

Research Article

Neonatal Exposure to Titanium Dioxide Nanoparticles Modulates the Redox Balance in Infantile and Adult Female Rats

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Abstract

Objectives: The aim of our study was to determine a possible relationship between the redox state of the organism and the exposure to TiO₂ Nanoparticles (NPs).

Design and methods: TiO₂ NPs (1% or 10% LD₅₀ TiO₂) were intraperitoneally administered to female Wistar rats from the Postnatal Day 4 (PND4) to PND7. Rats were sacrificed on the PND15 (infantile rats) or after the PND220 (adult rats). In plasma, Antioxidant Capacity (TEAC), concentration of protein carbonyls and lipoperoxides were measured. Hemolysates were used to determine the changes in catalase, Glutathione Peroxidase (GPx) and Superoxide Dismutase (SOD) activities.

Results: Young rats: 1% and 10% LD₅₀ TiO₂ induced the decrease in TEAC, lipoperoxide levels and Glutathione Peroxidase (GPx) activities and higher Superoxide Dismutase (SOD) activities in infantile rats, compared to controls. Adults: Significantly lower lipoperoxide levels, higher SOD and GPx activities were found in adults compared to young rats. 1% LD₅₀ TiO₂ significantly increased TEAC value and reduced SOD and GPx activities, but 10% LD₅₀ TiO₂ reduced TEAC compared to controls.

Conclusion: Our results demonstrate that neonatal exposure to TiO₂ NPs may modulate redox balance in female rats depending on the age, which might lead to alterations of their physiological functions.

Keywords: Nanoparticles; Titanium dioxide; Oxidative stress

Introduction

Nanoparticles (NPs) are unique in their size (smaller than 100nm), shape and surface. They are suitable for the Production of Nanomaterials (NMs) with special physicochemical properties [1], with wide applications in different fields of industry e.g. electronics, textile industry, cosmetics and medicine [2]. Nanosized Titanium Dioxide (TiO₂) particles are the most often used NPs for the production of NMs due to their special photocatalytic properties, high stability and whitening ability. Recent studies have demonstrated low toxicity of TiO₂ NPs which makes them suitable for their wide use in cosmetics (toothpastes, sunscreens) as a food additive and in biomedicine as e.g. a drug delivery system [3]. Despite the undeniable benefits of TiO₂ use, several studies have described also its high absorption, accumulation in tissues and associated increase of its negative properties [3,4]. Actually, when inhaled, it is classified as a carcinogen for human [5,6]. In addition, it has been reported that the highest concentrations of TiO₂ (anatase, 200-400 nm) after a single intravenous administration (250mg/kg) have been found in liver, spleen, lungs and kidneys of female Sprague-Dawley rats [7]. The same properties that make NPs exceptional in their use cause them to be associated with an increased incidence of adverse effects in environment and human health. It has been well documented that due to their size, insoluble NPs are able to pass through the biological membranes including the blood

- brain barrier [8]. As a general rule, the smaller the nanoparticle is, the more toxic effects it displays [9]. Negative effects of nanosized TiO₂ applications include also the redox imbalance characterized by the oxidative stress [10]. Oxidative stress is one of the main accepted hypotheses of side effects associated with toxicity of NPs on the cellular level. Reactive Oxygen Species (ROS) produced in the presence of NPs might interact with biologically important molecules e.g. nucleic acids, proteins and lipids and influence the activities of antioxidant enzymes. These reactions can lead to harmful effects on cells and can be one of the causes of diseases, ageing [11] and cell death [12,13]. ROS formation and redox imbalance force us to study the potential risk of application of TiO₂ NPs on the redox imbalance of the plasma of female rats. It is proven that in systemic circulation TiO₂ NPs can interact with components of plasma [14]. Our goal was to examine effects of intraperitoneal neonatal administration of TiO₂ NPs to female rats on antioxidant capacity of plasma and oxidative damage to plasma lipids and proteins by monitoring the levels of lipoperoxides and protein carbonyls. In addition, before the delivery to target organs, TiO₂ NPs travel through the whole body within the bloodstream. Erythrocyte, a prevailing cell in the blood, is susceptible to ROS toxic effects resulting in the membrane damage, deformation and agglutination [15]. Hence, our additional goal was to examine the changes in activities of Glutathione Peroxidase (GPx), Superoxide Dismutase (SOD) and Catalase (Cat) in lysates of erythrocytes of

female infantile and adult rats after neonatal exposure to TiO₂ NPs.

Materials and Methods

Choice of doses and study design

The experiments were carried out on one generation of the neonatal rat model described by the Newbold's group representing an ontogenic model consistent with the developmental stage from 0 to 28 postnatal days in human infants [16,17]. Anticipating that human exposure to TiO₂ is rather low, the neonatal female rats were short-term exposed to two low doses (1% and 10%) derived from the estimated value of LD₅₀ of TiO₂ NPs (administered by the single Intraperitoneal (i.p.) injection) calculated as 59.22mg/kg of Body Weight (bw), with the confidence interval from 55 to 70 mg/kg [18]. Two doses of TiO₂ NPs were used according to the Endocrine Disruptor Screening Program Test Guidelines (EDSPTG) OPPTS 890.1450 and the study by Newbold et al. [16] as investigation of the redox imbalance of the female rat organism in two different life stages is an integral part of reproductive and neuroendocrine toxicity study in female rats neonatally exposed to TiO₂ NPs.

Experiment 1: infantile female rats

Nulliparous female (n=20) and reproductive experienced male (n=10) Specific Pathogen Free (SPF) Charles River Wistar rats obtained from Breeding Facility VELAZ Prague (Czech Republic) were acclimatized to the new laboratory conditions for 7 days prior to mating. In total, 32 female rats were obtained from eleven litters [litter size ranged from 9 to 14 pups; litters delivered by Gestational Days (GD) 21-22] born to pregnant dams. Cross-fostering was done to minimize potential litter effects and standardized to 8 pups per dam with an equal or female predominance gender ratio to allow uniform breast-feeding and growth rates. Each treatment group of newborn female rats was Intraperitoneally (i.p.) injected daily with two different doses of TiO₂ NPs: 10% LD₅₀ TiO₂ = 592µg/kg b.w. (n=10) and 1% LD₅₀ TiO₂ = 59.2µg/kg b.w (n=11) in 10% (v/v) rat serum physiological solution or vehiculum (10% rat serum physiological solution, v/v, n=11) in the control group from the Postnatal Day 4 (PND 4) to the PND 7 in the dose volume of 10ml/kg b.w. Neonatally exposed infantile female rats were sacrificed on the PND 15 by decapitation under ketamine/xylazine anesthesia (60/10mg/kg b.w.) [Ketamin (Narketan) VETOQUINOL LTD, Czech Republic; Xylazine (Xylarium) Riemser Arzneimittel AG, Germany].

Experiment 2: adult female rats

Nulliparous female (n=20) and reproductive experienced male (n=10) specific pathogen free (SPF) Charles River Wistar rats were obtained from Breeding Facility VELAZ Prague (Czech Republic). A set of 59 female rats were obtained from 17 litters (litter size ranged from 12 to 19 pups; litters delivered by GD 21-22). Each litter was represented in the three treatment groups. Number of pups per dam was randomly reduced to 8 pups with an equal or female predominance gender ratio to allow uniform breast-feeding and growth. New-born female Wistar rats were i.p. injected daily with two different doses of TiO₂ NPs: 10% LD₅₀ TiO₂ = 592µg/kg b.w (n=20) and 1% LD₅₀ TiO₂ = 59.2µg/kg b.w. (n=19) in 10% (v/v) rat serum physiological solution or vehiculum (10% rat serum physiological solution, v/v, n=20) in the control group from the PND 4 to the PND 7 in dose volume of 10ml/kg b.w. Neonatally exposed adult female rats were sacrificed on the day of the first occurrence of estrus after PND 220 by decapitation

under ketamine/xylazine anesthesia (60/10mg/kg b.w.) [Ketamin (Narketan) VETOQUINOL LTD, Czech Republic; Xylazine (Xylarium) Riemser Arzneimittel AG, Germany].

Housing conditions

The animals were placed in plastic cages with wire lids, standard bedding (JRS Lignocel, Hygienic Animal Bedding and Germany) and enrichment (removed during nursing). They were housed in a ventilated animal room under controlled environmental conditions at 22 ± 2°C and 30 - 70 % relative humidity with 12h light:dark schedule (light from 6.00am). Standard laboratory chow (complete certified laboratory rodent chow M3, BONAGRO, CZ10174, Czech Republic) and tap water in glass bottles were available *ad libitum*. The study meets the WHO International Ethical Guidelines for Biomedical Research involving experimental animals and is in compliance with the Slovak Statutory order No 377/2012 Z. z. and No 436/2012 Z. z. (Collection of laws). The protocol of the study was covered by agreement from the Ethical committee in the Slovak Medical University and approved by the State Veterinary and Food Administration, Slovak Republic (C.k. Ro-1304/13-221/3). Animal care was in compliance with the Standard Operation Procedures (Good Laboratory Praxis) of the Department of Toxicology, the Slovak Medical University, Bratislava, and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123).

Preparation and characterization of TiO₂ NPs suspension

Titanium (IV) oxide nanopowder [$<100\text{nm}$ (BET), 99.9% metal basis, Sigma-Aldrich, Cat. No. 634662, CAS 13463-67-7, FW 789.9] was suspended in the physiological solution containing 10% (v/v) of rat serum (Sigma; pH=7.5) at concentration 59.22mg/10ml, sonicated (Sonopuls, Bandelin electronic, Germany) for 15min at 150W. Fresh suspension was prepared daily and immediately before administration, it was vortexed at the highest speed for 1min. Dynamic Light Scattering (DLS) was used to measure the average particle size, hydrodynamic size distribution, and the degree of aggregation and zeta potential of TiO₂ NPs in 10% rat serum physiological solution at concentration of 1mg/ml. DLS measurements were performed with a Malvern Instrument Zetasizer Nano equipped with a He-Ne laser ($\lambda=633\text{ nm}$, max 5mW) and operated at a scattering angle of 173°. The instrument incorporates a zeta potential analyser that uses electrophoretic light scattering for particles, molecules and surfaces, and a molecular weight analyser using static light scattering. The mean hydrodynamic diameter was calculated from the autocorrelation function of the intensity of light scattered from the particles. The temperature of standard measurements was set to 25°C or 37°C. Characteristics of tested TiO₂ NPs are summarized in (Table 1).

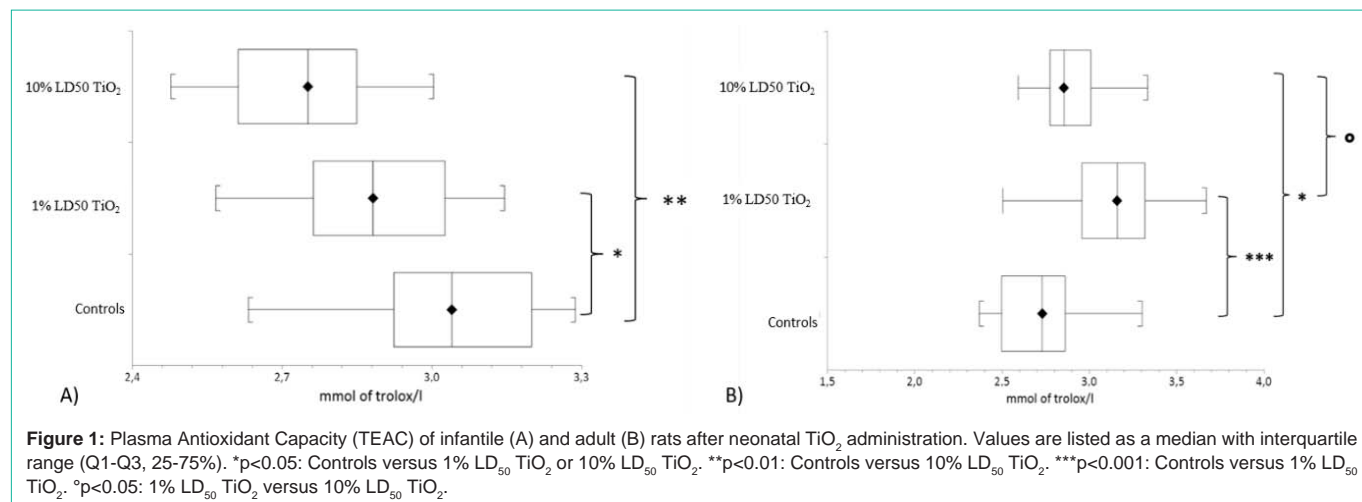
Plasma and hemolysate collection

Blood samples were collected after decapitation into commercial tubes with Li-Heparine as an anticoagulant. Subsequently, samples were centrifuged (1200xg, 5min, 4°C), separated plasma was aliquoted, shock frozen and stored until further analysis (at -80°C). Blood plasma aliquots were used for the determination of redox state parameters (antioxidant capacity, protein carbonyls and lipoperoxides) of the organism. Erythrocytes (0.5ml) were washed three times with 5ml of physiological solution. The suspension was centrifuged (660xg, 5min, 4°C) and hemolysed in chilled distilled water. Lysates of erythrocytes

Table 1: Characteristics of titanium dioxide nanoparticles.

T (°C)	Average size (nm, by DLS)	Size distribution (nm, by DLS)	PDI	Zeta potential (mV)
25	244±1	309±3	0.205±0.010	-13.81±0.35
37	257±8	28±17	0.223±0.032	-13.18±0.38

DLS: Dynamic Light Scattering; PDI: Polydispersity Index; Numbers Represent The Mean ± SEM.



were aliquoted, frozen and stored at -80°C. They were used for the evaluation of the hemoglobin level and determination of activities of antioxidant enzymes (glutathione peroxidase, catalase, superoxide dismutase).

Methods

Trolox equivalent antioxidant capacity (TEAC): For the screening of plasma antioxidant activity (TEAC), spectrophotometric method by Re et al. [19] was used. As a standard, trolox (water-soluble analogue of vitamin E) was used. The TEAC values are expressed in mmol of trolox/l of plasma.

Protein carbonyls: The level of protein carbonyls is associated with the amount of proteins present in plasma. The Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA) was used to measure the concentration of proteins in plasma samples. To evaluate protein carbonyl levels in the rat plasma, the commercial kit OxiSelect™ Protein Carbonyl ELISA Kit (Cell Biolabs, Inc., USA) was used. Protein carbonyl concentrations are expressed in nmol of carbonyls/mg of proteins.

Liperoxides: Concentration of liperoxides in plasma was measured spectrophotometrically according to El Saadani et al. [20]. Liperoxide levels are expressed in nmol/ml of plasma.

Catalase activity: To measure the activity of Catalase (Cat) in hemolysates, the method according to Bergmeyer [21] was used. The activity is expressed in μ kat/g Hemoglobin (Hb). Drabkin's reagent [22] was used to determine the hemoglobin concentration in the hemolysates.

Glutathione peroxidase activity: Glutathione Peroxidase (GPx) activity was determined in hemolysates using the commercial Glutathione Peroxidase Assay Kit (Cayman Chemical, USA). Activity of the enzyme is stated as nU/ml/mg Hb.

Superoxide dismutase activity: To evaluate Superoxide

Dismutase (SOD) activity in lysates of erythrocytes, SOD Assay Kit (Sigma-Aldrich Co., USA) was used. Enzyme activity is expressed in U/mg Hb, where activity is defined as the amount of enzyme able to inhibit the rate of chromagen reduction by 50%.

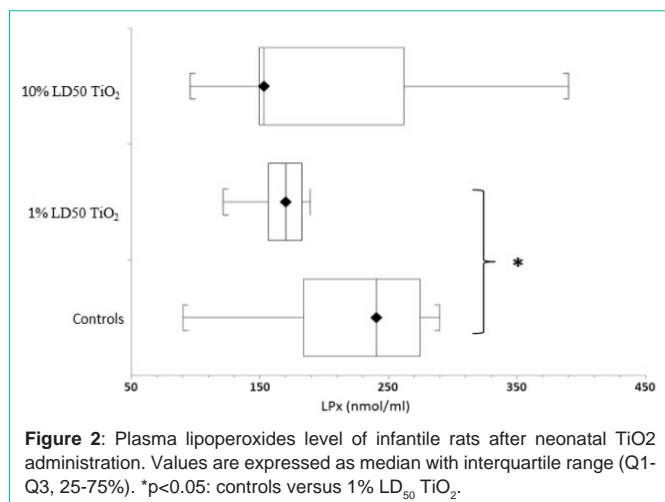
Statistical analysis

Stats Direct 3 Statistical software, version 2.3.7. (StatsDirect Ltd., UK), was used to perform statistical analyses. Significance level was set at p<0.05. Due to not normally distributed data, median with Interquartile Range (IQR) with minimal and maximal values was used. Mann-Whitney test was used to detect differences of particular variables between individual groups. The associations between parameters were analyzed by Spearman's correlations. Graphical representations of data were made using Box and Whisker graphics and charts of correlations were made using Spearman's rank correlation.

Results

Trolox equivalent antioxidant capacity (TEAC)

No age-related changes (p>0.05) were found in TEAC in vehicle-treated female rats (controls) [2.942 (2.899 – 3.195) nmol trolox/l in infantile rats vs. 2.850 (2.76-3.004) nmol trolox/l in adult rats]. In infantile rats (PND15) neonatal administration of 1% LD₅₀ TiO₂ [2.881 (2.727-2.930) nmol trolox/l] induced a significant decrease in TEAC compared to the control group [2.942 (2.889-3.195) nmol trolox/l], (p<0.05). Similarly, when compared to controls, the reduction in TEAC was even more evident after 10% LD₅₀ TiO₂ administration [2.724 (2.588-2.843) nmol trolox/l] (p<0.01) (Figure 1A). On the contrary, in adult rats when compared to associated controls [2.850 (2.760-3.004) nmol trolox/l], 1% LD₅₀ TiO₂ administration increased the TEAC [3.124 (2.935-3.298) nmol trolox/l] significantly (p<0.001). In contrast, compared to controls, relevant decrease was found after 10% LD₅₀ TiO₂ administration [2.721 (2.479 - 2.835) nmol trolox/l] (p<0.05) (Figure 1B).



Protein carbonyls

We have not observed any significant age-related differences ($p>0.05$) in protein carbonyl levels in infantile and adult control female rats. When compared to controls [2.073 (1.730-2.269) nmol carbonyls/mg proteins in infantile rats and 2.226 (2.075-2.413) nmol carbonyls/mg proteins in adult rats], neonatal administration of 1% LD₅₀ TiO₂ caused no significant differences in protein carbonyl concentrations in either infantile [1.984 (1.644-2.087) nmol carbonyls/mg proteins] or adult rats [2.173 (2.004-2.444) nmol carbonyls/mg proteins]. Similarly, no change in protein carbonyl levels was recorded after 10% LD₅₀ TiO₂ exposure [2.068 (1.767-2.648) nmol carbonyls/mg proteins in infantile rats and 2.329 (2.120-2.544) nmol carbonyls/mg proteins in adult rats] compared to corresponding controls.

Lipoperoxides

In contrast to TEAC and protein carbonyls, a significant decrease ($p<0.001$) was observed in lipoperoxide levels in adult control female rats [47.058 (34.708-86.876) nmol/ml] compared to infantile control female rats [239.121 (182.055-273.829) nmol/ml]. In the infantile group, neonatal administration of 1% LD₅₀ TiO₂ caused the significant reduction ($p<0.05$) in lipoperoxides [168.535 (155.226-176.520) nmol/ml] compared to infantile controls (Figure 2). Compared to controls, no significant difference was determined in

case of 10% LD₅₀ TiO₂ [for 10% LD₅₀ TiO₂ 152.884 (147.135-244.018) nmol/ml and for controls 239.121 (182.055-273.829) nmol/ml]. In adult rats, compared to corresponding controls, no difference in the concentration of lipoperoxides ($p>0.05$) was found in the both groups: 1% LD₅₀ TiO₂ [64.731 (35.985-74.739) nmol/ml] and 10% LD₅₀ TiO₂ [72.609 (40.031-100.397) nmol/ml].

Superoxide dismutase activity

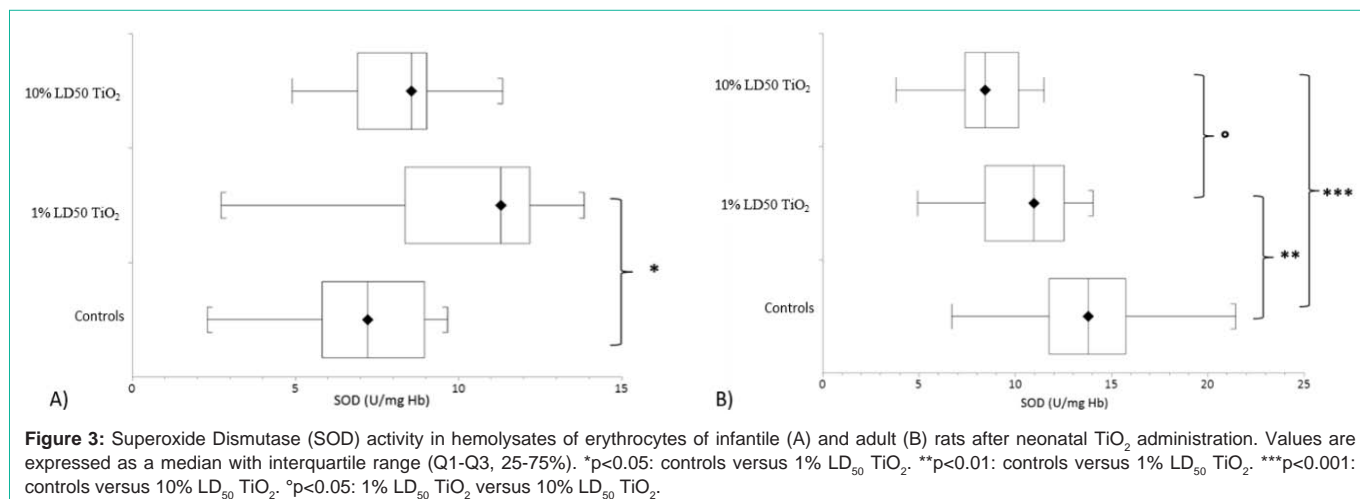
As with lipoperoxides, a significant difference ($p<0.001$) was found also in SOD activities of infantile [7.098 (5.564-8.737) U/mg Hb] and adult [13.342 (11.701-15.732) U/mg Hb] control rats. In infantile rats, SOD activity was increased significantly [11.069 (6.06-12.008) U/mg Hb] after 1% LD₅₀ TiO₂ administration in comparison to the control group ($p<0.05$). In the group of rats where elevated dose (10% LD₅₀ TiO₂) was administered [8.405 (6.803- 8.931) U/mg Hb] there was no significant increase compared to controls (Figure 3A). In contrast, in the group of adult rats, when compared to controls, already 1% LD₅₀ TiO₂ administration caused a significant reduction of SOD activity ($p<0.01$) [10.433 (8.319-12.493) U/mg Hb]. When compared to the control group, this inhibitory trend of SOD activities persisted also after the exposure to the higher dose of TiO₂ NPs [8.39 (7.272-10.110) U/mg Hb] ($p<0.001$) (Figure 3B).

Catalase activity

No age-related difference of catalase activity was detected [0.776 (0.586-0.900) U/mg Hb in the infantile group vs. 0.701 (0.551-0.840) U/mg Hb in adult rats] in female control rats ($p>0.05$). In infantile rats, neonatal administration of either 1% LD₅₀ TiO₂ [0.763 (0.504-0.937) U/mg Hb] or 10% LD₅₀ TiO₂ [0.757 (0.658-0.890) U/mg Hb] caused no significant changes ($p>0.05$) in catalase activity compared to corresponding controls. Likewise, in the group of adult rats, both doses of TiO₂ NPs caused no significant changes ($p>0.05$) of catalase activity [0.618 (0.452-0.829) U/mg Hb for 1% LD₅₀ TiO₂ and 0.616 (0.54-0.73) U/mg Hb for 10% LD₅₀ TiO₂].

Glutathione peroxidase activity

Glutathione peroxidase activity was significantly increased ($p<0.001$) in adult control rats [77.357 (70.257-92.981) nU/ml/mg Hb] when compared to the infantile control group [39.411 (31.622-50.897) nU/ml/mg Hb]. In infantile rats, compared to control female rats, 1% LD₅₀ TiO₂ administration caused no change ($p>0.05$) in



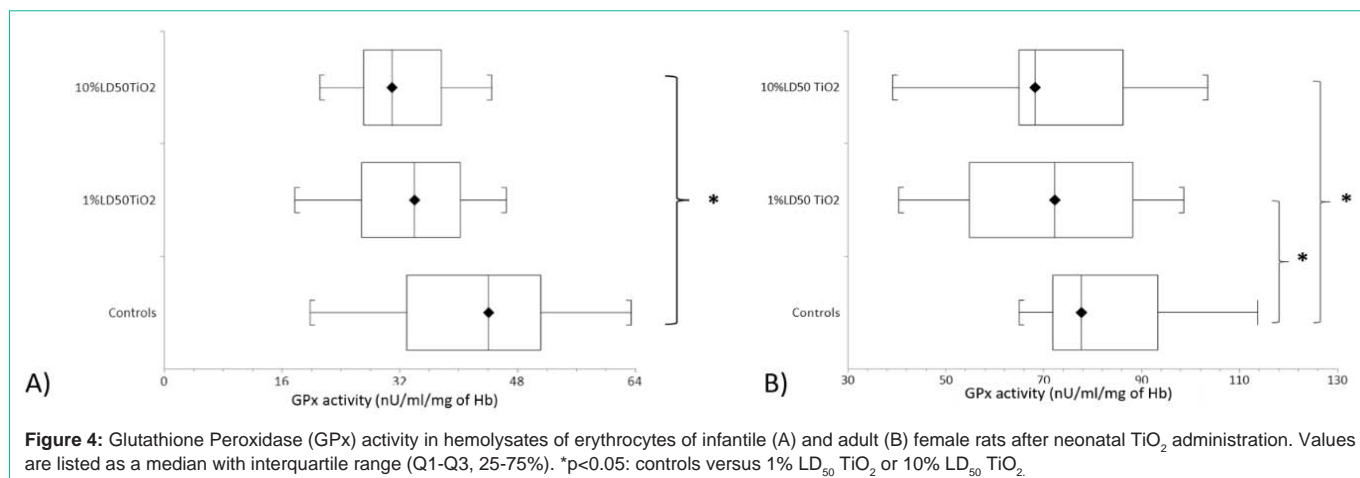


Figure 4: Glutathione Peroxidase (GPx) activity in hemolysates of erythrocytes of infantile (A) and adult (B) female rats after neonatal TiO_2 administration. Values are listed as a median with interquartile range (Q1-Q3, 25-75%). * $p < 0.05$: controls versus 1% LD_{50} TiO_2 or 10% LD_{50} TiO_2 .

Table 2: Correlations between activities of antioxidant enzymes.

Infantile rats			r	P
Controls	Cat	GPx	0.92	<0.001
1% TiO_2	Cat	GPx	0.853	<0.01
10% TiO_2	Cat	GPx	0.646	<0.05
	Cat	TEAC	-0.646	<0.05
	GPx	TEAC	-0.803	<0.05
Adult rats				
Controls	Cat	GPx	0.458	<0.05
	Cat	LPx	0.5	<0.05
1% TiO_2	Cat	GPx	0.82	<0.001
10% TiO_2	Cat	GPx	0.717	<0.05

Cat: Catalase; GPx: Glutathione Peroxidase; LPx: Lipoperoxides; TEAC: Trolox Equivalent Antioxidant Capacity.

GPx activity [34.008 (26.551-40.660) nU/ml/mg Hb]. After neonatal administration of 10% LD_{50} TiO_2 , GPx activity was significantly ($p < 0.05$) reduced [29.597 (24.415- 37.42) nU/ml/mg Hb] when compared to controls (Figure 4A). When compared to control female rats, an inhibition of GPx activity was even more pronounced in adult female rats. Already 1% LD_{50} TiO_2 administration reduced GPx activity significantly [71.809 (52.871-86.788) nU/ml/mg Hb] ($p < 0.05$) and this reduction was confirmed also after neonatal administration of 10% LD_{50} TiO_2 [68.088 (62.694- 80.706) nU/ml/mg Hb] ($p < 0.05$) (Figure 4B). Moreover, in infantile female rats but not in adults we have found an indirect association between plasma antioxidant capacity and activities of catalase and GPx indicating that increased oxidative stress leading to the depletion of antioxidant capacity in plasma stimulates activities of antioxidant enzymes. During the increased oxidative stress induced by TiO_2 NPs when free radicals attack biomolecules e.g. lipids, activities of both antioxidant enzymes are increased (Table 2).

Discussion

Since the NPs are able to pass through the biological membranes and enter the cytoplasm and nucleus, there is a strong probability of oxidative stress development in the cells [23]. In exposed cells, TiO_2 induces oxidative stress by increasing intracellular ROS concentration and damaging the antioxidant cellular response [24,25]. However,

TiO_2 NP compared to other metal NPs (CuO , ZnO , Fe_3O_4), seems to exhibit lower toxicity [26]. The aim of the current study was to examine effects of neonatal TiO_2 NPs administration to female rats on selected parameters of the redox imbalance in infantile and adult animals. Results of several studies [27,28] suggest the age-related decrease of antioxidant capacity. This decrease might be associated with the oxidative stress formation followed by the damage to biologically important molecules. In our study, we have observed age-related changes in SOD and GPx activities, and lipoperoxide levels in female rats. There was no significant difference in plasma antioxidant capacity between infantile and adult animals. When compared to controls, infantile rats showed the decrease in antioxidant capacity characterized by TEAC with increasing concentration of TiO_2 NPs. These results are consistent with finding of Meena et al. [29], who reported the increase in Reactive Oxygen Species (ROS) in brain of male Wistar rats after nano- TiO_2 treatment (25 and 50 mg/kg b.w., average particle size v PBS: 114nm), which might be the cause of TEAC reduction. Furthermore, as stated by his team as well as the team of Vasantharaja et al. [30], the ROS level increase triggers the cascade of oxidative damage to DNA, lipids and proteins of brain and liver and reduces the activities of antioxidant enzymes (SOD, GPx, and Cat) involved in the antioxidant defense mechanism. In our study, on the contrary, in adult individuals, 1% LD_{50} TiO_2 administration caused the increase in TEAC, but 10% LD_{50} TiO_2 administration had no effect on the antioxidant capacity. Huerta-García et al. [31] declare a strong induction of oxidative stress by TiO_2 NPs (size less than 100nm, aggregates 300nm and more, $20\mu\text{g}/\text{cm}^2$) in rat's and human's glial cells by changes in the cellular redox state and lipid peroxidation associated with an elevated expression of GPx, catalase, and SOD. Similarly, Hassanein [32] found an increased lipid peroxidation in male Sprague-Dawley rats intoxicated with TiO_2 NPs (300mg/kg b.w.) compared to controls. The same results were confirmed by Hou et al. [6], whose team claim, that the toxic effect of TiO_2 NPs is a dose- and time-dependent and putative mechanism leading to toxicity is oxidative stress through ROS over-production resulting in lipid peroxidation and the cell wall damage. In contrast, our results suggest the decrease in lipid peroxidation in infantile rats after TiO_2 NPs treatment, what could be the result of the ability of young organism to adapt to harmful conditions (higher oxidative stress induced by TiO_2 NPs raised antioxidative mechanisms). In adult animals no significant change was recorded. Since TiO_2 NPs

and nanomaterials have become an inseparable part of our lives in the form of food additives, drug delivery agents and cosmetics, it is obvious that the toxicity and potential harmful effects of these NPs have become the aim of the human interest. Although the number of published papers on this topic is increasing, the impact of NPs on human health is unclear and requires further investigation.

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