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Differential effect of aqueous *Desmodium gangeticum* root extract mediated TiO_2 nanoparticles on isolated mitochondria, cells and Wistar rats

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ABSTRACT

Objective: To study the renal toxic effect of titanium dioxide nanoparticles (TiNPs) prepared by chemical and green route.

Methods: TiNPs were prepared by chemical (sol gel technique) and green route (using aqueous extract of *Desmodium gangeticum* root by using titanium tetraisopropoxide as precursor). Thus prepared TiNPs were characterized using UV–visible spectrophotometry, X-ray diffractometry and evaluated its renal toxic impact in different experimental models *viz*., Wistar rats (100 mg/kg b.wt.; oral), LLC-PK1 cells (100 mg/mL) and isolated renal mitochondria (0.25, 0.5 and 1 mg/mL).

Results: Compared to the chemically synthesized TiNPs, *Desmodium gangeticum* synthesized nanoparticles showed less nephrotoxicity, determined by elevated serum renal markers like urea (62%), creatinine (35%), aspartate aminotransferase (61%) and alanine transaminase (37%) and the result was in agreement with cellular toxicity (measured by MTT assay and lactate dehydrogenase activity). Further toxicity evaluation at the level of mitochondria showed not much significant difference in TiNPs effect between two synthetic routes.

Conclusions: The biochemical findings in renal tissue and epithelial cell (LLC-PK1) supported by histopathology examination and isolated mitochondrial activity showed minor toxicity with TiNPs prepared by green route (TiNP DG) than TiNP Chem.

1. Introduction

Metal oxides with nanostructure have attracted considerable interest in many technology areas [1]. Among metal oxide nanoparticles, titanium dioxide (TiO_2) nanoparticles have been widely used as a pigment in food products, pharmaceutical preparations and as an antibacterial for water purification. It is also used in industries due to its high photo catalytic activity [2] and has been considered to be safe and non-toxic [3]. In a nanoscale, TiO_2 physicochemical properties are altered compared to finer particles, resulted in improved catalytic activity and toxicity as well. A recent study by Liu and his associates showed that titanium dioxide nanoparticles (TiNPs) exposure can cause pulmonary lesions, leading to its dysfunction [4]. Early reports suggest that the formation of free radicals such as superoxide and hydroxyl by TiO_2 is responsible for its organ toxicity at higher doses. In the human body, free radicals generation causes lipid peroxidation and DNA damage, which leads to cell death [5–7]. This constructs an emergent attention towards green synthesis of nanoparticles as an alternate method, which is proved to reduce the toxicity due to the bio-reducing agents from sources such as plants [8], algae [9], and bacteria [10] that gained importance due to the absence of toxic chemical agents used in conventional methods.

Works carried out at present suggest that metal oxide nanoparticle (NPs) toxicity is due to reactive oxygen species production that leads to oxidative stress. It is worthy to note that these nanoparticles, due to their relatively small size, binds to the cell surface and can localize in the subcellular organelle such as mitochondria, endoplasmic reticulum, and lysosomes, which eventually leads to the origin of death signals resulting in oxidative stress [11,12]. Among the subcellular organelle, mitochondria is a major target wherein the NPs impair the electron transport chain, cause ATP hydrolysis and leads to disruption of mitochondrial membrane [13–17]. The mechanism of NPs induced oxidative stress has been extensively reviewed by Manke *et al.* [18]. As a way forward, we have evaluated the

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effect of TiNPs synthesized by chemical and green routes on renal function and oxidative stress in Wistar rat. Additional emphasis has been laid on studying the sub-cellular stress experienced by the renal mitochondria under *in vivo* and *in vitro* conditions on exposure to TiNPs.

Desmodium species have been chosen for the green synthesis of TiNPs, since our previous studies reported that the extract of the root is a potent candidate with high antioxidant activity [19] and also preserves mitochondrial enzymes [20]. Thus, in the present study, TiNPs were synthesized using an aqueous extract of *Desmodium gangeticum* (*D. gangeticum*) root and compared with the conventional synthetic approach for renal toxicity in Wistar rat (*in vivo*) model, LLC PK1 cells (*in vitro*) and isolated renal mitochondria.

2. Material and methods

TiO_2 nanoparticles were prepared by using both chemical and green routes as previously described and were characterized for their bio-reduction confirmation, size, distribution and shape using UV–Vis spectroscopy, XRD, FTIR and SEM respectively as described elsewhere [21].

Eight to ten week old male Wistar rats weighted [(260 ± 15) g] were purchased from the Central Animal Facility, SASTRA University, India. All the procedures were approved by the Institutional Animal Ethics Committee (CPCSEA Approval No. 213/SASTRA/RPP/IAEC). Rats were treated with a single dose (100 mg/kg b.wt; *i.p.*) of TiNPs and blood was collected on 14th day and analyzed for serum creatinine, urea, and uric acid level to determine the nephrotoxicity. Serum transaminases were measured as a marker for hepatotoxicity using SPAN Diagnostics kits (Gujarat, INDIA). Kidneys were excised, weighed and stored at −80 °C for further analysis.

Renal tissue was homogenized and analyzed for oxidative stress markers like thiobarbituric acid reactive substances, glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase activities, using the methods as described by Kurian *et al.* [20]. Mitochondria were isolated and assayed for succinate dehydrogenase (SDH), malate dehydrogenase (MDH) and NADH dehydrogenase. Further, TiNPs (100 mg/mL) were incubated with renal epithelial cells (LLC PK1, purchased from NCCS, Pune) for 24 h to determine the cellular toxicity. Lactate dehydrogenase (LDH) enzyme level was used as a marker for cellular injury and MTT assay was carried out to determine the cell viability.

In order to study the direct effect of TiNPs, isolated normal renal mitochondria were incubated with different concentrations (0.25, 0.5 and 1 mg/mL) of both TiNP Chem and TiNP DG and SDH activity was determined. Swelling behavior was assessed to evaluate the mitochondria toxicity and expressed as ΔA_{540}/min/mg protein.

Experiments were carried out in triplicate and values were expressed as the mean ± standard deviation (SD). Statistical significance (5%) was evaluated by one-way analysis of variance (ANOVA) followed by Tukey test as *post hoc* test (Graph Pad, San Diego, CA).

3. Results

Prepared TiNPs were characterized using UV–visible spectrophotometry and X-ray diffractometry and the results were shown in Figure 1. Chemical and DG extract mediated TiNPs have shown absorption maxima at 320 nm and 380 nm respectively (Figure 1A and B). The average grain size of TiNP Chem and TiNP DG was calculated as 2.59 nm and 0.70 nm respectively, determined using XRD pattern (Figure 1C and D).

Figure 2 shows the tissue architectural changes in rat kidney, where the control rat kidney showed preserved glomeruli, tubules, interstitium and blood vessels. However the renal tissues from TiNPs treated animal showed perturbed histology, where the damage was more prominent in rat kidney treated with chemically prepared TiNPs. However, the overall kidney weight was unchanged across the experimental groups [TiNP Chem (0.004 10 ± 0.000 15) and TiNP DG (0.003 7 ± 0.000 1) treated rats, compared to control (0.003 8 ± 0.000 1)].

Table 1 represents serum and urine chemistry that describe the renal function. TiNP Chem treated rats showed significant ($P < 0.05$) increase in urea (62%), urea/creatinine ratio (43%) than TiNP DG (urea 46%; urea/creatinine 30%) when compared with normal control, indicating renal damage. ALP, another indicator of renal damage, was significantly ($P < 0.05$) elevated (50%) in TiNP Chem treated animal, along with 61% increase in AST activity. In order to assess the rate of glomerular filtration, urea and creatinine clearance was employed and the results showed that TiNPs prepared by both routes severely affect the clearance capacity of the glomerulus.

Table 2 describes the effect of TiNPs on the renal antioxidant enzyme, cellular viability, and mitochondrial ETC enzymes. Accordingly, the present study observed a two fold increase in lipid peroxidation level in renal tissues obtained from the rats treated with TiNP Chem, with a subsequent significant alterations in the antioxidant enzymes like catalase, SOD and GPx (60% *vs*. 38%; 26% *vs*. 16%; 34% *vs*. 11% respectively in TiNP Chem *vs*. TiNP DG, compared with the normal control), which shows a decline pattern except in SOD of TiNP DG.

Measured mitochondrial enzyme activity in the renal tissue, after TiNPs treatment was observed to exhibit similar pattern (decline) of changes in TiNPs treated animals irrespective of their production methods. Compared to the normal control rat, TiNP treated animal showed significant impairment in mitochondrial enzymes (Table 2).

In order to verify the cellular level toxicity of TiNPs, LLC PK1 (epithelial kidney cell line) was used, considering that proximal tubular epithelial cells are most susceptible to toxicants and the results are shown in Table 2. LDH level, a well-known biomarker for cellular cytotoxicity, was assayed in the culture media after 24 h incubation of cells with TiNPs. The enzyme leakage from cell to the medium was prominent in TiNP Chem treated cell and this result was re-confirmed with MTT assay (TiNP Chem: 68.00 ± 1.42 *vs*. TiNP DG: 95 ± 3 viable cells).

Next, we set up an *in vitro* experiment to confirm the renal toxicity at sub-cellular (organelle) level. Among other organelles, mitochondrion is considered to be one of the key players in free radical generation and also in deciding cell survival/death. Thus, mitochondria were isolated from normal rat kidney and incubated with TiNPs. The results confirmed the existence of impaired mitochondrial morphology assessed by increased swelling in TiNP Chem, compared to TiNP DG and normal control. Also, both TiNPs were found to exhibit dose dependent mito-toxicity with high significance in TiNP Chem treated group (69% *vs*. 39%), than TiNP DG group at 1 mg/mL concentration (Table 2).

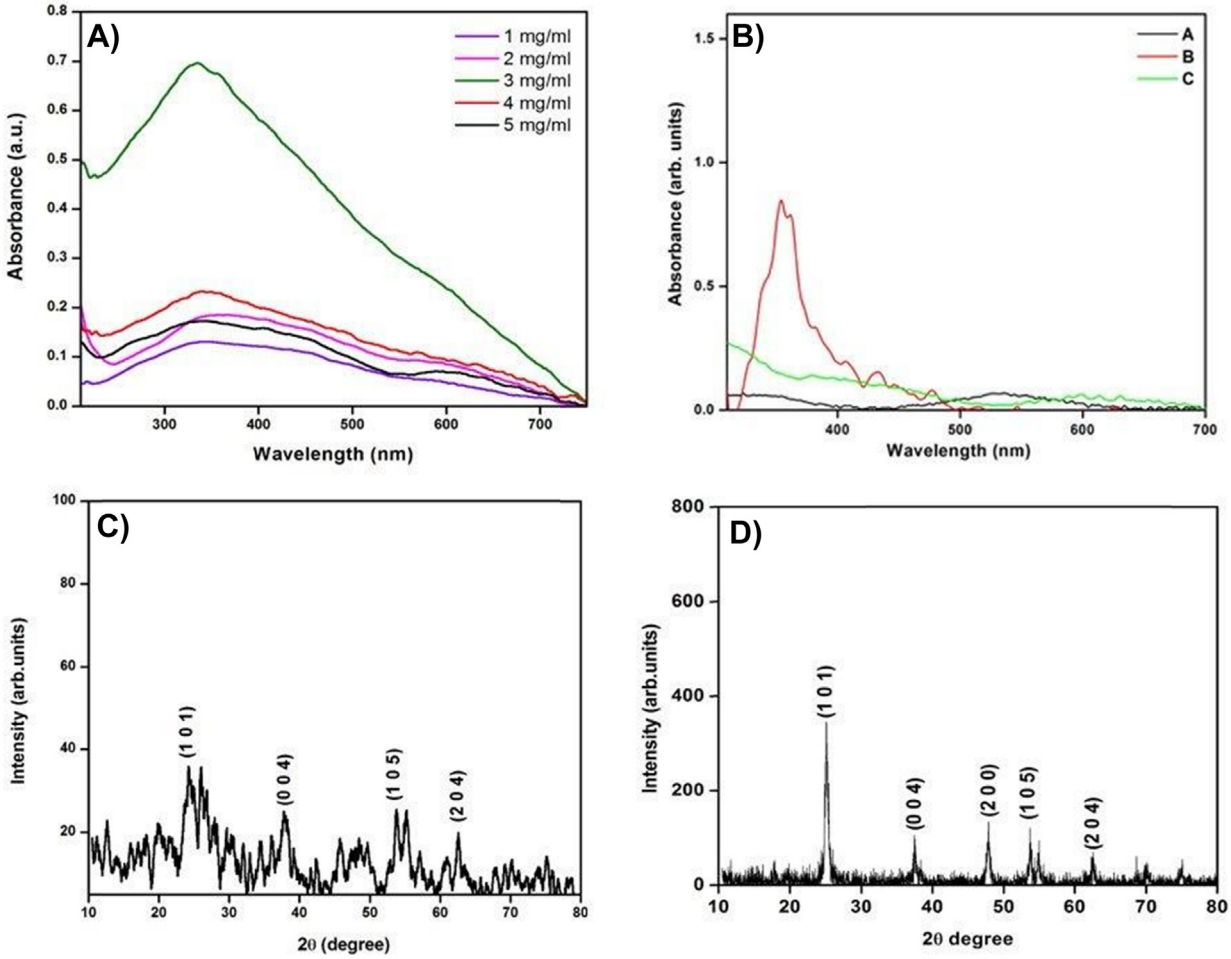

Figure 1. Characterization of Chemical and DG synthesized titanium dioxide nanoparticles.
A) and B) UV–Visible spectral analysis of TiNP Chem and TiNP DG, C) and D) XRD pattern of TiNP Chem and TiNP DG.

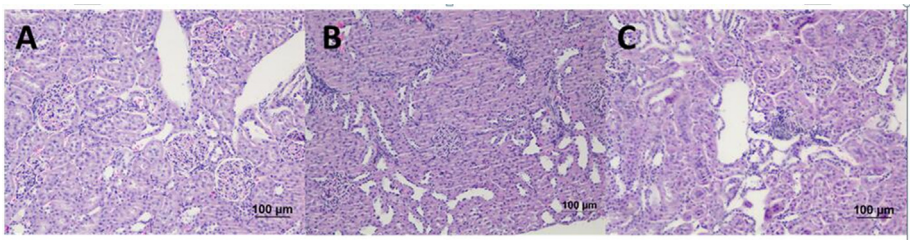

Figure 2. Histopathology and morphometric analysis.
H&E stained images of kidney from A) Normal control rat, B) TiNP Chem treated rat and C) TiNP DG treated rat. Scale bar is 100 µm for H&E image.

4. Discussion

The significant achievements in nanoparticle research especially in the field of health science revolutionizing the medical community due to its wide range of application not only in therapy but also in the diagnosis and other complex procedures. Despite this advancement, there is always a concern about the toxicity towards health and environment, which prevails over the minds of the scientific community. A wide acceptance for green nano science as a sustainable alternative for nanoparticle synthesis at least for the health related application paved the way for the present research. In this study, the toxic effect TiNPs synthesized by both chemical and green route in the three different biological experimental system (Wistar rat, renal epithelial cell, and isolated renal mitochondria) was evaluated. This study mainly focused on the toxic effect of TiNPs on renal tissue, as the kidney is considered to be the major site of chemical excretion, which results in its propensity to exhibit chemically induced toxicological effects at a higher rate than most other organs. TiNPs have unique physicochemical properties such as size, shape, conductance, redox status that can influence the degree of toxicity and its substantial usage in different fields demands extensive investigation [22]. In the present study, we prepared TiNPs by two different routes namely i) conventional chemical method (sol–gel), and ii) green method by *D. gangeticum* root aqueous extract. The major finding of the manuscript is that a) green synthesized TiNPs are better biocompatible and less toxicity than chemically prepared nanocrystals, and b) sub cellular level (organelle) toxicity of TiNPs in mitochondria from treated rats, was identified to be similar but differs with isolated mitochondria (TiNP DG showed less mitochondrial enzyme impairment than TiNP Chem).

Table 1
Blood & urine chemistry (mean ± SD, $n = 3$).

Parameters	Normal	TiNP Chem	TiNP DG
Serum urea (mg/dL)	16.31 ± 1.20	43.83 ± 4.20*	30.27 ± 3.40*
Serum creatinine (mg/dL)	0.45 ± 0.05	0.69 ± 0.06*	0.58 ± 0.07*
Serum uric acid (mg/dL)	3.150 ± 1.100	0.083 ± 0.002*	1.090 ± 0.004*
AST (U/L)	53.90 ± 3.00	137.00 ± 11.00*	58.00 ± 6.00#
ALT (U/L)	49.10 ± 7.00	77.50 ± 7.00*	34.30 ± 3.00#
ALP (U/L)	82.00 ± 7.10	167.00 ± 16.00*	80.00 ± 8.90#
Serum urea/ creatinine	36.24 ± 2.10	63.50 ± 19.10*	52.10 ± 20.10*
Serum uric acid/ creatinine	7.00 ± 1.00	0.12 ± 0.01*	1.87 ± 0.05*
Urea clearance (mL/min/kg b.wt)	8.45 ± 1.10	2.70 ± 1.10*	3.11 ± 3.60*
Creatinine clearance (mL/min/kg b.wt)	3.90 ± 0.80	1.60 ± 0.70*	3.70 ± 0.80

*$P < 0.05$ compared with Normal control; #$P < 0.05$ compared with TiNP Chem.

Table 2
Effect of TiNPs on antioxidant and mitochondrial enzymes in renal tissue, LLC PK1 cell viability and SDH activity on isolated mitochondria incubated with TiNPs at *in-vitro* system (mean ± SD, $n = 6$).

Parameters	Normal	TiNP Chem	TiNP DG
TBARS (nmoles MDA/mg protein)	0.061 ± 0.003	0.145 ± 0.007*	0.078 ± 0.003#
Catalase (mIU/mg protein)	35.00 ± 1.00	14.00 ± 0.70*	21.80 ± 0.59*
SOD (IU/mg protein)	14.00 ± 0.70	10.40 ± 0.82*	16.20 ± 0.81#
GPx (nM GSH oxidized/min/mg protein)	8.00 ± 0.40	5.30 ± 0.35*	7.10 ± 0.26#
MDH	25.00 ± 1.10	17.00 ± 1.90*	20.70 ± 0.06*#
SDH	18.0 ± 0.9	15.0 ± 1.1	16.0 ± 0.1*#
NADH dehydrogenase	40 ± 3	29 ± 5*	31 ± 5*#
LDH activity (mIU/mL)	128.6 ± 6.4	450.0 ± 22.5*	142.1 ± 5.1#
MTT (% cell viability)	96.00 ± 2.00	68.00 ± 1.42*	95.00 ± 3.00#
SDH activity in isolated mitochondria	17.1 ± 0.5	–	–
TiNPs (0.25 mg/mL)	–	12.25 ± 0.42	14.90 ± 0.87
TiNPs (0.5 mg/mL)	–	8.70 ± 0.36*	12.40 ± 0.52*
TiNPs (1 mg/mL)	–	5.30 ± 0.52*	10.50 ± 0.94*

Activity is expressed as µmol of succinate oxidized per min per mg protein for SDH; µmol of NADH oxidized per min per mg proteins for MDH and µmol of NADH oxidized per min per mg protein for NADH dehydrogenase. Lipid peroxidation (TBARS) and antioxidant enzymes like Catalase, SOD, GPx along with mitochondria enzymes like MDH, SDH and NADH were measured in renal tissue; LDH and MTT were determined in LLC-PK1 cells; SDH activity was measured in isolated mitochondria with different dose of TiNPs and expressed in U/mg protein.*$P < 0.05$ compared with Normal control; #$P < 0.05$ compared with TiNP Chem. TBARS: thiobarbituric acid reactive substances.

Few studies have shown that the toxicity of TiNPs is dose dependent, which may be manifested as inflammation, fibrosis, hyperplasia and even tumorigenesis [23]. In agreement with this report, toxicity was prominent in the animal treated with TiNPs prepared by conventional chemical route (measured via histology image and abnormal renal function markers in both blood and urine). The relative less toxicity of green synthesized nanoparticles may be attributed to the presence of phytochemicals in DG that may act not only as capping agent but also mediate renal protection via alkaloids [24,25]. One of the major mediators of tissue injury is reactive oxygen species and thus, we evaluated the endogenous antioxidant defense system in the renal tissues obtained from the animals exposed to nanoparticles. Our results suggested the presence of significantly enhanced oxidative stress in animals treated with TiNP Chem emphasizing likely kidney dysfunction. Numerous literatures have established that mitochondria is one of the major sources and targets of reactive oxygen species that leads to tissue injury [26]. But according to our result, we could not find any significant difference in the degree of toxicity, among TiNPs prepared by the two routes, although both showed a significant decline in mitochondrial enzyme activity as compared to the normal control. This observation suggests that the possible phytochemicals in TiNP DG, which act as capping agent may not be mitochondrial targeted. In order to reconfirm this finding, we isolated the mitochondria from normal rat kidney and incubated them with different doses of TiNPs and measured the mitochondrial enzyme activity. Interestingly, we found that mitochondria incubated with TiNP DG exhibited preserved enzyme activity than those incubated with TiNP Chem. These data collectively emphasize the penetrative effect of TiNP Chem at cell organelle level, that may have a distinct effect either protective or destructive nature. These findings in animal and cell organelle were further established in epithelial cells from kidney (LLC PK1) and we found similar results as described above. Based on the results, we conclude that TiNPs are more toxic to the renal tissue where as TiNPs prepared via *D. gangeticum* root aqueous extract impart less toxicity to the kidney.

Both chemical and green synthesized titanium dioxide nanoparticles are evaluated for its cytotoxicity in both LLC PK1 cells and Wistar rats. TiNPs prepared by green route may exhibit additional biological effect by reducing oxidative stress and by limiting the elevated nephrotoxic markers.

Conflicts of interest statement

The authors report no conflict of interest.

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