Toxicity of TiO₂ Nanoparticles to Escherichia coli: Effects of Particle Size, Crystal Phase and Water Chemistry

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Abstract

Controversial and inconsistent results on the eco-toxicity of TiO₂ nanoparticles (NPs) are commonly found in recorded studies and more experimental works are therefore warranted to elucidate the nanotoxicity and its underlying precise mechanisms. Toxicities of five types of TiO₂ NPs with different particle sizes (10–50 nm) and crystal phases were investigated using Escherichia coli as a test organism. The effect of water chemistry on the nanotoxicity was also examined. The antibacterial effects of TiO₂ NPs as revealed by dose-effect experiments decreased with increasing particle size and rutile content of the TiO₂ NPs. More bacteria could survive at higher solution pH (5.0–10.0) and ionic strength (50–200 mg L⁻¹ NaCl) as affected by the anatase TiO₂ NPs. The TiO₂ NPs with anatase crystal structure and smaller particle size produced higher solution pH and ionic strength. In addition, the antibacterial effects of TiO₂ NPs were investigated using different water chemistry parameters such as pH and ionic strength. The results showed that the anatase TiO₂ NPs have a higher toxicity than the rutile TiO₂ NPs due to their higher oxidative stress. These research results will shed new light on the understanding of the eco-toxicity of TiO₂ NPs.

Introduction

Due to their unique chemical and physical properties, titanium dioxide (TiO₂) nanoparticles (NPs) are produced at a large scale for industrial applications to meet with ever-increasing market demands [1]. The annual production of TiO₂ NPs is predicted to reach 2.5 million tons by 2025 [2]. The widely used TiO₂ NPs would find their way into aquatic environments [3–6] and interact with aquatic organisms [7]. Eco-toxicity of TiO₂ NPs is therefore received worldwide research attentions [8–16].

Bacteria, e.g., Escherichia coli (E.coli), as single cell organisms and ubiquitous in aquatic environments, are good model organisms for studying the eco-toxicity of NPs and the cell/organism-NP interaction. Many research works [8] have investigated the toxicity of various TiO₂ NPs toward E.coli, with a focus on the influencing factors such as: (1) Size. Many studies attributed the toxicity of TiO₂ NPs to their small particle size [17–22]. (2) Crystal structure. It is generally concluded that anatase TiO₂ NPs are more toxic than rutile NPs by inducing greater oxidative stress [15,23,24]. (3) Experimental matrix. Changes in water chemistry (e.g., pH and ionic strength) may influence the agglomeration and sedimentation characteristics of NPs and then their toxicity [12,21,25–28]. (4) Solar radiation, especially those in the UVA region, is also considered as a critical factor of aquatic nanotoxicity [13,29–35]. These researches substantially increased our knowledge on the eco-toxicity of TiO₂ NPs.

However, controversial and inconsistent results on the toxicity of TiO₂ NPs are commonly found in recorded studies and precise mechanisms of the nanotoxicity warrant more specific researches. For example, Adams et al. (2006) reported 44% reduction in the growth of E.coli by 1 g L⁻¹ TiO₂ NPs (66 nm, crystal structure not determined) [36]; Tong et al. (2013) [21] reported 70% reduction in the growth of E.coli by 10 mg L⁻¹ TiO₂ NPs while 30% reduction was observed by Planchon et al. (2013) [15] with the same TiO₂ NPs at 10 mg L⁻¹ (P25, consisting of an 80:20 ratio of anatase:rutile). So an acute lack of emphasis on the environmental and nanoparticle parameters prevents a meaningful comparative assessment from the hitherto available nanotoxicity data, and it highlights the necessity to provide additional eco-toxicological studies and physicochemical characterization of TiO₂ NPs to ensure consistency of research results.

This study is aimed to elucidate the roles of particle size and crystal structure in the toxicity of TiO₂ NPs using E.coli as a model organism. Five types of well characterized TiO₂ NPs with different particle sizes and crystal phases were examined. The toxicity assays were conducted at different concentrations of the NPs and various solution pHs and ionic strengths. In addition, the interactions between TiO₂ NPs and bacteria and the cell reactive responses were examined with transmission electron microscopy (TEM) and measurements of intracellular reactive oxygen species.
(ROS) and malondialdehyde (MDA) to address the toxicity mechanism. The results are believed to increase our understanding of the nanotoxicology.

**Materials and Methods**

1. Nanoparticles and characterizations

Five types of TiO\textsubscript{2} NPs were purchased and used in this study. They were anatase TiO\textsubscript{2} with particle sizes measured to be around 10 nm (TiO\textsubscript{2}-NP 10A), 25 nm (TiO\textsubscript{2}-NP 25A), and 50 nm (TiO\textsubscript{2}-NP 50A) and rutile TiO\textsubscript{2} of 50 nm (TiO\textsubscript{2}-NP 50R) and mixed anatase and rutile TiO\textsubscript{2} of 25 nm (TiO\textsubscript{2}-NP25 AR). TiO\textsubscript{2}-NP 10A and TiO\textsubscript{2}-NP 50R were from Hongsheng Material Sci & Tech Co., Zhejiang, China and the other three TiO\textsubscript{2} NPs from Wangjing New Material Sci & Tech Co., Zhejiang, China.

Morphologies of the NPs were examined using TEM (JEM-1230, JEOL Ltd., Tokyo, Japan). Powdered X-ray diffraction analysis (XRD, X’Pert Pro, Holland) was carried out to characterize the crystal structure of the NPs. Elemental compositions of the NPs were determined by using an X-ray energy dispersion spectroscopy (EDS, GENESIS 4000, EDAX Inc. America). Hydrodynamic diameters and zeta potentials of the NPs (50 mg L\textsuperscript{-1}) were measured with a Zetasizer (Nano ZS90, Malvern, UK) after being sonicated (100 W, 40 kHz, 30 min) into 100 mg L\textsuperscript{-1} NaCl solution at 25°C and various pH values. Points of zero charge (pHpzc) of the NPs were obtained from the zeta potential versus pH curves. Specific surface areas of the NPs were determined using the multi-point Brunauer-Emmett-Teller (BET) method (Quantachrome NOVA 2000e, America).

2. Dose-effect experiments

*E. coli* O111 (Genbank access no. GU237022.1) isolated from a sewage water was used as the test organism, as reported in our previous studies [37,38]. The bacteria were maintained in Luria Bertani (LB) solid plates at 4°C and inoculated in LB broth (pH 7.2~7.4) at 37°C overnight (12~16 h) at 150 rpm. The bacteria were separated from the broth by centrifugation at 3000 g for 15 min and washed twice with 0.85% physiology salt-water. The bacterium stock suspension was prepared by resuspending the bacterial pellets in 0.85% NaCl physiology salt-water with the cell concentration determined by the absorbance at 600 nm (OD\textsubscript{600}) being adjusted to 1.0.

The stock NP suspensions (500 mg L\textsuperscript{-1}) were obtained by sonicating (100 W, 40 kHz, 30 min) 50 mg of the TiO\textsubscript{2} NPs into 100 mL of ultra-pure water. The stock NP suspensions after sonication were diluted using ultra-pure water to the target test concentrations (10~500 mg L\textsuperscript{-1}).

One mL of the *E.coli* stock suspension was added into 100 mL of the test NP suspensions. The mixtures were placed on a shaker at 37°C and 150 rpm for 3 h with natural light. The bacteria in the resultant mixtures were spread on LB agar plates and incubated at 37°C for 24 h, and the colonies were counted. The percentage viabilities of the bacteria in the NP suspensions were calculated by dividing their colony forming units (CFU) mL\textsuperscript{-1} by that in the NP-free control. All treatments including the control were repeated in triplicate.

3. TEM Observations

TEM was used to observe the direct contact between the NPs and the bacterial cells. A drop of the bacteria exposed to the NPs (50 mg L\textsuperscript{-1}, 3 h) and the NP-free control were air-dried onto a
copper grid and was then imaged by the TEM. To observe the internalization and localization of the NPs in the cells and the changes in cellular structure as affected by the NPs, the NPs-treated and untreated bacteria were fixed in 2.5% glutaraldehyde, dehydrated in graded concentrations of ethanol, embedded in Epon resin, and stained with OsO4 [12,39]. Ultrathin sections were then cut and counterstained with Reynold’s and uranyl acetate for the TEM observation.

4. Reactive oxygen species (ROS) and lipid peroxidation measurements

The fluorescence probe 2’7’-dichlorodihydrofluorescein diacetate (H2DCFDA) was used to quantify the formation of intracellular ROS as described in our previous papers [12,39] with minor modifications. The bacterial cells after exposure to the test media were collected by centrifugation (8000 g, 5 min). The pellet was resuspended in 0.85% physiology salt-water containing 10 μM H2DCFDA and incubated on the shaker (150 rpm) for 30 min at 37°C. The bacteria were further pelleted and resuspended in 300 μL of 0.85% physiology salt-water. The fluorescence values were measured in a 96-well plate using a multifunctional microplate reader (M200 PRO, Ltd., Austria) with the excitation and emission wavelengths of 485 nm and 528 nm, respectively. Relative ROS levels were calculated by the fluorescence ratio of the treatments to the control.

The concentration of malondialdehyde (MDA) was determined as an indicator of lipid peroxidation as described in previous works [12,39]. Briefly, the exposed bacteria were mixed into 1 mL of 10% (wt/vol) trichloroacetic acid and left at room temperature for 10 min; the supernatant of the mixture was collected after centrifugation at 11,000 g for 40 min and then mixed with 1.5 mL of a freshly prepared 0.67% (wt/vol) thiobarbituric acid solution; the resultant mixture was incubated for 20 min in a boiling water bath and after cooling the absorbance was measured at 532 nm; and the MDA concentration was calculated using the Hodges’ equations.

5. Effects of pH and ionic strength

TiO2-NP 10A with a concentration fixed at 10 mg L\(^{-1}\) was selected as a type of representative NPs in examining the effect of water chemistry on the nanotoxicity. In the pH effect experiment, the suspension pHs were adjusted to 5.0, 7.0, 8.0 and 10.0 using 0.1 M HCl and NaOH; NaCl was added to maintain a constant background ionic strength (100 mg L\(^{-1}\)). For the ionic strength effect experiment, difference concentrations of NaCl (0, 50, 100, 150 and 200 mg L\(^{-1}\)) were added into the NP suspensions; the final suspension pH remained at about neutral without further adjustment. The bacterial exposure method in the pH and ionic strength effect experiments was the same as the above dose-effect experiments. Zeta potential of 1 mL of the stock bacterial suspension after being mixed into 100 mL of ultra-pure water at pH 5.0, 7.0, 8.0 and 10.0 was also measured by the Zetasizer.

6. Statistical analysis

Each treatment including the blank control was conducted in triplicate and the results were presented as mean ± SD (standard deviation). For each datum point, data were normalized by the reference without NPs. This representation thus strictly reports the incremental impacts of TiO2 NPs, excluding the medium stress. The Student’s t test was performed to analyze the significance of difference between two groups of data. Origin 8.0 was used to make graphs. The concentration resulting in 50% mortality (LC50) was calculated with SPSS 20.0.

| Sample   | Crystal phase, % | Zeta potential, mV | pH\(_{pzc}\) | hydrodynamic size, nm | \(S_{BET}\), m\(^2\) g\(^{-1}\) | TEM size, nm |
|----------|------------------|---------------------|-------------|-----------------------|-----------------|-------------|
| TiO2-NP10A | Anatase, 100     | 21.6                | 6.2         | 314±8                 | 324             | 11.0±3.4    |
| TiO2-NP25A | Anatase, 99.2    | 5.48                | 5.6         | 251±37                | 77              | 26.2±6.1    |
| TiO2-NP25AR | Anatase, 93.0    | 13.6                | 5.2         | 202±57                | 66              | 26.7±5.0    |
| TiO2-NP50A | Anatase, 98.8    | 9.34                | 6.0         | 486±12                | 105             | 57.1±14.0   |
| TiO2-NP50R | Rutile, 100      | 33.8                | 3.6         | 260±10                | 30              | 57.2±17.8   |

Note: the crystal phase was determined by the XRD; zeta potential and hydrodynamic size were measured in the toxicity test medium at pH 6.5 by the Zetasizer; \(pH\(_{pzc}\)\) was calculated from the zeta potential versus pH curves shown in Figure 2; \(S_{BET}\) (specific surface area) measured using the BET (Brunauer-Emmett-Teller) method; TEM size shows the NP size measured with the TEM images.

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Figure 2. Changes of zeta potentials of the TiO2 NPs against a solution pH.
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Results and Discussion

1. Characteristics of TiO₂ NPs

Selected properties of the TiO₂ NPs are listed in Table 1 with their TEM images shown in Figure 1. Big NP aggregates present in the TEM images and the measured large hydrodynamic diameters indicate the aggregation of the NP suspensions even after the sonication. TiO₂-NP 10A, having the smallest particle size (TEM size of 11.0±3.4 nm) among the five TiO₂ NPs, owned relative greater hydrodynamic diameter of 314±6 nm. Changes in zeta potentials of the TiO₂ NPs against a solution pH are shown in Figure 2. The calculated pH pzc of the NPs varied from pH 3.6 to 6.2, which could account for their negative zeta potentials (−33.8−−5.48 mV) in the neutral toxicity test medium. Specific surface areas of the TiO₂ NPs ranged from 30 m² g⁻¹ (TiO₂-NP 50R) to 324 m² g⁻¹ (TiO₂-NP 10A). Purities of the NPs determined by the EDS were all above 98.0% (Figure 3). XRD patterns of the TiO₂ NPs (Figure 3) confirmed the predominance of anatase phase of TiO₂-NP 10A, TiO₂-NP 25A, and TiO₂-NP 50A and the rutile nature of TiO₂-NP 50R; and TiO₂-NP 25AR was a mixture of anatase (93%) and rutile (7%).

2. Cell viability assessment

The particle dose, size and phase dependent reductions in the cell viability of E.coli upon exposure to the TiO₂ NPs for 3 h were observed through the plate count assay (Figure 4). The four anatase NPs were more or less toxic to E.coli and the viability of the bacteria exhibited a pronounced concentration-dependent decrease. The calculated 3 h LC₅₀ of the four anatase NPs had an order of TiO₂-NP 10A (17.0 mg L⁻¹), TiO₂-NP 25A (59.2 mg L⁻¹), TiO₂-NP 25AR (163 mg L⁻¹), TiO₂-NP 50A (304 mg L⁻¹). The enhancement of bactericidal effect of the NPs with decreasing particle size was observed throughout the various particle concentrations. TiO₂-NP 10A in the anatase phase with the minimum particle size and the largest BET surface area was determined to be the most toxic to E.coli. The presence of rutile phase in the NPs lowered the bactericidal activity in comparison to the pure anatase NPs. As shown in Figure 4, although similar in particle size, the toxicity of TiO₂-NP 25AR was much lower than that of TiO₂-NP 25A; the pure rutile TiO₂-NP 50R was nontoxic to the bacteria with concentration up to 500 mg L⁻¹, while the anatase TiO₂-NP 50A could inactivate half of the bacteria at 304 mg L⁻¹.

3. TEM observations of the direct NP-cell interactions

Nanoparticle-type-dependent bacterial cell membrane localizations of the TiO₂ NPs as well as morphological changes of the NPs-exposed cells were captured by the TEM images (Figure 5). The stronger NP-cell interaction was observed for the TiO₂ NPs with anatase crystal structure and smaller particle size. Numerous TiO₂-NP 10A aggregates with various sizes were observed tightly attached to the bacterial cell surfaces (Figure 5B). The big and tight NP-cell aggregate in Figure 5C indicates the strong...
interaction between TiO$_2$-NP 25A and the cells. Some of the TiO$_2$-NP 25AR aggregates were also observed attaching to the bacterial cells but some present away from the cells (Figure 5D), implying the relatively weaker NP-cell interaction as compared with the pure anatase TiO$_2$-NP 25A of the same size. A few big aggregates of TiO$_2$-NP 50A were observed loosely attached to the bacterial cell (Figure 5E), which suggests the much weaker interaction of TiO$_2$-NP50A than the smaller sized TiO$_2$-NP 25A and TiO$_2$-NP 10A with the cells. No obvious attachment between the TiO$_2$-NP 50R aggregates and the bacterial cells was observed (Figure 5F).

Figures 5G to 5L show TEM images of the sliced bacterial cells untreated or treated with 50 mg L$^{-1}$ of the TiO$_2$ NPs. The untreated (Figure 5G) and TiO$_2$-NP 50R-treated (Figure 5L) cells remained intact with unimpaired cell morphology and structure, indicating the nontoxicity of TiO$_2$-NP 50R. However, the NPs with smaller size and anatase phase were observed sticking to the cell surfaces (Figure 5H to 5K), which apparently induced cell distortion, plasmolysis and cell wall and membrane damage; penetration and internalization of the nanoparticles into the bacterial cells were also observed (Fig. 5H and 5I).

From the above TEM observations, it can be concluded that anatase TiO$_2$ NPs are more prone to attaching on the bacterial surfaces than rutile NPs, and the larger NPs interact weaker with cells compared to the smaller NPs. As particle size decreases, the ratio of surface area to mass increases and changes in the physicochemical properties (e.g., surface atom reactivity, electronic and optical properties) of the nanoparticles occur, consequently, the smaller particles tend to agglomerate to a greater extent, which can further influence their reactivity and binding characteristics [40]. The NP-cell attachment may inhibit the movement of substances in and out of bacterial cells, thereby causing homeostatic imbalance, cellular metabolic disturbance and even cell death [41]. Moreover, the NP-cell attachment would facilitate
the cell internalization of NPs and the intracellular ROS production [12]. If TiO\textsubscript{2} NPs are sufficiently small, they can penetrate in the cells, and then induce the potential photocatalytic process inside and adsorb and deactivate biomolecules such as proteins [22,42]. Therefore, the physical NP-cell attachment and interaction could substantially contribute to the observed nano-toxicity. Many studies [13,43–44] suggest that the antibacterial mechanism of NPs includes the disruption of bacterial cellular membrane. However, we do not know for sure yet why the anatase NPs had higher affinity to the cell surfaces than the rutile NPs, which could be possibly due to their different surface properties. It is indicated that the coordination and surface properties allow anatase but not rutile NPs after dispersion induce the generation of ROS [24].

4. Oxidative stress and lipid peroxidation induced by the NPs

Relative intracellular ROS productions following the exposures to the TiO\textsubscript{2} NPs (50 mg L\textsuperscript{-1}) are shown in Figure 6A. The produced ROS in the bacterial cells exposed to the anatase TiO\textsubscript{2} NPs was significantly (p < 0.05) higher than that in the blank control cells and increased with decreasing particle size; whereas the pure rutile TiO\textsubscript{2}-NP 50R had insignificant effect on the intracellular ROS production and TiO\textsubscript{2}-NP 25AR containing 7% rutile induced significantly lower intracellular ROS production compared with the pure anatase TiO\textsubscript{2}-NP 25A of the same particle size. The enhanced intracellular ROS would affect protein expression and function in the bacteria by interrupting translation and post-translational modification [45]. It has been indicated that TiO\textsubscript{2} NPs in anatase phase are capable of inducing generation of more ROS than that in the rutile phase [24,46] and thereby may cause higher cytotoxicity [47] including toward \textit{E. coli} [13,48–52]. The increased ROS generation in \textit{E.coli} exposed to the smaller and anatase TiO\textsubscript{2} NPs coincided with their enhanced bactericidal effects as shown in Figure 4, which suggests that the size and crystal phase of TiO\textsubscript{2} NPs played a critical role in the nanotoxicity and the nanotoxicity could be caused mainly by the elevated oxidative stress.

MDA productions in the \textit{E. coli} cells upon the exposures to the 50 mg L\textsuperscript{-1} TiO\textsubscript{2} NPs are shown in Figure 6B. Significantly higher MDA contents were observed in the NPs-treated cells compared with the blank control, indicating the cell membrane lipid peroxidation induced by the NPs. The MDA content was the highest in the TiO\textsubscript{2}-NP 10A treated cells and overall decreased with decreasing size of the anatase NPs, which was in the same order of the ROS production by anatase NPs. This implies that the lipid peroxidation could be mainly caused by the increased ROS.

Figure 7. The effects of pH (A) and NaCl (B) on the relative viability of \textit{E.coli} exposed to 10 mg L\textsuperscript{-1} TiO\textsubscript{2}-NP 10A for 3 h. Significant difference (p < 0.05) between two treatments is presented by different lowercase letters a, b and c. Error bars represent standard deviation (n = 3). doi:10.1371/journal.pone.0110247.g007
5. Effects of pH and ionic strength on the nanotoxicity

The bactericidal effect of the 10 mg L\(^{-1}\) TiO\(_2\)-NP 10A exhibited a significant dependence on the solution chemistry (Figure 7). No significant difference in the nanotoxicity was observed between pH 7.0 and 8.0, while the exposed bacteria presented significantly lower and higher viability at pH 5.0 and 10.0 as compared with that at pH 7.0, respectively (Figure 7A). Increasing the suspension pH from 5.0 to 10.0, the bacterial viability increased from 43.3% to 77.9%, indicating the decreasing nanotoxicity with increasing pH. It is generally considered that direct contact and adherence of NPs with the organism cell surfaces plays a critical role in the nanotoxicity [12]. Zeta potentials of the bacterial cells were all negative at the four pHs being −58.4, −56.7, −56.7 and −52.5 mV at pH 5.0, 7.0, 8.0 and 10.0, respectively; whereas zeta potential of TiO\(_2\)-NP 10A decreased from about 20 mV at pH 5.0 to lower than −40 mV at pH 10.0 (Figure 2). The positively-charge NPs at pH 5.0 could have a higher potential of contact and hetero-agglomeration with the negatively-charged bacterial cells through the electrostatic attraction and therefore had a higher antibacterial effect compared with the negatively-charged NPs at the three higher pHs.

It has been indicated that the antibacterial effect of TiO\(_2\) NPs (25 nm, P25) on *E.coli* was stronger at pH 5.5 versus 7.0 and 9.5 and the stronger antibacterial effect at the lower pH was attributed to the stronger accumulation of the NPs on the cell surfaces [35,34]. However, contradictory research result has also been reported. Planchnon et al. (2013) found a stronger adsorption of TiO\(_2\) NPs (25 nm, P25) on *E.coli* but a slightly lower toxicity at pH 5.0 versus 8.0, which was attributed to a better physiological state of *E.coli* bacteria at pH 5.0 (artificial water) versus 8.0 (surface water sample) [15]. Rincon and Pulgarin (2004) did not observe any difference in the *E.coli* deactivation rate by the TiO\(_2\) NPs (25 nm, P25) in the pH range of 4.0–9.0 [55]. The contradictory results on the pH effect may partly come from the difference in the crystal structure of the used TiO\(_2\) NPs, but the exact mechanisms remain to be studied.

It is observed that the addition of NaCl (50–200 mg L\(^{-1}\)) reduced the toxicity of TiO\(_2\)-NP 10A toward *E.coli* to an extremely significant extent (Figure 7B). Some studies suggest that NaCl introduced to the medium can decrease the toxicity effect on the bacteria by providing a barrier of steric hindrance between NPs and cells [15]. Li et al. (2013) found that saline ions promoted NP aggregation and reduced surface charge, and then inhibited the adsorption of NPs on bacterial surfaces, so higher saline ions could lead to higher cell viability [30]. Furthermore, ionic strength can also influence the tolerance of bacteria to toxicants [37,38]. The ionic strength of physiology salt-water (8.5 g L\(^{-1}\) NaCl) is isotonic and favorable for the bacterium survival. Hence, the bacteria were more tolerant to the NP suspensions at the higher ionic strengths (closer to the physiology salt-water).

Conclusions

The present study investigated the antibacterial effect of five types of TiO\(_2\) NPs with various crystal phase and particle size. A marked particle size and crystal phase dependent nanotoxicity was observed. Water chemistry, i.e. pH and ionic strength, could also significantly influence the bactericidal activity of the anatase TiO\(_2\) NPs. In conclusion, the TiO\(_2\) NPs with anatase phase and smaller particle size had higher affinity to the cell surfaces and induced heavier oxidative damage and toxicity to the bacterial cells, and the toxicity decreased with increasing pH (5.0–10.0) and ionic strength (50–200 mg L\(^{-1}\) NaCl). These findings substantiate the need to correlate the NP characterization and behavior in environmental matrices with the toxicological endpoints and to develop a common test strategy for the eco-toxicity study of NPs taking into consideration of various confounding factors relating to the NPs, bacterial cells, and the test environment in the near future.

Author Contributions

Conceived and designed the experiments: DHL XCL. Performed the experiments: XCL JYL SM GSL. Analyzed the data: XCL. Contributed reagents/materials/analysis tools: DHL KY MPT. Contributed to the writing of the manuscript: XCL DHL.

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