Nanoparticulat Printex 90 and Titanium Dioxide Stimulate Catecholamine Production in Ciliated Protozoan, *Tetrahymena thermophila*

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**Abstract**

The aim of this study was to find the effect of printex 90 and titanium dioxide nanoparticle on catecholamine homeostasis in *Tetrahymena thermophila*. These two nanoparticles were exposed for 20 hours and 40 hours respectively. *Tetrahymena* cell produce catecholamine which was extracted by solid phase extraction and then analyzed by HPLC-

**Keywords:** Nanoparticle; Printex 90; Titanium dioxide; Catecholamine

**Introduction**

Nanoparticles and nanomaterials are being rapidly produced in large quantities throughout the world due to their attractive usefulness. It has special properties such as small size (1-100 nm), high surface area and structure. Carbon black, printex 90 (CB) is a single walled carbon nanotube (SWCNT). It is extensively used in jet coloring of plastics and powder coatings due to good dispersibility and higher structure. On the other hand, titanium dioxide (TiO2) nanoparticle plays a special role in photocatalyst and catalytic support as well as a white pigment in the production of paints, paper, plastics, sunscreens and cosmetics [1,2]. Therefore, considering their wide range of applications, the impact of nanomaterials on human health and the environment makes it great interest. In the recent years, the increasing evidence of the adverse effects of nanoparticles, such as increase in respiratory and cardiovascular mortality, morbidity and worsening of asthma [3-5]. Many types of commercial nanoparticles, such as silica, TiO2, silver, chrysotile asbestos, carbon nanotubes, as well as some magnetic particles, have been investigated for their bio-safety, and these nanoparticles exhibited various levels of cytotoxicity in different cell lines [4,6]. However, the recent time it is also reported that upconversion nanoparticles composites are useful to control multifunctional drug delivery and reduce drawbacks of drug [7-9]. The mice treated with platinum(IV) pro-drug-conjugated nanoparticles under near-infrared (NIR) irradiation demonstrated better inhibition of tumor growth than that of the under direct UV irradiation [7]. A chemotherapy drug, doxorubicin, is loaded into the nanocomposite, forming an upconversion nanoparticles (UCNPs) together with iron oxide nanoparticles (IONPs) which enables novel imaging-guided and magnetic targeted drug delivery [8]. Subsequently, the combination of upconversion nanoparticles with the cisplatin (IV) prodruk will reduce not only the drawbacks of cisplatin but also give insight into its function of the central nervous system [16]. Furthermore, the nasal instillation of TiO2 nanoparticles produce oxidative stress in whole brain of exposed mice such as lipid peroxidation, protein oxidation and increased activities of catalase, as well as the excessive release of glutamic acid and nitric oxide and cause potential lesion in central nervous system of brain [17]. As far we know, many investigation of nanoparticle was conducted on vertebrates and mammalian cells. However, this study utilizes single cell ciliate protozoan, *Tetrahymena thermophila* as a model organism to test nanoparticles. In spite of this, few studies have been shown that nanoparticle causes toxicity to *Tetrahymena*. nCuO significantly decreases the proportion of unsaturated fatty acids (UFA) while it increases the relative amount of two saturated fatty acids by lowering membrane fluidity and inhibition of de-novo synthesis of fatty acid desaturases in *Tetrahymena* [18]. In addition, Ag, CuO and ZnO nanoparticle induces toxicity and decreases growth of *Tetrahymena* [19-21]. The aquatic organism, *Tetrahymena* synthesizes dopamine, noradrenaline and adrenaline at significant level although this organism does not contain adrenal medulla or nervous system [22]. As well, literature has recently been revealed that TiO2 nanoparticle causes cytotoxicity to the *Tetrahymena* [23]. However, our previous literature showed that the prenatal exposure of TiO2 causes the increase of dopamine and its metabolite in the different region of the brain in mice [24]. Therefore, the aim of the study was to explore...
the effect of printex 90 and TiO₂ nanoparticle on the catecholamine homeostasis in Tetrahymena thermophila as an ‘animal free testing organism’ in 96-well plates.

Materials and Methods

Two different nanoparticle (NP) such as printex 90 and TiO₂ were used in this study. Printex 90 was purchased from Evonik Degussa GmbH, Rellinghauser Straße 1–11, D-45128 Essen, Germany. The manufacturer reported that it is carbon black nanoparticle (CBNPs) with an average size of 14 nm. The specific surface area was determined to be 295–338 m²/gm corresponding to a theoretical average spherical particle size of 8.1-9.5 nm. It contains organic impurity less than 1%. The total carbon content was measured greater than 99 wt% with 0.82 nitrogen wt% and 0.01 hydrogen wt%. The literature also described that printex 90 particles in suspensions for exposure showed the average zeta-size was 140 nm and the hydrodynamic number size distribution had the major mode at 50–60 nm [25]. On the other hand, TiO₂ was purchased from Sigma- Aldrich, Taufkirchen, Germany. The manufacturer describes that it is a titanium (IV) oxide, anatase, 99.7% trace metals basis. Its average size is 25 nm. Its surface area, bulk density and density at 25°C were 45-55 m²/g, 0.04-0.06 g/mL and 3.9 g/mL respectively. The stock suspension of printex 90 and TiO₂ nanoparticles were prepared in proteose peptone yeast (PPY) extract cell culture medium separately and stabilized by ultra-sonication (Sonorex RK 510S, Bandelin, Berlin, Germany). Nanoparticles suspensions were test to unicellular ciliate protozoan cell, Tetrahymena thermophila SB210 for catecholamine homeostasis. The growth maintenance of this cell was described in the literature [23]. Tetrahymena cell was acclimatized in fresh PPY-medium by twice successive reseedings for producing exponential cell. The sterile stock suspension of nanoparticle was prepared in PPY-medium. It was ultra-sonicated for one hour prior to experiment for stabilization. 200 μl printex 90 nanoparticle suspension of different concentration and 50 μl exponential cells aliquot were added to different well of 96-well plates. The final volume of liquid in each well in 96-well plates is reached to 250 μl. As well, the initial Tetrahymena cell concentration in the 96-well plate was carefully maintained to 1x10⁴ cell/mL by adding adjusted 50 μl cell aliquots. Cell concentration was measured by Neubauer cell counter (Brand Wertheim, Germany). Due to the addition of different concentration of printex 90 nanoparticles in the 96-well plate were produced the final exposure concentrations of nanoparticle to 2.67, 4, 6, 9, 13.5, 15.8, 23.70, 35.56, 53.33, 80, 120, 180 mg/L respectively. In addition, TiO₂ nanoparticle was also tested as similar as printex 90. However, the final exposure concentrations of TiO₂ nanoparticle were calculated to 8, 16, 20, 24, 32, 40, 80, 100, 120, 160 and 200 mg/L respectively. Now, the prepared 96-well plates were placed into horizontal shaker at 40 rpm in dark for incubation and cultivation of cell at 32°C for 20 hours and 40 hours respectively. This slow shaking of 96-well plate aided nanoparticles to keep suspension in the PPY-medium. Nevertheless, nanoparticle tends to accumulate in the well plates due to the presence of complex PPY medium with the association of many ions. It is also recently reported upon a microscopic study that this accumulation of nanoparticle increases significantly when Tetrahymena species was cultivated in the PPY medium [23]. Tetrahymena cell internalize all free nanoparticles and finally exocytose them as larger aggregates into the culture medium, and this process continues until free small and visible nanoparticles are present the PPY-medium. However, the molecular mechanism of this particle internalization into Tetrahymena was not clear. In addition, the size distribution and zeta-potential of these two nanoparticles were not measured in PPY-medium in this study; size distribution of nanoparticles in PPY-medium was shown in our previous literature [23].

Results and discussion

Nanoparticles were exposed to Tetrahymena to study catecholamine homeostasis in 96-well plate. Three catecholamine compounds, namely noradrenaline (NA), adrenaline (A), and dopamine (DA), are known to be significantly synthesized in Tetrahymena and are quantitatively determined by HPLC–ECD using the ClinRep® HPLC complete kit [22]. This technique is very useful for the analysis of catecholamine at low concentration in several physiological fluids, eg. plasma and urine da 2006) [26]. A standard solution of catecholamine (ClinRep®) was used to prepare a standard chromatogram for the analysis of catecholamine through isocratic separation as illustrated in Figure 1a.

200 μL experimental aliquots from 96-well plates was aspirated and transfer into solid phase extraction (SPE) cartridge after 20 hours and 40 hours incubation respectively accordingly with 50 μl (=500 pg) internal standard (IS) solution (3,4 dihydroxybenzylamine, DHBA). Sample clean-up for catecholamine was carried out according to the manufacturer guide lines (ClinRep® HPLC Complete kit, Recipe, Germany). Catecholamine concentration was measured by HPLC-ECD as per description of literature [22]. The analytical precision, accuracy and the lower limits of determination were determined from biological control materials (ClinRep® HPLC Complete kit, Recipe, Germany). The recovery rate for the catecholamine in the control materials (blood plasma) was found above 80%. The quantitative data of the catecholamine was estimated using peak area of chromatogram as per standard formula of ClinRep® HPLC (Recipe, Germany).

The data from the all groups of nanoparticle dose were pooled and analyzed statistically using Two-tailed "Student t-distribution" test in order to compare with control to find significance of differences, where p value <0.05 was considered for significant result. Data of the control group (n=3) was compared to the data of each treatment (n=3) group for the catecholamine parameter of dopamine, adrenaline and noradrenaline separately.

![Figure 1: Chromatogram of catecholamine. (a) Standard chromatogram. Where, noradrenaline (NA), adrenaline (A), internal standard (IS) and dopamine (DA) respectively. (b) Catecholamine produced from Tetrahymena cell after 40 hour without exposure of nanoparticle. (c) Catecholamine produced from Tetrahymena with the exposure of printex 90 after 40 hours where nanoparticle concentration was 80 mg/L.](image-url)
Then the peak area of the desired compounds in the chromatogram of sample exposed by nanoparticles were identified and detected by comparison with peak area of standard chromatogram using retention time. However, in biological samples the retention time of analytes is slightly shifted due to the presence of some ions and trace metabolites but it does not affect the performance of catecholamine estimation. A chromatogram for normal synthesis of catecholamine in *Tetrahymena* was used to compare with the chromatogram of catecholamine exposed by nanoparticles as shown in Figure 1b. The exposed nanoparticles were internalized into the *Tetrahymena* cell and later exocytosed from the cell as bigger aggregates. However, during this discharge process of nanoparticles by the *Tetrahymena* cell turns to stimulate synthesis of noradrenaline, adrenaline and dopamine. This extra production of catecholamine from *Tetrahymena* cells with the exposure of printex 90 at 80 mg/L concentration after 40 hours was shown in Figure 1c. The comparison between Figure 1b and 1c clearly exhibited that printex 90 induces *Tetrahymena* cell for the additional synthesis of catecholamine. Similarly, all the exposure concentration of printex 90, as described in the methodology section, stimulated *Tetrahymena* for additional synthesis of noradrenaline, adrenaline and dopamine. Among these three compounds noradrenaline was most profoundly synthesized in the cell as compared to control. Most of the doses of printex 90 were significantly produced noradrenaline after 20 hours as presented in Table 1. However, the noradrenaline production was found to be very high and is not consistent for the printex 90 at 4, 80, 120 and 180 mg/L as compared to the other remaining doses. We can explain this irregular higher response of noradrenaline in response to printex 90; it may be caused due to the uneven sensitivity of electrode in detector of HPLC-ECD. Similarly, adrenaline and dopamine were also progressively synthesized in the cell with the exposure of printex 90. It is also showed that the level of adrenaline production was very low as compared to the noradrenaline and dopamine. Only two dose of printex at 80 and 120 mg/L were caused significant increase of adrenaline synthesis after 20 hours incubation. In addition, printex 90 was caused significant production of dopamine at 4, 80, 120 and 180 mg/L as presented in Table 1.

| mg/L | Noradrenaline | Adrenaline | Dopamine | Noradrenaline | Adrenaline | Dopamine |
|------|---------------|------------|----------|---------------|------------|----------|
|      | Control       | 49.8       | 5.64     | 1.13          | 0.43       | 12.17     | 2.90     |
| 2.67 | 60.82         | 6.08       | 0.95     | 13.14         | 2.19       | 68.09     | 1.12     |
| 4    | 82.89*        | 3.34       | 1.37     | 25.17*        | 1.81       | 79.99*    | 4.41     |
| 6    | 64.07         | 7.21       | 1.93     | 18.47         | 3.52       | 70.72     | 0.80     |
| 9    | 63.95         | 2.58       | 2.16     | 17.29         | 0.81       | 78.97*    | 5.42     |
| 13.5 | 64.16*        | 3.95       | 1.72     | 16.98         | 1.24       | 94.81*    | 5.15     |
| 15.8 | 59.49         | 6.16       | 0.97     | 13.36         | 2.42       | 92.73*    | 5.59     |
| 23.70| 65.83*        | 5.15       | 0.73     | 14.45         | 1.34       | 75.75*    | 1.12     |
| 35.56| 60.04         | 1.52       | 1.09     | 12.52         | 0.90       | 74.19     | 2.92     |
| 53.33| 68.17*        | 3.43       | 1.91     | 16.31         | 0.91       | 105.8*    | 2.14     |
| 80   | 94.86*        | 4.10       | 2.6*     | 23.92*        | 1.20       | 106.6*    | 3.99     |
| 120  | 96.34*        | 2.24       | 2.9*     | 28.56*        | 0.86       | 95.41*    | 2.77     |
| 180  | 101.85*       | 3.87       | 1.65     | 28.59*        | 1.41       | 74.35     | 5.35     |

Table 1: Effect of printex 90 nanoparticle on catecholamine homeostasis in *Tetrahymena thermophila* after 20 hour and 40 hour exposure. (*) indicates a significant difference of analytes as compared to the control.
In a brief, this study showed that nanoparticle induces chemical signal which is associated to stimulate catecholamine production in the aquatic model organism Tetrahymena as illustrated in Figure 2. Tetrahymena was more responsive to printex 90 than TiO₂ for elevating catecholamine synthesis, although, Tetrahymena cell does not contain nervous system. Therefore, this study concluded that nanoparticles can be considered as new chemical factor to stimulate catecholamine production in Tetrahymena. Furthermore, this study recommends catecholamine as an important parameter for studying the neurotoxicity of nanoparticle in different model experimental mammals and other cell lines which potentially synthesize catecholamine. This can also find the bridge of studying the mechanism of toxicity associated with nanoparticles and also in addition to explore the target enzymes of analytes as compared to the control.

Table 2: Effect of TiO₂ nanoparticle on catecholamine homeostasis in Tetrahymena thermophila after 20 hour and 40 hour exposure. (*) indicates a significant difference of analytes as compared to the control.

| mg/L | 20 hour exposure of TiO₂ | 40 hour exposure of TiO₂ |
|------|-------------------------|-------------------------|
|      | Noradrenaline | Adrenaline | Dopamine | Noradrenaline | Adrenaline | Dopamine |
|      | nmol | SD | nmol | SD | nmol | SD | nmol | SD | nmol | SD | nmol | SD |
| Control | 50.25 | 3.54 | 5.10 | 1.04 | 32.58 | 2.87 | 55.40 | 2.19 | 5.13 | 1.66 | 27.03 | 0.84 |
| 8     | 63.12* | 2.76 | 5.94 | 0.77 | 39.82 | 0.91 | 50.30 | 2.79 | 5.29 | 1.89 | 23.83 | 6.48 |
| 16    | 66.18* | 0.63 | 8.76 | 2.10 | 47.66* | 3.79 | 44.42* | 1.18 | 3.88 | 0.42 | 21.94 | 4.26 |
| 20    | 56.26 | 6.31 | 8.01 | 2.57 | 46.35 | 5.70 | 62.75* | 1.71 | 4.64 | 2.89 | 33.29* | 1.12 |
| 24    | 74.15* | 4.36 | 9.51 | 2.31 | 54.84* | 4.24 | 60.01 | 2.01 | 4.20 | 3.43 | 20.17 | 3.88 |
| 32    | 71.09* | 1.20 | 10.54* | 0.87 | 50.92* | 2.95 | 56.87 | 2.38 | 3.38 | 2.64 | 30.23 | 3.14 |
| 40    | 85.18* | 2.48 | 11.69* | 0.81 | 48.31* | 4.41 | 56.97 | 2.99 | 4.69 | 0.79 | 36.89* | 2.29 |
| 60    | 70.29* | 4.82 | 7.01 | 0.57 | 47.07* | 1.16 | 51.28 | 0.63 | 3.44 | 0.45 | 28.01 | 4.08 |
| 100   | 65.20* | 1.54 | 3.84 | 0.23 | 33.62 | 2.15 | 42.46 | 6.25 | 5.68 | 1.58 | 20.11* | 2.01 |
| 120   | 57.67 | 2.18 | 4.34 | 0.41 | 41.32* | 0.87 | 49.71 | 7.35 | 5.51 | 3.17 | 23.05 | 3.58 |
| 160   | 71.03* | 3.95 | 4.50 | 2.38 | 40.54 | 3.49 | 52.26 | 2.27 | 6.22 | 1.39 | 28.72 | 3.51 |
| 200   | 64.10* | 0.91 | 5.76 | 1.25 | 49.48* | 1.35 | 40.99* | 1.33 | 3.22 | 0.26 | 23.50* | 0.85 |

Figure 2: Stimulation of catecholamine synthesis in the Tetrahymena thermophila cell with the exposure of nanoparticles.

at higher concentrations, due to the depletion of cell number. This unconquered effect of nanoparticles was explained in the literature [23]. The authors were reported that higher concentration of TiO₂ particle causes cytotoxicity to cells. As a result; due to the presence of lower number of cells were collectively produced smaller amounts of noradrenaline, adrenaline and dopamine.

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