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Anemia and genotoxicity induced by sub-chronic intragastric treatment of rats with titanium dioxide nanoparticles

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HIGHLIGHTS

- anatase TiO_2 nanoparticles (TiO_2 NPs) exposure alter the hematopoietic system in rats.
- TiO_2 NPs caused also a macrocytic anemia.
- TiO_2 NPs are genotoxic *in vivo* at 100 and 200 mg/kg BW of an intragastric treatment in rats.

ABSTRACT

Titanium dioxide nanoparticles (TiO_2 NPs) are widely used for their whiteness and opacity. We investigated the hematological effects and genotoxicity of anatase TiO_2 NPs following sub-chronic oral gavage treatment. TiO_2-NPs were characterized by X-ray diffractometry (XRD), X-ray photoelectron spectroscopy (XPS), and transmission electron microscopy (TEM). Wistar rats were treated with anatase TiO_2 NPs by intragastric administration for 60 days. Hematological analysis showed a significant decrease in RBC and HCT and a significant increase in MCV, PLT, MPV and WBC at higher doses. Furthermore, abnormally shaped red cells, sometimes containing micronuclei, and hyper-segmented neutrophil nuclei were observed with TiO_2 NPs treatment. The micronucleus test revealed damage to chromosomes in rat bone marrow at 100 and 200 mg/kg bw; the comet assay showed significant DNA damage at the same doses.

Abbreviations

TiO_2 NPs: Titanium dioxide nanoparticles

bw: Body weight

RBC: Red blood cells

HGB: Hemoglobin

HCT: Hematocrit

MCHC: Mean corpuscular hemoglobin concentration

MCH: Mean corpuscular hemoglobin

MCV: Mean corpuscular volume

MPV: Mean platelet volume

PLT: Platelets

WBC: White blood cells

TEM: Transmission electron microscopy

XRD: X-ray diffraction

XPS: X-ray photoelectron spectroscopy

EDX: energy-dispersive X-ray (EDX) analysis

SIPHAT: Pharmaceutical Industrial Society of Ben Arous Tunisia

% DNA-T: DNA in tail

TL: Tail length

TM: Tail moment

%PCE: Percentage of polychromatic erythrocytes

MN-PCE: Micronucleated polychromatic erythrocytes

ANOVA: One way analysis of variance

Keywords: Anemia; DNA damage; Genotoxicity; Hematopoietic system; Rat; Titanium dioxide nanoparticles.

1. Introduction

Since the advent of nanotechnology, titanium dioxide nanoparticles (TiO_2 NPs) have been widely used in several applications, such as food colorants, drug additives, paints, paper, inks, cosmetics, sunscreens, antibacterial materials, and electronics, for their whiteness and opacity [1]. Food is an important route of human exposure to TiO_2 NPs. At least 36% of the TiO_2 particles present in food are in nano-sized form [2]. Gastrointestinal absorption may be an important route for TiO_2 NPs, since drug carriers, food products, water, and beverages may contain TiO_2 NPs. However, the effects of TiO_2 NPs in the gastrointestinal tract are little investigated [3].

After absorption of TiO_2 NPs, the systemic circulation can distribute the particles to all organs and tissues in the body [1]. After reaching the systemic circulation, TiO_2 NPs can interact with plasma-proteins, coagulation factors, platelets and red or white blood cells [4]. The influence of TiO_2 NPs on the hematological system has not been studied thoroughly. Recently, Duan et al. (2010) suggested that intragastric exposure to TiO_2 NPs in mice results in damage to the hemostasis blood system.

Because of their small size and high surface area, together with other physico-chemical characteristics, NPs may have unpredictable genotoxic proprieties [5]. Many authors have focused on the interactions of TiO_2 NPs exposure with genetic material. If small enough, NPs may pass through cellular membranes and gain access to the nucleus, where they may interact directly with DNA, causing damage. Additionally, if NPs are able to accumulate within a cell but not necessarily gain access to the nucleus, they may still come into direct contact with DNA during mitosis, when the nuclear membrane breaks down, providing an opportunity for DNA aberrations to arise [5]. The genotoxicity of TiO_2 NPs has been intensively studied in vitro [6-11] *and in vivo* [12-16] , but few studies have evaluated the genotoxic potential of TiO_2 NPs exposed by oral administration [17-19]. Overall, these studies evaluated short-term and/or high-dose oral exposure of TiO_2 NPs to animals. With increasing application of TiO_2 NPs in the food industry, there is an urgent need to evaluate the genotoxicity of long-term and low-dose oral exposure to TiO_2 NPs, more consistent with human exposure.

We have investigated the hematological characteristics and genotoxicity of long-term and low-dose oral exposure of rats to TiO_2 NPs. Rats were exposed to anatase TiO_2 NPs by intragastric administration, daily for 60 days. Hematological parameters were measured and blood smears were analyzed. Genotoxicity (damage to chromosomes or mitotic apparatus)

was evaluated by the bone marrow micronucleus test, and the comet assay was used to assess DNA damage in peripheral blood.

Materials and methods

1.1. Chemicals

The TiO_2 NPs product used in this study was "titanium dioxide" produced by AZ Tech (Italy) and marketed in Tunisia as a paint ingredient in the ceramic sector. The CAS Registry Number for TiO_2 is 13463-67-7. Suspensions of dispersed TiO_2 particles were prepared in distilled water, treated by ultrasonic vibration for 30 min, and mechanically vibrated for 5 min before each use. Distilled water was used as a vehicle control.

2.1. Characterization

The structural properties and chemical bonding states of the TiO_2-NPs were investigated using X-ray diffraction (XRD, Bruker D5005 diffractometer, using CuKα radiation (λ=1.5418 Å)) and X-ray photoelectron spectroscopy (XPS). Crystallite sizes (G, in Å) were estimated from the Debye-Sherrer equation [20]: Eq. (A.1): $G = 0.9\lambda / B\cos\theta_B$

where λ is the X-ray wavelength (1. 5418 Å), θ_B is the maximum of the Bragg diffraction peak and B is the line width at half maximum.

The morphologies of TiO_2-NPs were studied by transmission electron microscopy (TEM). The compositions of the samples were examined by energy-dispersive X-ray (EDX) analysis available with TEM.

1.2. Animals and treatment

Male Wistar rats aged 4 months were obtained from SIPHAT (Pharmaceutical Industrial Society of Ben Arous, Tunisia). The animals were randomly assigned to control (n=6) or treatment groups (3 groups, n=6 per group) and housed at 21±5 °C with a 12h light/dark cycle and 55% humidity. The rats were fed with a standard commercial pellets diet from Sico Sfax, (Tunisia) and provided drinking water ad lib. They were maintained during the whole experimental period in accordance with the guidelines for animal care of the "Faculty of Medicine of Monastir", Tunisia.

Doses

The animals were randomly divided into a control group and three experimental groups (50, 100, and 200 mg/kg bw TiO_2 NPs). They were weighed and the volume of TiO_2 NPs suspension was calculated for each rat. Fresh TiO_2 suspensions were administered intragastrically every day for 60 d. Doses were prepared from a suspension, 10 ml/kg of the rat's weight. Each animal received TiO_2 NPs daily and each control animal received the same volume of distilled water. The intragastric doses were selected based on the intake of dietary TiO_2 particles in the UK, which has been estimated to be about 5 mg/person/day [3], equivalent to approximately 0.1 mg/kg body weight (bw) per day [19]. In this study, 500 × the potential human exposure dose (50 mg/kg bw) was used as the low-dose TiO_2 NPs exposure.

Growth state, eating, drinking and activity, and any symptoms or mortality were observed and recorded daily during. After 60 d, and 24 h after the last TiO_2 NPs dose, all rats were weighed, anesthetized with ether, and blood samples were collected by cardiac puncture for haematological parameters, blood smears, and comet assay. Rats were then euthanized and one femur from each rat was removed for bone marrow preparation. All four tests were performed on the same animals of each group.

1.3. Haematological parameters and blood smear preparation

The blood samples were collected in tubes containing EDTA anticoagulant. Red blood cells (RBC), white blood cells (WBC), hemoglobin (HGB), platelets (PLT), thrombocytocrit (PCT), hematocrit (HCT), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV) and mean platelet volume (MPV) were quantified in an automatic hematological assay analyzer (9 parameters system) (Medonic Cell Analyzer-Vet CA530, Boule Medical, Stockholm, Sweden) [21] at "Fattouma Bourguiba University Hospital" in Monastir (Laboratory of Hematology).

Blood smears were prepared immediately from samples taken with anticoagulant and were stained with Giemsa (Biopur Quimica, Argentina). These same samples were used for hematological studies.

1.4. Micronucleus test

The six rats of the different groups (control, 50, 100, and 200 mg/Kg bw TiO2 NPs) were used for the bone-marrow micronucleus assay. The animals were processed for enumerating micronuclei in the erythrocytes as described previously [22] with modifications, as recommended by Bhilwade et al. [23]. In brief, the bone marrow was flushed in a 5-ml

centrifuge tube containing fetal bovine serum and centrifuged at 400×g for 5 min. The cell pellet was mixed thoroughly, the bone-marrow smears were made on clean glass slides and stained as follows: 5-min incubation in undiluted May–Gruenwald (0.25% in methanol), 3 min in diluted May–Gruenwald solution (1:1, May–Gruenwald/distilled water), rinsed in distilled water three times and then stained with diluted Giemsa (1:6 of the Giemsa stock/distilled water) for 10 min, followed by thorough washing with distilled water. The slides were dried, cleared for 5 min in xylene, and mounted. Two slides were made per animal. Coded slides were scored for the percentage of polychromatic erythrocytes (% PCE) and for the incidence of micronucleated polychromatic erythrocytes (MN-PCE) at 100× magnification under oil immersion. The percentage of polychromatic erythrocytes (% PCE) was determined by analyzing 500 erythrocytes (polychromatic and normochromatic erythrocytes). Simultaneously, at least 2000 polychromatic erythrocytes (PCE) with or without micronuclei were scored per animal to determine the percentage of MN in PCE.

1.5. Alkaline comet assay

For detection of total DNA strand breaks in peripheral blood leukocytes, the alkaline comet assay (single-cell gel electrophoresis) was used [24]. For this assay, aliquots (about 60 µl) of heparinized whole blood from six rats from the different groups were collected and mixed with 1.2% low-melting agarose solution (60 µl) prepared in PBS (without Ca^{2+}/Mg^{2+}) at 37°C,and evenly layered on fully-frosted slides. After agarose solidification, the slides were kept in lysis buffer (2.5 M NaCl, 100 mM EDTA, 1% Triton X-100,10 mM Tris–HCl, pH 10.0 and 10% DMSO) for 3 h at 4°C. The slides were removed from the lysis solution and placed on a horizontal electrophoresis apparatus in electrophoresis buffer (10 N NaOH, 200 mM EDTA, pH10) at room temperature for 15 min to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was carried out for 20 min at 25 V (0.90 V/cm), 300 mA.

Afterwards, the slides were washed gently in neutralizing buffer (0.4 M Tris–HCl, pH 7.5) to remove alkali and detergents, stained with ethidium bromide, and observed under a Nikon Eclipse TE 300 fluorescence microscope (Nikon, Tokyo, Japon) at 40× magnification. Images of 50-60 individual cells per slide were acquired with a digital imaging system. These images were analyzed with COMET ASSAY IV software and different parameters of DNA damage, such as tail length, tail intensity, and tail moment (TM), were obtained. We used TM (considered to be the optimum parameter) to represent DNA damage [25].

1.6. Statistical analysis

The data were expressed as means ± SD based on the indicated number in the experiment (n = 6). Data were analyzed using the Statistical Package for Social Sciences (SPSS, version 21). Statistical significance was determined using one way analysis of variance (ANOVA) following by Tukey's post hoc comparisons. The differences were considered significant at $p \leq 0.05$.

2. Results

2.1. Characterization of nanoparticles

Fig. 1 depicts the XRD patterns of the TiO_2-NPs. The diffraction peaks matched very well with JCPDS Card No.: 21-1272, indicating the body-centered tetragonal anatase phase of TiO_2. The diffraction peaks at $2\theta = 25.25°$ (101), 37.82° (004), 47.98° (200), 53.59° (105), 55.03° (211) and 62.36° (204) all correspond to the lattice plane of anatase phase only. The absence of the diffraction peaks at $2\theta = 27.5°$ (110) and 30.8° (121) corresponding to rutile and brookite phases confirms that the TiO_2-NPs were pure anatase nanoparticles.

Using the Debye-Sherrer equation, the X-ray diffraction spectrum indicated that the anatase crystals had sizes in the range 5-10 nm.

Fig. 2 shows the XPS survey spectra of the TiO_2-NPs. Major characteristic transition peaks for Ti, O and C are indicated. The $2p_{3/2}$ peak for Ti has a binding energy of 453.8 eV for metal, 455.1 for TiO, 458.5 for TiO_2, and 459.2 for anatase/rutile TiO_2 [26]. We note the appearance of the photoelectron peak for Ti at 459.5 eV, which is in the form of TiO_2, corresponds to anatase-phase, also supported by structural studies. We also observed the O1s peak at 532.8 eV. The minor peak might be due to oxygen contamination. Also, some oxygen bonded with hydrogen cannot be excluded. In addition, contamination by carbon is evident, as a peak for this element is already visible at 285.8 eV for the TiO_2.

Fig. 3 shows the TEM image and EDX analysis of representative nanoparticles. The morphologies of the sample are nearly spherical, with diameters 5-12 nm. This clearly shows that the average particle size is nanoscale and it is in accordance with the results of the XRD. An EDX result gives strong K diffraction peaks at 4.5, 4.9 and 0.5 keV, corresponding to elemental Ti and O.

2.2. Hematological parameters

The hematological study results (Table 1) indicate that RBC, HCT, HGB and MCH of the TiO_2 NPs treated rats were gradually reduced, while MCV, PLT, MPV, WBC and MCHC of these rats were gradually elevated with increasing doses of TiO_2 NPs. Except for HGB, MCH

and MCHC, all the parameters mentioned above from the higher doses TiO_2 NPs groups showed significant differences from those of the controls (p<0.05 or 0.01).

2.3. Blood smears

Blood smears from control animals were normal (Fig. 4a). In the treated groups with 50, 100, and 200 mg/kg bw TiO_2 NPs, the blood smears had abnormal pathology changes compared with the control group. TiO_2 NPs produced poikilocytotic (abnormally shaped) hypochromatic red cells, sometimes containing micronuclei fragments or Heinz bodies, present in about 60% of red cells of rats treated with 200 mg/kg bw. Lymphocytes and neutrophils from rats treated with 100 and 200 mg/kg bw TiO_2-NPs groups also had abnormally shaped nuclei (Fig. 4c, d, e) and hypersegmented nuclei (Fig. 4f).

2.4. Micronucleus test

To assess the effect of TiO_2 NPs exposure on genotoxicity in rats, the micronucleus assay was used. As shown in Fig. 5-A, 200 mg/kg bw showed a statistically significant decrease in the percentage of polychromatic erythrocytes (PCE) compared to the control group. The frequency of micronuclei in bone marrow polychromatic erythrocytes (MN-PCE; Fig. 5B) showed a statistically significant dose-dependent increase in 100 and 200 mg/kg bw of anatase TiO_2 NPs. In the 200 mg/kg bw group, a highly significant (p<0.001) increase in the number of MN-PCE was observed. This increase was approximately 2.6-fold compared to the 100 mg/kg bw group and 3.4-fold compared to the 50 mg/kg bw group.

2.5. Comet assay

DNA damage, in terms of TM, observed in rats treated with 50, 100 or 200 mg/kg bw of anatase TiO_2 NPs, was measured by the alkaline comet assay (Fig. 6). As with micronucleus frequency in the bone marrow, DNA damage also showed a statistically significant increase in 100 and 200 mg/kg bw treated animals.

3. DISCUSSION

Hematological characteristics are an effective and sensitive index of physiological and pathological changes in animals and humans [27]. In order to evaluate the physiological and pathological state of rats due to intragastric administration of TiO_2 NPs for 60 d, hematological parameters were measured. Treatment with higher doses of TiO_2 NPs caused a significant increase (p <0.05 or 0.001) in MCV, PLT, MPV and WBC and a significant decrease (p<0.05 or 0.001) in RBC, HCT and HGB. Similar results were also observed in

mice by Duan et al. [27]. The reduction in RBC, HCT and HGB might be due to the inhibition of erythropoiesis and hemosynthesis and to an increase in the rate of erythrocyte destruction in hemopoietic organs. The observed decrease of RBC, HCT, HGB and the increase of MCV in rats exposed to TiO_2 NPs were a manifestation of swollen erythrocytes and macrocytic anemia. In this study, the TiO_2 NPs treated animals also exhibited significantly higher WBC than the control animals. Increase of leucocytes might indicate the activation of the defense and immune systems of the body [28, 29]. In addition, there was inflammation in the tissues of various animal organs, including lungs, kidneys, liver, and brain, previously described [27, 28, 30-33]. The increase of PLT and MPV suggested a possible effect of TiO_2 NPs on blood coagulation, causing severe damage of platelets but improving the metabolic function of the bone marrow.

The current study focused on the effect of anatase TiO_2 NPs on the bone marrow, where hematopoiesis takes place. Damage to bone marrow DNA may cause diminished presence or increased level of affected cells in the circulated blood. In the present study, we demonstrated that TiO_2 NPs induced cytotoxicity in the bone marrow of rats exposed only to 200 mg/kg bw for 60 d. This reduction in the percentage of PCE indicates that TiO_2 NPs were reaching the bone marrow and inhibiting the hematopoietic system. Treatment with higher doses of TiO_2 NPs caused genotoxicity to bone marrow cells by inducing significant levels of MN in PCE, which may originate from lagging acentric chromosomes or chromatid fragments caused by unrepaired DNA breaks. Furthermore, our results with the comet assay showed a significant increase in DNA damage in peripheral blood leukocytes. Consistent with this, Trouiller et al. [17] reported that anatase TiO_2 NPs were genotoxic and clastogenic in mice exposed to 500 mg/kg bw TiO_2 NPs in drinking water for 5 d. Sycheva et al. [18] found that TiO_2 NPs could lead to a significant increase in MN-PCE frequency and induced DNA damage in bone marrow cells of male CBAB6F1 mice after a 7 d exposure to 1000 mg/kg bw TiO_2 NPs via oral gavage.

Our results confirm previous in vivo studies on genotoxicity induction by TiO_2 NPs [17, 18] and show for the first time a consistent dose-dependent increase in MN in bone marrow PCE and DNA damage in peripheral blood leukocytes via intragastric administration of 100 and 200 mg/kg bw TiO_2 NPs for 60 d in male rats. Furthermore, a recent study [19] showed that intragastric administration of 50 and 200 mg/kg TiO_2 NPs for 30 d can clearly exert genotoxicity to the *in vivo* test systems and significantly enhance the ratio of cells positive for γ-H_2AX foci in the bone marrow of rats, but no obvious differences in MNPCE frequency and PCE/NCE ratio were observed. Nevertheless, the greatest difference between

our study and the previous one [19] concerns TiO_2 time exposure. In the present study, cytotoxicity and genotoxicity of TiO_2 NPs were proved by a micronucleus test when rats were exposed to 60 d of treatment while Chen et al. [19] saw bone marrow genotoxicity with γ-H_2AX foci cells ratio only after only 30 d treatment.

Several studies reported the genotoxicity of TiO_2 NPs *in vitro* in cell culture systems [6-11, 34]. However, other researches showed no genotoxic effects of TiO_2 NPs *in vivo* when intravenously administrated [16, 35, 36] or inhaled [14, 15] for short exposure times. These disparities may be due to differences in the test conditions, such as exposure time, concentrations, animal model, the dispersal of the particles, and the physicochemical characteristics of TiO_2 used by the investigators.

4. Conclusions

In conclusion, our data clearly demonstrate that anatase TiO_2 NPs exposure alters the hematopoietic system in rats after sub-chronic intragastric administration. Furthermore, our results show the genotoxic effects of anatase TiO_2 NPs *in vivo* at 100 and 200 mg/kg bw. Low-dose TiO_2 NPs, used in the present study, did not induce genotoxicity. However, it is interesting to note a significant dose-response relationship in rat bone marrow cells by the micronucleus test at the highest doses. According to these findings, the application of TiO_2 NPs as food and drug additives might prudently be controlled.

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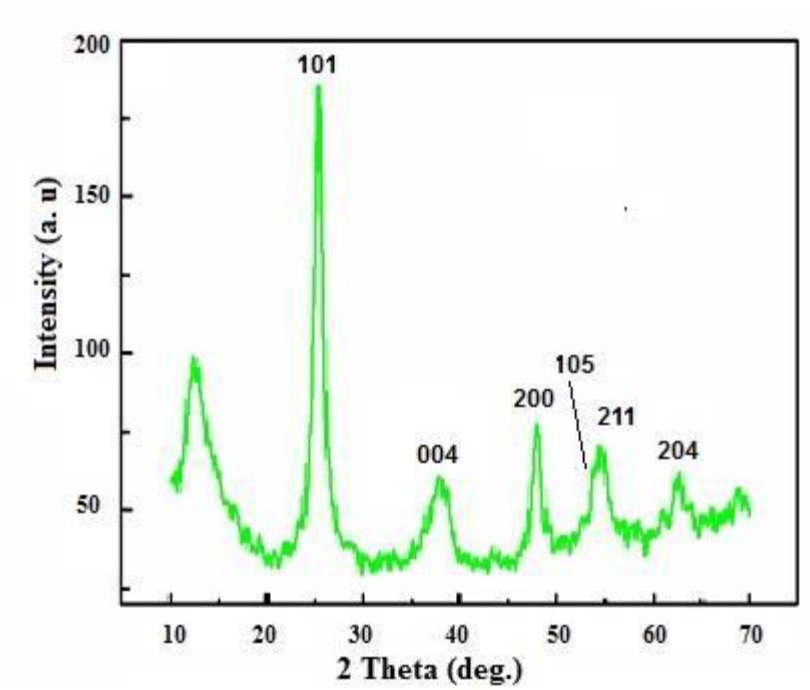

Fig 1

Fig 1. X-ray diffraction (XRD) pattern of the TiO_2 nanoparticles.

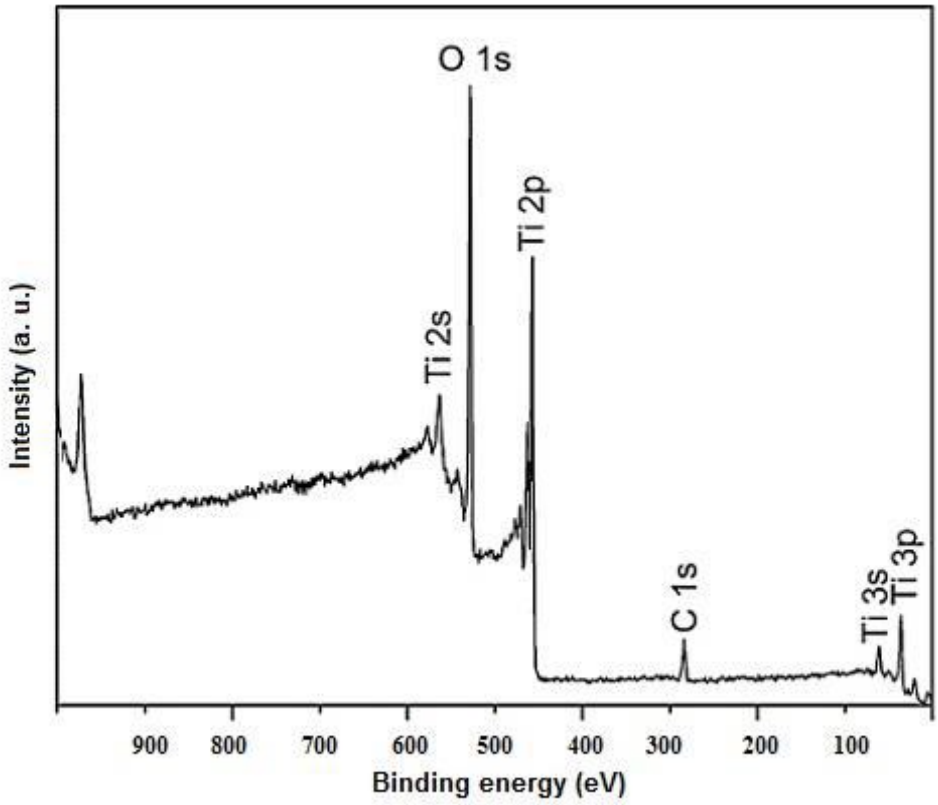

Fig 2

Fig 2. XPS spectra of TiO_2 nanoparticles.

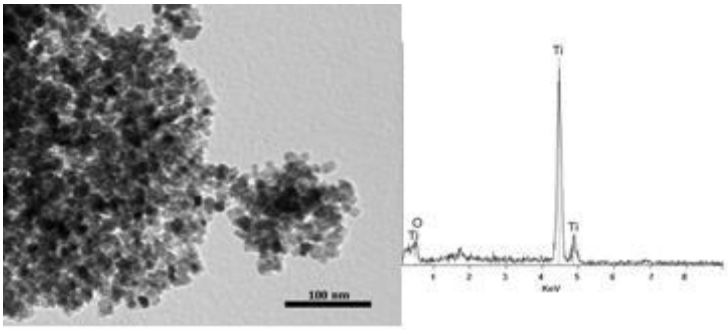

Fig 3

Fig 3. The TEM photograph showing the general morphology and EDX analysis of TiO_2 nanoparticles.

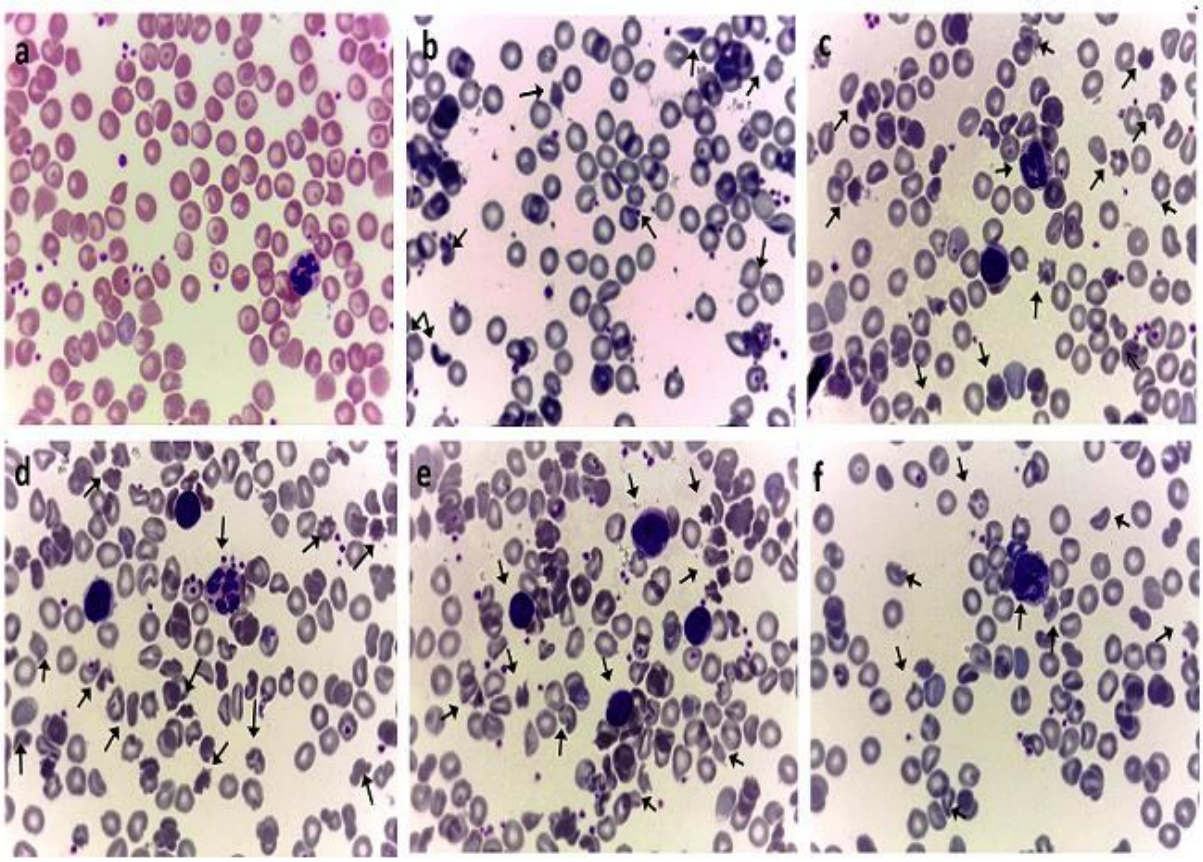

Fig 4

Fig 4. Blood smears examined and photographed under light microscope Leica DM750, provided with a camera Leica ICC50 (magnification 1000×) of control and treated rats with intragastric administration of TiO_2 NPs for 60 d. (a) Control group; (b) 50 mg/kg bw group: the arrow indicates abnormally shaped red cells; (c) 100 mg/kg bw group: the arrow indicates abnormally shaped red cells and hypersegmented neutrophils; (d, e, f) 200 mg/kg bw group: the arrow indicates abnormally shaped red cells and hypersegmented neutrophils.

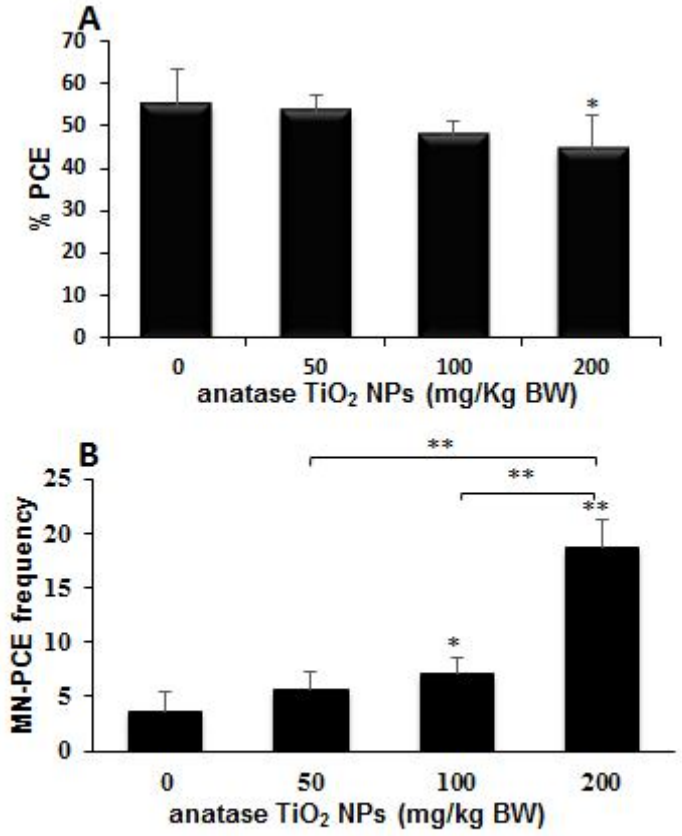

Fig 5

Fig 5. The genotoxic effects of anatase TiO_2 NPs in bone marrow cells of rats after intragastric exposure to anatase TiO_2 NPs for 60 d. (A) Percentage of polychromatic erythrocytes (% PCE) and (B) the micronucleated polychromatic erythrocytes (MN-PCE) frequency. Values represent means ± SD.

*: significantly different from the control (no anatase NP TiO_2) at 5%, **: significantly different from the control at 1% confidence level, [: significantly different between groups.

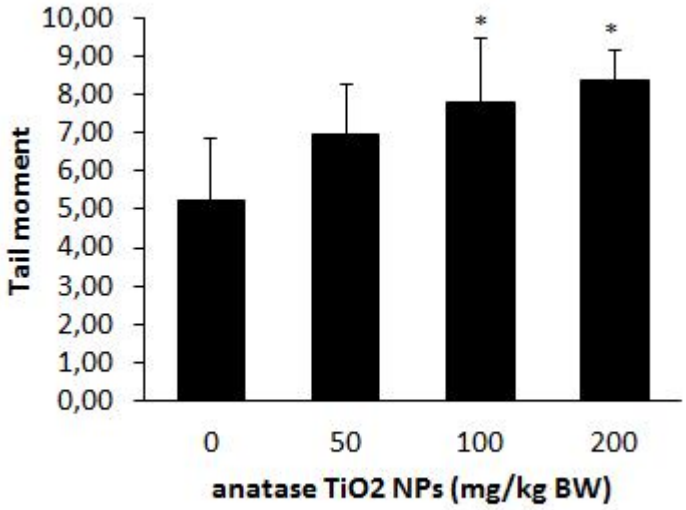

Fig 6

Fig 6. Single-cell gel electrophoretic analysis of spontaneous DNA damage in peripheral blood leukocytes in rats treated with an intragastric administration of anatase TiO_2 NPs for 60 d. Values represent means ± SD. *: significantly different from the control (no iO_2) at 5%.

Tables

Table 1 Hematological parameters in rats by intragastric administration with anatase TiO_2 NPs for consecutive 60 days.

Indexes	Anatase TiO_2 NPs			
	0	50	100	200
WBC (10^9/L)	10.56 ± 0.21	13.00 ± 0.99*	19.52 ± 1.68**	24.86 ± 1.59**
RBC (10^{12}/L)	7.48 ± 1.45	6.40 ± 0.41**	6.08 ± 0.45**	5.39 ± 0.29**
HGB (g/L)	14.28 ± 0.27	10.08 ± 2.99	12.35 ± 0.18	10.28 ± 4.14
MCV (fL)	54.72 ± 1.45	56.16 ± 1.77	60.07 ± 1.81**	70.69 ± 2.85**
MCH (pg)	19.00 ± 0.57	18.80 ± 0.50	18.30 ± 0.44	18.18 ± 0.56
MCHC (g/L)	34.94 ± 0.55	34.84 ± 0.49	34.77 ± 0.75	35.68 ± 1.51
PLT (10^9/L)	512.16 ± 18.01	542.20 ± 44.62	640.80 ± 43.73**	725.40 ± 37.07**
HCT (%)	41.53 ± 1.24	35.88 ± 0.55**	33.35 ± 0.59**	31.96 ± 1.62**
MPV (fL)	6.66 ± 0.13	7.28 ± 0.35*	7.92 ± 0.41**	8.68 ± 0.41**

Values represent means ± SD.

*: significantly different from the control (no anatase TiO_2-NPs) at 5%, **: significantly different from the control at 1% confidence level.