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Evaluation of distribution, redox parameters, and genotoxicity in Wistar rats co-exposed to silver and titanium dioxide nanoparticles

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ABSTRACT

The increasing production of silver nanoparticles (AgNPs) and titanium dioxide nanoparticles (TiO_2NPs) has resulted in their elevated concentrations in the environment. This study was, therefore, aimed at determining the distribution, redox parameters, and genotoxic effects in male Wistar rats that were treated with either AgNP or TiO_2NP individually, as well as under a co-exposure scenario. Animals were exposed via oral gavage to either sodium citrate buffer (vehicle), 0.5 mg/kg/day TiO_2NP, 0.5 mg/kg/day AgNP or a mixture of TiO_2NPs and AgNPs. Exposure lasted 45 days after which rats were sacrificed, and tissue biodistribution of Ag and Ti measured. The blood concentration of glutathione (GSH) and activities of glutathione peroxidase (GPx) and catalase (CAT) were determined while the genotoxicity was analyzed using the comet assay in peripheral blood and liver cells. The tissue concentrations of Ag followed the order; blood > liver > kidneys while for Ti the order was kidneys > liver > blood. There was no significant change in the measured redox parameters in animals that were exposed to TiO_2NPs. However, there was a significant increase in GSH levels accompanied by a reduction in the GPx activity in AgNP-treated and co-exposed groups. The individual or co-exposure to TiO_2NP and AgNP did not markedly induce genotoxicity in blood or liver cells. Data showed that TiO_2NP did not produce significant oxidative stress or genotoxicity in rats at the dose used in this study while the same dose level of AgNPs resulted in oxidative stress, but no noticeable adverse genotoxic effects.

Introduction

Nanoparticles (NPs) are structures having one or more external dimensions in the size range 1–100 nm (European Union commission 2011) that possess unique physical and chemical properties which contributed to their wide biological and environmental applications (Alaraby et al. 2016; Blum et al. 2015; Carneiro and Barbosa 2016; Kermanizadeh et al. 2016; Lee et al. 2015; Liu 2006). Due to their larger surface area compared with particles of the same material, NPs may have a higher toxic potential, increased chemical reactivity, and higher ability to cross the cell membrane (Engler-Chiurazzi et al. 2016; Hanot-Roy et al. 2016; Schreiber et al. 2016). Silver (AgNP) and titanium dioxide (TiO_2NP) nanoparticles are known for their biological and biomedical properties; while AgNPs exhibit antimicrobial properties (Singh and Ramarao 2012), TiO_2NPs are employed in cancer photodynamic therapy, drug delivery systems, cell imaging, biosensors for biological assay, and genetic engineering (Yin et al. 2013). In addition to their biomedical applications, AgNPs and TiO_2NPs are commonly present in consumer products such as cosmetics, food additives, kitchen utensils, and toys (Behra et al. 2013; Tassinari et al. 2014).

These nanostructures may penetrate the organism through different routes, including oral (Choi et al. 2015; Tassinari et al. 2014), dermal (Crosera et al. 2009) or inhalation (Bakand et al. 2012). AgNPs and TiO_2NPs may be distributed to various tissues such

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as liver, kidneys, spleen, lungs, and heart (Shi et al. 2013; Wang et al. 2007) and finally, interact with biological systems and potentially induce toxic effects (Hawkins et al. 2015; Shakeel et al. 2015).

The literature is rich with various adverse effects attributed to TiO_2NP and AgNP. In this respect, investigators showed that oral administration of AgNP may lead to liver (Van Der Zande et al. 2012), intestinal (Shahare and Yashpal 2013), and neuronal (Hadrup et al. 2012) damage, while oral administration of TiO_2NP was found to result in endocrine disruption and spleen toxicity (Tassinari et al. 2014). These harmful effects might be related to adverse biological outcomes exerted by TiO_2 NP or Ag NP associated with oxidative stress, reactive oxygen species (ROS) production, and depletion of the cellular antioxidant defense capacity (Shakeel et al. 2015; Vasantharaja et al. 2015; Völker et al. 2015; Walters et al. 2016). Furthermore, TiO_2NP induced DNA damage possibly due to the generation of free radicals, and this was demonstrated by both *in vitro* and *in vivo* genotoxicity studies using different experimental models (Asare et al. 2016; Biola-Clier et al. 2017). Similarly, genotoxic effects attributed to AgNP were previously reported as evidenced by elevation in levels of DNA strand breaks in lungs and testes of exposed mice (Asare et al. 2016).

Taken together, it is evident that considerable attention has been given to assess the adverse influence of AgNP or TiO_2NP individually but combined toxic effects under a co-exposure scenario are largely unknown which is the purpose of the present study. In this investigation, adult male Wistar rats were exposed via oral gavage for 45 days to 0.5 mg/kg of either of AgNP or TiO_2NP individually, as well as to a mixture. Subsequently, the biodistribution of NP and consequent oxidative stress and genotoxic effects were determined.

Materials and methods

Chemicals

The NP of TiO_2 (CAS 13463-67-7) and Ag (CAS 7440-22-4) used in are commercially available products and purchased from Sigma-Aldrich (St. Louis, MO, USA).

Nanoparticle characterization

To assess the average hydrodynamic radius and size distribution of the particles, dynamic light scattering (DLS) measurements were performed with a Zetasizer Nano ZS90 DLS equipment (Malvern Instruments Ltd., England). A cuvette QS 3 mm was employed as a sample container. An X-ray diffractometer (XRD 6000, Shimadzu) was utilized to determine crystalline phases of the NP. Data were collected from 20 to 80° 2θ at a step width of 0.5°, 10 sec per step, at 40 kV, 200 mA, and CuKα radiation (λ = 1.540560 Å). Silicon was used as external standard. The size of the NPs were also characterized by transmission electron microscopy (TEM) (Figure 1), using a JEM-100CXII transmission electron microscope (JEOL, Japan) with an accelerating voltage ranging from 100 to 200 kV.

Animal maintenance and treatments

Male Wistar rats weighing 200–220 g obtained from University of São Paulo Central Animal Facility (Ribeirão Preto, SP, Brazil) were used. All animal procedures were reviewed and approved by the Ethics Committee in the Use of Animals from the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo (approval number 141763530), and carried out according to the guidelines from the National Council of Animal Experimentation Control. Rats were housed in an animal room at a controlled temperature (22°C–24°C) and 12 hr light/dark cycle with food and water provided *ad libitum*. Animals were randomly assigned to 4 different groups (n = 6/group): Group I (control): received vehicle; Group II (TiO_2NP): received TiO_2 (0.5 mg/kg); Group III (AgNP received Ag (0.5mg/kg); Group IV (AgNP + TiO_2NP): received AgNP + TiO_2NP (0.5 mg/kg). The vehicle used was sodium citrate buffer 0.1 M, pH 4.5. The treatment was given daily by oral gavage for 45 days.

After 45 days of treatment, animals were euthanized by overdose of ketamine (300 mg/kg) and xylazine (30 mg/kg), decapitated and had their blood and organs (liver and kidney) excised. Aliquots of the total blood were used for comet

assay and hemoglobin measurement performed right after anesthesia; blood samples were also stored at −80°C in heparinized micro centrifuge tubes until analysis. Organs were stored in 10% (v/v) formalin.

Determination of metals in tissues and blood

The total Ti and Ag in kidneys, liver, and blood were determined using an inductively coupled plasma mass spectrometer (ICP-MS) (NexIon 300D, PerkinElmer, SCIEX, Norwalk, CT, USA). The sample introduction system was composed of a quartz cyclonic spray chamber and a Meinhard® nebulizer connected by Tygon® tubes to the ICP-MS's peristaltic pump (set at 20 rpm). For kidneys and liver, the method proposed by Palmer et al. (2006) was adopted. Briefly, 50–75 mg of each tissue was weighed and transferred to a conical tube. Then, 1 ml 50% (v/v) tetramethylammonium hydroxide (TMAH) solution was added to the samples, incubated at room temperature for 12 hr and the volume made up to 10 ml with a solution containing 0.5% (v/v) nitric acid (HNO_3), 0.01% (v/v) Triton® X-100. Analytical calibration standards were prepared daily over the range of 0–20 µg/L in a diluent containing 5% (v/v) TMAH, 0.5% (v/v) HNO_3, 0.01% (v/v) Triton® X-100. Correlation coefficient for the calibration curves was always better than 0.9999.

The total Ti and Ag in whole blood were measured using the method described by Palmer et al. (2006). Calibration against matrix-matching was used. Bovine whole blood (base blood) was then homogenized and diluted 50-fold with a solution containing 0.01% (v/v) Triton® X-100 and 0.5% (v/v) HNO_3. The concentration ranges of curves were from 0 to 20 µg/L. Rat blood samples were prepared with the same dilution factor (1:50) with 0.01% (v/v) Triton® X-100 and 0.5% (v/v) HNO_3 and analyzed against the matrix-matching curve. The correlation coefficient was always better than 0.9999. Reference materials (for tissues: NIST SRM 1577b (bovine liver); for blood: NIST SRM 966 (toxic metals in bovine blood) were analyzed to verify the accuracy of data, and measured values were in agreement with the certified values.

Glutathione peroxidase (gpx) activity

The activity of the antioxidant enzyme GPx was determined spectrophotometrically by using the method of Paglia and Valentine (1967). Briefly, 880 µl of the system solution (0.15 mM NADPH, 1 mM GSH, 0.1 U/ml GR, 100 mM sodium azide, in potassium phosphate buffer, pH 7) was transferred into each of the two cuvettes labeled blank and sample. Thereafter 20µl water was placed into the blank cuvette but 20 µl diluted blood sample (40-fold diluted with the potassium phosphate buffer) into the sample cuvette. Later, 100 µl hydrogen peroxide (H_2O_2) (0.4 mM) was added to both the blank and sample cuvettes, and the change in absorbance monitored over 2 min at 340 nm using UV/visible spectrophometer. The GPx activity in the blood was expressed as µmol NADPH/min/ml blood.

Glutathione (GSH) assay

Glutathione (GSH) was determined by the method described by Ellman (1959). Blood (0.3 ml) was hemolyzed using 10% (v/v) Triton X-100 (0.1 ml) and, after 10 min, precipitated with 0.2 ml 20% (w/v) trichloroacetic acid (TCA). The precipitated blood samples were centrifuged at 4500g for 10 min, and 50 µl of supernatant aliquots was mixed with 900µl potassium phosphate buffer (1M, pH 7.4). This was followed by addition of 50 µl 10 mM 5-5′-dithio-bis(2-nitrobenzoicacid) (DTNB) and absorbance of the reaction product was read at 412 nm. GSH levels were expressed as µmol/ml blood.

Catalase (CAT) activity

CAT activity was assayed by measuring the decreased rate in H_2O_2 absorbance using a spectrophotometer at 240 nm (Aebi 1984). The total blood was diluted 60-fold in 50 mM phosphate buffered saline, after which an aliquot of 20 µl diluted blood was added to 1910 µl the buffer and then 70 µl H_2O_2. CAT activity was monitored by the decomposition of H_2O_2 for 5 min. Data were expressed in κ/g Hb. The hemoglobin content of blood was determined using a commercially available kit, hemoglobin test (Doles

Reagents, Goiania, Brazil) following manufacturer's instructions.

Comet assay

The genotoxicity of AgNP and TiO_2NP was assessed as previously described by Singh et al. (1988) and Tice et al. (2000). Blood samples were collected in tubes with K2 EDTA preservative (Labtest, Lagoa Santa, Brazil), mixed in 0.5% (w/v) low melting point agarose, and spread on 1.5% (w/v) agarose pretreated microscope slides. For liver samples, approximately 0.4 g liver was placed in 2 ml chilled Hank's solution in a Petri dish and sliced into fragments to obtain a cell suspension. The cell viability was determined by the trypan blue dye exclusion method in order to verify a possible effect of cytotoxicity on DNA migration. Cell suspensions (80 µL) were mixed with 240 µl 0.5% low melting agarose and spread onto microscope slides precoated with 1.5% normal melting agarose. After agarose solidification (20 min, 4°C), slides were subjected to lysis (overnight, 4°C), DNA unwinding (20 min, 4°C), electrophoresis (20 min, 0.85 V/cm, 300 mA, 4°C), neutralization (5 min, pH 7.5, 4°C), and fixation (ethanol, 2 min). Immediately before analysis, slides were stained with GelRed (1:10,000 v/v). Tail intensity (% DNA in tail) was analyzed in 150 nucleoids per animal using Comet Assay IV software (Perceptive Instruments, Suffolk, UK) and a fluorescence microscope (Axiostarplus, Zeiss) equipped with 515–560 nm excitation and 590 nm barrier filters. Cell viability was determined in cell suspensions by trypan blue exclusion method, and all results were higher than 90%.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5.0 (GraphPad, Carlsbad, CA, USA). Statistical evaluation was performed by One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. The level of statistical significance was set at $p < 0.05$.

Results and discussion

The widespread applications of NP in various fields have contributed significantly to their immense release into the environments (Carneiro and Barbosa 2016; Kermanizadeh et al. 2016). Human exposure to these substances especially AgNP or TiO_2NP are increasing considerably due to their uses as materials for food packaging, textile fabrics, medicines and in a large variety of personal care and cosmetic products (Carneiro and Barbosa 2016; Zhao and Castranova 2011). Notwithstanding the anticipated high exposure levels, the specific interactions of AgNP or TiO_2NP in humans at biochemical and cellular levels are still poorly understood (Behra et al. 2013; Shakeel et al. 2015). Furthermore, toxicity studies are necessary to assess the NP distributions in the body, as well as the underlying mechanisms of action.

Firstly, the distribution and disposition of individually AgNP (TEM size: 91.71 ± 1.6 nm; hydrodynamic diameter: 287.99 ± 24.15 nm) or TiO_2NP (TEM size: 41.99 ± 1.63 nm; hydrodynamic diameter: 447.67 ± 6.43 nm) were determined and then co-exposure consequences in rat tissues given that the nanomaterials have small size and large surface area that may penetrate organs and cells leading to harmful effects (Behra et al. 2013; Schilling et al. 2010). Our results demonstrated that the concentrations of AgNP were highest in blood, followed by liver, and lowest in kidneys in animals that were treated with only AgNP. In contrast, in the rats administered a mixture of AgNP and TiO_2NP, the highest concentrations of AgNP were found in kidneys, followed by liver and then blood (Figure 2). In agreement with our results, Van der Zande et al. (2012) showed that following oral treatment of rats with 90 mg AgNP/kg bw/day for 28 days, the concentrations of Ag was higher in liver compared to kidneys and other organs. It is noteworthy to mention that Van der Zande et al. (2012) did not determine the levels of Ag in blood. In another similar study, administration of more than 125 mg/kg to rats for 90 days resulted in discrete liver damage (Kim et al. 2010). In contrast to these findings, Garcia et al. (2016) observed the highest Ag concentrations in kidneys of rats treated with AgNP. Indeed, orally administered Ag may accumulate in the glomerular basement membrane, leading to detrimental consequences (Garcia et al. 2016).

The tissue biodistribution of TiO_2NP was quite different from the trend noted with AgNP. The levels of Ti were highest in kidneys compared to blood and liver in rats exposed to TiO_2NP alone and those administered the mixture of TiO_2NP and AgNP (Figure 3). Wang et al. (2007) reported enhanced susceptibility of liver and kidneys to toxic effects in mice orally exposed to TiO_2NP (5g/kg). In addition, oral administration of 50 and 100 mg/kg bw TiO_2NP to Wistar rats predominantly affected the kidneys with a significant increase in blood urea nitrogen (BUN) and uric acid levels indicating TiO_2NP induced kidney damage (Vasantharaja et al. 2015). The differences in the biodistribution patterns of TiO_2NP and AgNP in tissues of rats in this study might be attributed to differences in their kinetics in the body. The higher level of AgNP in the blood may be an indication of slower clearance rate of the toxicant from the blood stream. Generally, a toxicant that enters an organism through ingestion traverses the gastrointestinal tract (GIT) to the blood from where substances are distributed to other tissues (Lehman-McKeeman 2015).

The mechanisms by which NP produce cell and tissue damage remain unclear. Studies demonstrated that excessive production of free radicals, oxidative stress, and changes in the redox status of the cell may be associated with toxicity of AgNP and TiO_2NP (Gagne et al. 2013; Rizk et al. 2017; Valerio-García et al. 2017). In the present study, various redox parameters such as levels of GSH, and activities of GPx and CAT after low-dose AgNP or TiO_2NP exposure both individually and in mixture were evaluated. Glutathione (GSH) is a major endogenous non-enzymatic thiol which exerts many biological roles, including protection against reactive oxygen and nitrogen species (ROS and RNS) (Lushchak 2012). No significant differences were observed in GSH levels in TiO_2NP exposed rats compared to controls (Figure 4A). However, treatment with AgNP alone or the mixture of TiO_2NP and AgNP significantly elevated the levels of GSH compared to controls. The results of this study corroborate findings from previous studies in which cellular levels of GSH were altered following exposure to AgNP (Arora et al. 2009; Choi et al. 2010; Genter et al. 2012; Krawczynska et al. 2015). GPx and CAT are antioxidant enzymes involved in the cell redox control that converts hydrogen peroxides to H_2O and O_2 (Halliwell and Chirico 1993). When compared to control, the activity of GPx decreased significantly in rats receiving AgNP alone or concomitant with TiO_2NP (Figure 4B). In contrast, CAT activity rose significantly in rats compared to controls only after co-exposure (AgNP + TiO_2NP) (Figure 4C). The reduced GPx but increased CAT activities noted following AgNP administration were similar to the observations in the freshwater bivalve *Sphaerium corneum* in which GPx activity was markedly diminished after 28 days exposure to AgNP (0–500 µg Ag/L) (Völker et al. 2015). The lower GPx activity may be attributed to strong affinity of Ag ions to thiol groups of GSH resulting in decreased activity of the enzyme using this cofactor ((Völker et al. 2015).

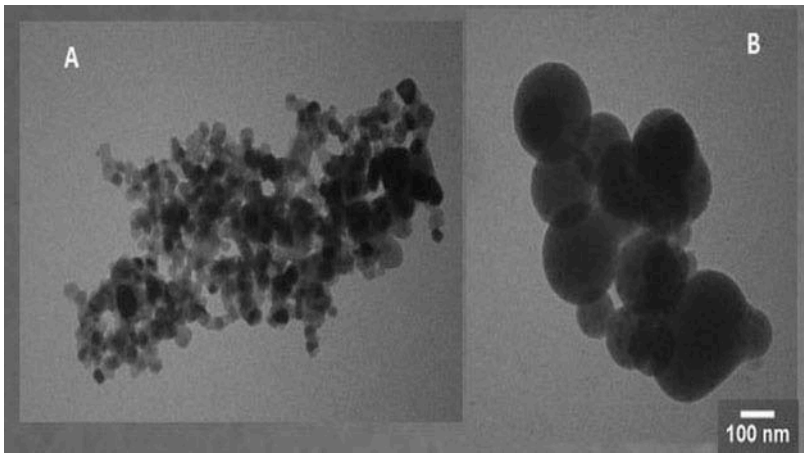

Figure 1. Typical TEM image of A) TiO_2NP and B) AgNP. (mag: 100,000).

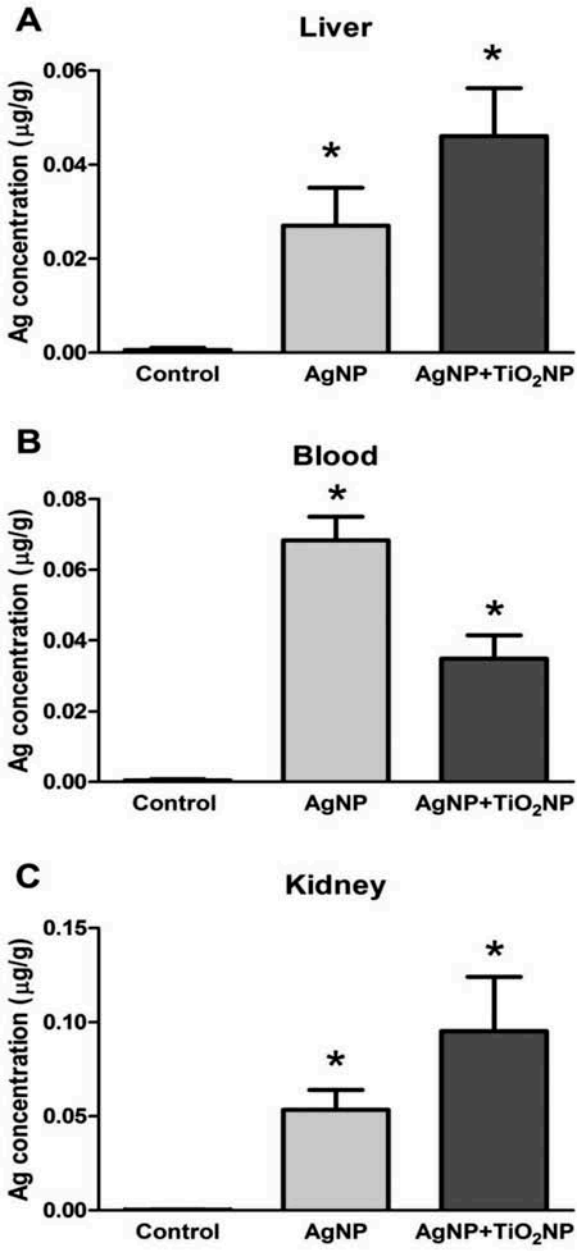

Figure 2. Silver (Ag) concentrations after administration of AgNP or AgNP + TiO_2NP in A) Liver. B) Blood. C) Kidney. Data are expressed as mean ± SEM.
*Significant from control at $p \leq 0.05$.

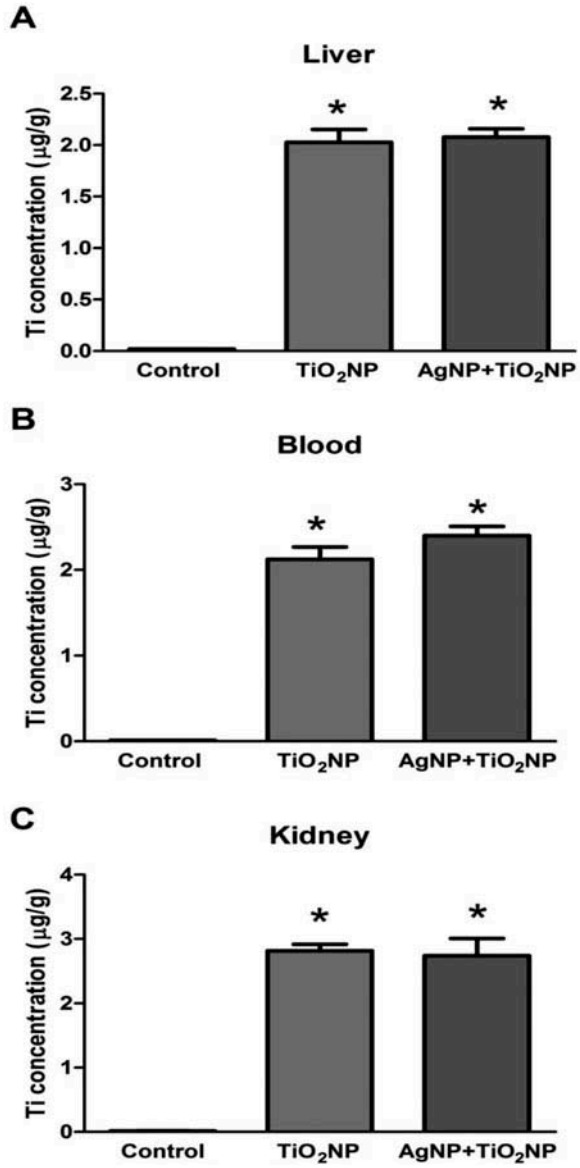

Figure 3. Titanium (Ti) concentrations after administration of TiO_2NP and AgNP + TiO_2NP in A) Liver. B) Blood. C) Kidney. Data are expressed as mean ± SEM.
*Significant from control at $p \leq 0.05$.

The results on redox state provided by this study reinforce the hypothesis that exposure to AgNP, even at low doses, may exert an impact upon the redox system. In contrast to other Ag^+ ion sources, such as silver nitrate or silver sulfadiazine, AgNP are able to generate a sustained release of Ag^+ ions, due to their higher surface to volume ratio (Ahlberg et al. 2016). Apparently, at the dose, size and form used in the present study, AgNP were able to release Ag^+ ions that potentially reacted with cell components and induced formation of free radicals, thereby affecting cell anti-oxidative mechanisms. On the other hand, this was not detected for TiO_2NP alone. In addition, when combined with TiO_2NP, redox state may have been impacted in a more pronounced way by AgNP. Additional studies are however needed to elucidate the mechanisms underlying the effects found after AgNP exposure both alone and concomitant with TiO_2NP.

The mechanisms by which NP produce genotoxicity are not completely understood, but may be associated with direct interaction with DNA or the excess production of ROS or release of metallic

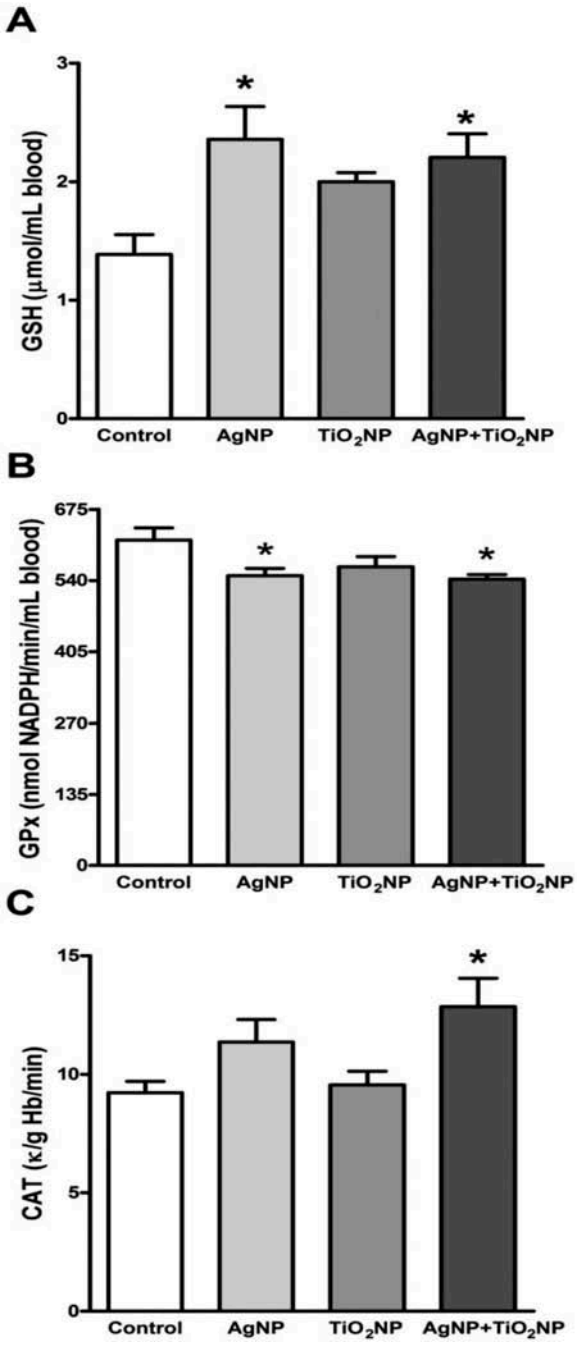

Figure 4. Redox parameters in Wistar rats exposed to citrate buffer (control), AgNP, TiO_2NP, and mixture of AgNP and TiO_2NP. A) GSH levels (µmol/ml blood), B) GPx activity (µmol NADPH/min/ml blood), C) CAT activity (κ/g hb/min). Data are expressed as mean ± SEM.

*Significant from control at $p \leq 0.05$.

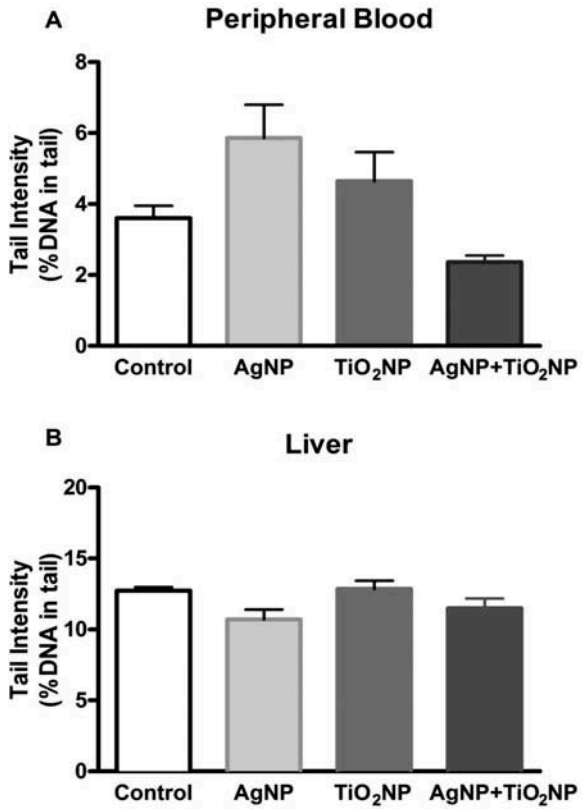

Figure 5. Genotoxic effects (% DNA in the tail) in Wistar rats exposed to citrate buffer (control), AgNP, TiO_2NP and mixture of AgNP and TiO_2NP in A) Peripheral blood cells. B) Liver cells.

*Significant from control at $p \leq 0.05$.

ions when in solution (Magdolenova et al. 2014). DNA damage produced by AgNP, TiO_2NP or both compounds administered simultaneously was assessed by measuring % DNA in tail of nucleoids obtained by the comet assay using liver and peripheral blood cells. The results are as shown in Figure 5A and 5B. In both tissues, (liver and peripheral blood), there was no significant change in % DNA in the tail compared to negative control, although a numerical rise in % DNA in tail was observed for AgNP in peripheral blood (5.86 ± 0.93) and TiO_2NP (4.64 ± 0.82) compared with control (3.6 ± 0.35).

In agreement with our results, Dobrzynska et al. (2014) found that, after intravenous (i.v.) administration of 5–10 mg/kg/bw 20nm AgNP or 5mg/kg/bw 21nm TiO_2NP, there were no marked differences in tail intensity evaluated by comet assay of bone marrow leukocytes from Wistar rats. In a similar study, Patlolla et al. (2015) also examined 10 nm AgNP genotoxicity in bone marrow of Sprague-Dawley rats at concentrations ranging from 5 to 100 mg/kg/bw. At lower doses (5 and 25 mg/kg/bw), Patlolla et al. (2015) did not observe any marked alterations in % DNA in tail, which corroborates our findings. However, increased % DNA in tail was reported for 50 and

100 mg/kg/bw treatment. Suzuki et al. (2016) did not find DNA damage in the liver of mice exposed for 4 weeks to 2–50 mg/kg/bw 21 nm TiO_2NP.

Since there are many differences between nanomaterials employed in biological studies (different shapes, diameters, surface areas and charges) coupled with the fact that nanometals tend to agglomerate when dispersed in solution (Schluesener and Schluesener 2013), it is difficult to find consistent information when comparing different studies as it relates to evaluation of potential genotoxic effects. In a study in which male 8-oxoguanine DNA glycosylase knockout mice and wildtype mice were administered a single i.v. injection of 5 mg/kg/bw of either AgNP (20 nm) or AgNP (200 nm) and sacrificed after 7 days, significant elevated DNA strand breaks levels were observed in groups exposed to the AgNP (200 nm) but not in those treated with AgNPs (20 nm), thus emphasizing the role of particle size in nanomaterials toxicity (Asare et al. 2016). While one may conclude that both AgNP and TiO_2NP are non-genotoxic especially from the results of this study, caution needs to be taken since the doses used in the present investigation were quite low, bearing in mind that genotoxicty due to AgNP exposure was reported at higher concentrations (Patlolla et al. 2015). To the best of our knowledge, there are limited numbers of *in vivo* studies addressing the genotoxic potential effects of low dose exposure to TiO_2NP and AgNP; and therefore, additional studies are encouraged to better understand the effects of low dose exposure to these nanomaterials.

Conclusions

The differential patterns in the tissue biodistribution of AgNP and TiO_2NP in this study is interesting. Following subchronic oral administration, AgNP accumulate to a greater extent in blood, followed by liver and then kidneys while TiO_2NP accumulate more in kidney. These results indicated the potential differences in kinetics of the two NP within the body. The higher level of AgNP in the blood might be an indication of slower clearance rate of the nanomaterial in the blood stream. Furthermore, at the dosage tested in this study, TiO_2NP did not alter the redox status of exposed rats, and did not induce genotoxicity. However, in rats that were administered AgNP and the mixture of AgNP and TiO_2NP, measured redox parameters were altered but again no evidence of significant genotoxicity was observed. Although, oxidative stress was numerically higher in rats that administered the mixture of compounds, data suggest that AgNP were predominantly responsible for the effects noted with co-exposure. Further studies are required to better understand the uptake, metabolism, oxidative and genotoxicity effects of NP *in vivo*.

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