

Original Article

Effect of nanoparticles injected into larvae on spermatogenesis in the pupal testis of the sweet potato hornworm, *Agrius convolvuli* (L.)

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(Received December 14, 2014; Accepted December 22, 2014)

ABSTRACT — Lepidopteran species fly freely in the environment and their larvae feed on the leaves of host plants which may be exposed to nanomaterials. As an ecological model of nanoparticle exposure, 5th instar larvae of the sweet potato hornworm (*Agrius convolvuli*) were subcutaneously injected with suspensions of 10 μ L (100 μ g/mL) titanium dioxide nanoparticles (TiO₂-NPs), 10 μ L (100 μ g/mL) zinc oxide nanoparticles (ZnO-NPs) or saline (control) and the effects on spermatogenesis were examined in the pupal testis. During the larval wandering stage, larval tissues (except the testis) underwent extremely rapid histolytic changes. Pupation and emergence were not affected by the injection. On pupal day 4, there was a significant decrease in testis weight and the number of sperm bundles in the ZnO-NPs group. Electron microscopic observation revealed that cyst cells surrounding the spermatogenic cells took on small agglomerates of TiO₂-NPs or ZnO-NPs by phagocytosis. As spermatogenesis advanced in the nanoparticle-injected groups, vacuoles of various sizes were found in the nuclei of spermatocytes, the nuclear chromatin of spermatids was uncondensed and some vacuoles were found in the nuclei of sperm bundles. A possible mechanism for this is that abnormal vacuoles disturbed the chromatin condensation, resulting in the decrease of sperm bundles. Toxicity of manufactured TiO₂-NPs and ZnO-NPs were demonstrated detrimental to insect spermatogenesis.

Key words: Hornworm, Pupae, Spermatogenesis, Injection, Titanium dioxide, Zinc oxide

INTRODUCTION

Due to their wide range of uses in nanotechnology, nanomaterials are inevitably discharged into the environment and the potential for the environmental health hazard is drawing increasing attention (Gottshalk *et al.*, 2009; Gottshalk and Nowack, 2011; Klaine *et al.*, 2008; Mueller and Nowack, 2008; Nowack and Bucheli, 2007; Oberdörster *et al.*, 2005; Warheit *et al.*, 2008). When titanium dioxide nanoparticles (TiO₂-NPs) and zinc oxide nanoparticles (ZnO-NPs) are used in cosmetics, they do not penetrate normal human skin and are therefore

assumed to pose no risk to human health (Nohynek *et al.*, 2010).

The reproductive toxicity of nanomaterials has been investigated mainly in mammals (Bai *et al.*, 2010; Kyjovska *et al.*, 2013; Yamashita *et al.*, 2011; Yoshida *et al.*, 2010). We previously reported that anatase-type TiO₂-NPs (25-70 nm) injected during the maternal period caused testicular dysfunction in male offspring (Takeda *et al.*, 2009) and that the shift of small agglomerates of rutile-type TiO₂-NPs (below 200 nm in size) from the mother's body to the offspring testis has been demonstrated (Kubo-Irie *et al.*, 2014).

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Little research has been reported the reproductive toxicity on insects. In the complete metamorphosis, the larval tissues (except the testis) undergo extremely rapid histolysis and spermatogenesis progresses rapidly in the pupal testis (Shimoda *et al.*, 2007). Here, we examine the effects of TiO₂-NPs and ZnO-NPs injected into larvae on spermatogenesis in the pupal testis of the sweet potato hornworm (*Agrius convolvuli*).

MATERIALS AND METHODS

Insects

Agrius convolvuli eggs were obtained from a laboratory colony of National Institute of Agrobiological Science (Tsukuba, Ibaraki, Japan). Larvae are maintained in an insectarium using an artificial diet containing sweet potato leaf powder. Non-diapause pupae were produced by rearing larvae at 27°C under a 16 hr light, 8 hr dark photoperiod (Fig. 1a).

Schematic of the experiment

On larval day 3, 5th instar larvae were subcutaneously injected with 10 µL (100 µg/mL) suspension containing either TiO₂-NPs or ZnO-NPs. The suspension was sonicated for 30 min before injection by the micro-syringe with a fine-glass needle (130 µm in inner diameter) (ITO Co., Shizuoka, Japan). Larvae and pupae were maintained individually in a plastic cup. Testis and body weights of the pupae were measured on pupal day 4 (PD4) (Fig. 1b). According to Shimoda *et al.* (2007), the testes of PD4 pupae are characterized as being composed entirely of spermatogenic cysts; spermatogonia containing less than 32 nuclei, spermatocytes with 64 or 128 nuclei and sperm bundles with 256 filiform nuclei. The control group received phosphate buffered saline (PBS), which was injected in the same manner.

Characterization of TiO₂-NPs and ZnO-NPs

Anatase-type TiO₂-NPs and ZnO-NPs in white pow-

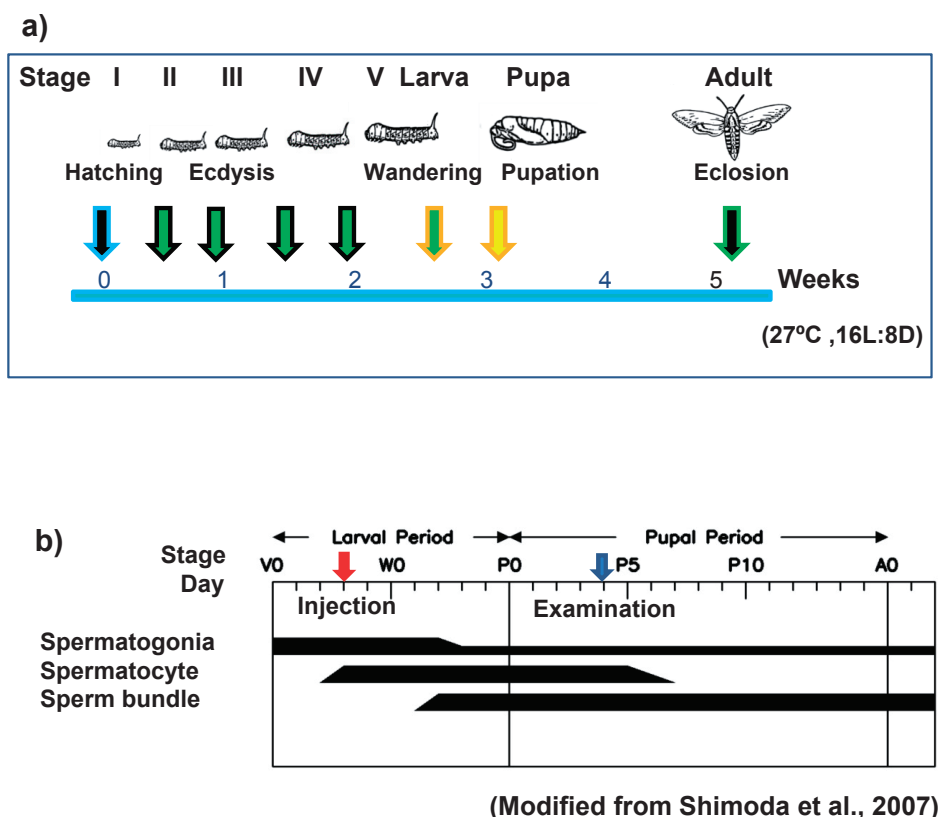


Fig. 1. Schematic of the *in vivo* insect experiment. a) The life cycle of the hornworm (*Agrius convolvuli*) is 36-37 days under the condition of 27°C temperature under 16 hr light, 8 hr dark cycle. b) Schematic of the experiment. Fifth instar larvae were subcutaneously injected on larval day 3 and the testes were examined on pupal day 4 (PD4).

der form were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Tayca Co. (Osaka, Japan), respectively. They were suspended at 100 µg/mL in PBS and sonicated for 30 min before the injection. A drop of the suspension was placed on formvar-coated copper grids, which were fully drained and allowed to air dry. Particle size and shape were observed by transmission electron microscopy (TEM) (JEM 1200 EX II, JEOL, Ltd., Tokyo, Japan) and size distribution was analyzed by dynamic light scattering (DLS) measurement using an LB-550 (Horiba, Ltd., Tokyo, Japan).

Tissue preparation for optical microscopy

Supravital staining with Hoechst 33342 (H342, 5 µg/mL; Sigma-Aldrich) dye was used to evaluate the development of spermatogenic cells in the testes. Testes from PD0-PD14 pupae were dissected and their contents were spilled into a modified Kiev solution, in which spermatogenic cells were stained by incubation with H342 for 10 min. The stained cells were mounted on glass slides and observed under a Leica 5000B microscope equipped with fluorescence optics (Leica Mikrosysteme Vertrieb GmbH, Vienna, Austria). Furthermore, the testes of six PD4 pupae were fixed in 10% neutral buffered formalin then dehydrated with an ethanol series and xylene, embedded in paraffin, cut into 5 µm sections and mounted on glass slides. Following paraffin removal and rehydration, the sections were stained with hematoxylin and eosin (HE).

The number of spermatogonial cysts, spermatocytic cysts and sperm bundles were counted in five slides with 500 x 600 µm in area from each testis (n = 6). All images were captured with an attached Leica DFC 300FX digital camera (Leica Microsystems Digital Imaging, Cambridge, UK). Student's *t*-test was used for statistical analysis. KyPlot version 5 (Kyens Lab, Tokyo, Japan) was used to perform the analysis. All values are expressed as mean ± standard error. $P < 0.05$ was considered statistically significant.

Tissue preparation for TEM

The dissected testes of the pupae were placed directly in the primary fixative, 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer at pH 7.4 and left overnight, then post-fixed with 2% OsO₄ for 1 hr. Samples were dehydrated through graded levels of ethanol and embedded in Quetol 812 (Nissin EM, Tokyo, Japan). Ultrathin-sections (85 nm thickness) were made with the ultra-microtome EMUC6 (Leica Microsystems K.K., Tokyo, Japan), stained with uranylacetate and lead citrate and then observed under TEM.

RESULTS

Characterization of TiO₂-NPs and ZnO-NPs

The TEM micrographs demonstrated the shapes and sizes of TiO₂-NPs and ZnO-NPs. They formed small agglomerates of various sizes in which TiO₂-NPs were spherical and ZnO-NPs were either spherical or rod-shaped (Fig. 2a and b). There were two peaks in the agglomerate sizes of TiO₂ and ZnO in the suspensions analyzed by dynamic light scattering (DLS) measurement (Fig. 1c and d). The large agglomerates were cut off using by the micro-syringe.

Effects of NPs injection on PD4 body and testis weight

Larval development was not affected by the subcutaneous injection of TiO₂-NPs or ZnO-NPs. The pupation of all groups occurred within 2 days. Adult moths that emerged from the pupae survived after the experiment. The weight of the testes and body of pupae at PD4 were compared among the TiO₂-NPs, ZnO-NPs and control groups. There was no significant difference between these groups in body weight, but there was a decrease in testis weight between the NP-injected groups (TiO₂-NPs, 17.0 ± 2.9 mg; ZnO-NPs, 16.4 ± 3.7 mg) and the control group (18.9 ± 3.2 mg). The testes weight in the ZnO-NPs group, however, was significantly decreased in comparison to the other groups (n = 10, $P < 0.05$) (Table 1).

Effects of TiO₂-NPs and ZnO-NPs on spermatogenesis

Observation under supravital staining with H342 using an optical microscope equipped with fluorescence optics revealed that the pupal testis was composed entirely of spermatogenic cysts at PD4 (Fig. 3a) and mostly occupied by sperm bundles at PD10 (Fig. 3b). Histological examination of the pupal testis was performed under optical microscope on PD4 with HE-stained sections. The testis was enveloped with a thick layer of testicular wall and consisted of several follicles. Cysts of spermatogonia were found adjacent to the testicular wall. Cysts of spermatocytes and sperm bundles were centralized in the lumen. Each follicle of the control and TiO₂-NPs groups (Fig. 3c and d) was occupied by spermatogenic cells, whereas the spermatogenic cells of the ZnO-NPs group were dispersed in the compartment (Fig. 3e).

Spermatogenic cells, spermatogonia, spermatocytes and sperm bundles were defined for counting on the basis of morphology. The total numbers of spermatogenic cells in each testis were 430 ± 80.4, 401.8 ± 80.2 and 261.3 ± 69.4 in the control, TiO₂-NPs and ZnO-NPs

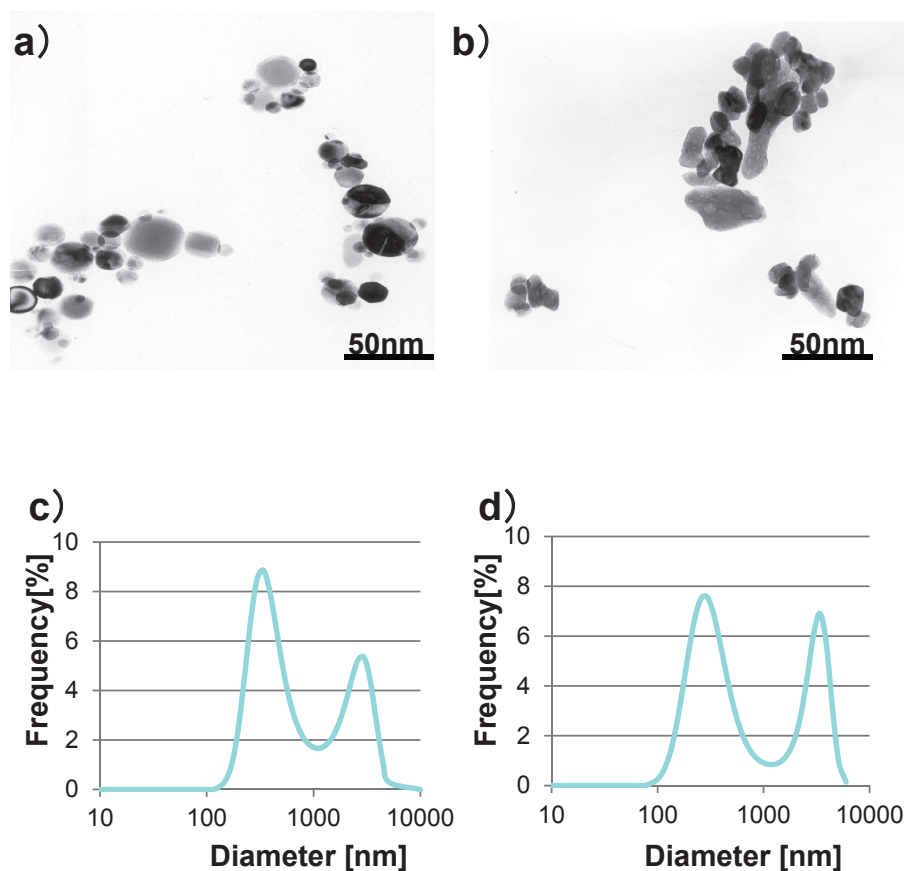


Fig. 2. TEM micrographs of nanoparticles. a) Small agglomerates of TiO₂-NPs were spherical. b) Agglomerates of ZnO-NPs were either spherical or rod-shaped. Two peaks of agglomerate sizes were observed by dynamic light scattering measurement: 477.8 nm and 3.6 μm of TiO₂-NPs (c) and 408.7 nm and 2.9 μm in ZnO-NPs (d).

Table 1. Effect of TiO₂-NPs and ZnO-NPs on weight of body and testis.

	Control (n = 10)	TiO ₂ -NPs (n = 10)	ZnO -NPs (n = 10)
Body (g)	4.92 ± 0.43	4.46 ± 0.30	4.97 ± 0.38
Testes (mg)	18.0 ± 3.21	17.00 ± 2.90	16.40 ± 3.71*

Values are mean ± standard error, * P < 0.05

groups, respectively. There were no significant differences between the three groups in the numbers of spermatogonia or spermatocytes. However, there was a significant decrease in the number of sperm bundles in the ZnO-NPs group (n = 6, P < 0.01) (Fig. 3f).

TiO₂-NPs and ZnO-NPs found in cyst cells

Under TEM, the surface of cyst cells showed phagocytic activity, indicating the presence of microvilli and numerous phagosomes in the cytoplasm (Fig. 4a and c).

Electron-dense particles were found around the surface in the TiO₂-NPs group. At high magnification (X 100,000), particles composed of a few nanoparticles were recognized as spherical, the same as the original shape of TiO₂-NPs (Fig. 4 b). Particles in the phagosomes were spherical or rod-shaped; similar to the original shape of ZnO-NPs (Fig. 4d).

Ultrastructural alteration of spermatogenesis

Observation under TEM showed that a layer of cyst

NPs affected on spermatogenesis in the testis of insect pupae

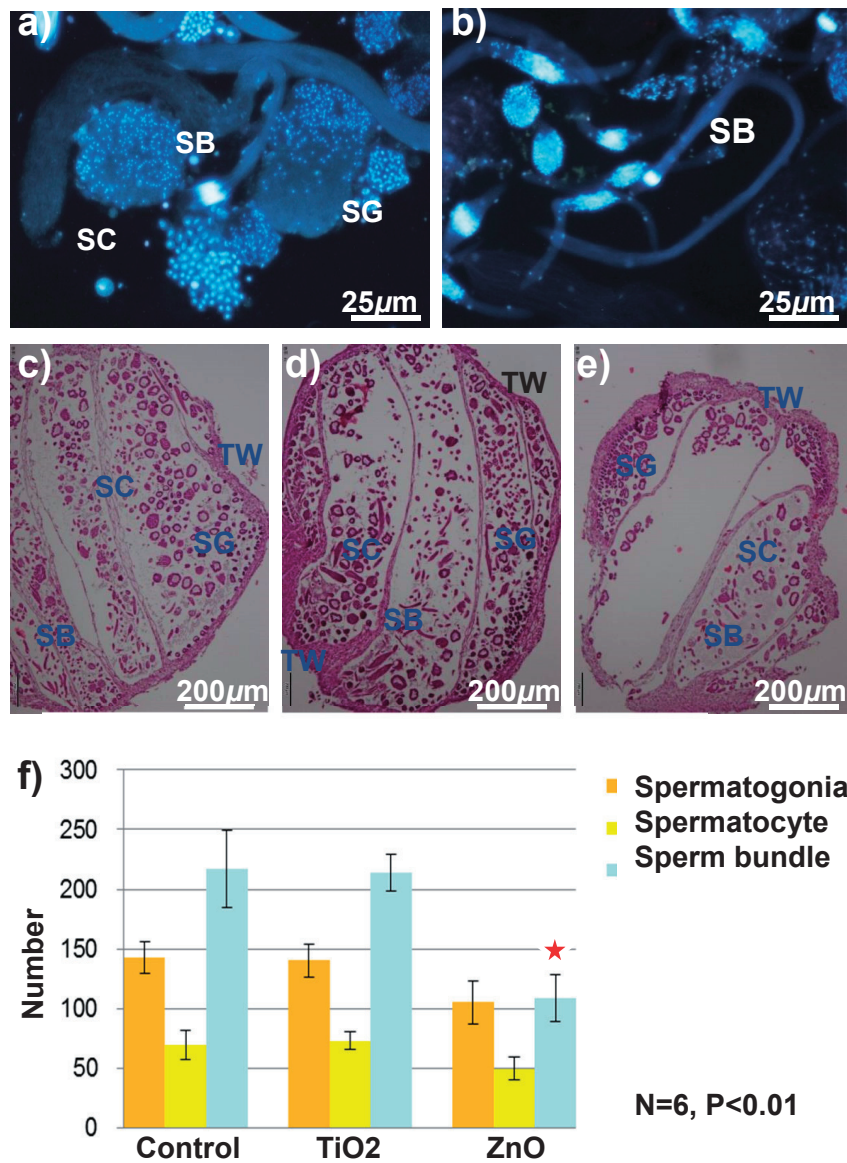


Fig. 3. Optical microscopic observation of effects on spermatogenesis of exposure to different NPs. a) The testis of pupae at pupal day 4 (PD4) of supravital staining with H342 under an optical microscope equipped with fluorescence optics. The testis was composed of spermatogenic cysts; spermatogonium containing less than 32 nuclei, primary spermatocytes with about 64 nuclei and sperm bundles with filiform nuclei. b) The same staining at PD10, the testis was mostly occupied by sperm bundles. c) The testis of the control group at PD4 of HE-stained sections under optical microscope. d) The TiO_2 -NPs group. e) The ZnO-NPs group. f) The number of spermatogenic cells in sperm bundles was significantly decreased in the ZnO-NPs group. Data are presented as mean \pm standard error; the asterisk indicates significant difference. $P < 0.01$; $n = 6$. SG: spermatogonia; SC: spermatocyte; SB: sperm bundle; TW: testicular wall.

cells surrounded the developing spermatogenic cells. In a cyst of spermatocyte metaphases, nuclear chromatin aggregates to form chromosomes on PD4. The appearance of the synaptonemal complex in the nucleus showed a primary spermatocytic cyst. No vacuoles were found in

the nucleus in the control group (Fig. 5a). Similar to the control group, nuclear chromatin began to form aggregates but vacuoles of various shapes and sizes were found in the nuclei of spermatocytic cysts in the TiO_2 -NPs and ZnO-NPs groups (Fig. 5b and c).

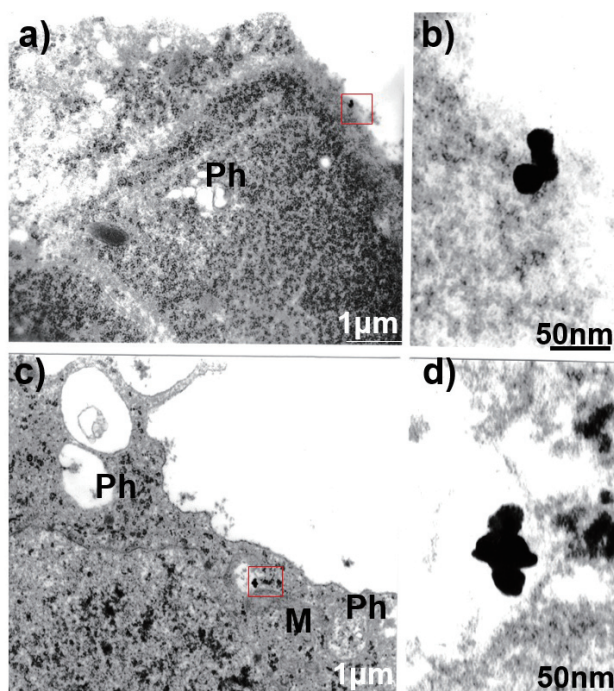


Fig. 4. Nanoparticles found in a cyst cell under TEM. a) The TiO_2 -NPs group: Electron dense particles (in the box) were found in the cyst cells of the pupal testis at PD4. In the magnified figure (X 100,000) in the box of b), small particles were found to be composed of spherical nanoparticles (the original shape of TiO_2 -NPs). c) The ZnO-NPs group: A small agglomerate (in the box) was found in the phagosome of a cyst cell. In the magnified figure (X 100,000) in the box of d), the particles in the phagosome were either spherical or rod-shaped (the original shape of ZnO-NPs). M: mitochondria; Ph: phagosome.

In the control group, the early spermatid had dispersed chromatin in the nucleus on PD4 (Fig. 6a). As spermiogenesis progressed, nuclear chromatin appeared to be fibrous in shape (Fig. 6b) and was completely condensed in the late spermatid (Fig. 6c). In the TiO_2 -NPs (Fig. 6d and e) and ZnO-NPs (Fig. 6f and g) groups, the early spermatid nuclear chromatin disintegrated and some vacuoles remained in the nuclei of the sperm bundles.

DISCUSSION

We report for the first time that TiO_2 -NPs and ZnO-NPs induced detrimental effects on insect spermatogenesis but that the injection of these nanoparticles into 5th instar larvae did not disturb pupation and emergence. In

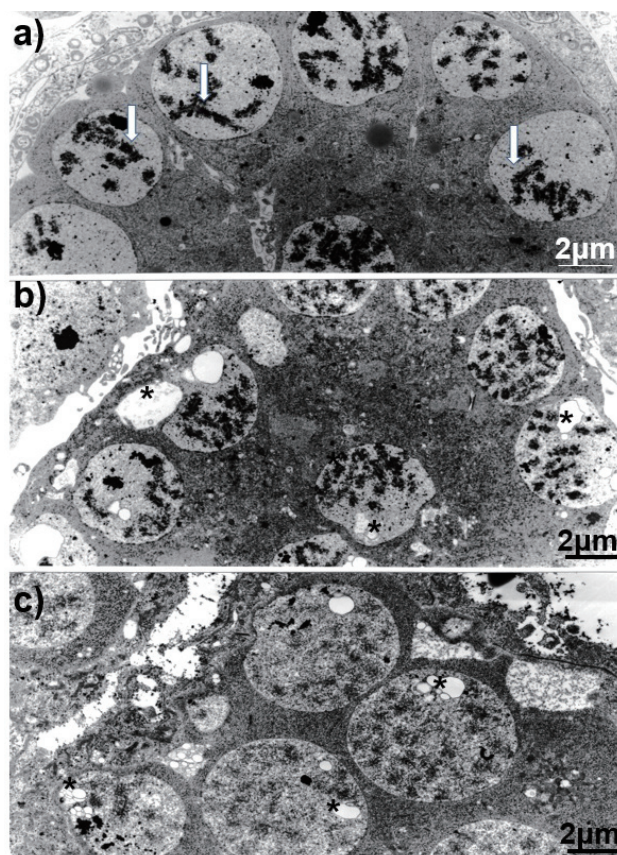


Fig. 5. Ultrastructural alteration of spermatocytes. a) In a cyst of spermatocyte metaphases, nuclear chromatin aggregated to become a chromosome. The appearance of the synaptonemal complex (white arrows) in the nucleus showed in the cysts of primary spermatocytes. There were no vacuoles in the control group. b) Vacuoles of various shapes and sizes (asterisks) were found in the nuclei of the spermatocytic cysts of the TiO_2 -NPs group. c) In the ZnO-NPs group, vacuoles of various shapes and sizes (asterisks) were found in the nuclei. N: Nucleus; Cy: cyst cell.

the pupae on PD4, spermatogonia and spermatocyte numbers did not differ among the control, TiO_2 -NPs and ZnO-NPs groups. However, there was a significant decrease in the number of sperm bundles in the ZnO-NPs group. This indicates that ZnO-NPs are more toxic than TiO_2 -NPs. The toxicity of TiO_2 -NPs, ZnO-NPs and SiO_2 -NPs in water suspensions has also been evaluated in bacteria (*Bacillus subtilis*, *Escherichia coli*) and eukaryotes (*Daphnia magna*). The nanoparticles were revealed to be hazardous to all test organisms, with toxicity increasing in line with concentration. The toxicity of three compounds increased

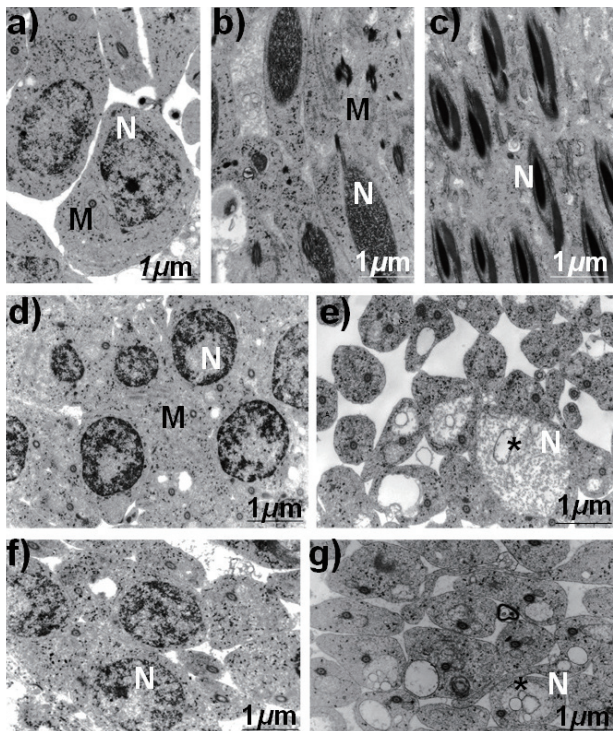


Fig. 6. Ultrastructural alteration of spermiogenesis. a) In the control group, the early spermatid has dispersed chromatin in the nuclei. b) As spermatogenesis progresses, the nuclear chromatin appears fibrous in shape. c) The nuclear chromatin completely condensed in the late spermatid. d) In the TiO_2 group, the early spermatid of nuclear chromatin is disintegrated. e) Some vacuoles (asterisk) are found in the uncondensed chromatin in the nuclei of sperm bundles. f) In the ZnO-NPs group, the early spermatid of nuclear chromatin is disintegrated. g) Some vacuoles (asterisk) found in the nuclei of sperm bundles. M: mitochondria; N: nucleus.

from SiO_2 -NPs to TiO_2 -NPs to ZnO-NPs (Adams *et al.*, 2006). ZnO-NPs, unlike SiO_2 -NPs and TiO_2 -NPs are soluble, which may play an important role in the toxicity of ZnO-NPs in water environments. TiO_2 -NPs and ZnO-NPs have also been reported to exert harmful effects on the coelomocyte cells of the earthworm *Eisenia fetida* at soil levels of higher than 1.0 gkg^{-1} (Hu *et al.*, 2010). The report noted that the toxicity of ZnO-NPs is greater than that of TiO_2 -NPs.

Spermatogenesis is a complex process involving mitotic cell division, meiosis and the process of spermiogenesis. The regulation of spermatogenesis involves both endocrine and paracrine mechanisms among germ cells,

Leydig cells and Sertoli cells (de Krester *et al.*, 1998). Testicular oxidative stress was shown to increase germ cell apoptosis and subsequent hypospermatogenesis in mammals (Turner and Lysiak, 2008). The damage induced in the seminiferous epithelium by TiO_2 -NPs was caused by the nano-size of the particles, which can induce ROS or oxidative stress in mice (Trouiller *et al.*, 2009). It is known that nanoparticles induce oxidative stress because their large surface area allows them to create active oxygen species easily.

In our previous work, small agglomerates of TiO_2 -NPs were found in the Sertoli cells of testicular epithelium and a detrimental effect on spermatogenesis was observed (Takeda *et al.*, 2009; Kubo-Irie *et al.*, 2014). In the present study, we found that nano-sized particles were taken up through phagocytosis in the cyst cells of the pupal testis in the TiO_2 -NPs (Fig. 4a and b) and ZnO-NPs groups (Fig. 4c, and d). The function of the cyst cells, which surround and support spermatogenic cells in moth testes, resembles that of the Sertoli cells of the testicular epithelium in mammals. In TiO_2 -NPs and ZnO-NPs groups, vacuoles of various sizes in the nuclei of spermatocytes and spermatids were observed. A possible mechanism for this is that abnormal vacuoles disturbed the chromatin condensation, resulting in the decrease of sperm bundles. To the authors' knowledge, this is the first report to show toxicity of manufactured TiO_2 -NPs and ZnO-NPs on insect spermatogenesis.

ACKNOWLEDGMENT

This work was supported in part by Grant-in Aid for Health and Labor Sciences Research Grants, Research on Risk of Chemical Substances from the Ministry of Health, Labor and Welfare and a NEX-Supported program for the Strategic Research Foundation at Private Universities (Ken Takeda, 2011-2015).

Conflict of interest---- The authors declare that there is no conflict of interest.

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