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RESEARCH ARTICLE

Eugenol attenuates TiO_2 nanoparticles-induced oxidative damage, biochemical toxicity and DNA damage in Wistar rats: an in vivo study

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Abstract

Titanium dioxide nanoparticles (TiO_2 NPs) are widely used in food, edible dyes, and other commercial products. Human exposure to TiO_2 NPs has raised concerns regarding their toxic potential. Various studies have evaluated the TiO_2 NPs-induced toxicity, oxidative damage to the cellular components, and genotoxicity. In the present study, we examined whether co-treatment with the dietary antioxidant eugenol can attenuate or protect against TiO_2 NPs-induced toxicity. We exposed the adult male Wistar rats to TiO_2 NPs (150 mg/kg body weight) by intraperitoneal injection (i.p.) either alone or as co-treatment with eugenol (1-10 mg/kg body weight) once a day for 14 days. The untreated rats were supplied saline and served as control. Titanium (Ti) accumulation in various tissues was analyzed by inductively coupled plasma mass spectrometry. Serum levels of liver and kidney biomarkers and oxidative stress markers in the liver, kidney, and spleen were determined. A significant increase in hydrogen peroxide level confirmed that oxidative stress occurred in these tissues. TiO_2 NPs induced oxidation of lipids, and decreased glutathione level and antioxidant enzyme activity in the kidney, liver, and spleen of treated rats. TiO_2 NPs also increased the serum levels of alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, albumin, and total cholesterol and decreased the blood urea nitrogen, uric acid, and total bilirubin in serum, which indicates oxidative damage to the liver and kidney. In eugenol and TiO_2 NPs co-treated rats, all these changes were mitigated. Single-cell gel electrophoresis (comet assay) of lymphocytes showed longer comet tail length in TiO_2 NPs-treated groups, indicating DNA damage while tail length was reduced in eugenol and TiO_2 NPs co-treated groups. Thus, it seems that eugenol can be used as a chemoprotective agent against TiO_2 NPs-induced toxicity.

Keywords TiO_2 nanoparticles · Oxidative damage · Biochemical toxicity · DNA damage · Eugenol · Wistar rats

Introduction

Nanotechnology is a branch of science that deals with synthesis, characterization, and application of nanomaterials, materials having at least one dimension in the nano range (1–100 nm), which are widely used in industrial products, agriculture, and medicine (Dubchak et al. 2010; MubarakAli et al. 2013; He et al. 2019). Owing to their high surface area to volume ratio, nanoparticles (NPs) have different physical, chemical, and biological properties from their bulk materials, and these properties facilitate their use in a wide range of applications (Thakkar et al. 2010; Schmid 2011). NPs have wide biomedical applications, for instance, silver nanoparticles are used as antimicrobial (Hamida et al. 2020a, b) and anticancer agents (Bin-Meferij and Hamida 2019), carbon nanotubes are used in drug delivery for effective transport and controlled release of drugs (Bianco et al. 2005), quantum dot NPs are used to locate malignant cells in the body (Pathak et al. 2019), iron oxide NPs are used in resonance imaging and diagnosis of tumors (Park et al. 2008), while copper oxide NPs (Hassan et al. 2019), zinc oxide NPs (Fouda et al. 2018), and selenium NPs (Salem et al. 2020) have anticancer activities.

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Titanium dioxide (TiO_2) nanoparticles (NPs) occur as bright white pigment commonly used in cosmetics, plastics, ceramics, paints, inks, pharmaceuticals, tooth-paste, tableted drugs, and even for whitening skim milk and brightening foods (Xie et al. 2011; Mohammed and Safwat 2020). TiO_2 NPs have anti-bacterial, anti-fungi, and anti-cancer activities, and are also used in photodynamic therapy (PDT), drug delivery, cell imaging, and biosensors for biological assay (Behnam et al. 2018). TiO_2 NPs have been used as delivery agent of anticancer drugs such as daunorubicin, doxorubicin, and cisplatin (Xu et al. 2015; Ren et al. 2013; Liu et al. 2015).

Despite these widespread applications, studies have reported that upon exposure to humans, TiO_2 NPs accumulate in the lungs, alimentary tract, liver, heart, spleen, kidneys, and cardiac muscle and induce oxidative damage, genotoxicity, and apoptosis in these tissues (Baranowska-Wojcik et al. 2020). TiO_2 NPs destroy the villi in the human small intestine (Faust et al. 2014), affect heart rate (Kan et al. 2014), and are neurotoxic to the human brain (Allen 2016; Feng et al. 2015; Wang et al. 2008; Márquez-Ramírez et al. 2012). TiO_2 NPs also induce hepatotoxicity (Liu et al. 2009; Xu et al. 2013; Shakeel et al. 2016), renal damage (Liang et al. 2009; Zhao et al. 2010), inflammation in spleen (Chen et al. 2009), genotoxicity in bone marrow cells (Sycheva et al. 2011; Dobrzyńska et al. 2014), and compromise immune response (Duan et al. 2010) in various animal models.

Eugenol (4-allyl-1-hydroxy-2-methoxybenzene) is a major ingredient of essential oil derived from traditional spices such as clove, cinnamon, nutmeg, and basil. It possesses a multitude of health promoting bioactivities including antiseptic, antibacterial, anti-inflammatory, and antioxidant as well as analgesic activities, and therefore is used for medicinal purposes (Lee and Shibamoto 2001; Tiku et al. 2004; Pramod et al. 2010; Rao and Gan 2014). Antioxidant activity of eugenol has also been demonstrated against thioacetamide (Yogalakshmi et al. 2010), triton (Venkadeswaran et al. 2014; Elbahy et al. 2015), lipopolysaccharide (Huang et al. 2015), TPA (Tetradecanoylphorbol-13-acetate) (Kaur et al. 2010), cisplatin (Sakat et al. 2019), and arsenic trioxide (As_2O_3) (Binu et al. 2018) toxicity. Intraperitoneal LD50 of eugenol was reported to be 500–630 mg/kg body weight (bw) and oral as 2650–3000 mg/kg bw in rats (Vijayasteltar et al. 2016).

To the best of our knowledge, no previous studies have been conducted on the role of eugenol in counteracting TiO_2 NPs-induced toxicity. The antioxidant potential of eugenol has been found to be 5 times more powerful than vitamin E (Nagababu et al. 2010). Therefore, the present study was done to investigate the TiO_2 NPs-induced oxidative stress, biochemical toxicity, and DNA damage in male Wistar rats, and a trial to explore the possibility that a potent antioxidant such as eugenol can reduce the TiO_2 NPs-induced oxidative damage and thereby, lower the toxicity of these NPs.

Materials and methods

Titanium dioxide nanoparticles (CAS Number: 13463-67-7, CAT Number: 634662) and eugenol (CAS Number: 97-53-0, CAT Number: 35995) were purchased from Sigma-Aldrich Co, USA. Trichloroacetic acid (TCA) (CAS 76-03-9, CAT 100807), potassium phosphate (CAS 7778-77-0, CAT 104873), potassium iodide (CAS 7681-11-0, CAT 105043), maleic acid (CAS 110-16-7, CAT 800380), tris(hydroxymethyl)aminomethane (Tris) (CAS 77-86-1, CAT 102408), n-butanol (CAS 71- 36-3, CAT 100988), hydrogen peroxide (H_2O_2) (CAT 107209), thiobarbituric acid (CAS 504-17-6, CAT 108180), pyridine (CAS 110-86-1, CAT 109728), and ethidium bromide (CAS 1239-45-8, CAT 111615) were purchased from Merck, India. NaOH (CAS 1310-73-2, CAT 68151), low melting point agarose (CAS 9012-36-6, CAT 91466), pyrogallol (CAS 87-66-1, CAT 71646), succinic acid (CAS 110-15-6, CAT 96330), Triton X-100 (CAS 9002-93-1, CAT 64518), EDTA (CAS 6381-92-6, CAT 40088), Tris-HCl (CAS 1185-53-1, CAT 99438), NaCl (CAS 7647-14-5, CAT 33205), and dithiobisnitrobenzoic acid (CAS 69-78-3, CAT 32363) were purchased from Sisco Research Laboratory (SRL, Mumbai, India). Histopaque (CAT 10771) was purchased from Sigma-Aldrich Co, USA

All the kits for biochemical analysis were purchased from Siemens Ltd., Gujarat, India.

Animals

Wistar rats (adult males, 180–200 g body weight (bw)) were used for the experiments. All animals were kept under standard conditions (room temperature of 18 ± 25 °C; relative humidity 45–55%, 12:12 h light/dark cycle and given food and water ad libitum. The animals were acclimatized with the laboratory conditions for 15 days before treatments were started, in the animal house facility of Department of Zoology, Aligarh Muslim University, Aligarh. Institutional Animal Ethics Committee (IAEC) Aligarh Muslim University, Aligarh, (714/GO/Re/S/02/CPCSEA) has approved this work and experimental guidelines set by CPCSEA, New Delhi, were followed.

TiO_2 NPs characterization, preparation and exposure

The characteristics of the TiO_2 NPs as described by the manufacturer are as follows: Titanium (IV) oxide -nanoparticles, rutile and anatase mixture, < 100 nm particle size (BET), 99.5 % trace metal basis. The NPs were further characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD), and dynamic light scattering (DLS) methods. Hydrodynamic diameter of the NPs and zeta potential was measured using dynamic light scattering on a Malvern

Instrument Zetasizer (Malvern Instruments, UK) equipped with He-Ne (633 nm λ laser). To prepare stock solutions, 10 mg of TiO_2 NPs was dispersed in 1 ml of normal saline (Rossi et al. 2019) and sonicated in an ultra-sonic water bath (Elmasonic S 30H, Elma, Frequency 37 kHz) for 15 min. The suspensions were diluted to required doses in fresh saline and stirred on vortex agitator to obtain uniformity before use. The stock solution and the dilutions were freshly prepared every day for 14 days (the duration of the experiment).

Animal experimental studies

A schematic representation of experimental design is depicted in Fig. 1. The studies were divided into two parts:

In the first part, rats were treated with different doses of TiO_2 NPs. The purpose of this was to analyze the accumulation of titanium (Ti) in various tissues and also to determine a dose of TiO_2 NPs at which significant biochemical disturbance in serum and oxidative damage in liver is induced, when administered intraperitoneally (i.p.) for 14 days. Fifty rats were randomly grouped into five groups, with each group containing 10 rats: Group 1 (control) rats were given normal saline, groups 2–4 were treated with TiO_2 NPs doses of 50, 100, and 150 mg/kg bw/day, respectively. Rats in 5th group were given the bulk TiO_2 (150 mg/kg bw/day). All treatments were given for 14 days through intraperitoneal injection. Titanium content in tissues, serum biochemical analysis and hydrogen peroxide (H_2O_2) production, lipid peroxidation (LPO), catalase (CAT), and superoxide dismutase (SOD) activities were determined in liver homogenate.

In the second part of the study, rats were co-treated with different doses of eugenol. TiO_2 NPs dose of 150 mg/kg bw was used based on the results of our preliminary study and various other previous studies (Liu et al. 2009, 2010a, b). Eugenol doses chosen were based on earlier studies which reported 10 mg/kg bw dose of eugenol to be safe that confers protection against oxidative damage (Yogalakshmi et al. 2010; Prasad 2013; Abd El Motteleb et al. 2014; Huang et al. 2015).

Fifty rats were randomly grouped into five groups, with each group containing 10 rats. All treatments were done for 14 days through intraperitoneal injection.

Group 1 (control): Rats given normal saline
Group 2 (NP group): Rats treated with TiO_2 NPs (150 mg/kg body weight (bw)/day)
Group 3 (NP + E1 group): Rats co-treated with TiO_2 NPs (150 mg/kg bw/day) and eugenol (1 mg/kg bw/day)
Group 4 (NP + E5 group): Rats co-treated with TiO_2 NPs (150 mg/kg bw/day) and eugenol (5 mg/kg bw/day)
Group 5 (NP + E10 group): Rats co-treated with TiO_2 NPs (150 mg/kg bw/day) and eugenol (10 mg/kg bw/day)

Biochemical analysis of serum and oxidative stress markers were measured in the liver, kidney, and spleen. The comet assay was performed in lymphocytes to determine DNA damage.

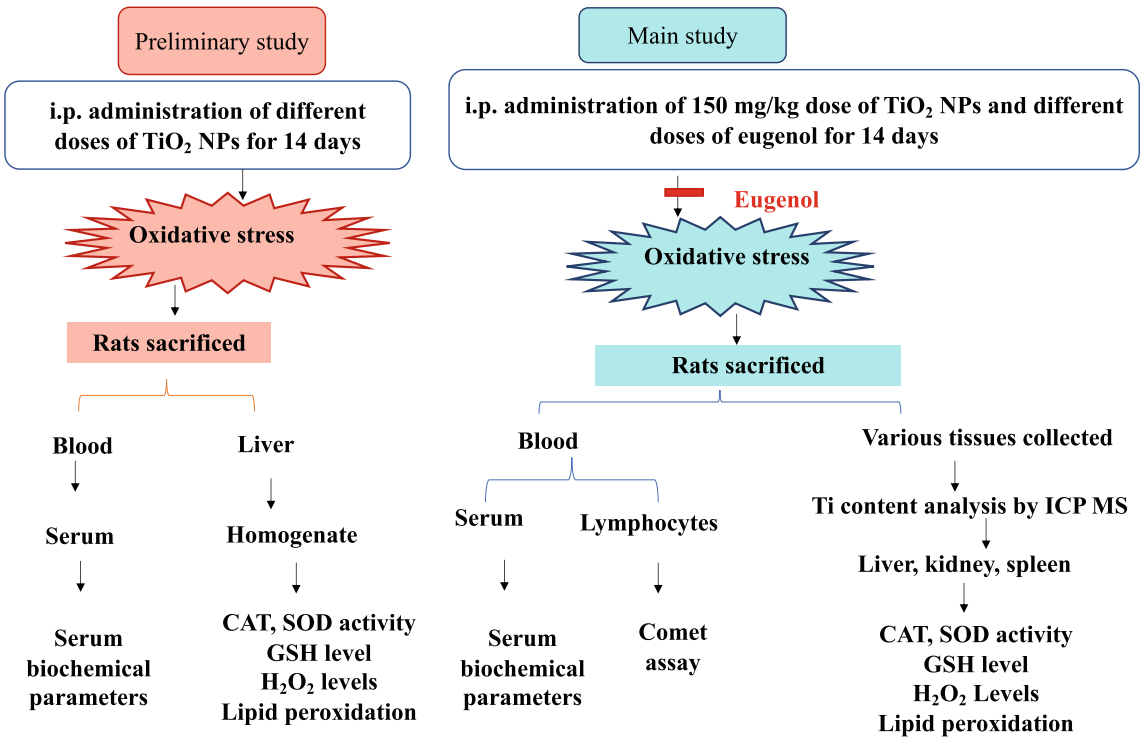

Fig. 1 Schematic representation of the experimental design. CAT, catalase; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; ICP-MS, inductively coupled plasma mass spectrometry; i.p., intraperitoneal; SOD, superoxide dismutase; Ti, titanium; TiO_2 NPs, titanium dioxide nanoparticles

A dose of 150 mg/kg bw in rats is equivalent to 24.3 mg/kg bw of humans, calculated by using the equation described in an earlier report (Nair and Jacob 2016): HED (mg/kg) = Animal does (mg/kg) × (Animal K_m/ Human K_m), where HED refers to Human Equivalent Dose. i.p. route of administration was chosen based on previous studies (Alarifi et al. 2013; Hilal et al. 2018; Younes et al. 2015; Valentini et al. 2019; Meena and Paulraj 2012; Turner et al. 2011) and co-treatment mode of NPs and antioxidant was chosen based on results of a previous study (Parveen et al. 2014).

Preparation of samples

The rats were anesthetized 24 h after the final treatment using CO_2 inhalation in cages of size 10" × 19" × 9" with CO_2 flowrate of 5.6 L/minute and then sacrificed by abdominal venesection method. Euthanasia with CO_2 is approved by the American Veterinary Medical Association (AVMA). After sacrifice, blood was immediately collected via heart puncture and centrifuged for 10 min at 2500*g* at 4 °C in a clinical centrifuge, and the serum was taken in aliquots. The liver, kidney heart, lung, brain, and spleen were collected from each rat; one portion was used for titanium content analysis and the other part was processed for determining oxidative damage and protein estimation by Lowry method (Lowry et al. 1951). Homogenates prepared were preserved at − 80 °C for further analysis.

Titanium content analysis in tissues

About 0.3 g of each harvested tissue was used for analysis of titanium content by ICP-MS (Element XR, Thermo Fisher Scientific, Germany) using 20 ng/ml indium as internal standard. Briefly, the tissues were put in nitric acid overnight; the digested tissues were then mixed with H_2O_2 and heated in an oven at 160 °C until the solution turned colorless. The final solution was adjusted to 3 ml with 2 % nitric acid prior to analysis with ICP-MS. The results are expressed in nanogram (ng) per gram of tissue.

Biochemical analysis of serum

Biochemical parameters including alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), albumin, total bilirubin, and total cholesterol (liver function markers) as well as uric acid (UA) and blood urea nitrogen (BUN) (kidney function markers) were analyzed in serum of untreated and TiO_2 NPs-treated samples using commercial kits (Siemens Ltd., Gujarat, India) following the manufacturer's instructions. All analysis was done using a clinical biochemical autoanalyzer (7180 Hitachi, Japan).

Oxidative stress markers

Estimation of H_2O_2 level

H_2O_2 levels were estimated by the method as described in an earlier report (Lone et al. 2017). Briefly, 1 g of tissue sample from each animal was homogenized in 0.1% trichloro acetic acid (TCA), centrifuged at 12000*g* for 15 min at 4 °C. The reaction mixture consisting of 0.5 ml supernatant, potassium phosphate buffer (10 mM, pH 7.0), and potassium iodide (1 mM) was read spectrophotometrically at 390 nm. H_2O_2 concentrations were expressed as μmol/mg of protein.

Estimation of GSH content

GSH content was determined by using 5,5′-dithiobisnitrobenzoic acid after precipitating the protein as described in an earlier report (Beutler 1984).

Determination of malondialdehyde level

Lipid peroxidation (LPO) in the tissue sample was estimated from the malondialdehyde (MDA) levels using protocol described in an earlier report (Mehrotra and Trigun 2012). Briefly, to 0.5 ml of diluted tissue homogenate, 1 ml of Tris-Maleate buffer (0.2 M, pH 5.9) was added and the mixture was incubated in a 37 °C water bath for 30 min. Thiobarbituric acid reagent was then added and incubated for 10 min in a 100 °C water bath. The mixture was allowed to cool, 3 ml of pyridine: n-butanol (3:1 v/v) and then 1 ml NaOH (1 N) was added and kept at room temperature. After 10 min, the mixture was read spectrophotometrically at 548 nm. Lipid peroxidation levels were expressed as nmol MDA/mg protein.

Determination of anti-oxidant enzymes

Superoxide dismutase (SOD) activity in tissue homogenates was measured by the method of Marklund and Marklund (1974). The reaction was started by adding 0.05 ml sample to the reaction mixture consisting of 2.85 ml Tris-succinate buffer (50 mM, pH 8.2) and 0.1 ml 8 mM pyrogallol. The decrease in absorbance was followed at 420 nm for 3 min at an interval of 30 s. A reaction mixture containing 50 μl distilled water instead of sample was used as reference. SOD activity was expressed as U/mg protein.

Catalase (CAT) activity was determined tissue homogenates as described by Aebi (1984). Sample (0.1 ml) was mixed with 1.9 ml potassium phosphate buffer (50 mM, pH 7.0), followed by addition of 1 ml of 30 mM H_2O_2 solution. H_2O_2 is converted to H_2O and O_2 that results in decrease in

absorbance at 240 nm which was followed for 5 min. CAT activity was expressed as U/mg protein.

Comet assay

Genotoxicity was studied by single-cell gel electrophoresis (comet assay) in lymphocytes isolated from rats treated with TiO_2 NPs in absence and presence of eugenol. Diluted blood (1:1 with normal saline) was layered on histopaque and centrifuged for 20 min at 530*g* in a laboratory centrifuge; the buffy layer at interface was taken, washed thrice, and suspended in saline to get a lymphocyte suspension. The lymphocytes were then mixed with 0.5% low melting point agarose (LMPA) and spread on glass slides pre-coated with 1% agarose. After another coat of 0.5 % LMPA, the slides were left at 4 °C to solidify the gel. Lymphocytes were lysed by putting slides in lysis buffer (2.5 M NaCl, 1.0 % Triton X-100, 100 mM EDTA, 10 mM Tris-HCl, pH 10.0) for 2 h at 4 °C. The slides were placed in alkaline electrophoretic solution (0.42 M NaOH, 1.5 mM EDTA, 0.9 % NaCl) for 30 min at 4 °C to unbind the DNA. After electrophoresis for 20 min at 300 mA and 25 V, slides were kept in neutralization buffer and the DNA stained with ethidium bromide (20 μg/ml) for 10 min. DNA damage was examined under a CX41 fluorescence microscope (Olympus, Japan) at × 100 magnification (Singh et al. 1998; Maheshwari and Mahmood 2020). Ethidium bromide-stained comet slides were observed under a CX41 fluorescence microscope (Olympus, Japan). Three slides were prepared for each animal in each group. Using Komet 5.5 software, USA, the tail lengths of 100 cells per slide were measured (100 × 3 = 300 cells per animal per group). Since each group contained 6 animals, so 300 × 6 = 1800 nuclei were counted per group. The mean values of each group with respect to tail length were calculated and reported.

Statistical analysis

One-way ANOVA was used for statistical analysis of data. Prior to ANOVA, Shapiro-Wilk test confirmed normality of data while Bartlett's test checked homoscedasticity. We have compared the difference among individual sample results, that difference/error has been reported as standard error of the mean (SEM). Tukey's test was used to compare differences between samples. The results were presented as mean ± SEM. We have chosen SEM and not SD because SD indicates how accurately the mean represents sample data, not the difference among sample results. The level of statistical significance was set at $p < 0.05$. All analysis was performed using R version 3.5.2 (R Core Team 2018) and figures were created using R packages ggplot2 (Wickham 2016) and cowplot (Wilke 2019).

Results

Nanoparticle characterization

The nanoparticles were characterized by XRD (Fig. 2a) and SEM (Fig. 2b). No spurious peak was observed in the XRD pattern of TiO_2 NPs, which is an indication of crystallographic purity. TiO_2 in the anatase phase was confirmed by diffraction peaks of 101 and 200 at 2θ values of 25.3° and 48° respectively. Using Scherer's formula ($D = 0.89\lambda/\beta\cos\theta$, where, D is particle diameter size, λ is wave length of X-ray (0.1540 nm), β is FWHM (full width at half maximum), θ is diffraction angle) (Bin-Meferij and Hamida 2019), the average grain size was calculated to be 14.5 nm. SEM micrographs revealed spherical shape of TiO_2 NPs with an average size of 50 nm, approximately. To understand the NP characteristic in the exposure medium (normal saline, pH 7), hydrodynamic diameter of the NPs and zeta potential was measured. The NPs tended to agglomerate with a wide particle size distribution ranging in between 16 and 118 nm; however, most of the particles dispersed within 50 nm in normal saline (Fig. 2c). At pH 7 of the suspension medium (normal saline), the Zeta potential of the NPs was found to be – 20.2 mV.

Analysis of titanium content

Levels of titanium (Ti) content in various tissues are shown in Fig. 3. Ti content accumulation increased in rat tissues with the increase of TiO_2 dose, the order of accumulation being liver > kidneys > spleen > lung > brain > heart. Since Ti accumulated highest in the liver, kidney, and spleen, these tissues were chosen for the further study. Also, the levels of Ti in the tissues of rats treated with 150 mg/kg bw bulk TiO_2 were significantly lower than those of 150 mg/kg bw NPs-treated ones, indicating that TiO_2 in the nano form penetrated the rat tissues more easily than in the bulk form.

Effect of different doses of TiO_2 NPs on serum biomarkers and oxidative stress in the liver

In TiO_2 NPs-treated rats, a statistically significant increase in ALT, AST, ALP, albumin, and total cholesterol ($p < 0.05$ or $p < 0.01$) with a decrease in the bilirubin, UA, and BUN serum levels were observed as compared to control (Table 1). The NPs treatment decreased the activity of CAT and SOD (Fig. 4a, b), with an increase in the lipid peroxidation and hydrogen peroxide levels (Fig. 4c, d) in the liver. At 150 mg/kg bw dose of NPs, these changes were more statistically significant than bulk TiO_2 and 50 and 100 mg/kg bw doses of TiO_2 NPs.

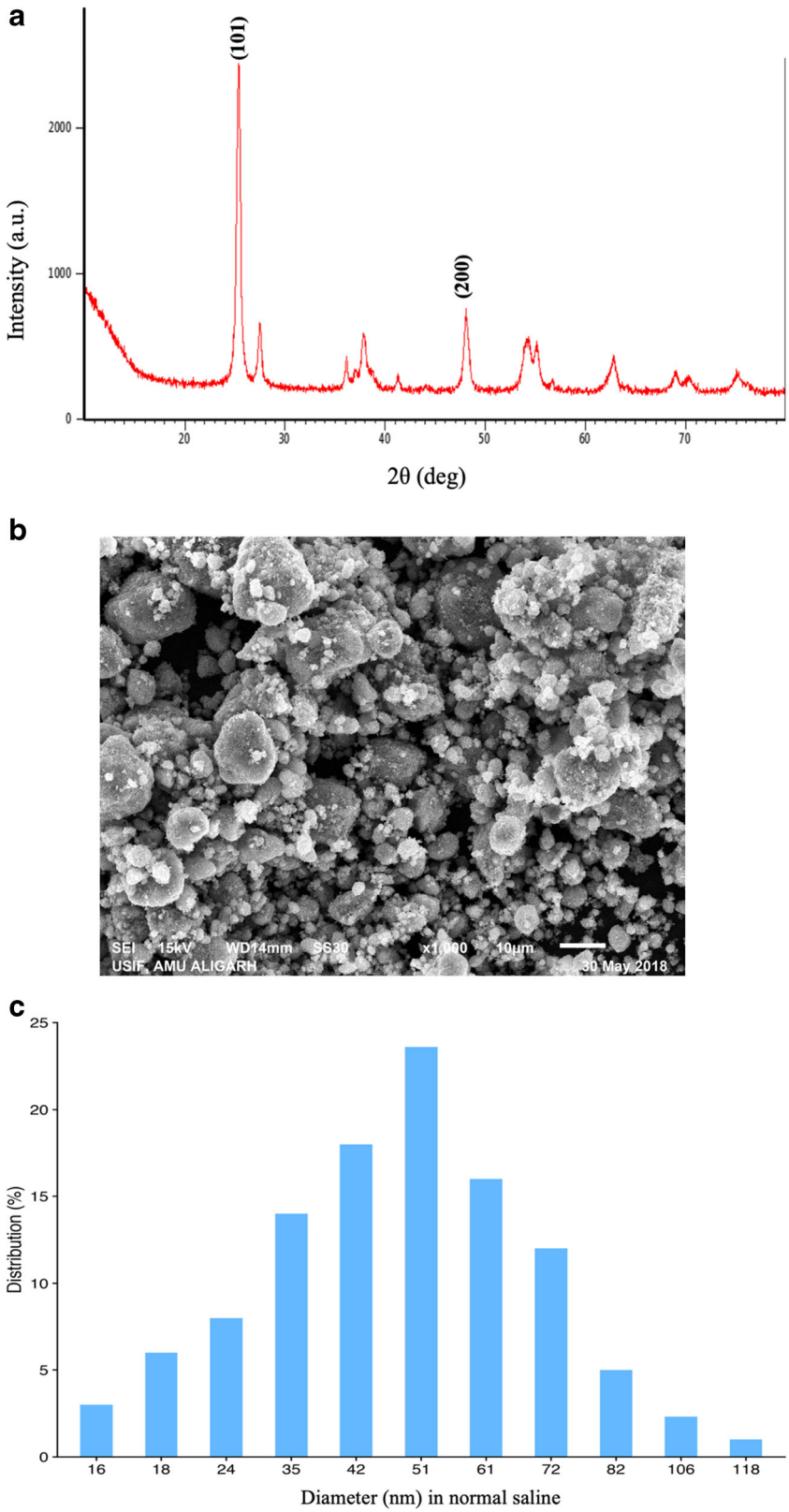

Fig. 2 **a** X-ray diffraction pattern of TiO_2 NPs; **b** SEM image of TiO_2 NPs. Scale bar, 10 μm; **c** DLS characterization of TiO_2 NPs in normal saline

Table 1 The changes of biochemical parameters in the blood serum of Wistar rats induced by intraperitoneal administration of different doses of TiO_2 NPs and 150 mg/kg bw of bulk TiO_2 for 14 days

	TiO_2 NP dose (mg/kg bw)				
	0	50	100	150	Bulk TiO_2
ALT (U/l)	39.8 ± 1.63	47 ± 2.072*	55 ± 2.52$^{\#}$	68.3 ± 2.30$^{\#}$	59.23 ± 0.16*^{,a}
AST (U/l)	44.6 ± 0.40	62 ± 0.25*	78 ± 0.81$^{\#}$	94.3 ± 0.04$^{\#}$	84.12 ± 0.12*^{,a}
ALP (U/l)	128.8 ± 6.38	149 ± 6.12*	186 ± 8.55$^{\#}$	222.1 ± 10.90$^{\#}$	189 ± 6.04$^{\#,a}$
BUN (mg/dl)	18.6 ± 0.46	17.8 ± 0.40*	17.2 ± 0.37*	16.8 ± 0.24*	17.01 ± 0.37*^{,a}
UA (mg/dl)	5.1 ± 0.44	3.4 ± 0.17*	3 ± 0.16$^{\#}$	2.7 ± 0.12$^{\#}$	2.93 ± 0.14*
Albumin (g/dl)	2.63 ± 0.12	2.77 ± 0.13*	2.84 ± 0.14*	3.1 ± 0.17*	2.95 ± 0.11*^{,a}
Bilirubin (mg/dl)	0.8 ± 0.04	0.73 ± 0.03*	0.7 ± 0.03*	0.67 ± 0.03*	0.72 ± 0.02*
Total cholesterol (mg/dl)	45.3 ± 2.07	47.2 ± 2.09*	49.7 ± 2.28*	51.8 ± 2.38*	49.2 ± 2.17*^{,a}

Values represent mean ± SEM, $n = 10$; * Significantly different from the control at $p < 0.05$; # Significantly different from the control at $p < 0.01$; [a] Significantly different from the 150 mg/kg NP-treated group at $p < 0.05$

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *ALP* alkaline phosphatase, *BUN* blood urea nitrogen, *UA* uric acid

Effect of eugenol co-treatment on serum biomarkers

As shown in Table 2, rats treated with TiO_2 NPs had significant elevated levels of ALT, AST, ALP, albumin, and total cholesterol and decreased bilirubin levels in serum, suggesting damage to the liver while low serum levels of UA and BUN indicate kidney damage ($p < 0.05$ or $p < 0.01$). In contrast, co-treatment with eugenol for 14 days restored the biochemical parameters in serum towards normal level. The 10 mg/kg bw dose of eugenol was much more effective than its lower doses (Table 2).

Effect of eugenol co-treatment on oxidative stress markers in the liver, kidney and spleen

In comparison to control group, a statistically significant increase in H_2O_2 (Fig. 5a–c) and MDA levels (Fig. 5d–f) was found in the liver, kidney, and spleen of TiO_2 NPs-treated group, suggesting that TiO_2NPs induce oxidative stress. Co-treatment with eugenol significantly declined H_2O_2 and MDA levels ($p < 0.05$ or $p < 0.01$), with eugenol being more effective at the highest dose used (10 mg/kg bw). TiO_2 NPs also inhibited CAT (Fig. 6a–c), SOD activity (Fig. 6d–f), and GSH

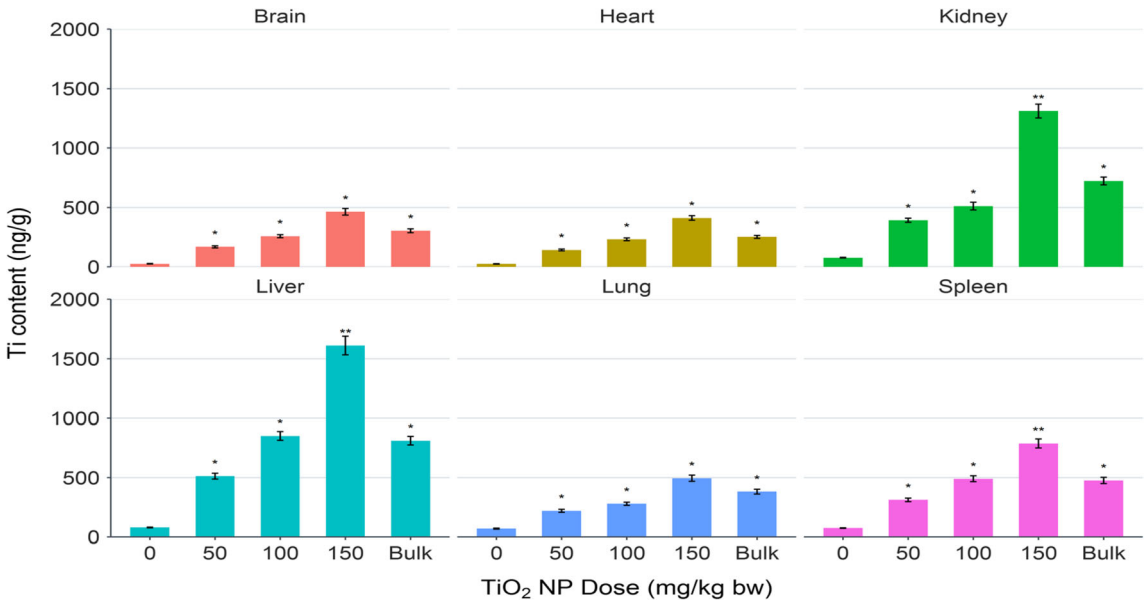

Fig. 3 Levels of titanium in different rat organs after intraperitoneal administration of various doses of TiO_2 NPs and bulk TiO_2 (150 mg/kg bw) for 14 days. The values represent mean ± SEM where $n = 10$. * Significantly different from the control at $p < 0.05$, ** significantly different from the control at $p < 0.01$

Table 2 The changes of biochemical parameters in the blood serum of Wistar rats after TiO_2 NPs (150 mg/kg bw) treatment alone or cotreatment with TiO_2 NPs (150 mg/kg bw) and different doses of eugenol for 14 days

Parameter	Group				
	Control	NP	NP + E1	NP + E 5	NP + E10
ALT (U/L)	41.2 ± 1.33	70.3 ± 3.20[a]	68.2 ± 2.36	56 ± 2.21[c]	48.22 ± 1.41[d]
AST (U/L)	44.2 ± 0.20	93.8 ± 0.04[a]	90.1 ± 0.02	76 ± 0.06[c]	52.3 ± 0.02[d]
ALP (U/L)	126.7 ± 6.28	223.1 ± 10.42[a]	219 ± 9.11	182 ± 6.32[c]	149 ± 9.37[d]
BUN (mg/dl)	18.8 ± 0.42	16.4 ± 0.28[b]	16.6 ± 0.34	17.24 ± 0.45	18.1 ± 0.42[c]
UA (mg/dl)	5.02 ± 0.42	2.5 ± 0.12[a]	2.8 ± 0.08	3.7 ± 1.01	4.6 ± 0.08[d]
Albumin (g/dl)	2.7 ± 1.19	3.17 ± 1.39[b]	3.12 ± 1.17	2.98 ± 1.29[c]	2.87 ± 1.10[c]
Bilirubin (mg/dl)	0.79 ± 0.06	0.65 ± 0.07[b]	0.66 ± 0.06	0.70 ± 0.05[c]	0.74 ± 0.04[c]
Total cholesterol (mg/dl)	45.8 ± 2.11	52.01 ± 2.29[b]	51.7 ± 2.54	50.2 ± 1.67	47 ± 1.376[c]

Values represent mean ± SEM, $n = 10$; [a] Significantly different from the control at $p < 0.01$; [b] Significantly different from the control at $p < 0.05$; [c] Significantly different from the NP group at $p < 0.05$; [d] Significantly different from the NP group at $p < 0.01$

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *ALP* alkaline phosphatase, *BUN* blood urea nitrogen, *UA* uric acid

level (Fig. 7a–c) in the liver, kidney, and spleen. However, co-treatment with eugenol significantly improved these changes towards normal levels ($p < 0.05$ or $p < 0.01$, Fig. 6a–e, Fig. 7).

Assessment of TiO_2 NPs-induced DNA damage and effect of eugenol co-treatment

The biochemical and oxidative stress results indicated that eugenol offered more protection at the highest dose used (10 mg/kg bw). Therefore, only NP + E10 group among eugenol-treated groups was evaluated for comet assay. The extent of DNA damage, measured as length of comet tails, is presented in Table 3. Statistically significant increase in DNA damage in terms of tail length was detected in rats exposed to TiO_2 NPs (150 mg/kg bw). The comet tail length was significantly reduced in eugenol co-treated group as compared to the TiO_2 NPs-treated group (Fig. 8).

Discussion

Due to increasing use of TiO_2 NPs in industrial and daily use products, human exposure is inevitable which leads to accumulation of NPs in various organs resulting in oxidative

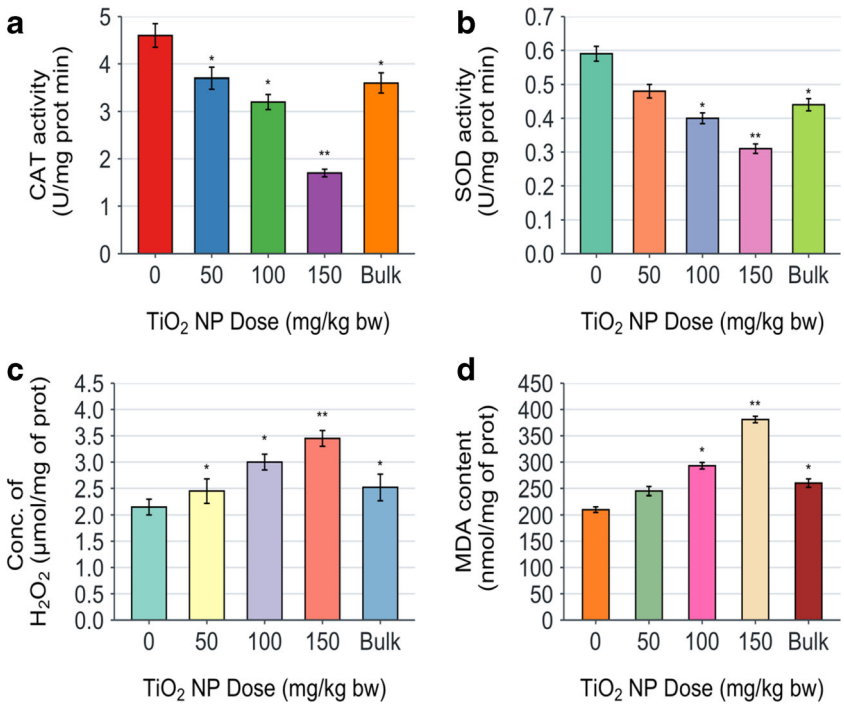

Fig. 4 Effect on the antioxidant activity in the liver of rats after intraperitoneal injection of TiO_2 NPs and bulk TiO_2 (150 mg/kg bw) for 14 days. The values represent mean ± SEM where $n = 10$. * Significantly different from the control at $p < 0.05$, ** significantly different from the control at $p < 0.01$

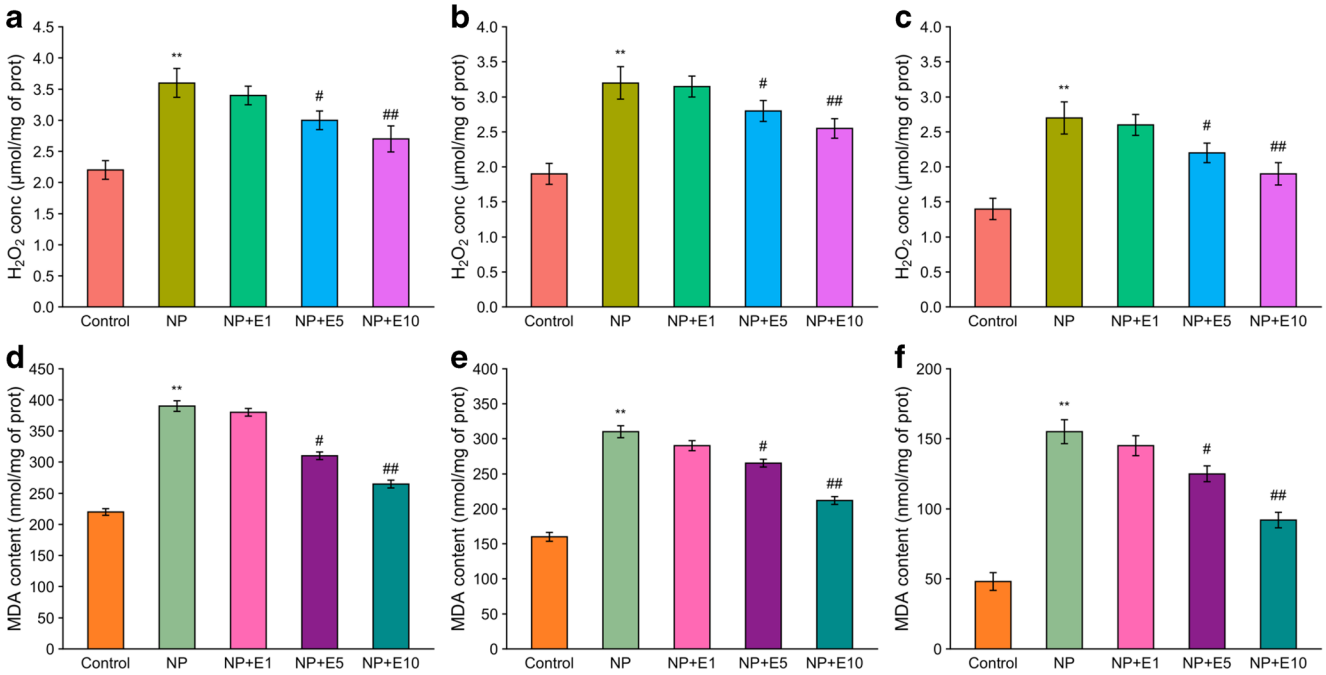

Fig. 5 Effect of TiO_2 NPs and eugenol treatment on the level of H_2O_2 concentration in the liver (**a**), kidney (**b**), and spleen (**c**) and level of MDA in the liver (**d**), kidney (**e**), and spleen (**f**) of Wistar rats. The values represent mean ± SEM where $n = 10$. ** $p < 0.01$ (control versus TiO_2 NPs-treated group). ## $p < 0.01$ (TiO_2 NPs-treated group versus eugenol co-treated group). # $p < 0.05$ (TiO_2 NPs-treated group versus eugenol co-treated group)

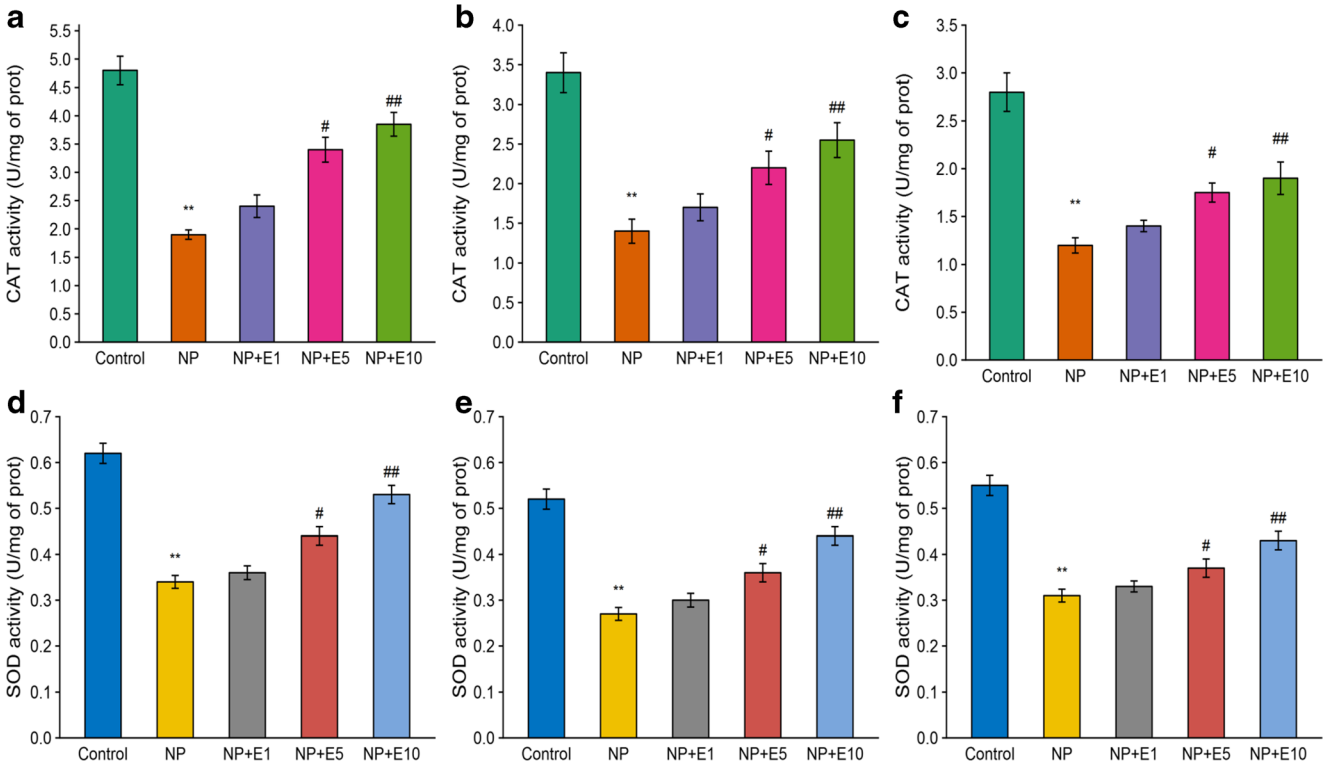

Fig. 6 Effect of TiO_2 NPs and eugenol treatment on the activity level of CAT in the liver (**a**), kidney (**b**), and spleen (**c**), and activity level of SOD in the liver (**d**), kidney (**e**), and spleen (**f**) of Wistar rats. The values represent mean ± SEM where $n = 10$. ** $p < 0.01$ (control versus TiO_2 NPs-treated group). ## $p < 0.01$ (TiO_2 NPs-treated group versus eugenol co-treated group). # $p < 0.05$ (TiO_2 NPs-treated group versus eugenol co-treated group)

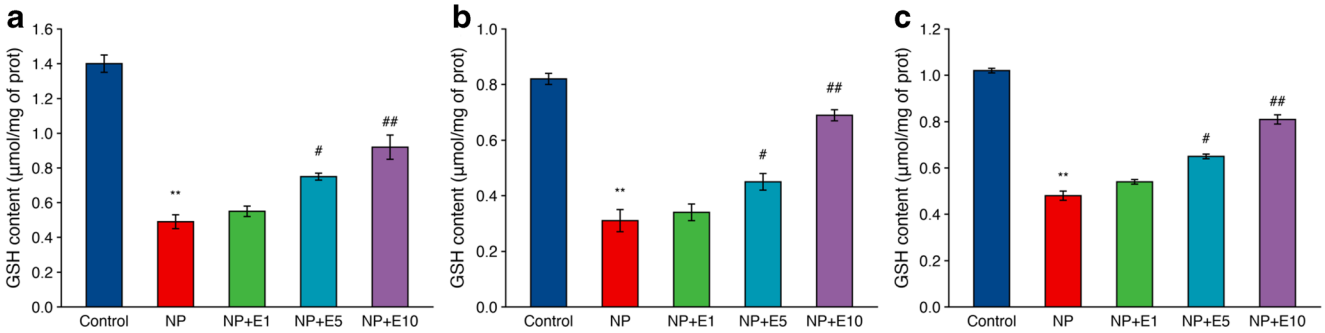

Fig. 7 Effect of TiO_2 NPs and eugenol treatment on the level of GSH in the liver (**a**), kidney (**b**), and spleen (**c**) of Wistar rats. The values represent mean ± SEM where $n = 10$. ** $p < 0.01$ (control versus TiO_2 NPs-treated group). ## $p < 0.01$ (TiO_2 NPs-treated group versus eugenol co-treated group). # $p < 0.05$ (TiO_2 NPs-treated group versus eugenol co-treated group)

damage, apoptosis, genotoxicity, and chromosomal instability (Baranowska-Wojcik et al. 2020). Studies have reported accumulation of TiO_2 NPs and ROS-mediated toxicity in rat tissues following intraperitoneal administration (Chen et al. 2009; Liu et al. 2009). Various natural antioxidants such as idebenone, carnosine, vitamin E, quercetin, and thymoquinone as well as plant extract proanthocyanidin from grape seeds and moringa seed extract have been investigated for their protective action against TiO_2 NPs-induced ROS and oxidative damage (Azim et al. 2015; Gonzalez-Esquivel et al. 2015; Hassanein and El-Amir 2017; Kandeil et al. 2020; Mohammed and Safwat 2020). Eugenol is a major ingredient of cloves which have been reported to boost immune function, promote healthy digestion, and fight against inflammations (Al-Okbi et al. 2014).

It has been reported that after entering the body through any route, NPs mostly accumulate in the liver thereby causing damage to liver cells (Xie et al. 2010; Nishimori et al. 2009). Consistent with this, we also found Ti accumulation highest in the liver in the present study. NPs, being extremely small in size, easily cross biological barriers and get readily absorbed into the vascular system in the gastrointestinal tract and are transported to the liver via portal system and then to different body organs (Singh et al. 2009; Afaq et al. 1998). Liu et al. (2009) also reported highest accumulation of TiO_2 NPs in the liver of mice.

Table 3 Mean comet tail lengths of lymphocytes isolated from the blood of Wistar rats after treatment with TiO_2 NPs (150 mg/kg bw) alone or cotreatment with eugenol (10 mg/kg bw) for 14 days. Three comet slides were prepared for each animal in each group. The tail lengths of 100 cells per slide were measured (100 × 3 = 300 cells per animal per group, or 300 × 6 = 1800 cells per group). The mean values of each group with respect to tail length were calculated and reported

Group	Mean tail length (μm)
Control	0.941 ± 0.49
NP	18.246 ± 2.55*
NP + E10	3.241 ± 0.45#

Values represent mean ± SEM. * Significantly different from the control at $p < 0.01$; # Significantly different from NP group at $p < 0.01$

TiO_2 NPs treatment to rats disrupted the oxidative/antioxidative balance which led to a state of oxidative stress in the liver, kidney, and spleen. All cells of aerobic organism are well equipped with antioxidant defense system to neutralize the oxidant-mediated damage. Glutathione (GSH) is a well-known non-enzymatic antioxidant which mostly exists in millimolar range (Sies 1999). The enzymatic antioxidants such as SOD and CAT convert ROS into less damaging materials, thus keeping ROS level low and preventing oxidative damage to cells (Hamida et al. 2020a, b; Sies 2000). Sulfhydryl groups in GSH possess high reducing power to regulate complex thiol-exchange system which is important in antioxidant defense (Dickinson and Forman 2002). Reduction in GSH levels due to TiO_2 NPs exposure might be due to direct oxidation of sulfhydryl groups in GSH by TiO_2 NPs-induced ROS which diminishes its activity (Jugan et al. 2012). Decreased SOD and CAT activities might be due to direct inhibition by NPs or ROS mediated modification in the enzyme active site. Decrease in CAT and SOD activities and GSH levels is a sure sign of increased H_2O_2 (ROS) levels in cells (Yuan et al. 2018). This burst in ROS causes oxidative damage to DNA, proteins, and lipids including those constituting the cell membrane (Abdelazeim et al. 2020) which is evident by increased DNA damage and lipid peroxidation in TiO_2 NPs-treated groups in the present study. It is well known that LPO affects cell membrane by inducing depolarization, inhibiting membrane-bound enzymes, altering protein transport, and eventually leading to loss of membrane integrity. Our findings are in agreement with the previous studies which reported reduction in CAT and SOD activities and GSH content as well as increase in lipid peroxidation in the liver and kidney of rodents following administration of TiO_2 NPs (Liu et al. 2010a, b; Meena and Paulraj 2012). Hamida and colleagues (Hamida et al. 2020a, b) also reported a decrease in GSH content due to

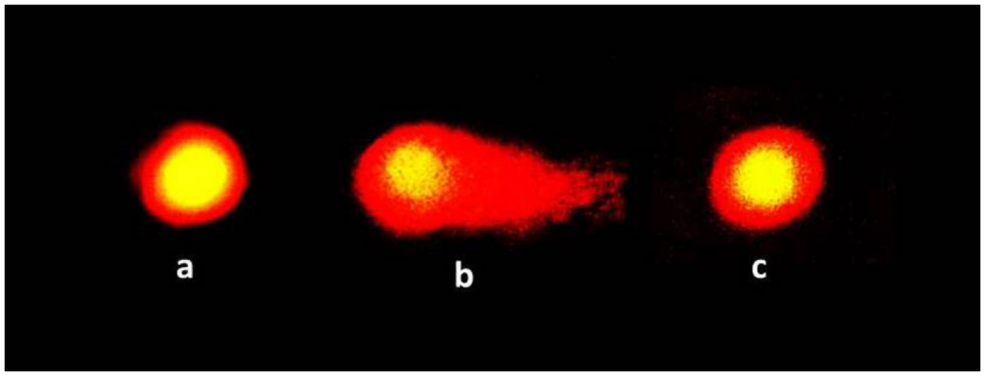

Fig. 8 Comet pictures illustrating the effects of TiO_2 NPs with or without co-treatment with eugenol on the extent of DNA damage. **a** Control group; **b** NP group, treated with TiO_2 NPs at the dose of 150 mg/kg bw; **c** NP + E10 group, co-treated with TiO_2 NPs (150 mg/kg) and eugenol (10 mg/kg)

novel silver nanoparticles. Wang et al. (2011) and Li et al. (2010) reported that TiO_2 NPs induced ROS production and lipid peroxidation in the spleen of mice.

Activities of enzymes ALP, ALT, and AST and levels of albumin, total bilirubin, and total cholesterol in serum are often used as indicators of liver function (Liu et al. 2009; Meena and Paulraj 2012). Oxidative injury to liver will cause leakage of cellular enzymes into the blood and result in their elevated activities in blood. It is therefore quite understandable that in the present study, a significant increase in ALT, ALP, and AST and disturbances in the normal levels of albumin, total bilirubin, and total cholesterol in serum due to TiO_2 NPs was observed which is an indication that high doses of TiO_2 NPs could severely damage the liver. These results are in agreement with the report of Duan et al. (2010). Increased serum cholesterol level indicates the impaired lipid metabolism and TiO_2 NPs-induced oxidative damage to the liver. NPs exposure leads to increased level of low-density lipoproteins which has direct association with cardiovascular disease (Duan et al. 2010). Reduced UA and BUN levels in serum indicate impaired kidney function (Liu et al. 2009) while increased serum albumin is a sign of nephrotoxicity and changes in liver cells (Mahdieh et al. 2015).

TiO_2 NPs have been reported to be genotoxic in a wide variety of in vivo and in vitro models (reviewed by Wani and Shadab 2020). TiO_2 NPs treatment damaged DNA in A549 cells (Kansara et al. 2015) and HEK 293 cells (Meena et al. 2012). Even E171, a TiO_2 food additive, promotes DNA damage (Dorier et al. 2017; Proquin et al. 2017). These reports suggest the DNA damaging ability of TiO_2 NPs is due to induction of oxidative stress. We also speculate that DNA damage observed in the present study could be due to NP-induced strand break or genotoxic mechanism associated with inflammation and/or oxidative stress generated by these NPs.

In the present study, co-treatment with eugenol significantly reduced lipid peroxidation and H_2O_2 levels in the liver, kidney, and spleen as well as restored their GSH content, CAT, and SOD activities towards normal. The reduction in oxidative stress following the administration of eugenol implied the possible protective effect of the antioxidant in these tissues, which is in agreement with the report of Kandeil et al. (2020). Various studies have reported that eugenol significantly enhanced the antioxidant enzymes as well as reduced lipid peroxidation, thereby lowering the oxidative stress (Yogalakshmi et al. 2010; Venkadeswaran et al. 2014; Elbahy et al. 2015; Huang et al. 2015; Kaur et al. 2010; Sakat et al. 2019; Binu et al. 2018). It seems that eugenol treatment increased the GSH content in tissues that reduced the H_2O_2 levels and consequently lipid peroxidation. This may be due to antioxidant activity of eugenol which prevented GSH from oxidative damage by TiO_2 NPs, leading to increased GSH levels as eugenol doses increased. In agreement with this, Jugan et al. (2012) showed that exposure of TiO_2 NPs led to increase in ROS levels which reduced significantly on addition of glutathione, indicating the antioxidant activity of glutathione. Maheshwari et al. (2018) also reported that antioxidant activity of 3,4-dihydroxybenzaldehyde lowered arsenic (III)-induced ROS generation and increased the level of antioxidants in human red blood cells. As a result of reduction in oxidative stress due to eugenol, there is an in increase in antioxidant enzyme activities, DNA as well as tissues are protected from oxidative damage and thus liver and kidney serum biomarkers do not show significant deviation from their normal levels. This may be the reason why DNA damage in eugenol co-treated rats was significantly lower than NP-alone–treated rats. Harb et al. (2019) also reported that eugenol administration caused an increase in the activity of antioxidant enzymes as well as reduction of cholesterol in hypercholesterolemic rats. Fen et al. (2018) reported that 10–20 mg eugenol/kg body weight dose confers protection against oxidative damage. Thus, eugenol reduces TiO2 NPs-induced oxidative stress and damage which is consistent with previous study (Mohammed and Safwat 2020).

Eugenol reduces the toxic effect of TiO_2 NPs as seen in this study. However, the mechanism of its action is not clear.

Antioxidants like glutathione reduce the toxic effects of NPs by involving glutathione S transferase (GST); glutathione conjugates with NPs, an enzymatic process catalyzed by GST (Jugan et al. 2012). We speculate that eugenol may act via a similar mechanism—it conjugates with the NPs, thereby preventing them from causing toxicity. However, no such report exists anywhere in the scientific literature and hence needs to be validated. Nevertheless, such a claim may prove helpful while the underlying mechanism of the protective action of eugenol is investigated.

Conclusions

The present study provides conclusive evidence of biochemical toxicity and oxidative stress induced by TiO_2 NPs in the liver, kidney, and spleen of rats, besides genotoxicity in the rat blood cells. Results from the present study show an increase in H_2O_2 and LPO, and decrease in CAT, SOD activity, and GSH content in the liver, kidney, and spleen of TiO_2 NPs-treated rats. As is evident from the literature published till date, this is the first study to explore the potential of eugenol in reducing the TiO_2 NPs toxicity by modulating and restoring oxidative modifications towards normal and preventing genotoxic damage. Thus, eugenol seems to be an effective chemoprotectant that can be used to protect against toxicity induced by TiO_2 NPs exposure.

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Author contributions MRW and GGHAS conceived and designed the work and performed data analysis. MRW and NM performed the experiments and wrote the manuscript. All the authors read and approved the manuscript.

Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Ethics approval Institutional Animal Ethics Committee (IAEC), Aligarh Muslim University, Aligarh, (Registration number 714/GO/Re/S/02/CPCSEA) has approved this work.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare that they have no conflict of interest.

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