Toxicity of nano-Zinc Oxide on Planarians (*Dugesia japonica*) with Neurobehavioral endpoints

Kiattipan Kanjana and Surisak Prasarnpun*

Department of Biology, Faculty of Science, Naresuan University,
Phitsanulok 65000 Thailand
*Corresponding author E-mail: surisakp@nu.ac.th

ABSTRACT

The research aims to study the effects of Zinc Oxide nanoparticles (ZnO NPs) on planarians by using locomotion behaviors as the endpoints. Sub-lethal doses of ZnO NPs of various concentration, *i.e.*, 100, 300, 500, 1000, and 2000 ppm were applied to the experimental groups, while the control groups were in artificial pond water (APW). Ten planarians per replicates, and three replicates were conducted. Planarians were observed morphological alterations and locomotion activities under stereo-microscope. Locomotion velocity (LV) and cumulative locomotion (CLM) were quantified as the number of gridlines crossed or re-crossed by planarians per minute over a 30-minute observation period. Data were collected every minute. Four patterns of planarian morphology, *i.e.*, irregular-shaped, screw-like, elongated, and bridge-like were found. However, irregular-shaped was found only in control group and planarians exposed to low concentration of ZnO NPs (100 ppm). In addition, bridge-like pattern had shorter latency time of response when ZnO NPs concentrations were increase. Compare to the control, the LV of the planarians exposed to ZnO NPs reduced significantly ($p < 0.05$) in every concentration leading to CLM in all groups of planarians exposed to ZnO NPs was also reduced significantly. Since morphological alterations are as the results of muscle contractions under the control of the nervous system, it is concluded that ZnO NPs toxic to planarians and had neurobehavioral effects.

Keywords: *Planaria, nano-Zinc Oxide, Toxicity, Neurobehavior*

INTRODUCTION

Nanotechnology is the most an emerging innovative field of the 21st century, which can lead to a new revolution in every field of science (Rico *et al*., 2011). This technology deals with nanoparticles that are atomic or molecular aggregates characterized by size less than 100 nm. These are actually modified form of basic elements derived by altering their atomic as well as molecular properties of elements. Several metal oxide nanoparticles are produced with possible future applications. Among variety of metal nanoparticles, zinc oxide nanoparticles (ZnO NPs) are considered to be one of the best exploited at nanodimentions, and very much important due to their vast area of utilization. It appears as a white powder and is nearly insoluble in water. The powder ZnO is widely used as an additive in

numerous materials and products including ceramics, glass, cement, rubber (such as car tyres), lubricants, paints, ointments, adhesives, plastics, sealants, pigments, food additive for source of Zn nutrient, batteries, ferrites, retardants, solar cells, and drug delivery (Golovina, 2012; Choi and Choy, 2014; Feng *et al*., 2015). In the Earth crust, ZnO is present as Zincite minerals but mostly ZnO NPs used for commercial purposes is produced synthetically (Sabir *et al*., 2014). Nowadays, one of the more frequently used of ZnO NPs is in the manufactured of sunscreens.

The increasing use of ZnO NPs has raised public concern to know about toxicological and environmental effects of ZnO NPs. Toxicological studies carried out on ZnO NPs in the last ten years showed that ZnO NPs have potential health as well as environmental risk. ZnO NPs can impose serious toxicity on bacteria, *Daphnia magna*, freshwater microalga, mice, rats, and even human cells (Brayner *et al*., 2006; Franklin *et al*., 2007; Heinlaan *et al*., 2008; Han *et al*., 2011; Xie *et al*., 2012; Ben-Slama *et al*., 2015; Feng *et al*., 2015). To study toxicology, planarians are one of the best characterized animal models for researches. Planarians are the first example of organisms displaying cephalization including a primitive brain with many features common to vertebrate nervous system. In addition, nearly every neurotransmitter found in mammals is present in planarians (Buttarelli *et al*., 2008). In fact, the planarian nervous system is more similar to vertebrate nervous systems than to invertebrate nervous systems, in term of both morphology and physiology. Thus, planarian worms are being rediscovered as useful organisms in neurobiology and pharmacology. As mentioned, because of the similarity of the planarian nervous system to vertebrate nervous system, this allows for the observation of behavioral responses in planarians comparable to vertebrate behavioral responses. In recent years, nanomaterials have been reported to be able to produce biological effects on the CNS of many animal models, such as mice and rats (Wohlfart *et al*., 2012; Powers et al., 2012). However, the biological fates of ZnO NPs are still unclear since some studies suggest that the primary bioavailable form of ZnO NPs in tissue is ionic zinc rather than the particulate form. This issue needs to be urgent studied. However, vertebrate experimental protocols require more preparation in terms of permission and ethical concerns. It is therefore, using of planarians for studying toxicology is a suitable model to investigate behavioral responses to the exposure of NPs acting on the nervous system, and can be representatives of other higher animals. Two specific behavioral endpoints have proven useful in pharmacological experiments: the observation of changes in motility (Raffa *et al.*, 2001), and the induction of planarian seizer-like movements (Rawl *et al*., 2011). Both responses are easily quantified and have been used to study the effects of a wide variety of agents and drugs. Herein, the research aims are to investigate the concentration effects of ZnO NPs for their toxicity by neurobehavioral observations without the usual considerations needed when working with vertebrate animals.

MATERIALS AND METHODS

Animals and Chemicals

Planarian worms (*Dugesia japonicum*) were kindly supplied from *The Institute of the Promotion of Teaching Science and Technology (IPTST)*. General laboratory reagents were purchased from Sigma-Aldrich (St. Louis, MO). The experimental nano ZnO was purchased from Ajax FineChem Pty Ltd.

General procedures

Planarians were transferred to Artificial Pond Water (APW) which contains: 6 mM NaCl, 1 mM $NaHCO_3$, 0.6 mM$CaCl_2$. The APW was adjusted pH to 6.9. Planarians were left to acclimate to the laboratory conditions for at least one week before being used. The worms with body length approximately1-1.5 cm were used within 2 weeks of arrival, and the APW was changed daily. Prior to the experiments, planarians were not fed for a week. All the experiments described in this work were in APW at room temperature. Ten planarians per replicates, and 3 replicates were conducted. The control groups were in APW while the other experimental groups were in APW with varying concentrations of ZnO NPs as the followings: 100, 300, 500, 1000 and 2000 ppm, respectively.

Locomotor experiments

To measure planarian locomotion, we followed the modified behavioral protocol of Pagan *et al*. (2006). This is a simple, yet useful procedure that can be used to study the effects of experimental compounds on planarian locomotion behavior. By using a small paintbrush, a worm was gently transferred to an APW-rinsed petri dish which was placed on a grid of 1 cm^2 square, followed by adding 5 ml of APW with or without ZnO NPs as indicated experimental design. Planarian motility was observed under stereo microscope, and measured by counting each time the worm crossed or re-crossed a square, minute by minute, over a period of 8 min, but observations on morphological alterations were checked until 30 min. Each worm was used only once. The data were graphed as cumulative crosses vs. time, and fit to a linear equation. Locomotion velocity (LV) was calculated and graphed as well as the latency time to response different concentrations of ZnO NPs were also observed.

Data analysis

The locomotion velocity, accumulative locomotion, and also latency time, were expressed as the mean ± S.E.M. One-way *ANOVA* was applied for testing the significant at $p < 0.05$.

RESEULTS

The results of the study showed planarians kept in APW had continuous movement during the observation period with normal morphology as shown in Figure 1(a). In contrast, planarians exposed to ZnO NPs of all concentrations exhibited obvious behavioral changes, hyperkinesia in response to exposure of ZnO NPs. In the extent experimental groups, alterations of body morphology and locomotors were found.

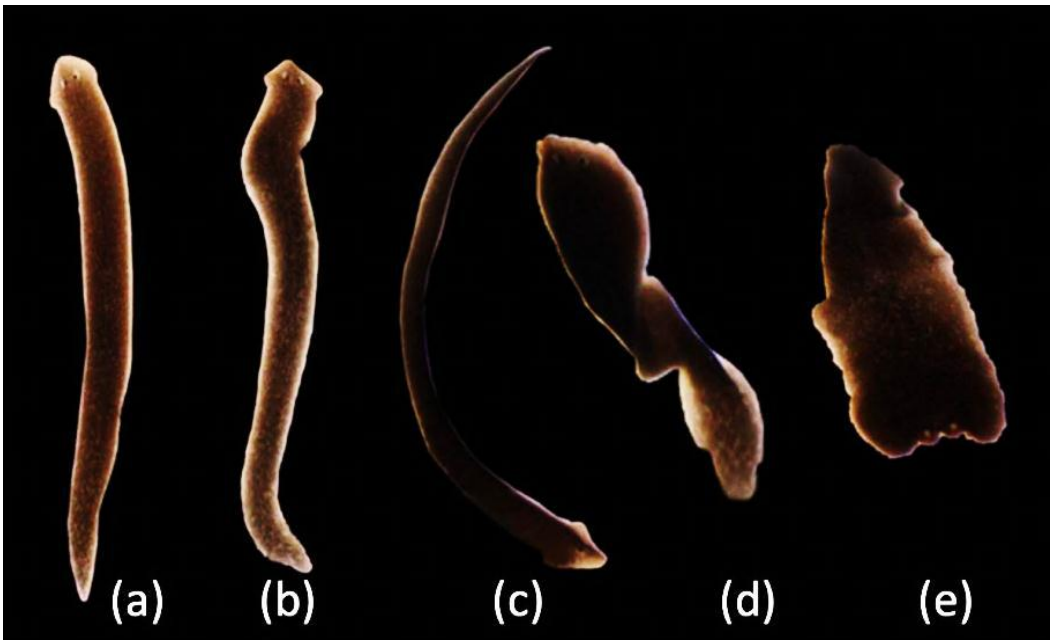

Figure1. Four patterns of motor behavior observed in planarians: (a.) normal locomotion; (b) elongated body; (c) bridge-like or C-shaped locomotion; (d) screw-like locomotion; (e) irregular-shaped locomotion.

The locomotors was categorized into 4 patterns; irregular, elongated, screw-like, and bridge-like or C-shape as shown in Figure 1 (b-e). Behavioral changes were dose-dependent manners, *i.e*., irregular shape locomotor activity was found only in lower concentration of ZnO NPs (100 ppm), but disappeared in higher concentrations (data shown in Table 1 and Figure 2). Higher doses caused the enhancement of the extent locomotion patterns; elongated, screw-like, and bridge-like as shown in Table 2-4 and Figure 2. The dose-dependent manners also revealed the shorter latency time once the concentration of ZnO NPs was increase (Table 1-4 and Figure 2).

Comparable to the controls, locomotion Velocity (LV) of planarians exposed to ZnO NPs was reduced in a dosed-dependent manner as well (Figure 3). As a result, cumulative locomotion of planarians in all higher doses was reduced too (Figure 4). These two figures also show clear concentration-dependence on the ZnO NPs ability to slow down the worms. Normal motor behavior was restored after 30-45 min in APW was also observed.

Table1. Latency time of irregular shape pattern of response of planarian in various concentrations of ZnO NPs

Concentration of ZnO (ppm)	Latency time to response of Irregular shaped locomotor pattern (minutes)
	Mean ± SE (n=4)
100	2.27 ± 0.01
300	0.00 ± 0.00
500	0.00 ± 0.00
1000	0.00 ± 0.00
2000	0.00 ± 0.00

Table2. Latency time of screw-like pattern of response of planarian in various concentrations of ZnO NPs (Values are means ± SE. Different letters in the same column indicate values that are statistically different at $P < 0.05$)

Concentration of ZnO (ppm)	Latency time to response of screw-like locomotor pattern (minutes)
	Mean ± SE (n=4)
100	9.76 ± 1.44^{a}
300	3.65 ± 0.43^{b}
500	2.64 ± 0.71^{b}
1000	5.50 ± 1.77^{b}
2000	1.54 ± 0.32^{b}

Table3. Latency time of elongate pattern of response of planarian in various concentrations of ZnO NPs (Values are means ± SE. Different letters in the same column indicate values that are statistically different at $p < 0.05$)

Concentration of ZnO (ppm)	Latency time to response of elongate locomotor pattern (minutes)
	Mean ± SE (n=4)
100	16.79 ± 1.14^{a}
300	11.92 ± 1.06^{b}
500	7.51 ± 0.33^{cd}
1000	8.96 ± 0.78^{ce}
2000	13.22 ± 2.00^{bf}

Table4. Latency time of bridge-like pattern of response of planarian in various concentrations of ZnO NPs (Values are means ± SE. Different letters in the same column indicate values that are statistically different at $P < 0.05$)

Concentration of ZnO (ppm)	Latency time to response of bridge-like locomotor pattern (minutes)
	Mean ± SE (n=4)
100	47.08 ± 5.99^{a}
300	44.73 ± 2.64^{a}
500	31.30 ± 2.17^{b}
1000	16.82 ± 1.49^{cd}
2000	20.99 ± 1.99^{ce}

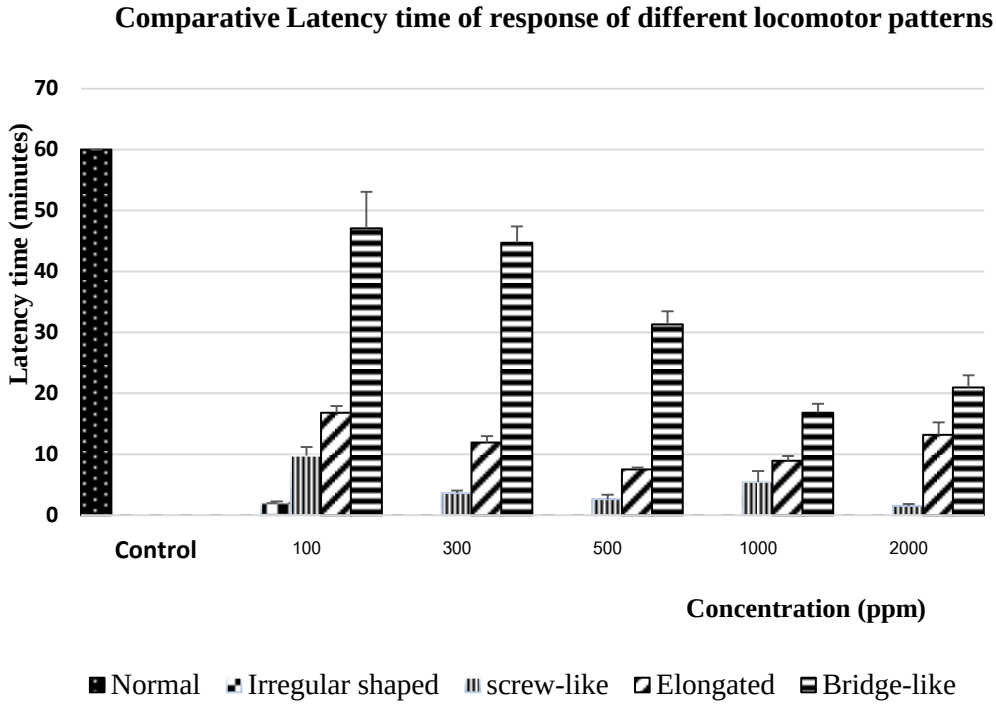

Figure2. Comparative of concentration-dependent responses with different morphological and behavioral patterns, and latency time of responses in planarian exposed to ZnO NPs. Note that the irregular shape disappeared in higher concentrations.

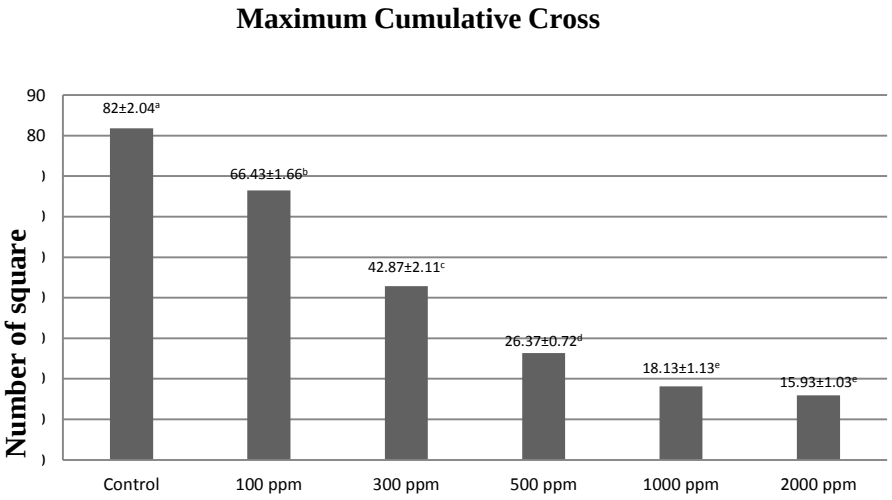

Figure3. Cumulative locomotor activities of planarians over the course of 8 min observations in both non-treated and treated planarians with ZnO NPs. (Values are means ± SE. Different letters on different bars indicate values that are statistically different at $p < 0.05$)

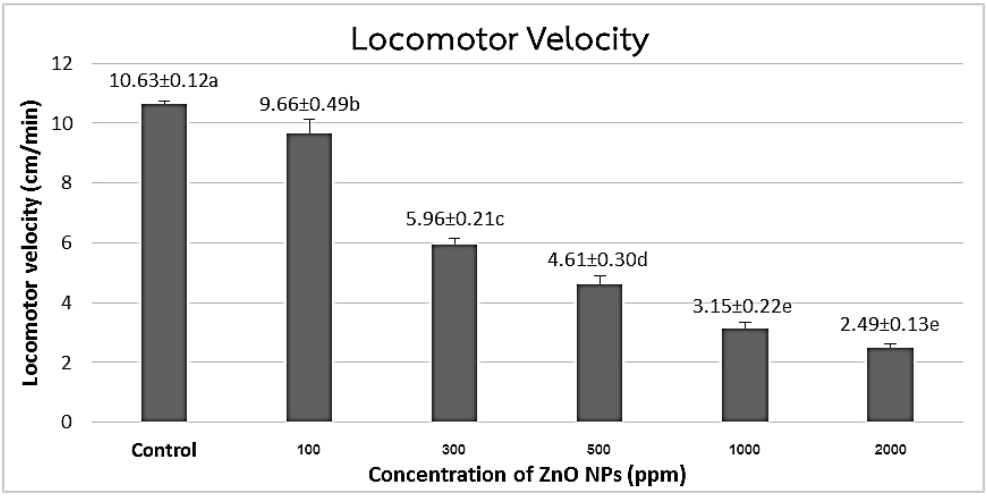

Figure4. Concentration-dependent locomotor velocity of planarians exposed to different concentrations of ZnO NPs. (Values are means ± SE. Different letters on different bars indicate values that are statistically different at $p < 0.05$)

DISCUSSION AND CONCLUSION

The impact of NPs on animal behavior has received considerable attention in recent years. Herein, we report the effect of ZnO NPs on selected aspects of planarian motility. To our knowledge, there were not any previous reports on toxicity of ZnO NPs in lower animals. Most reports were on rodents and mammals. This should be useful data to fulfill the gap of knowledge. Planarians display two main types of locomotor movements, gliding and crawling. Gliding is modulated by ciliary action, thought to be independent from the nervous system. Crawling is mainly modulated by muscular contraction when the worms are stimulated. Dopamine (DA) and serotonin (5-HT) are two of the major neurotransmitters found in freshwater planarians. DA modulates the locomotor activity of planarians and 5-HT controls planarian regeneration and mediates the regulation of some neuromuscular functions. In addition, acetylcholine (ACh) and dopamine interactions have also been reported involved the behavioral changes of planaria (Buttarelli *et al*., 2000). Reduction of cholinergic transmission seems to play a pivotal role in determining hyperkinesia in planaria was previously reported by Buttarelli *et al*., (2000).

Distribution of nanoparticles after penetration through the living things body wall was also of interest. Cellular and sub-cellular locations of manufactured nanoparticles were questioned and attracted many researchers to find out. *In vitro* studies have helped to highlight the existence of nanoparticles in neurons, astrocytes, and microglial cells, but potentially all cell types in the brain could be involved. Once the nanoparticles penetrated into neurons and glial cells, they may be directed to the lysosomes or persist in the cytoplasm offering to the opportunity to interact with other organelles. Electron microscopy studies have shown the presence of nanoparticles within the glial and neuronal cells. For instance, AgNPs of 20 nm were found mostly in the lysosomes of astrocytes (Locht *et al*., 2011; Haase *et al*., 2012). Silica-based nanoparticles engineered for nanomedicine tools were detected in the endoplasmic reticulum (ER) and in the cytoplasm of microglial cells (Ducray *et al*., 2017), suggesting that the subcellular location can be highly reliant on the dimension of the nanoparticle. The proven possibility of nanoparticles to reach the nervous system, together with the subcellular detection of nanoparticles within the cells of the nervous system, attention for the study of the possible effects on the function of neurons had been taken. Recently, Shang *et al*. (2014) reported the interactions of nanoparticles with live cells by quantitative fluorescent microscopy. The conclusion of their experiments was that NPs with diameters of less than 10 nm were observed to accumulate at the plasma membrane before being internalized by the cells. In contrast, larger NPs (100 nm) were directly internalized without prior accumulation at the plasma membrane, regardless of their surface charges. They suggested that the distinct size dependence to the requirement of a sufficiently strong local interaction of the NPs with the endocytic machinery in order to trigger the subsequent internalization.

Recently, investigations of nanoparticles on behavior and mechanosensory organ of invertebrate have been done by Sabat *et al*. (2016). The research has been observed in *Drosophila melanogaster* focusing on larva crawling and climbing

behavior of hatched flies. It was concluded that titatniun dioxide nanoparticles generate oxygen species which can modify multiple signaling pathways and thus can alter the development and behavioral pattern of the fly.

Since neurons ensure the transfer of information, it is key to note that variations in electrical activities have been documented including studies on neurons isolated from the hippocampus (Xu *et al*., 2009; Zhao *et al*., 2009; Liu *et al*., 2012a) and primary murine cortical networks (Gramowski *et al*., 2010). More information on studies on the effects of variety nanoparticles on the functions of neurons has been reviewed by Bencsik *et al*. (2018). These studies indicated that silver nanoparticles (50-100 nm, 10 µg/ml) inhibited postsynaptic currents in neurons from the CA1 region of the hippocampus (Zhaowei *et al*., 2009), and that ZnO nanoparticles (20-80 nm, 10 µg/ml) are able to increase the entry of Na^+ and the output of K^+ from the neurons of the CA3 region, enhancing their excitability while CuO nanoparticles had small effects on transient outward potassium current (Liu *et al*., 2012b).NPs, such as Cd, have been reported by Wu *et al*. (2014) to disturb the neurotransmitter levels and their metabolic enzyme activity in freshwater planarians. Hyperkineathesias of planarians as a result of NPs in dose-dependent manner were also reported by Hagstorm *et al*. (2016). Some studies have indicated that ZnO NPs affected functions of different cells or tissues, including neurons (Song *et al*., 2008; Osmond and McCall, 2010) which were suggested to modulate synaptic transmission (Zhao *et al*., 2009). In summary, this study verified that ZnO NPs could ameliorate the behavior of planarians through disturbing of neurotransmitter balance, and may affect the biosynthetic pathway. More investigations will be required for the better understanding of the neuronal effects of nanosized materials and their mechanisms.

ACKNOWLEDGEMENT

We are very grateful for the financial and institutional support from the Department of Biology, Naresuan University. We also gratefully acknowledge *the IPTST* for its kindly supplied of experimental animals.

REFERENCES

Ben-Slama, I., Mrad, I., Rihane, N., Mir, LE.,Sakly, M and Amara, S. (2015). Sub-acute oral toxicity of zinc oxide nanoparticles in male rats. *Journal of Nanomedicine and Nanotechnology*, 6(3), doi 1000284.

Bencsik, A., Lestaevel, P. and Canu, I.G. (2018). Nano- and neurotoxicology: An emerging discipline. *Progress in Neurobiology*, 160, 45-63.

Brayner, R., Ferrari-Iliou, R., Brivois, N., Djediat, S., Benedetti, MF. and Fievet, F. (2006). Toxicological impact studies based-on *Escherichia coli* bacteria in ultrafine ZnO nanoparticles colloidal medium. *Nanoscience Letters*, 6(4), 866-870.

Buttarelli, FR., Pellicano, C. and Pontieri, FE. (2008). Neuropharmacology and behavior in planarians: Translation to mammals. *Comparative Biochemistry and Physiology, Part C. Toxicol Pharmacol*., 147, 399-408.

Choi, SJ. and Choy, JH. (2014). Biokinetics of Zinc Oxide nanoparticles: toxicokinetics, biological fates, and protein interaction. *International Journal of Nanomedicine*, 9(suppl. 2), 261-269.

Ducray, AD., Stojilikovic, A., Moller, A., Stoffel, MH., Widmer, HR., Frenz, M. *et al*. (2017). Uptake of silica nanoparticles in the brain and the effects on neuronal differentiation using different *in vitro* models. *Nanomedicine*, 13, 1195-1204.

Feng, X., Chen, A., Zhang, Y., Wang J., Shao, L. and Wei, L. (2015). Application of dental nanomaterials: Potential toxicity to the central nervous system. *International Journal of Nanomedicine*, 10, 3547-3565.

Franklin, NM., Rogers, NJ., Apte, SC., Batley, GE., Gadd, GE. *et al*. (2007). Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and $ZnCl_2$ to a freshwater microalga (*Pseudokirchneriella subcapitata*): the importance of particle solubility. *Environmental Science and Technology*, 41(24), 8484-8490.

Golovina, N. (2012). Toxicity of nanoparticles. *Marie Curie Initial Training Network Environmental Chemoinformatics (ECO) Final Report*, 16 pp.

Gramowski, A., Flossdorf, J., Bhattacharya, K. Jonas, L., Lantow, M., Rahman, Q. *et al*. (2010). Nanoparticles induce changes of the electrical activity of the neuronal networks on microelectrode array neurochips. *Environmental Health Perspect*ive., 118, 1363-1369.

Haase, A., Rott, S., Mantion, A. Graf, P., Plendl, J., Thunemann, AF. *et al*. (2012). Effects of silver nanoparticles on primary mixed neural cell cultures: uptake, oxidative stress and acute calcium responses. *Toxicological Sci*ence., 126, 457-468.

Hagstorm, D., Escartin, OC. and Collins, ES. (2016). Planarian brain regeneration as a model system for developmental neurotoxicology. *Regneration*, 3(2), 65-77.

Han, D., Tian, Y., Zhang, T., Ren, G. and Yang, Z. (2011). Nano-zinc oxide damages spatial cognition capability via over-enhanced long-term potentiation in hippocampus of Wistar rats. *Journal of Nanomedicine*, 6, 1453-1461.

Heinlaan, M., Ivask, A., Blinova, I., Dubeourguier, HC. and Kharu, A. (2008). Toxicity of nanosized and bulk ZnO, CuO and TiO_2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. *Chemosphere*, 71(7),1308-1316.

Liu, Y., Guan, W., Ren, G. and Yang, C. (2012a). The possible mechanism of silver nanoparticle impact on hippocampal synaptic plasticity and spatial cognition in rats. *Toxicology Lett*ers, 209, 227-231.

Liu, Y., Zhang, T., Ren, G. and Yang, Z. (2012b). Nano-Ag inhibiting action potential independent glutaminergic synaptic transmission but increasing excitability in rat CA1 pyramidal neurons. *Nanotoxicology*, 6, 414-423.

Locht, LJ., Perdersen, MO., Markholt, S, Bibby, BM., Larsen, A. *et al*. (2011). Metallic silver fragments caused massive tissue loss in the mouse brain. *Basic Clinical and Pharmacological Toxicology*, 109, 1-10.

Osmond, MJ. and McCall, MJ. (2010). Zinc oxide nanoparticles in modern sunscreens: an analysis of potential exposure and hazard. *Nanotoxicology*, 4(1), 15-41.

Pagan, OR., Rowlands, AL. and Urban, KR. (2006). Toxicity and behavioral effects of dimethylsulfoxide in planaria. *Neuroscience Letters*, 407, 274-278.

Powers, CM., Bale, AS., Kraft, AD. *et al*. (2013). Developmental neurotoxicity of engineered nanomaterials: identifying research needs to support human health risk assessment. *Toxicological Science*, 134(2), 225-242.

Raffa, RB., Holland, LJ. and Schulingkamp, RJ. (2001). Quantitative assessment of dopamine D2 antagonist activity using invertebrate (Planaria) locomotion as a functional endpoint. *Journal of Pharmacological and Toxicological Methods*, 45, 223-226.

Rawls, SM., Patil, T., Tallarida, CS., Baron, S., Kim, M., Song, K. *et al*. (2011). Nicotin behavioral pharmacology: clues from planarians. *Drug and Alcohol Dependent*, 118, 274-279.

Rico, CM., Majumdar, S., Duarte-Gardea,M., Peralta-Videa, R. and Gardea-Torresdey. (2011). Interaction of nanoparticles with edible plants and their possible implication in the food chain. *Journal of Agricultural and Food Chemistry*, 59(8), 3485-3488

Sabat, D., Patnaik, A, Ekka, B., Dash, P. and Mishra, M. (2016). Investigation of titania nanoparticles on behavior and mechanosensory organ of *Drosophila melanogaster*. *Physiology & Behavior*, 167, 76-85.

Sabir, S., Arshad, M. and Chaudhari, SK. (2014). Zinc Oxide nanoparticles of Revolutionizing Agriculture: Synthesis and Applications. *The Scientific World Journal*, DOI 925494, 8pp.

Shang, L., Nienhaus, K., Jiang, X, Yang, L., Landfester, K., Mailänder, V. *et al*. (2014). Nanoparticle interactions with live cells: Quantitative fluorescence microscopy of nanoparticle size effects. *Beilstein Journal of Nanotechnology*, 5, 2388-2397.

Song, W., Wu, C., Yin, H., Liu, X., Sa, P. and Hu, J. (2008). Preparation of PbS nanoparticles by phase-transfer methods and application to Pb-selective electrode based on PVC membrane. *Analytical Letters*, 41(15), 2844-2859.

Wohlfart, S., Gelperina, S. and Kreuter, J. (2012). Transport of drugs across the blood-brain barrier by nanopartilces. *Journal of Control Release*, 161(2), 264-273.

Wu, JP., Lee, HL. And Lee, MH. (2014) Cadmium neurotoxicity to a freshwater planarian. *Archives of Environmental Contamination Toxicology*, 67, 639-650.

Xie, Y., Wang, Y., Zhang, T., Ren, G. and Yang, Z. (2012). Effects of nanoparticle zinc oxide on spatial cognition and synaptic plasticity in mice with depressive-like behaviors. *Journal of Biomedical Science*, 19, 14.

Zhaowei, L., Guogang, R., Tao, Z. and Zhuo, Y. (2009). Action potential changes associated with the inhibitory effects on voltage-gated sodium current of hippocampal CA1 neurons by silver nanoparticles. *Toxicology*, 264, 179-184.

Xu, LJ., Zhao, JX., Zhang, T., Ren, GG. and Yang, Z. (2009). *In vitro* study on influence of nanoparticles of CuO on CA1 pyramidal neurons of rat hippocampus potassium currents. *Environmental Toxicol*ogy, 24, 211-217.

Zhao, J., Xu, L., Zhang, T., Ren, T. and Yang, Z. (2009). Influences of nanoparticle zinc oxide on acutely isolated rat hippocampal CA3 pyramidal neurons. *Neurotoxicology*, 30(2), 220-230.