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EFFECTS OF N-TIO$_2$ AND BISPHENOL A ON CELLULAR STRESS INDICES OF THE FRESHWATER BIVALVE *UNIO TUMIDUS*

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Freshwater organisms including mollusks are occasionally exposed to nanoparticles rapidly intervening into environment from increased industrial and domestic use. However, their effects on freshwater mollusks remain unclear. We determined the effect of n-TiO_2 (1.25 μM) alone and in combination with bisphenol A (BPA, 0.88 nM) as well as of $TiCl_4$ (Ti, 1.25 μM) and BPA (as the positive controls) on selected parameters of oxidative stress, metabolic profile, immune, endocrine and nervous systems and genotoxic manifestations in widespread bivalve, *Unio tumidus*. It was shown that n-TiO_2 and BPA caused quite similar response and evoked in mollusks neuro-endocrine disorders, oppression of immunity and acidosis (only for n-TiO_2), exposure-specifically activated antioxidant system and genotoxicity. Meanwhile co-exposure (n-TiO_2+BPA) and Ti provoked the strong response of mollusks that is differed from the responses to BPA and n-TiO_2 alone. The most prominent signs included no endocrine disruption effect for n-TiO_2+BPA group, and harsh oxidative stress and genotoxicity (DNA fragmentation and nuclear abnormalities) for both (n-TiO_2+BPA) and Ti groups. These findings show that n-TiO_2 and BPA at realistic environmental concentrations significantly affected different functional and molecular parameters of freshwater bivalves and may pose a serious risk to mussel health. Obviously, n-TiO_2 acts as a catalyst of BPA decomposition and eliminate endocrine disruption effect inherent for BPA, but enhances oxidative stress response in mollusks.

Keywords: nanoparticles, TiO_2, bisphenol A, endocrine disruption, oxidative stress, *Unio tumidus.*

Titanium dioxide nanoparticles (n-TiO_2) have been widely using as a component in cosmetics, pharmaceuticals, food colorants, sunscreens and toothpastes [7]. Also, TiO_2 is considered as the most promising catalyst for photodegradation of bisphenol A (BPA), a representative endocrine disruptor, due to its excellent photocatalytic activity, low cost, and high stability [5]. However, extensive utilization of n-TiO_2 increases the risk of its interaction with other environmental pollutants and then appearance of unpredictable toxic effects for animals and humans. Meanwhile this issue is poorly studied despite several intermediate compounds, among them 4-isopropylphenol, hydroquinone and phenol, should be highly toxic to biota [4].

Surface water is anticipated to represent the ultimate sink for nanomaterials and organic compounds from non-point sources, where their chemical interactions and consequent fate may be critical in determining the biological effects. Filter-feeding organisms named mollusks should be primary affected by varieties of pollutants due to high rate of accumulation ability and potentially play an pivotal role in their uptake by endo- and phagocytosis, biotransformation and transfer through food chain [2,3,6]. The response of mollusks toward n-TiO_2 has been extensively studying on last decades in marine mussels then in freshwater bivalves such point remains unclear. Thereby the aim of present study was to estimate the molecular and cellular effects of

n-TiO_2 alone and in combination with BPA on the widespread freshwater mollusk, *Unio tumidus*. Based on our previous experience [1,2,3] we've filled asset up with selected parameters of oxidative stress (evaluated by the activities of Mn- and Cu,Zn- superoxide dismutase (SOD), tissue level of total glutathione (GSH), oxyradicals and sign of oxidative lesion protein carbonyls (PC)), metabolic profile (level of lactate and pyruvate), immune (phenoloxidase-like (PhO) activity), endocrine (vitellogenin-like protein, Vtg-LP) and nervous (cholinesterase, ChE) systems. The possible genotoxic effect of studied exposures was investigated by micronuclei in hemocytes and DNA strand break in digestive gland of mollusks.

Materials and Methods

Adult *Unio tumidus* (Unionidae) (8±1 cm length, and 42±5 g weight) were collected in early autumn from 0.5 to 1 m depth in a pristine site located in the upstream portion of Seret River (49°49' N, 25°23' E) and transported to the laboratory. One group was exposed to the tap water only and was considered control (C), other groups were exposed to nano-sized titanium dioxide (n-TiO_2, 1.25 μM, $M_{(size)}$ <150 nm, *Sigma*) and BPA (0.88 nM) separately and in combination, and also $TiCl_4$ (Ti, 1.25 μM, positive control) during 14 days with the replacing of water medium each two days.

The activities of Mn- and Cu,Zn-SOD (EC 1.15.1.1), ChE (EC 3.1.1.7), level of GSH, PC, lactate and pyruvate were determined in digestive gland by spectrophotometric methods, and oxyradicals and DNA strand breaks in digestive gland by fluorescence methods that were described in details in [1-3]. The phenoloxidase-like activity (PhO, EC 1.14.18.1) was determined spectrophotometrically by recording the formation of *o*-quinones [6]. Level of Vtg-LP was determined in hemolymph as alkali labile phosphates as it was described in [2,3]. Genotoxic effect of exposures was assessed in the immune cells (hemocytes) by the frequency of the micronuclei (MN) and other abnormalities [2,3]. For all traits and all experimental treatment groups, sample size was 8. The data are presented as M±SD. All statistical calculations were performed with Statistica v. 10.0. Differences were considered significant if the probability of Type I error was < 0.05.

Results and Discussion

We have shown that n-TiO_2 and BPA treatments had endocrine disruption effect according to two-fold elevation of Vtg-LP in hemolymph of male mollusks (Table). Except that exposures provoked neurotoxic (inhibition of ChE, most prominent in n-TiO_2 group) and genotoxic (manifested as increasing of MN and also other abnormalities for BPA-group) effects, oppression of immunity (inhibition of PhO activity) and shifting to anaerobiosis (only for n-TiO_2). Meanwhile exposure-specificity slight activation of antioxidant defense related to cellular thiols in n-TiO2 group and to Cu,Zn-SOD in BPA group consistently with no oxidative lesions was recorded. Under combine exposure (n-TiO_2+BPA) no endocrine disruption effect for males was shown. It should be served as an evidence of BPA decomposition by n-TiO_2. Besides that, co-exposure exerted the most prominent oxidative stress (due to calculation of integral index of oxidative stress, which was decreased in the sequence n-TiO_2+BPA (3.1 RU) >Ti (1.4 RU) > n-TiO_2 (0.43 RU) ~BPA (0.41 RU)), genotoxicity and immune system oppression. Obviously, intermediate compounds appeared under photocatalytic reaction of BPA decomposition, primary hydroquinone and phenol, stimulated oxidative stress and then exhibit toxic effects for bivalves which has shown *in vitro* for vertebrate and human cell cultures [4].

Ti provoked the strongest response of mollusks almost differ from n-TiO_2 which has allowed us to confirm lack of degradation of n-TiO_2 in water surrounding. Ti has typified by deep oxidative stress which characterized by simultaneous elevation of antioxidants (GSH, Cu,Zn-, Mn-SOD) and oxidative lesions (PC, OR), activation of energy metabolism, genotoxicity (DNA

fragmentation and nuclear abnormalities) and endocrine disruption sign (~2-fold elevation of Vtg-LP). Thereby in this study, we have identified a number of molecular and physiological changes in freshwater bivalves in response to TiO_2 and BPA exposure and may pose a serious risk to mollusks health. n-TiO_2 can serve as a catalyst of BPA decomposition and eliminate endocrine disruption effect but enhances oxidative stress response in mollusks compare to single-exposure effect.

Table

Effect of n-TiO_2 alone and under co-exposures with Bisphenol A on the cellular stress parameters and toxicity in the digestive gland and hemolymph of *Unio tumidus*

Parameters	Control	n-TiO_2	Ti	BPA	n-TiO_2+BPA
Total GSH, μmol·g^{-1} FW	1.5±0.1	3.1±0.1*	1.6±0.3	0.9±0.1*	1.4±0.2
Mn-SOD, RU·mg^{-1} proteins	2.7±0.3	2.6±0.3	3.8±0.8*	2.6±0.8	1.3±0.3*
Cu,Zn-SOD, RU·mg^{-1} proteins	1.0±0.2	0.6±0.2*	2.0±0.4*	2.5±0.5*	3.6±0.1*
PC, μmol·g^{-1} FW	0.52±0.04	0.52±0.06	0.64±0.05*	0.16±0.02*	0.10±0.02*
ChE, nmol·min^{-1} mg^{-1} proteins	2.8±0.3	1.9±0.4*	2.3±0.3*	2.3±0.3*	2.4±0.4
DNA strand-break, ng mg^{-1} proteins	75.8±16.2	100.4±18.2	137.7±23.1*	92.7±11.2	135.2±21.1*
Oxyradical, RFU mg^{-1} proteins	109.2±6.9	73.0±4.7*	127.9±6.9*	111.6±16.1	67.5±5.3*
Micronuclei, per 1000 cells	1.5±0.3	3.5±0.5*	2.5±0.4*	4.5±0.7*	2.5±0.4*
Other abnormalities, per 1000 cells	7.0±0.8	10.0±1.3	10.0±1.2	13.2±1.7*	13.0±1.6*
Phenoloxidase, μmol/(minЧg FW)	0.73±0.07	0.47±0.05*	0.79±0.05	0.56±0.11*	0.49±0.04*
Vtg-LP, μg P_i·mg^{-1} proteins	2.9±0.1	6.9±1.5*	6.6±2.0*	5.6±1.3*	2.0±0.2*
Lactat, nmol·mg^{-1} protein	232.3±29.8	398.7±56.1*	569.0±35.1*	244.5±45.5	461.0±90.6*
Piruvate, nmol·g^{-1} protein	78.4±6.2	66.2±12.1	141.8±30.3*	173.2±17.3*	87.5±8.7

Note: the asterisks indicate that the values are significantly different from the respective control values ($p<0.05$).

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ВПЛИВ H-TIO$_2$ ТА БІСФЕНОЛУ А НА ПОКАЗНИКИ КЛІТИННОГО СТРЕСУ У ПРІСНОВОДНОЇ ДВОСТУЛКИ *UNIO TUMIDUS*

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Прісноводні організми, у тому числі молюски, зазнають впливу наночастинок, які постійно потрапляють у навколишнє середовище в результаті збільшення їх промислового і побутового використання, проте їх вплив на прісноводних молюсків практично не з'ясований. Ми досліджували вплив нано-TiO_2 (1,25 мкМ) окремо та в суміші з бісфенолом А (БФА, 0,88 нМ), а також $TiCl_4$ (Ti, 1,25 мкМ) і БФА (позитивний контроль) за показниками окисного стресу, метаболічного стану, імунної, ендокринної та нервової систем і генотоксичності у двостулкових молюсків *Unio tumidus*. нано-TiO_2 і БФА викликають подібну відповідь у молюсків, зокрема нейро-ендокринні розлади, пригнічення імунітету і ацидоз (тільки за дії нано-TiO_2) та видоспецифічно активують антиоксидантний захист і сприяють появі ознак генотоксичності. Поєднана дія (нано-TiO_2 + БФА), а також Ti викликають відповідь у молюсків, відмінну від окремої дії чинників. Найбільш істотні прояви включають відсутність ендокринних розладів за сумісної дії нано-TiO_2 та БФА та істотний окисний стрес і генотоксичність (фрагментація ДНК та ядерні аномалії) для нано-TiO_2+BPA та Ti груп. Одержані дані свідчать, що нано-TiO_2 і БФА в реальних екологічних концентраціях істотно впливають на різні функціональні і молекулярні показники прісноводних двостулок і можуть становити серйозну небезпеку для їх стану. нано-TiO_2 може виступати каталізатором розкладу БФА і усувати ефект ендокринних розладів, характерний для окремої дії чинника, але посилює окисний стрес у молюсків.

Ключові слова: наночастинки, TiO_2, бісфенол А, ендокринні розлади, окисний стрес, *Unio tumidus.*

ВПЛИЯНИЕ H-TIO$_2$ И БИСФЕНОЛА А НА ПОКАЗАТЕЛИ КЛЕТОЧНОГО СТРЕССА У ПРЕСНОВОДНОЙ ДВУСТВОРКИ *UNIO TUMIDUS*

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Пресноводные организмы, в том числе моллюски, подвержены влиянию наночастиц, которые постоянно попадают в окружающую среду в результате их усиленного промышленного и бытового использования, однако их влияние на пресноводных моллюсков практически не изучено. Мы исследовали влияние нано-

TiO_2 (1,25 мкМ) отдельно и в смеси с бисфенолом А (БФА, 0,88 нМ), а также $TiCl_4$ (Ti, 1,25 мкм) и БФА (положительный контроль) на показатели окислительного стресса, метаболического состояния, иммунной, эндокринной и нервной систем и генотоксичности у двустворчатых моллюсков *Unio tumidus*. нано-TiO_2 и БФА вызывают подобный ответ у моллюсков, в частности нейро-эндокринные расстройства, угнетение иммунитета и ацидоз (только при действии нано-TiO_2) и видоспецифично активируют антиоксидантную систему и способствуют появлению признаков генотоксичности. Совместное действие нано-TiO_2 и БФА, а также Ti вызывают ответ у моллюсков, отличающийся от отдельного действия факторов. Наиболее существенные проявления включают отсутствие эндокринных расстройств при совместном действии нано-TiO2 и БФА, заметный окислительный стресс и генотоксичность (фрагментация ДНК и ядерные аномалии) для обеих нано-TiO_2+BPA и Ti групп. Полученные данные свидетельствуют, что нано-TiO_2 и БФА в реальных экологических концентрациях существенно влияют на различные функциональные и молекулярные показатели пресноводных двустворок и могут представлять серьезную опасность для их состояния. Нано-TiO_2 может выступать катализатором разложения БФА и устранять эффект эндокринных расстройств, характерных для отдельного действия фактора, но усиливает окислительный стресс у моллюсков.

Ключевые слова: наночастицы, TiO_2, бисфенол А, эндокринные нарушения, окислительный стресс, *Unio tumidus.*