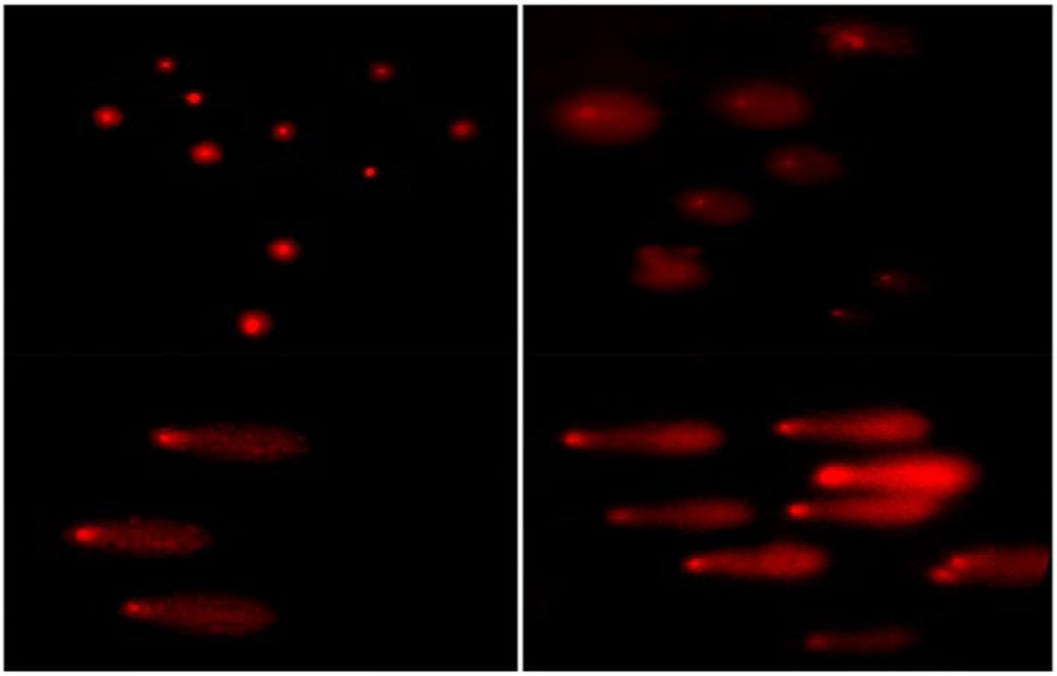

Fig. 2 : Representative photo for the observed DNA damage

Table 1: Effect of acute treatment (single injection) with titanium dioxide (TiO_2) nanoparticles alone and in combination with chlorophyllin (CHL) on the frequencies of micronucleated polychromatic erythrocytes (MNPCEs) and polychromatic to normochromatic erythrocytes (PCEs/NCEs) ratio.

Group	Treatment (dose mg/kg)	Sampling time (hr)	MnPCE s/2000 PCE s cells/animal					MNPCE s/1000 Mean ± SD	PCE s/NCE s Mean ± SD
			1	2	3	4	5		
1	Negative control		17	11	10	9	8	5.50 ± 1.77	1.00 ± 0.02
2	$CHL_{(200)}$	24	11	14	12	13	11	6.10 ± 0.65	0.98 ± 0.03
3	$TiO_{2\,(50)}$	24	72	69	80	88	76	38.50 ± 3.70^{a***}	0.71 ± 0.05^{a***}
4	$TiO_{2\,(50)}$	48	35	54	39	41	40	20.90 ± 3.60^{a***b***}	0.81 ± 0.03^{a***b**}
5	$TiO_{2\,(200)}$	24	94	95	104	98	98	48.90 ± 1.94^{a***}	0.58 ± 0.05^{a***}
6	$TiO_{2\,(200)}$	48	77	64	65	64	70	34.00 ± 2.81^{a***b***}	0.74 ± 0.03^{a***b**}
7	$TiO_{2\,(200)}$	24	127	127	134	130	131	64.90 ± 1.49^{a***}	0.47 ± 0.06^{a***}
8	$TiO_{2\,(200)}$	48	84	83	98	93	86	44.40 ± 3.23^{a***b***}	0.63 ± 0.03^{a***b**}
9	$CHL_{(200)}$+$TiO_{2\,(50)}$	24	11	13	15	12	13	6.40 ± 0.74^{a***}	1.00 ± 0.02^{a***}
10	$CHL_{(200)}$+$TiO_{2\,(200)}$	24	32	27	32	35	27	15.30 ± 1.75^{a***c***}	1.00 ± 0.01^{a***}
11	$CHL_{(200)}$+$TiO_{2\,(200)}$	24	49	60	48	56	51	26.40 ± 2.53^{a***c***}	0.92 ± 0.02^{a**b**}

a: statistically compared with negative control group
b: statistically compared with comparable group treated with TiO2 alone at 24 hour.
c: Statistically compared with comparable group treated with TiO2 alone.
*; ** and ***: significantly different at P< 0.05, P< 0.01 and P< 0.001, respectively, using student t-test.

3.2 Comet assay

Significant elevations in DNA damage parameters (tail length, %DNA in tail and tail moment) were observed in groups treated with TiO2 alone at different doses and different sampling times (24 and 48 hour) in liver, brain and bone marrow (Table 2 and Fig. 2).

Regression analysis (Fig. 3) showed a dose dependent increase in DNA damage as indicated by the strong positive correlations observed between %DNA in tail and different TiO_2 doses at both sampling times. CHL alone generally did not cause any significant change, however, co-administration of CHL and TiO_2 resulted in significant reductions in DNA damage parameters.

3.3 Hepatic oxidative stress markers

Results were summarized in table 3. CHL alone (group 2) did not induce any significant change in MDA level. MDA level was significantly increased ($p<0.001$) in groups treated with TiO2 alone (groups 3 and 7) by about 42% and 147% compared with negative control. Significant reduction in MDA level was observed in groups treated with TiO2 suspended in CHL solution (groups 9 and 11) by 38% and 34% respectively. CHL alone (200 mg/kg) did not induce any statistical significant

change in GSH level. On the other hand, significant decrease (P<0.001) in the GSH level was observed in groups 3 and 7 treated with TiO2 alone in a dose dependent manner by 30% and 47%, respectively, compared with negative control group. This decreased level was returned to control level when mice coadministered CHL.

Its worth mentioning that administration of CHL alone resulted in slight elevation in activities of SOD, CAT and GPx which reached a statistically significant level (p<0.05) only in case of SOD. Treatment with TiO2 alone (500 and 2000 mg/kg b.w.) resulted in a decreased activities of SOD (31% and 51%), CAT (44% and 77%) and GPx (60% and 87%) when compared with that of the negative control level. Co-administration of TiO2 and CHL increased the activities of SOD, CAT and GPx to the negative control level and even higher in case of SOD.

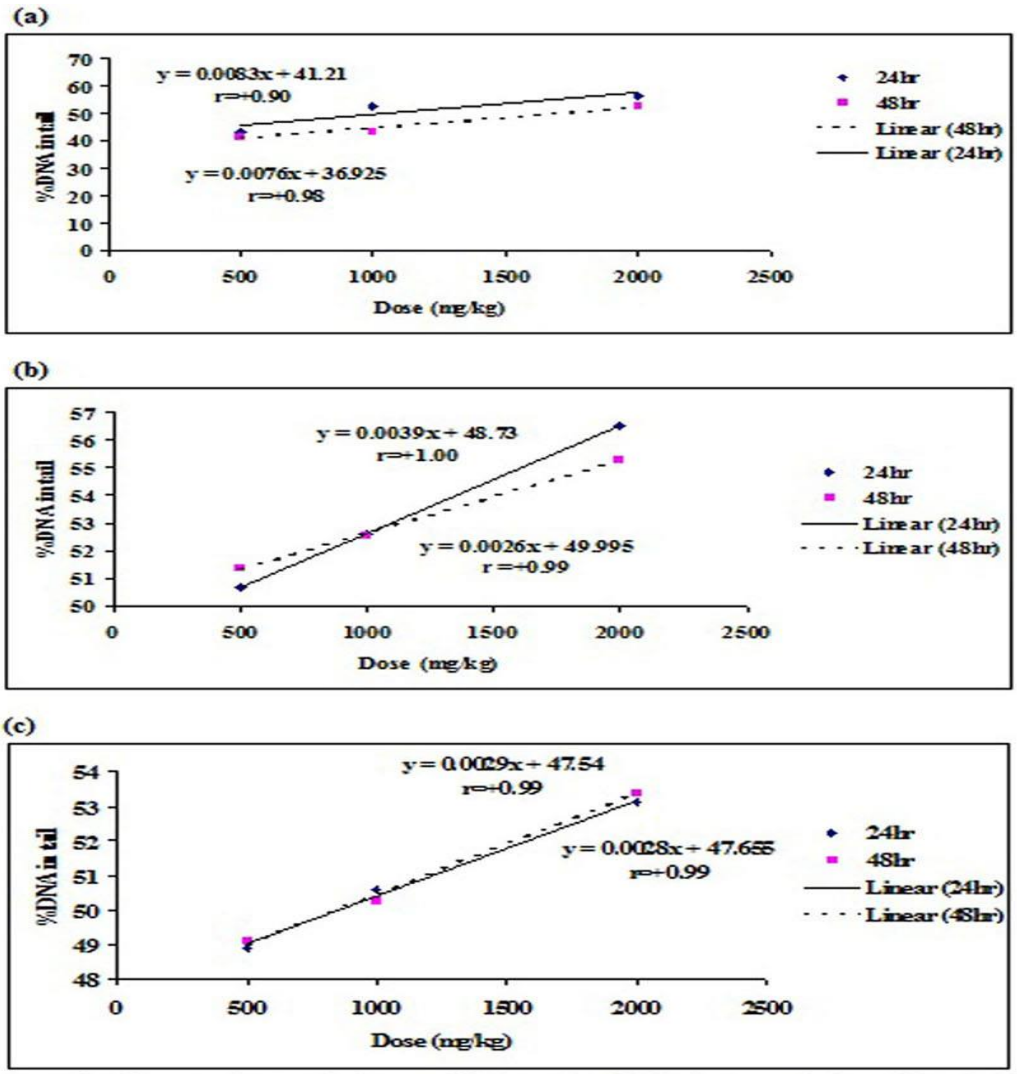

Fig. 3: Regression lines and correlation coefficients for DNA damage induced by the TiO2 nanoparticles tested doses after 24 and 48 hour sampling times in bone marrow (a), brain (b) and liver (c) cells.

Table 2: Effect of single i.p. injection with titanium dioxide (TiO2) nanoparticles alone and in combination with chlorophyllin (CHL) on DNA damage (comet assay) in bone marrow, brain and liver cells of mice.

Group No.	Treatment (dose mg/kg)	Sampling time (hr)	Bone marrow			Brain			Liver		
			Tail length (µm) Mean± SD	%DNA in Tail Mean± SD	Tail moment Mean± SD	Tail length (µm) Mean± SD	%DNA in Tail Mean± SD	Tail Moment Mean± SD	Tail length (µm) Mean± SD	%DNA in Tail Mean± SD	Tail Moment Mean± SD
1	Negative control	24	3.32 ± 0.55	15.78 ± 1.04	0.69 ± 0.21	1.74 ± 0.41	24.33 ± 6.15	0.60 ± 0.21	1.34 ± 0.53	19.71 ± 5.48	0.34 ± 0.13
2	$CHL_{(200)}$	24	3.28 ± 0.22	16.36 ± 1.91	0.62 ± 0.19	$1.46 \pm 0.24^{a*}$	24.24 ± 3.88	0.62 ± 0.14	1.31 ± 0.22	$14.64 \pm 3.05^{a**}$	0.32 ± 0.07
3	$TiO2_{(800)}$	24	$12.14 \pm 3.37^{a***}$	$43.14 \pm 0.88^{a***}$	$5.85 \pm 1.66^{a***}$	$5.99 \pm 0.24^{a***}$	$50.68 \pm 0.68^{a***}$	$3.42 \pm 0.29^{a***}$	$4.50 \pm 0.37^{a***}$	$48.93 \pm 1.04^{a***}$	$2.47 \pm 0.32^{a***}$
4	$TiO2_{(800)}$	48	$10.67 \pm 1.36^{a***}$	$41.61 \pm 4.46^{a***}$	$5.42 \pm 0.51^{a***}$	$5.96 \pm 0.25^{a***}$	$51.34 \pm 2.38^{a***}$	$3.30 \pm 0.29^{a***}$	$4.89 \pm 0.62^{a***}$	$49.10 \pm 1.34^{a***}$	$2.58 \pm 0.34^{a***}$
5	$TiO2_{(1000)}$	24	$23.45 \pm 3.29^{a***}$	$52.77 \pm 6.72^{a***}$	$13.80 \pm 2.73^{a***}$	$7.65 \pm 0.68^{a***}$	$52.62 \pm 1.17^{a***}$	$4.16 \pm 0.12^{a***}$	$6.18 \pm 0.42^{a***}$	$50.58 \pm 1.06^{a***}$	$3.22 \pm 0.29^{a***}$
6	$TiO2_{(1000)}$	48	$17.22 \pm 1.26^{a***b**}$	$43.27 \pm 4.86^{a***b*}$	$10.15 \pm 0.99^{a***b*}$	$7.58 \pm 1.05^{a***}$	$52.58 \pm 3.17^{a***}$	$4.32 \pm 0.69^{a***}$	$5.98 \pm 0.50^{a***}$	$50.27 \pm 3.80^{a***}$	$3.23 \pm 0.48^{a***}$
7	$TiO2_{(2000)}$	24	$28.93 \pm 2.53^{a***}$	$56.63 \pm 5.37^{a***}$	$17.96 \pm 3.05^{a***}$	$9.78 \pm 1.17^{a***}$	$56.52 \pm 3.32^{a***}$	$6.06 \pm 1.10^{a***}$	$8.67 \pm 0.25^{a***}$	$53.13 \pm 1.09^{a***}$	$4.91 \pm 0.15^{a***}$
8	$TiO2_{(2000)}$	48	$20.74 \pm 1.54^{a***b***}$	$52.64 \pm 5.12^{a***}$	$12.21 \pm 1.39^{a***b**}$	$9.88 \pm 0.56^{a***}$	$55.27 \pm 1.71^{a***}$	$5.77 \pm 0.58^{a***}$	$8.57 \pm 0.17^{a***}$	$53.39 \pm 2.30^{a***}$	$4.82 \pm 0.18^{a***}$
9	$CHL_{(200)}+TiO2_{(800)}$	24	$2.22 \pm 0.15^{a***c***}$	$14.64 \pm 1.44^{a***}$	$0.43 \pm 0.05^{a***c***}$	$1.69 \pm 0.08^{a***}$	$23.88 \pm 2.54^{a***}$	$0.62 \pm 0.08^{a***}$	$1.31 \pm 0.21^{a***}$	$20.20 \pm 4.46^{a***}$	$0.32 \pm 0.09^{a***}$
10	$CHL_{(200)}+TiO2_{(1000)}$	24	$6.11 \pm 0.12^{a***c***}$	$34.32 \pm 1.62^{a***c***}$	$2.16 \pm 0.23^{a***c***}$	$2.31 \pm 0.43^{a***c***}$	$32.94 \pm 5.87^{a***c***}$	$1.08 \pm 0.38^{a***c***}$	$2.43 \pm 0.27^{a***c***}$	$34.07 \pm 2.31^{a***c***}$	$0.83 \pm 0.07^{a***c***}$
11	$CHL_{(200)}+TiO2_{(2000)}$	24	$16.97 \pm 2.35^{a***c***}$	$46.70 \pm 3.62^{a***c***}$	$10.31 \pm 1.56^{a***c***}$	$3.32 \pm 0.57^{a***c***}$	$35.29 \pm 4.47^{a***c***}$	$1.22 \pm 0.21^{a***c***}$	$2.34 \pm 0.22^{a***c***}$	$34.38 \pm 1.59^{a***c***}$	$0.88 \pm 0.14^{a***c***}$

Results are expressed as mean ± S.D. a: statistically compared with negative control group in the same column; b: statistically compared with comparable group treated with TiO2 alone at sampling time 24 hour in the same column; c: statistically compared with comparable group treated with TiO2 alone in the same column. *; ** and ***: significantly different at P< 0.05, P< 0.01 and P< 0.001, respectively, using student t-test.

Table 3: Effect of single i.p. injection of mice with titanium dioxide (TiO2) nanoparticles alone and in combination with chlorophyllin (CHL) on the hepatic biochemical markers of oxidative stress (MDA, GSH, CAT, SOD and GPx) 24 hour later.

Group No.	Treatment (dose mg/kg)	MDA (nmol/g) Mean±SD	GSH (mmol/g) Mean±SD	SOD (U/g) Mean±SD	CAT (U/g) Mean±SD	GPx (U/g) Mean±SD
1	Negative control	36.00 ± 7.30	0.14 ± 0.01	470.00 ± 22.00	1.76 ± 0.17	7.59 ± 0.53
2	$CHL_{(200)}$	36.00 ± 6.10	0.14 ± 0.01	$500.00 \pm 1.00^{a*}$	1.77 ± 0.15	7.63 ± 0.35
3	$TiO2_{(800)}$	$51.00 \pm 7.80^{a***}$	$0.10 \pm 0.01^{a***}$	$324.00 \pm 40.00^{a***}$	$0.98 \pm 0.22^{a***}$	$3.19 \pm 0.56^{a***}$
7	$TiO2_{(2000)}$	$89.00 \pm 8.60^{a***c***}$	$0.07 \pm 0.01^{a***c***}$	$228.00 \pm 9.00^{a***c***}$	$0.40 \pm 0.17^{a***c***}$	$0.97 \pm 0.07^{a***c***}$
9	$CHL_{(200)}+TiO2_{(800)}$	$37.00 \pm 2.50^{b*}$	$0.13 \pm 0.00^{b***}$	$495.00 \pm 13.00^{b***}$	$1.73 \pm 0.13^{b***}$	$7.67 \pm 0.30^{b***}$
11	$CHL_{(200)}+TiO2_{(2000)}$	$59.00 \pm 5.60^{a***b**}$	$0.13 \pm 0.00^{b***}$	$528.00 \pm 10.00^{a***b***}$	$1.74 \pm 0.28^{b***}$	$7.59 \pm 0.31^{b***}$

MDA: malondialdehyde; GSH: reduced glutathione; CAT: catalase; SOD: superoxide dismutase and GPx: glutathione peroxidise. Results are expressed as mean ± S.D. a: statistically compared with negative control group; b: statistically compared with comparable group treated with TiO2 alone; c: statistically compared with comparable group treated with TiO2 with different dose. *; ** and ***: significantly different at P< 0.05, P< 0.01 and P< 0.001, respectively, using student t-test.

4. Discussion

Although nano-TiO2 is being used increasingly in various commercial products e.g. production of paper, plastics, cosmetics and paints thereby increasing the occupational and other environmental exposure of these nanoparticles to humans and other species, its genotoxicity has been evidenced in several

studies both in vitro and in vivo **(Gurr *et al.*, 2005; Wang *et al.*, 2007; Trouiller *et al.*, 2009; El ghor et al., 2014; Mohamed, 2015)**.

Thus, the present study was designed to evaluate the genotoxic effects TiO2 nanoparticles <100 nm and the possible modulation of the potential toxicity of these nanoparticles by CHL to shed more light on the mechanism of TiO_2 genotoxicity.

Results of the present study confirmed the non-genotoxic effect of CHL (Table 1 & 2) that has been reported by several studies **(Bez *et al.*, 2001; Botelho *et al.*, 2004; Osowski *et al.*, *2010)*** as indicated by non-significant changes in the induction of both micronuclei and DNA damage. The CHL reported capacity to decline the basal reactive oxygen species (ROS) levels (Table 3) compared with the control level both in vitro and in vivo may explain decreases in DNA damage observed in some instances **(Kumar *et al.*, 2004)**.

In the present study animals were treated with TiO_2 nanoparicles by the i.p. route to avoid excessive loss of nano-titanium by oral administration and we expected that nano-sized TiO_2 could accumulate in, and transport to, other tissues after intraperitoneal injection depending on the previous studies that indicated that nanosized titanium particles could cross the small intestine by persorption and further distribute into blood, brain, lung, heart, kidney, spleen, liver, intestine and stomach **(Chen *et al.*, 2009*)***.

The observed nano titanium induced DNA damage in this study may be directly or indirectly via oxidative stress and/or inflammatory responses. The proven direct

The observed elevations in micronuclei and DNA damage (Table 1 & 2) induced by nan-sized TiO2 at the tested doses confirmed the genotoxicity of nano-TiO2 reported in previous studies **(Gurr *et al.*, 2005; Wang *et al.*, 2007; Trouiller *et al.*, 2009)**. Micronuclei induction by nano TiO2 reflects the extent of induced chromosomal changes leading to formation of micronuclei either from a broken centric or acentric part(s) of chromosomes or from intact whole chromosomes lagging behind at the anaphase stage of cell division and failed to be incorporated into one of the daughter nuclei **(Rahman *et al.*, 2002)**.

The chromosomal and DNA damage in mice bone marrow, liver and brain tissues observed in the present work in consistent with study of **Trouiller *et al*. (2009)** who reported that nan-sized TiO2 catalyze oxidative DNA damage and chromosomal damage in multiple organs of mice. In addition, the cytotoxic effect of TiO2 nanoparticles was confirmed by the reported significant reduction in PCEs\NCEs erythrocytes ratio and is, in agreement with previous studies **(Rahman *et al.*, 2002; Wang *et al.*, 2007; El ghor et al., 2014; Mohamed, 2015)**.

chemical interaction between TiO2 nanoparticles and DNA through the DNA phosphate group **(Zhu *et al.*, 2007; Li *et al.*, 2008)** and its direct binding to DNA or repair

enzymes leading to the generation of strand breaks **(Jha *et al., 1992;* Hartwig, 1998)**. On the other hand, the observed elevations of hepatic MDA level in our study (Table 3) confirmed the previously reported naon-TiO2 induced DNA damage mechanism indirectly through inflammation **(Chen *et al.,* 2006; Grassian *et al.,* 2007)** and generation of reactive oxygen species (ROS) **(Gurr *et al.,* 2005; Long *et al.,* 2006*;* Kang *et al.,* 2008)** as enhanced production of ROS results in oxidative stress that cause lipid peroxidation and can damage DNA.

As a marker of lipid peroxidation elicited by ROS, dose-dependent increased in the MDA production was observed in this study (Table 3). A possible explanation for the enhancement of MDA concentration may be decreased formation of antioxidants which in view of augmented activity of ROS allows a consequent increase in MDA production Possible ROS that could be formed by nano-TiO2 are hydroxyl radicals (•OH), superoxide radical anions (O2−), hydrogen peroxide (H2O2) and singlet oxygen (1O2) **(Reeves *et al.,* 2008)**. These species can be identified by means of electron spin resonance (ESR), using the technique of spin trapping **(Chen *et al.,* 2009*)***.** •OH radicals are the predominant radical species generated and play the major role in producing the genotoxic effects in terms

(Naziroglu *et al.,* 2004*;* Naghizadeh *et al.,* 2008). Also, the inverse correlation between cell viabilities decline versus ROS level elevation demonstrated by **Yang *et al.* (2008)** further proved that oxidative stress was probably a key route by which TiO2 nanoparticles induced the observed cytotoxicity.

of oxidative DNA damage **(Reeves *et al.,* 2008)**. Moreover, nano TiO2 induced oxidative DNA damage has been proved by the determination of increased 8-hydroxy-deoxyguanosine (8OHdG), a good marker of oxidative DNA lesion **(Schins *et al.,* 2002; Papageorgiou *et al.,* 2007)**, in mouse livers after TiO2 nanoparticles treatment **(Trouiller *et al.,* 2009)**.

It is here worth mentioning that GSH is an important antioxidant defense mechanism which regulates the enzymatic and non-enzymatic detoxification of ROS generated by many compounds (**Venkatesan, 1998)**, its role in the formation of conjugates with electrophilic drug metabolites most often formed by cytochrome P450 linked monooxygenase is well established **(Sener *et al.,* 2003; Ajith *et al.,* 2007*).*** Moreover, the role of GSH in DNA synthesis **(Wang and Ballatori, 1998)** and its involvement in the repair of DNA damage has been reported (**Pendyala *et al.,* 1997)**. Therefore, decreased cellular GSH levels recorded in the present study (Table 3) increases the sensitivity of organs to oxidative and chemical injury.

The recorded decrease in SOD, CAT and GSH-Px activities by nano-titanium (Table 3) further confirmed that oxidative stress is a key route for DNA damage indirect mechanism as SOD, CAT and GSH-Px enzymes are among the endogenous antioxidant enzymes that play a pivotal role in the elimination of the superoxide radical and hydrogen peroxide and thus interrupt the propagation of lipid peroxidation reactions. In this process, GSH-Px requires endogenous reduced glutathione as a co-substrate. Thus, these enzymes can act as protectors that guard against oxidative damage induced by ROS **(Mansour *et al.*, 2002*)***.

CAT is an oxidoreductase enzyme, which transforms H2O2 into H2O and O2 and protects cells from damage induced by ischemia reperfusion by scavenging ROS **(Shen *et al.*, 2007*)***. SOD and GPx called antioxidant enzymes protect the cells against reactive oxygen radicals (singlet oxygen: 1O, superoxide radical: O2 -, hydroxyl radical: -OH and hydrogen peroxide: H2O2). SOD catalyses the dismutation of two superoxide radicals to O2 and H2O2 while GPx detoxifies H2O2 to H2O and O2, and converts lipid hydroperoxides to nontoxic alcohols **(Watson *et al.*, 1999*)***.

Additionally, The functional groups of SOD (NH_2 and ε-amino groups of lysine and the SH group of cysteine) are highly prone to oxidative damage. Conversion of SH groups into disulphides and other oxidized species (e.g. oxyacids) is one of the earliest events observed during radical-mediated oxidation of proteins **(Uchida *et al.*, 1999*)***.

Nano-TiO2 induced hepatotoxicity observed in this study confirmed the previous study of **Chen *et al.* (2009)** who showed that some neutrophilic cells were found indicating that nano-TiO2 particles induced inflammation in liver tissues. Moreover, our study confirmed the previously described neurotoxicity of nanosize TiO2 reported by **Long *et al.* (2007) and Renping *et al.*, (2010)** that TiO2 stimulates ROS in brain microglia and damages neurons in vitro.

The observed stability of TiO2 genotoxicity after 24 and 48 hours sampling times in liver and brain may be attributed to the previously reported long retention half-time of TiO2 *in vivo* as a result of its difficult excretion which directly lead to difficult clearance of TiO2 *in vivo* and particle deposition in liver causing hepatic lesion **(Chen *et al.* 2009)**. On the contrary, a decreased genotoxicity and cytotoxicity of nano-sized TiO2 was observed in case of bone marrow cells (frequency of MNPCEs, PCEs/NCEs ratio and parameters of comet assay) (Table 3). This may be explained based on the rapid division of bone marrow cells leading to the elimination of damaged cells from the pool of cells which in turn was reflected in decreased level of toxicity.

Furthermore, this study confirmed the nano-TiO2 dose-dependent toxicity as

evidenced by regression analysis (Fig. 3 & 5) and may be attributed to the previously reported long retention half-time of TiO2 *in vivo* as a result of its difficult excretion directly leading to difficult clearance of TiO2 *in vivo* and particle deposition in liver causing hepatic lesion **(Chen *et al*. 2009)**. Also, titanium contents in lung, kidney and liver (expect heart) subsequently increased gradually with time to various degrees as well.

The results of the present study revealed that CHL co-treatment significantly decreased the micronuclei and oxidative DNA damage induction (Table 1 & 2) and inhibited ROS generation (Table 3) by nano TiO2 under the experimental conditions used. These results confirmed the antigenotoxic effects of CHL that have been previously described by several studies **(Bez *et al.*, 2001; Negraes *et al.*, 2004; Hayatsu *et al.*, 2009; Osowski *et al.*, 2010)**.

Despite its precise mechanism of action remain unclear yet. CHL protective effect may be attributed to its ability to scavenge free radicals, binding to the active site of mutagen, adsorbing or absorbing toxic compounds **(Sarkar *et al.*, 1996; Hayatsu *et al.*, 2009*)*** and formation of complex with mutagen **(Dashwood, 1997)**. Our study confirmed the reported antioxidative property of CHL **(Kamat *et al.*, 2000; Kumar *et al.*, 2004*;* Zhang *et al.*, 2008)** based on the observed MDA depletion and restoring antioxidant defense system GSH level and SOD, CAT and GPx activities (Table 3). Similar results were observed by **Boloor *et al.* (2000)** who showed that CHL inhibits lipid peroxidation by scavenging free radicals and restores depleted levels of SOD and GSH suggesting the role of CHL in quenching the ROS formed.

Therefore, the obtained results from this part of experiment suggest the possible involvement of cellular GSH as a mechanistic step in CHL mediated protection against nano sized TiO_2 induced genotoxicity that may be due to significantly increased GSH concentration. Moreover, the elevated level of GSH could effectively provide thiol group for the possible GST mediated detoxification reactions **(Ajith *et al.*, 2007)**. Moreover, our results suggest that increased SOD, GSH and CAT activities may be attributed to reactive oxygen products such as superoxide anions and H_2O_2 generated by nano TiO_2 particles. These data indicate that CHL may provide protection in part by improving activities of the endogenous antioxidant enzymes, which scavenge ROS and reduce their effects.

In conclusion, single injection of nano-TiO2 induced genotoxicity in liver, brain and bone marrow cells in a dose dependent manner mainly via ROS generation as evidenced by scavenging protective effect of CHL in mice.

5. Acknowledgement

We would like to thank Faculty of science, Cairo University, Egypt for kindly providing chemicals. Also, thanks for Dr. Ameria (Cairo University, Egypt) for her kindly assistant in Comet assay learning.

6. Author contributions

All authors designed the present study. H.M and R.M executed the experimental work, performed data analyses and wrote the manuscript. HM revised the final manuscript.

7. Conflict of interest

All authors declared no conflict of interest.

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