Protective effect of TiO₂ nanowhiskers on Tetra Sulphonatophenyl Porphyrin (TSPP) complexes induced oxidative stress during Photodynamic Therapy

Fawad Ur Rehman^{1, §}, Chunqiu Zhao^{1, §}, Hui Jiang¹, Matthias Selke², Xuemei Wang^{1,*}

¹State Key Laboratory of Bioelectronics, School of Biological Science and Medical Engineering, Southeast University, Sipailou 2, Nanjing 210096, China.

²Department of Chemistry and Biochemistry, California State University, Los Angeles, CA 90032, USA

[§]These authors contributed equally to this work.

*Corresponding author email address: xuewang@seu.edu.cn

Abstract:

Background: Tetra Sulphonatophenyl Porphyrin (TSPP) is well known photosensitizer for photodynamic therapy; nevertheless, its well-known adverse effects hamper its potential use. Recently, nano TiO_2 's potential role in biomedical has been defined for various disease theranostics, including cancer and other infections. Thus, in this contribution we have explored the possibility of utilizing TiO_2 nanowhiskers as novel strategy to lower TSPP adverse effects both *in vitro*, and *in vivo*.

Methods: Various concentrations of TSPP, TiO_2 -TSPP, and TiO_2 were injected to three different rat groups, while fourth group was kept as control. Toxic effects were evaluated on excretory and circulatory system by using histopathology, fluorescent microscopy, complete blood cells count (CBC) and serum enzymes.

Results: In complete blood cells count, all cells were significantly ($p < 0.01$) affected by the various concentration and treatment groups. The various dose concentrations and treatment also significantly ($p < 0.01$) affected the serum enzyme parameters including AST, ALT, LDH, Creatinine and BUN level. The low concentration of TSPP- TiO_2 was found to be the safest, on the bases of serum enzyme parameters, CBC, histopathology, and fluorescent microscopic analysis. The MTT assay was used to evaluate *in vitro* cytotoxicity, and the results demonstrated maximum viability in illuminated TSPP- TiO_2 nanowhiskers group when compared with TSPP treated group.

Conclusions: It was evident that increase in concentration of TSPP increased the toxic effects; however, the TiO_2 nanowhiskers combination with TSPP decreased these adverse effects.

Moreover, TSPP (0.1 mM) combined with TiO₂ nanowhiskers (0.6 mM) was safer than TSPP (0.1 mM) alone.

Key words: Tetra sulphotanophenyl porphyrin, Photodynamic therapy, TiO₂ nanowhiskers, Toxicity, Serum enzymes, Fluorescent microscopy

Introduction:

Potential biomedical applications of nanomaterials have been very extensively explored. Their role in various diseases theranostics is inevitably improved, especially in cancer. Titanium Dioxide (TiO₂) is a widely available material used in feed additives, cosmetics, sunscreens, photovoltaic cells, etc. [1]. Nano TiO₂ has recently become popular in photodynamic therapy for cancer, microbial infections and autoimmune diseases [2]. Lower rates of toxicity have been reported with the use of TiO₂, compared with other nanoparticles [3]. Its porous nature, large surface area, lower toxicity and excellent biocompatibility makes it suitable PD-therapeutic candidate for various diseases [4, 5].

Photodynamic therapy (PDT) is an important alternative to traditional therapies for various malignant, invasive or non-invasive maladies [6-8]. In general, PDT uses oxygen, light and a photosensitizer [9], where photosensitizer is either injected systemically, or introduced locally to the *in vivo* subject. When photosensitizer is excited by visible light in the presence of molecular oxygen, it produces singlet oxygen (¹O₂) and possibly other reactive oxygen species (ROS) e.g. OH[•], HO₂[•], O₂^{•-} etc. These ROS and ¹O₂ result in the oxidation of the surrounding molecules and initiate apoptosis or necrosis of the target tissue, i.e., neoplasm, dermal maladies or microbial

infections and exposed body cavities [10-12]. Unfortunately, there exist numerous well-documented problems for broader applications of PDT[13], especially with first generation PD-therapeutic agents such as severe and prolonged photosensitivity and neurotoxicity [14]. The increased production of ROS and $^1\text{O}_2$ leads to the loss of cell membrane potential and integrity, leading to an increased ionic influx that destroys the cellular organelle and initiate apoptosis by activating caspase 3 or necrosis [15, 16].

The cytotoxic effects of PDT are initiated by the excitation of a photosensitizer from its ground state to excited state. Following intersystem-crossing to the triplet excited state, the photosensitizer reacts with substrates of surrounding tissues in the presence of oxygen. It may do so either directly by electron transfer or hydrogen atom abstraction to form free radicals (Type I mechanism) or by energy transfer to ground state oxygen thereby generating $^1\text{O}_2$ (Type II mechanism)[17]. Both pathways may occur simultaneously and their products are very much reactive to the surrounding environment [6]. There are many studies showing the toxic effects of PDT via lipid peroxidation [18, 19], initiated within a few minutes after photoactivation. All $^1\text{O}_2$ and other ROS produced by the photosensitizer are the main cause of alteration in DNA, lipids and proteins leading to cellular necrosis or apoptosis [6].

To date, porphyrin derivatives are the most extensively used photosensitizers for PDT [6, 20]. Tetra Sulphonatophenyl Porphyrin (TSPP) (Additional information figure 1) is among the porphyrins whose potential as a PDT agent has been explored in cancer [6] and other inflammatory diseases. Nevertheless, the existence of some well-known adverse effects limits the potential therapeutic use of TSPP in biomedicine. TSPP enters the cell via endocytosis and

localizes in cellular organelle, i.e., lysosomes, endosomes and endoplasmic reticulum, where it interferes with cellular signal pathways, thus inducing apoptosis or necrosis [21].

Based on the above considerations, in this study we have explored the possibility that adjuvant use of TiO₂ nanowhiskers could mitigate the adverse effects of TSPP, and our findings demonstrate the protective role of TiO₂ nanowhiskers when combined with TSPP for PDT, both *in vivo* and *in vitro*.

Materials and methods

MTT assay for cytotoxicity

The MTT assay protocol was followed as earlier reported [22]. Briefly, Fibroblast primary cell culture was obtained from the SD- rats. In each well of 96 well plates, 200 µl of cells suspension containing 5.1×10^5 cells ml⁻¹ were incubated at 37° C temperature, 5 % CO₂ and 95 % humidity. After 24 hours cells were treated with TSPP and TSPP combined TiO₂ nanowhiskers. After a further three hour incubation period, one group (TSPP & TSPP-TiO₂) was illuminated (IL-T) with visible light while the other group, designated as NL-T, served as a no drug, no light control. TSPP concentration included 1 mM, 0.8 mM, 0.4 mM, 0.2 mM, 0.1 mM, 80 µM, 40 µM, 20 µM, 10 µM and TiO₂ concentration in combination with 0.1 mM TSPP was 1 mM, 0.75 mM, 0.5 mM, 0.25 mM, 0.1 mM, 75 µM, 50 µM, 25 µM, 10 µM. The cells were again incubated for 24 hours and after adding MTT reagent, the reading was taken by computer assisted microplate ELISA reader and data was stored in MS-Excel. Experiments were repeated three times and mean values were taken as standard.

Selection of animals

Four groups of male SD-rats were selected (12 (3x4) rats in each group except control i.e. three rats), named as illuminated porphyrin (IP), illuminated porphyrin with TiO₂ nanowhiskers (ITP) treated with green light (500-550 nm), non-illuminated TiO₂ (NT) and control group (N0) without porphyrin and TiO₂. The average weight of each adult rat was 180±20 g. Rats were provided with standard light of 12/24 hours with standard pallet food and water at ad-libitum.

All the rats were kept under the guidance of National institute of biological science and animal care research advisory committee, Southeast University and research was conducted under the guidance of animal research ethics of Southeast University, Nanjing China.

Porphyrin preparation and injection

Tetra Sulphonatophenyl Porphyrin (TSPP) was purchased from the ABI chemicals and TiO₂ nanowhiskers (Additional information in figure 2) were generously provided by Dr. Xiao Hua Lu (College of Chemical Engineering, Nanjing University of Technology, Nanjing 210009 China). The TSPP was dissolved in deionized ultrapure water to achieve the concentrations of 0.1, 0.2, 1.0 and 2.0 mM and injected to IP sub groups (A, B, C, and D). Similarly, TiO₂ was dissolved in ultrapure water to obtain 0.6, 1.2, 6.2 and 12.5 mM concentration and injected to the NT sub groups (A, B, C, and D). Moreover, TiO₂ was mixed together with TSPP to form the final solution named as A (0.1 mM TSPP + 0.6 mM TiO₂), B (0.2 mM TSPP + 1.2 mM TiO₂), C (1.0 mM TSPP + 6.2 mM TiO₂) and D (2.0 mM TSPP + 12.5 mM TiO₂), and injected it to ITP sub groups. The N0 group was kept as control with placebo.

Two hours post injection all sub groups of IP and ITP were illuminated with visible light (500-550 nm) for one hour every day, while the other groups (NT, N0) were kept untreated.

Sample collection All sampling was done on day 29 of the experiment.

Blood sampling and biochemical assay

Blood samples were collected into 5 ml syringes through intracardiac injection technique, while animals were under general anesthesia [23]. Three ml of each collected sample was transferred to a non-EDTA tube for quantitative analysis of markers of hepatic and renal function (AST, ALT, LDH, CRT, BUN). Sample analysis was done by using Cobasintegra system by kinetic and kinetic Jaffe reaction. The remaining 2 ml of each collected blood sample was transferred to an EDTA tube for CBC analysis.

Histopathology sample and tests

Post euthanasia, lungs, liver, kidneys, and spleen were harvested from each animal, washed in PBS, and preserved in 10 % formalin for a minimum of 24 hours to ensure dehydration. 3-4 μ m tissue section slides were then prepared using paraffin embedding techniques, and subsequently stained using H&E techniques [24].

Florescence imaging:

The liver, kidney, spleen and lungs were removed from all groups and observed under florescence microscope (PerkinElmer[®] IVIS Lumia XRMS, Series III), to evaluate the concentration of the TSPP and TSSP with TiO₂, within vital excretory and circulatory organs.

Statistical analysis

Data was recorded by using MS-Excel. The SPSS version 18 software was used to statistically analyze the data by the analysis of variance (ANOVA) and Duncan Multiple Range (DMR) test to rank the means of dependent variables. The probability value < 0.05 was considered as significant.

Results

MTT cytotoxicity assay:

In the MTT assay, it was noted among photoactivated groups that cell viability was negatively correlated with TSPP concentration, cell viability decreased when photoactivated, i.e., from 1 to 0.4 mM of TSPP, the cell viability was 15, 38 and 71 percent (Fig 1A). Surprisingly, when TSPP was combined with TiO₂ nanowhiskers, the cell viability remained 100 percent in the TSPP-TiO₂ group when compared with TSPP alone (IL-T1, IL-T2, IL-T3) and overall viability for all TSPP-TiO₂ concentrations remained above 80 percent. Moreover, in non-illuminated cell cultures (NL-T) the cytotoxic effects of TSPP with TiO₂ nanowhiskers were much lower than the TSPP alone and the cell viability remained more than 85 % (Fig 1B).

Gross lesions

Immediately post sacrifice, animal organs were examined for gross lesions. Severe hepatomegaly was noticed with higher doses of TSPP in the IP group. Gross lesions of petechial pinpoint hemorrhages were noticed in IP (B) and higher doses. Moreover, necrotic lesions were found common in TSPP treated groups (B, C and D) and its severity increased with the increase in dose concentration. Kidney and spleen color was found to be darker in TSPP treated group as compared to ITP. Moreover, chromodacryorrhea, a reddish porphyrin color discharge from the

eyes was frequently observed in higher concentration TSPP sub groups (Additional information in figure 3).

Biochemical assay

All treatments had significant ($p < 0.01$) effect on the AST and ALT in IP, ITP, NT and N0 groups. In the IP group the AST and ALT mean $\pm$ standard deviation (SD) level was 58.50 ± 10.362 (SD) and 251.58 ± 43.129 (SD) U/L respectively, compared to values of ITP (i.e. AST; 33.33 ± 2.807 (SD) and ALT; 112.75 ± 11.331 (SD) U/L), and remained also higher than other treatment groups (i.e. NT and N0). The treatment also had a significant ($p < 0.01$) effect on LDH mean value, i.e., IP (1143.33 ± 327 (SD) IU/L), ITP (556 ± 90 (SD) IU/L), NT (1532.25 ± 429 (SD) IU/L) and N0 (731.67 ± 77 (SD) IU/L) (Fig 2 e, f, g.).

CRT level was significantly ($p < 0.01$) affected by the various treatments. The CRT level was found to be higher in IP (56.17 ± 19.61 (SD) IU/L) when compared to ITP (38.83 ± 7.38 (SD) IU/L). The effect of treatments on BUN was also highly significant ($p < 0.01$) with the highest mean value (7.217 ± 1.20 (SD) mmol/L) for IP group followed by ITP (4.850 ± 1.19 (SD) mmol/L), N0 (4.267 ± 0.37 (SD) mmol/L) and NT (4.183 ± 0.70 (SD) mmol/L) (fig 3 e, f).

There also existed a highly significant difference ($p < 0.01$) among various concentrations in treated sub groups. Comparing the sub groups various concentration mean, it was found that ALT, AST and LDH mean values were lower in ITP-A (i.e. 33.00 ± 2.00 (SD) U/L, 136.67 ± 7.37 (SD) U/L, 1401.67 ± 65.04 (SD) IU/L, respectively), compared to the values of IP-A (65.67 ± 1.15 (SD) U/L, 184.33 ± 3.05 (SD) U/L, 689.00 ± 4.00 (SD) IU/L, respectively) (Table 1).

Blood cells count

The treatment had a highly significant effect ($p < 0.01$) on WBC, RBC, hemoglobin (HGB), platelets, neutrophils and lymphocytes. WBC mean value was higher in IP group (i.e. 14.11 ± 2.31 (SD) $10^9/L$) as compared with the ITP group (13.52 ± 4.50 (SD) $10^9/L$), whereas NT and N0 groups mean was 17.80 ± 3.44 (SD) $10^9/L$ and 10.37 ± 0.60 (SD) $10^9/L$, respectively (Fig 4 c). The RBC mean was higher in ITP (8.14 ± 0.70 (SD) $10^{12}/L$) as compared with IP (6.44 ± 1.83 (SD) $10^{12}/L$) (Fig 4 b). The highest mean values for HGB and platelets were found among the IP group at 17.98 ± 1.20 (SD) g/L and 453 ± 114 (SD) $10^9/L$ respectively, compared with 16.31 ± 0.74 (SD) g/L, 564 ± 99 (SD) $10^9/L$ respectively, in the ITP group, while NT (16.83 ± 14 (SD) g/L, 611 ± 57 (SD) $10^9/L$ respectively) and N0 (14.16 ± 1.10 (SD) g/L, 531 ± 90 (SD) $10^9/L$ respectively) (Fig 4 a, d). The lowest mean neutrophil and lymphocyte counts were found in the ITP group at 1.96 ± 1.53 (SD) $10^9/L$ and 4.47 ± 1.53 (SD) $10^9/L$, respectively. The treatment effect on basophils, monocytes and eosinophils remained non-significant ($p > 0.05$).

The various concentrations also had significant effect ($p < 0.01$) on all blood cells count except RBC, for which the probability value was > 0.05 (Table 2).

Histopathology findings

Liver

The pinpoint hemorrhages and necrotic lesions were found in all IP sub groups, and its severity increased with an increase in concentration of porphyrin. Bile duct hyperplasia was more prevalent at higher concentrations, i.e., 2.0 mM of IP-D and ITP-D groups, while none was observed in the NT group. Moreover, liver was severely infiltrated by inflammatory cells in IP group with all concentrations and relatively lower inflammatory cell numbers were found in the ITP and NT (A, B), however, in higher concentration groups it remained the same as in IP group.

Among animals in the ITP-A group, no evidence of hepatic necrosis or hemorrhage was present. Hepatic necrosis was noted in animals from the higher dose ITP groups, though it was less prominent than those among the IP. In the ITP sub groups C and D, the histopathology changes remained same as in IP sub groups (C and D). In NT the severity of necrotic changes increased with increase in the dose rate, however, the NT-A was normal. Sinusoidal dilatation and congestion were prominent in IP, ITP (B, C, D) and NT (B, C, D), while sub group A in ITP and NT remained unaffected (Fig 2 a-d).

Kidney

In IP all sub groups and ITP (C and D) severe congestion and hemorrhages were found, while ITP (A and B) and lower concentration of NT were less affected. Tubular casts were found in all groups with higher concentration, i.e., C and D in IP, ITP and NT. Glomerular size was decreased and space between the Bowmans capsule and Glomerulus increased in all the treated groups except ITP (A) and N0 (Fig 3 a-d).

Spleen

In general, spleen remained less affected by the lower concentration (A and B) in all treated groups. However, lymphoid aggregates and germinal center within splenic nodules were affected by higher concentration of IP, NT (C, D) and ITP (A, B). Lesser histopathological lesions were observed in ITP (A and B), IP (A) and NT (A and B) (Fig 5 e-h).

Lungs

Lungs were affected in all treated groups with higher concentrations; however, in all subgroups of IP, ITP (B, C, D) and NT (B, C, D) more severe congestion, inflammatory cells and damaged bronchiolar epithelium were observed and severity increased with increase in dose concentration. The IL-TP (A) remained less affected with lower severity (Fig 5 a-d).

Fluorescent imaging

Intensity of fluorescent signal in target organ was directly proportional to drug accumulation. The kidneys were affected in all IP sub groups, and less fluorescence was observed in ITP (C, D). The liver was also showing higher fluorescence in all IP sub groups and ITP-D. In lungs mild fluorescence was observed in ITP-D, IP (A, B and C) and strong fluorescence in higher concentration group IP-D. Spleen remained non-fluorescent in all treated groups. All the organs in NTP and N0 group were found non-fluorescent (Fig 6).

Discussion

The aim of this study was to explore the potential of TiO₂ nanowhiskers in mitigating TSPP-PDT adverse effects on excretory and circulatory system *in vivo* as well as *in vitro*.

We estimated cytotoxicity by MTT and found that in TSPP treated group cell viability was much lower as compared to the combined effect of TSPP with TiO₂ nanowhiskers, i.e., the viability remained 100 % for relatively higher doses. Moreover, in a non-illuminated group of TSPP, the overall viability was higher and toxicity was lower than illuminated group. The main reason for lower viability and higher cytotoxicity in the illuminated TSPP group was that ¹O₂ and ROS were generated at once, when visible light (500-550 nm) was passed through the cells that induced apoptosis or necrosis. Surprisingly, in TSPP-TiO₂ combined group the cell viability was relatively higher and TiO₂ nanowhiskers combination had a protective effect after photoactivation. This protection may be attributed to the TiO₂ nanowhiskers porous nature that

adsorbed TSPP and slowly release into the surrounding environment, thus help in lowering adverse effects without compromising the theranostics properties.

Kidney and liver are believed to be the major excretory organs for removal of any metabolite from the body. AST and ALT along with LDH are considered as special biomarkers for liver functionality [24]. The liver functional enzymes (AST, ALT) were elevated by the higher doses of TSPP and increased with the increase in concentration of TSPP. Nevertheless, it was found that AST and ALT level were much lower in TiO₂ nanowhiskers-TSPP combined than TSPP treated group. In liver, histopathology studies revealed severe inflammation in TSPP and higher concentration TiO₂ treated groups, which is in agreement with earlier studies [25, 26]. When specific light passes through porphyrin within the *in vivo* environment, reactive oxygen species (ROS) are released [27], and these ROS damage surrounding tissues resulting in necrosis or apoptosis, with hemorrhages in severe conditions as gross lesions [10, 28]. It has been experimentally proven that ROS reside in target tissues for more than 18 hour post photoactivation [29]. Hence, a cascade of reaction is initiated by PDT leading to cytotoxic affects [6]. In vital organs fluorescent microscopy we found subtle fluorescence generated in the TiO₂ nanowhiskers-TSPP combined groups that indicated lesser accumulation of TSPP in the vital organs (i.e. lungs, liver, kidney and spleen) and vouch the protective role of the TiO₂ nanowhiskers.

In PDT, the photosensitizer introduced into the cell can affect multiple cell targets organelle including mitochondria, lysosome, endoplasmic reticulum, cell membrane and nuclei. In case of TSPP, the drug localizes to endosomes, lysosomes and endoplasmic reticulum [21]. When TSPP is combined with TiO₂ nanowhiskers, the drug readily adsorbed in the pores of TiO₂ nanowhiskers, thus ensuring slow release of TSPP and resulting in lesser quantum yield than

singly used TSPP. Through scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS), it was evident that TiO₂ nanowhiskers remain in the cytoplasm of the cell, ensuring lesser uptake of TSPP by the vital cellular organelle, i.e., endoplasmic reticulum, lysosomes and endosomes (Additional information in figure 4).

Normally cellular injuries are prevented by SOD and dithiothreitol (DTT), which are initiated by ROS lipid peroxidation [30], whereas ¹O₂ is neutralized by sodium azide (NaN₃) [31]. The higher concentration will lead to greater cytotoxic effects that will lead to necrosis in the accumulated cells and surrounding tissues [32-34]. Toxic effects on the kidney were evaluated by creatinine and blood urea nitrogen (BUN) level in blood plasma as well as fluorescence microscopy and histopathology investigation of both kidneys. The severity of adverse effects increased with the increase in TSPP concentration that ensures more damage to surrounding tissues. The creatinine and BUN level is directly correlated to the glomerular filtration rate (GFR)[35], here the serum creatinine level is lower in TiO₂ combined TSPP than TSPP alone treated groups; moreover, the histopathology findings also demonstrate severe adverse effects on the glomerulus by increasing the space between glomerulus and bowmen capsule.

The spleen is considered a vital organ for blood cells production and maturation. However, our results revealed that the spleen was least susceptible of all organs to toxic effects. Earlier studies indicated that PDT significantly altered the erythrocytic antioxidative enzymes, i.e. CAT decreased while SOD and GPx were increased, resulting in cell membrane damage and cell death [6, 36].

Photosensitizer's localization in any tissue plays vital role to exert toxic effects as it generates local ROS with a short half-life [37, 38]. The lifespan of singlet oxygen generated in the cell is ~

3 μ s [17, 39], which is sufficient to interfere with the cell signals and lead to cell apoptosis or necrosis. Despite the long lifespan of $^1\text{O}_2$ within the cell, its cytotoxic effects are limited to the surrounding environment due to the viscosity gradient between the intra and inter-cellular environment [39]. As a result, PS localization in non-target tissue such as lungs, liver, kidney and spleen is expected, due to the fact that these organs play vital role eliminate any foreign substance from the body and maintain homeostasis.

To the best of our knowledge these findings are the first report to considerably enhance the PDT efficiency and decrease TSPP toxic effects through combining the TSPP with TiO_2 nanowhiskers. These results suggest that TiO_2 nanowhiskers can very efficiently decrease the toxic effects of TSPP on the vital body organs for excretory and circulatory system.

Conclusion

In summary, our observations demonstrate that the severe adverse effects resulting from relatively higher concentration of TSPP could be considerably decreased when combined with TiO_2 nanowhiskers. These findings suggest that *in vivo*, 0.1 mM TSPP combined with 0.6 mM TiO_2 nanowhiskers are more safe and efficient than TSPP alone and other combinations used in this study. Moreover, the MTT assay also revealed that when TSPP was combined with TiO_2 nanowhiskers, its cytotoxic effects were minimized on the normal cells in terms of increased viability. We suggest that TSPP combined with TiO_2 nanowhiskers can be more efficiently used in PDT and bio-imaging of cancer and other diseases theranostics.

Competing interests

All the authors declare that they have no competing interests.

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Table 1 Different photosensitizer's concentrations effecting circulatory and excretory system biomarkers mean value

Treatment	AST** (U/L)	ALT** (U/L)	CRT** (IU/L)	BUN** (mM/L)
IP-A	65.66 ^a	184.33 ^c	47.33 ^{b,c}	7.067 ^{a,b}
IP-B	62.00 ^a	282.33 ^a	41.33 ^{b,c,d,e,f}	6.833 ^{a,b,c}
IP-C	47.00 ^b	252.00 ^b	50.67 ^b	7.067 ^{a,b}
IP-D	59.33 ^a	287.67 ^a	85.33 ^a	7.900 ^a
ITP-A	33.66 ^c	102.67 ^g	35.00 ^{e,f,g}	3.800 ^e
ITP-B	35.33 ^c	109.67 ^{ef}	38.33 ^{c,d,e,f,g}	5.200 ^{b,c,d}
ITP-C	31.67 ^c	117.67 ^e	33.00 ^{f,g}	4.967 ^{c,d}
ITP-D	32.67 ^c	121.00 ^e	49.00 ^{b,c}	5.433 ^{b,c,d}
NT-A	33.00 ^c	136.67 ^d	34.33 ^{e,f,g}	3.467 ^e
NT-B	36.67 ^c	134.00 ^d	39.00 ^{c,d,e,f,g}	4.233 ^e
NT-C	34.67 ^c	116.00 ^{ef}	28.00 ^g	4.600 ^e
NT-D	36.00 ^c	108.00 ^{ef}	37.00 ^{d,e,f,g}	4.433 ^e
N0	29.67 ^c	76.67 ^h	45.00 ^{b,c,d,e}	4.267 ^e

^{a, b, c, d, e, f, g}; superscripts level showing means difference with in a same column (from Duncan Multiple Range Test). Whereas *AST* stand for aspartate aminotransferase, *ALT*; alanine transaminase, *CRT*; creatinine and *BUN* for blood urea nitrogen in the treated groups. Moreover, **IP-A** group stands for 0.1 mM, **IP-B** (0.2 mM), **IP-C** (1.0 mM), **IP-D** (2.0 mM) concentration of TSPP, the **ITP-A** stands for 0.1 mM TSPP, 0.6 mM TiO₂, **ITP-B** (0.2 mM TSPP, 1.2 mM TiO₂), **ITP-C** (1.0 mM TSPP, 6.2 mM TiO₂), **ITP-D** (2.0 mM TSPP, 12.5 mM TiO₂), similarly **NT-A** stands for 0.6 mM, **NT-B** (1.2 mM), **NT-C** (6.2 mM) and **NT-D** (12.5 mM) TiO₂ nanowhiskers only. The **N0** shows control group without treatment. ** shows the significance level with probability value < 0.01.

Table 2 Various photosensitizer's concentrations effecting WBC, RBC, HGB and Platelets mean in the treated SD rats groups.

Treatment	WBC** (10⁹/L)	HGB** (g/L)	PLT** (10⁹/L)	RBC (10¹²/L)
IP-A	14.37 ^{a,b,c,d}	17.80 ^{a,b,c}	439.67 ^{d,e}	6.55 ^{a,b,c}
IP-B	14.25 ^{a,b,c,d}	16.66 ^{b,c}	448.67 ^{d,e}	7.14 ^{a,b,c}
IP-C	13.33 ^{b,c,d}	18.93 ^a	511.67 ^{c,d,e}	5.84 ^{a,b,c}
IP-D	14.49 ^{a,b,c,d}	18.53 ^{a,b}	412.00 ^e	6.24 ^{c,d}
ITP-A	8.96 ^d	16.03 ^{c,d}	532.67 ^{c,d,e}	7.81 ^d
ITP-B	13.35 ^{b,c,d}	16.76 ^{b,c}	500.33 ^{c,d,e}	8.34 ^{a,b,c}
ITP-C	13.39 ^{b,c,d}	16.26 ^c	517.33 ^{c,d,e}	7.70 ^{a,b,c}
ITP-D	18.37 ^{a,b}	16.20 ^c	706.33 ^a	8.71 ^{a,b}
NT-A	15.13 ^{a,b,c}	16.00 ^{c,d}	553.33 ^{b,c,d,e}	7.58 ^{a,b,c}
NT_B	16.54 ^{a,b}	17.46 ^{a,b,c}	614.67 ^{a,b,c}	7.59 ^{a,b,c}
NT-C	19.59 ^a	16.53 ^{b,c}	582.67 ^{a,b,c,d}	7.56 ^{a,b,c}
NT-D	19.95 ^a	17.30 ^{a,b,c}	693.33 ^{a,b}	8.83 ^a
N0	10.37 ^{c,d}	14.16 ^d	531.00 ^{c,d,e}	8.17 ^{a,b,c}

^a, ^b, ^c, ^d, ^e; superscripts level showing means difference with in a same column (from Duncan Multiple Range Test). Whereas *WBC* stands for white blood cells, *HGB*; hemoglobin, *PLT*; platelets and *RBC* for red blood cells in treated groups. Moreover, **IP-A** group stands for 0.1 mM, **IP-B** (0.2 mM), **IP-C** (1.0 mM), **IP-D** (2.0 mM) concentration of TSPP, the **ITP-A** stands for 0.1 mM TSPP, 0.6 mM TiO₂, **ITP-B** (0.2 mM TSPP, 1.2 mM TiO₂), **ITP-C** (1.0 mM TSPP, 6.2 mM TiO₂), **ITP-D** (2.0 mM TSPP, 12.5 mM TiO₂), similarly **NT-A** stands for 0.6 mM, **NT-B** (1.2 mM), **NT-C** (6.2 mM) and **NT-D** (12.5 mM) TiO₂ nanowhiskers only. The **N0** shows control group without treatment. ** shows the significance level with probability value < 0.01.

Figure titles and legends:

Figure 1 *In vitro* Cell viability (%) with light and without light after treatment with TSPP and TiO₂

(A) Cell viability %, when treated with TSPP (Red line a) only and TSPP in combination with TiO₂ nanowhiskers (Blue line b) in the presence of visible light. Whereas, **IL-T1** represents 1.06 mM TSPP only and 12.5 mM TiO₂ with 0.1 mM TSPP; **IL-T2** (0.85 mM TSPP only and 9.4 mM TiO₂ with 0.1 mM TSPP); **IL-T3** (0.42 mM TSPP only and 6.3 mM TiO₂ with 0.1 mM TSPP); **IL-T4** (0.1 mM TSPP only and 3.13 mM TiO₂ with 0.1 mM TSPP); **IL-T5** (0.1mM TSPP only and 1.25 mM TiO₂ with 0.1 mM TSPP); **IL-T6** (0.08 mM TSPP only and 0.94 mM TiO₂ with 0.1 mM TSPP); **IL-T7** (0.04 mM TSPP only and 0.63 mM TiO₂ with 0.1 mM TSPP) **IL-T8** (0.02 mM TSPP only and 0.31 mM TiO₂ with 0.1 mM TSPP) and **IL-T9** (0.01 mM TSPP only and 0.13 mM TiO₂ with 0.1 mM TSPP) as various concentrations.

(B) Cell viability %, when treated with TSPP (Red bars) only and TSPP in combination with TiO₂ nanowhiskers (Blue gradient bars) in the absence of visible light. Whereas, **NL-T1** represents 1 mM TSPP only and 12.5 mM TiO₂ with 0.1 mM TSPP; **NL-T2** (0.85 mM TSPP only and 9.4 mM TiO₂ with 0.1 mM TSPP); **NL-T3** (0.42 mM TSPP only and 6.3 mM TiO₂ with 0.1 mM TSPP); **NL-T4** (0.1 mM TSPP only and 3.31 mM TiO₂ with 0.1 mM TSPP); **NL-T5** (0.02 mM TSPP only and 0.313 mM TiO₂ with 0.1 mM TSPP) as various concentrations.

Figure 2 Liver histopathology and serum enzyme markers after various treatments (TSPP, TSPP-TiO₂, TiO₂ and Control) in normal SD- rats

Here in this figure *Pv* stands for Portal vein, *E*; epithelium, *Ic*; inflammatory cells, while *p* represents the probability value for significance. The fig (a) shows (0.1 M/ml) TSPP (tetra sulphonatophenyl porphyrin) effect on hepatic tissue with damaged epithelium in portal vein,

sinusoidal dilatation and inflammatory cells in hepatic parenchyma, **(b)** shows the TSPP with TiO_2 (0.1 mM TSPP- 0.6 mM TiO_2) group remain almost normal, **(c)** shows the effect of TiO_2 alone on the hepatic tissue and damaged portal vein epithelium with higher concentration of (1.2, 6.2 and 12.5 mM), **(d)** is control with normal hepatic tissue (Magnification 10X). Moreover in this figure **(e)** demonstrate the AST (Aspartate aminotransferase) serum level mean for all groups, **(f)** shows the serum ALT (Alanine transaminase) mean for all treated groups and **(g)** is showing the LDH (Lactate dehydrogenase) mean difference among all treatment groups.

Figure 3 Kidney histopathology and serum bio-markers after various treatments (TSPP, TSPP- TiO_2 , TiO_2 and Control) to SD- rats

Here in this figure, *G* stands for glomerulus, *B*; bowmans capsule, *S*; space between glomerulus and bowmans capsule, *T*; tubules, *p*; probability value, whereas **(a)** represents the kidney from (0.1 mM) TSPP (tetra sulphonatophenyl porphyrin) treated group with larger space between bowmans capsule and glomerulus, **(b)** shows kidney treated with TSPP- TiO_2 (0.1 mM TSPP- 0.6 mM TiO_2) having less effected glomerulus, **(c)** is a (0.6 mM) TiO_2 treated kidney and **(d)** is showing normal kidney (Magnification 40X). Figure **(e)** is demonstrating CRT (creatinine) mean value difference among various treatment groups and **(f)** demonstrate the BUN (blood urea nitrogen) mean value for all treated groups.

Figure 4 Blood parameters (hemoglobin, Red blood cells, White blood cells and Platelets count) after various treatments (TSPP, TSPP-TiO₂, TiO₂ and Control) in normal SD- rats

Here, *HGB* stands for hemoglobin, *RBC*; red blood cells, *WBC*; white blood cells, *PLT*; platelets and *p* is showing the probability value for significance. The figure (a) shows mean difference among various treated group for HGB, (b) demonstrate the mean value of RBC among various treated groups, (c) is showing WBC mean difference among various treated groups and (d) is showing mean value for platelet count in various treated groups.

Figure 5 Lungs and Spleen histopathology after various treatments (TSPP, TSPP-TiO₂, TiO₂ and Control) in normal SD- rats

In this figure *Br* stands for bronchiole, *Al*; alveoli, *Ep*; epithelium, *pn*; pneumocysts, *Sn*; splenic nodules, *Gc*; germinal center, *Wp*; white pulp and *Rp* for red pulp. The figure (a) is showing (0.1 mM) TSPP (tetra sulphonatophenyl porphyrin) treated lungs with broken epithelium in bronchioles and impaired alveoli, (b) is the lung from TSPP-TiO₂ (0.1 mM TSPP- 0.6 mM TiO₂) treated group rats with less damaged bronchioles and alveoli, (c) is the lung treated with higher concentration (12.5 mM) TiO₂ having damaged bronchiole epithelium and broken alveoli having TiO₂ nanowhiskers (arrow), whereas (d) is showing normal bronchiole with intact epithelium and prefect shaped alveoli (Magnification 40X). The figure (e) is a spleen from (0.1 mM) TSPP treated group with impaired splenic nodules, (f) is a spleen from TSPP-TiO₂ (0.1 mM TSPP- 0.6 mM TiO₂) treated group with normal splenic nodules, (g) is (12.5 mM) TiO₂ treated spleen with impaired splenic nodules and (h) is normal spleen from control group (Magnification 4X).

**Figure 6 Fluorescent microscopic images of Lungs, Liver, Kidney and Spleen
after various treatments (TSPP, TSPP-TiO₂, TiO₂ and Control) given
to SD- rats**

In this figure K stands for kidney, L; liver, S; spleen, lg; lungs, H; higher TSPP concentration and L shows lower TSPP concentration. The C is control group with no treatment given and TiO₂ is a group treated with TiO₂ nanowhiskers without fluorescence. The ITP-A is a (0.1 mM TSPP, 0.6 mM TiO₂) TSPP-TiO₂ treated rats group with no fluorescence, ITP-B is (0.2 mM TSPP, 1.2 mM TiO₂) TSPP-TiO₂ treated group with low fluorescence in kidneys, ITP-C is (1.0 mM TSPP, 6.2 mM TiO₂) TSPP-TiO₂ treated group with low fluorescence in kidneys and ITP-D is (2.0 mM TSPP, 12.5 mM TiO₂) TSPP-TiO₂ treated group, showing lower fluorescence in kidney, lungs and liver, while spleen is non-fluorescent in all TSPP-TiO₂ treated groups. Similarly IP-A (0.1 mM) and IP-B (0.2 mM) is showing low fluorescence in lungs, liver and kidneys when treated with TSPP. IP-C (1.0 mM) is showing relatively higher fluorescence in liver and kidneys when treated with TSPP only, whereas IP-D (2.0 mM) demonstrates highest fluorescence in all organs except spleen with subtle fluorescence.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6