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In vivo toxicity evaluation of boron nitride nanosheets with different sizes by silkworm model

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Abstract

Boron nitride nanosheets (BN NSs), a novel material with great potential in biomedical applications, have attracted great attention due to its extraordinary properties. A crucial issue is the toxicity of BN NSs, which depends greatly on various factors, including size. The size may affect viability of cells due to the interactions between BN NSs and cell membranes. In this study, two kinds of silkworms (qiufeng × baiyu, Nistari 7019) were used as models to investigate the toxicity of BN NSs with different sizes (BN NSs-1: thickness of 41.5 nm, average diameter of about 200 nm; BN NSs-2: thickness of 48.2 nm, average diameter of about 500 nm) from the levels of animal entirety (silkworm mortality, silkworm growth, cocoons) and tissues. The results show that exposure to different sized BN NSs causes no obvious adverse effects on the growth or tissues of silkworm. This study has performed size-dependent in vivo toxicity

evaluation of BN NSs and provided safety information to enrich the database for better application of BN NSs. Further studies should be carried out to discover the biosafety of diverse sizes and shapes BN NSs.

Keywords

boron nitride nanosheets; silkworm model; toxicity evaluation; in vivo; size effect

Introduction

Boron nitride nanosheets (BN NSs) are a kind of novel material, known as white graphene with two-dimensional morphology and sheet-like structure. They have gained particular interest due to their extraordinary physical, chemical, thermal, morphological and electrical conductivity properties [1-3], which are widely and commonly used in many areas of industry, as transistors, solid lubricant, thermal conductors, etc. [4-6]. However, it raised the safety concerns about BN NSs, which have the potential for widespread human exposure. For safer applications of BN NSs, it is essential to evaluate the toxicity which is conducted to protect human health and the environment. Only a few studies are focused on the toxicity evaluation of BN NSs [1, 3, 7]. Liu et al. [7] analyzed the adverse effects of BN NSs on HepG2 cells, the results showed that low exposure concentrations of BN were cytotoxic, which could act as a chemosensitizer and inhibit the transmembrane transporter activity. Zhang et al. [1] studied the toxicity of BN NSs to bacterial membranes, demonstrating that liquid molecules from both outer and inner membranes could be damaged. In our preliminary in vivo study, the results showed that BN NSs, with a thickness at about 200 nm and an average diameter of 1.8 μm , caused no obvious adverse effects on the growth, silk properties or tissues of silkworm [3]. Many factors may have effects on the toxicity, i.e.

shape, size, charge, surface environment, purity, production method [8-10]. Among all the factors above, nanoparticle size is one of the most important factors [8], as size is directly connected with the cellular internalization process by cellular uptake. Hence, it is necessary to study the size effect on the in vivo toxicity of BN NSs by using a suitable model.

Silkworm (*Bombyx mori*) is an ideal invertebrate animal model with a short growth cycle, which is favored by researchers in many fields, such as pharmaceutical [11], environmental [12], classical genetics [13]. Compared to the typical mammalian models such as rabbit, rat and mouse, silkworm will not involve ethical problems but share the similar pharmacokinetics and median lethal doses with mammals [14-16]. Compared with the non-mammal models such as *Drosophila*, zebra fish and *Salamandra laurenti*, there is not biosafety issues since silkworm can't survive outside, which is also suitable for handling during injection [17-18]. Different strains of silkworm have different sensitivity to the analytes [19-20], it is better to choose more than one strain of silkworms for affirming the result.

In this study, the size effect on the in vivo toxicity of BN NSs were evaluated by using two strains of silkworm (qiufeng × baiyu, Nistari 7019) as model animals, through characterizing their growth status and cell morphology. The silkworm was characterized through the larvae length and weight, cocoon length, and microstructures of several tissues (midgut, fat body and silk gland). The results show that BN NSs with different sizes (BN NSs-1: thickness of 41.5 nm, average diameter of about 200 nm; BN NSs-2: thickness of 48.2 nm, average diameter of about 500 nm) cause no obvious hazards to the growth and tissues of silkworms, which is in consistent with our former research. It is the first study investigating the size effect on the in vivo toxicity of BN NSs which will enrich the toxicology database and shed light on the safety

evaluation of BN NSs. In the future, more different sizes and shapes of BN NSs need to be studied with more strains of silkworm for better application of BN NSs.

Results and Discussion

Characteristics of the two sized BN NSs

Characteristics of the two sized BN NSs are shown in Fig. 1 and 2, respectively. The scanning electron microscopy (SEM) images show that BN NSs-1 (Fig. 1a, b) and BN NSs-2 (Fig. 2a, b) both have sheet-like structures, indicating the existence of BN NSs. The scanning electron microscopy-energy-dispersive X-ray spectra (SEM-EDS) results reveal that B and N elements account for the majority of the materials (Fig. 1c and 2c), further confirming the existence of BN NSs. The thickness of both BN NSs was characterized by SEM, the results exhibit that BN NSs-1 have a thickness of about 41.5 nm, while that of BN NSs-2 is about 48.2 nm. The diameter of both BN NSs was determined by transmission electron microscope (TEM) (Fig. 1d and 2d), the results show that BN NSs-1 and BN NSs-2 have a diameter near 200 nm and near 0.5 μm , respectively. The dynamic light scattering (DLS) was also carried out to get the hydrodynamic size, which demonstrate that BN NSs-1 have a diameter ranging from 164.2 nm to 458.7 nm, with an average diameter of 270.7 nm, BN NSs-2 have a diameter ranging from 458.7 nm to 712.4 nm, with an average diameter of 562.2 nm. The details on the DLS are shown in Fig. S1 in the supporting information.

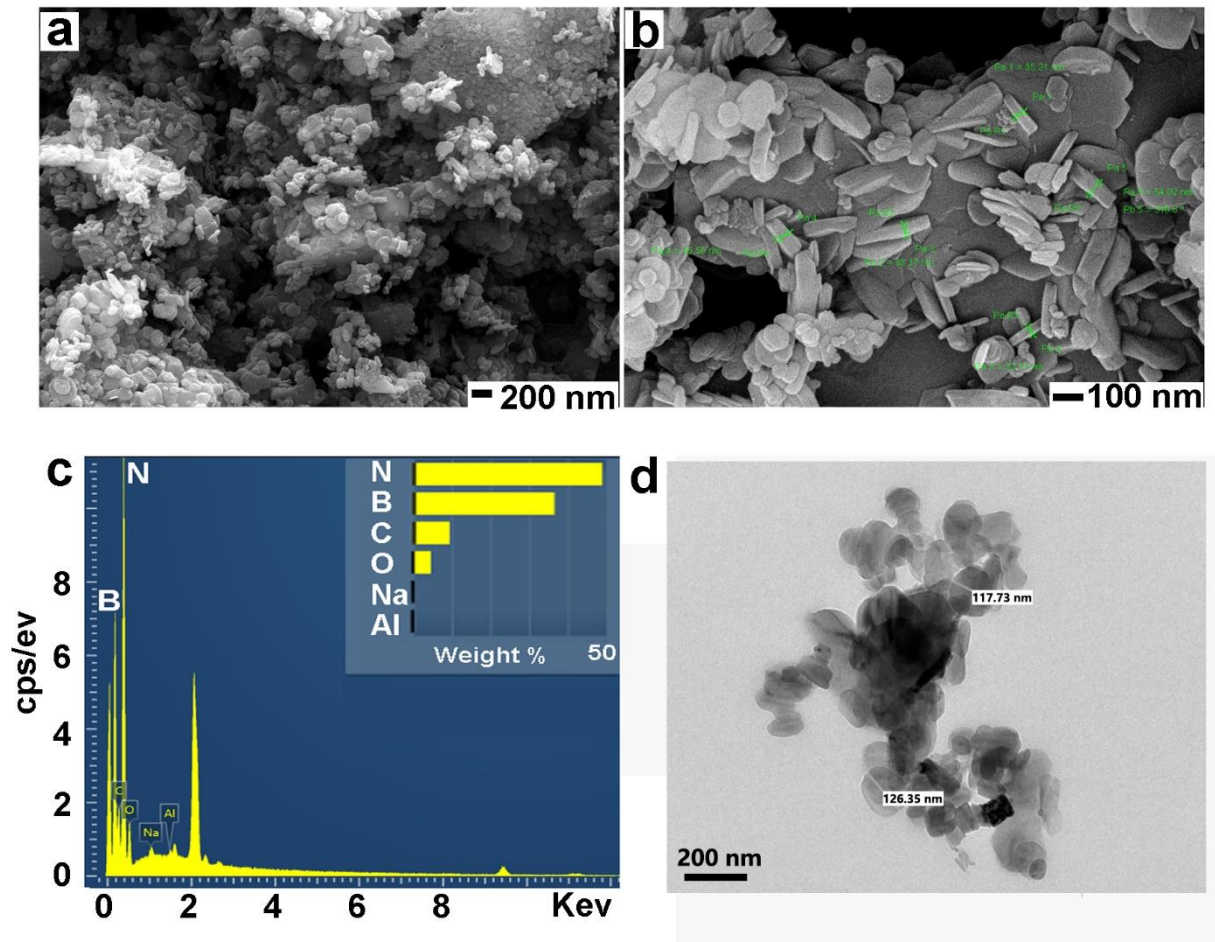


Figure 1: a, b) SEM images of BN NSs-1 taken along different directions, at different magnifications. c) EDS of BN NSs-1. d) TEM images of BN NSs-1.

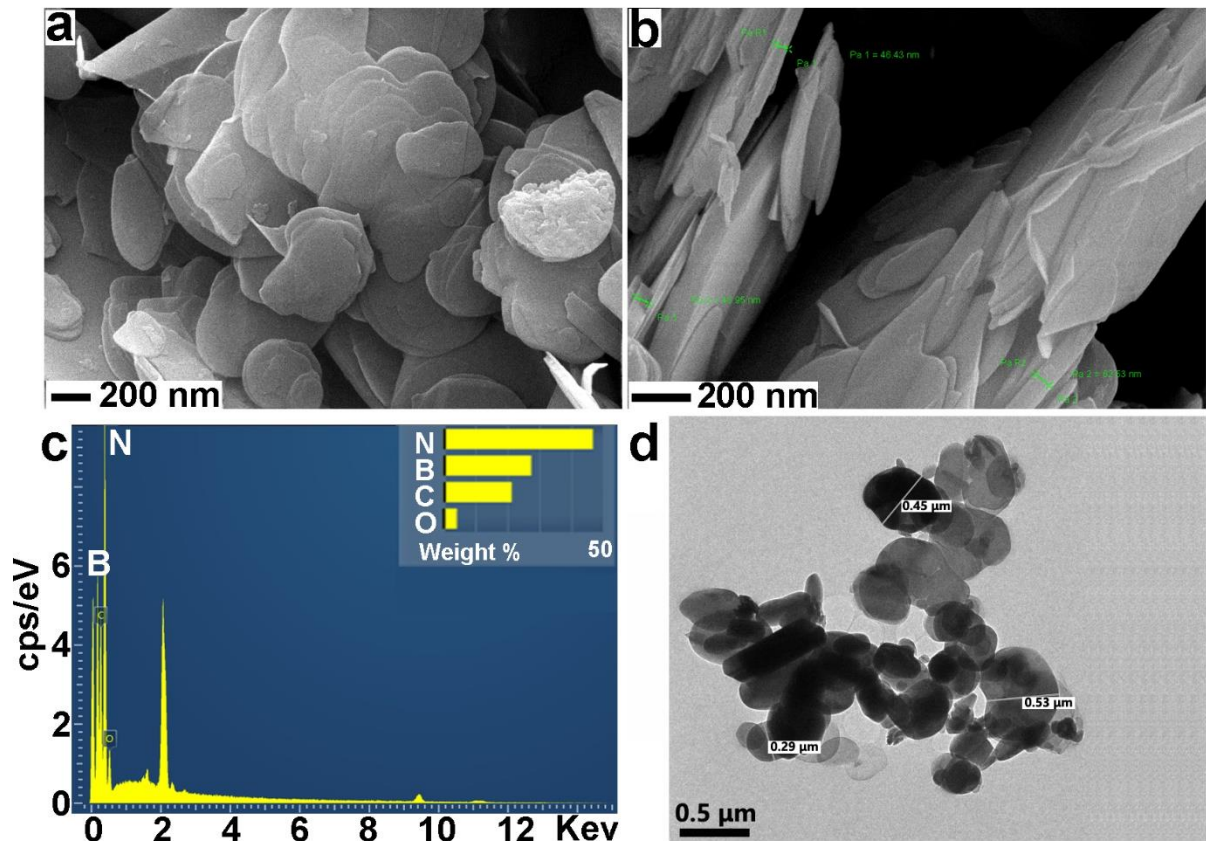


Figure 2: a, b) SEM images of BN NSs-2 taken along different directions, at different magnifications. c) EDS of BN NSs-2. d) TEM images of BN NSs-2.

The influence of two sized BN NSs on the death rate and growth of silkworm larvae

In this assay, 60 silkworms (qiufeng × baiyu) and 60 silkworms (Nistari 7019) were grouped into 3 sections evenly, called as control, G1 and G2 respectively, according to their diet components (control: only mulberry leaves, G1: mulberry leaves sprayed by BN NSs-1, G2: mulberry leaves sprayed by BN NSs-2). In our previous research, inductively coupled plasma mass spectrometry (ICP-MS) experiment was carried out to indicate whether BN NSs were absorbed and accumulated in silkworm body. The results showed that despite the continuous intake of BN NSs, it does not induce significant accumulation of B element in silkworm organs but can be easily cleared by silkworm [3]. Some previous researches indicate that BN NSs are thermally and

chemically stable for a long time under high temperature conditions as 850 °C, so it is impossible to degrade in the silkworm [27-29]. The death rate of each group was recorded every 24 hours (from the 1st day of the intake of BN NSs until cocooning).

In order to study the influence of the two BN NSs on the growth status of silkworm, the appearance, average weight (AW) and average length (AL) of silkworm larvae were observed every 24 hours. All the results are shown in Fig. 3 (qiufeng × baiyu) and 4 (Nistari 7019). Fig. 3a-c and 4a-c show that silkworm larvae from all the groups have similar appearance and color. Fig. 3d-e and 4d-e show that the AW and AL of all the groups are comparable. Fig. 3f-g and Fig. 4f-g show that the growth trend (weight and length) of silkworm larvae from G1 and G2 are basically consistent with the control. Then the SPSS software was used to analyze the data and to verify whether they obey the normal distribution, such as Skewness Kurtosis Test. The previous researches show that when confidence level is 0.05 ($\alpha=0.05$), the Skewness value falls within the range between -1.095 and +1.095, and Kurtosis value falls within the range between -2.191 and +2.191, it can be considered as approximately normal distribution [24-25]. The results in the Fig. S2-S5 show that most of the data of silkworm larvae (qiufeng × baiyu, Nistari 7019), including silkworm weight and length, obey the normal distribution, only a few data of silkworm larvae (qiufeng × baiyu) weight at 0 h (control), silkworm larvae (Nistari 7019) weight at 48 h (control), silkworm larvae (qiufeng × baiyu) length at 0 h (control), and silkworm larvae (Nistari 7019) length at 48 h (control), do not obey the normal distribution. Then the nonparametric test, such as Kruskal-Wallis Test [26], were used to verify whether there are significant differences among silkworms treated with two sized BN NSs and control. From the data of qiufeng × baiyu in Table S6, we can see that there are no significant differences among three groups of silkworm weight (0 h); as the feeding time is extended to 24 h, there are significant differences; as to 48h, there are no significant differences; 72 h and 96 h, there are significant

differences. The same phenomenon appears on silkworm length of qiufeng × baiyu shown in Table S18. For Nistari 7019 showed in Table S12, there are no significant differences among three groups of silkworm weight at 0 h and 24 h; as the feeding time is extended to 48 h, 72 h, 96 h, there are significant differences. The silkworm length of Nistari 7019 have the same phenomenon with qiufeng × baiyu shown in Table S24. From the results of statistical analysis, it can be seen that there is significant differences among three groups of silkworms. The reason might be from the inconsistency of silkworm species, which have different sensitivities to different nanomaterials [30]. All the raw data are shown in Table S1-24. The results showed that no silkworms died during the whole process, the food intake speed and growth rate of each group were also similar, indicating that despite the significant differences from different sensitivities to nanomaterials, the two different sized BN NSs are of no or low toxicity to silkworm within a certain dose. The experiments on food intake speed of silkworms from different groups were carried out and the raw data were shown in Table S25 in the supporting information.

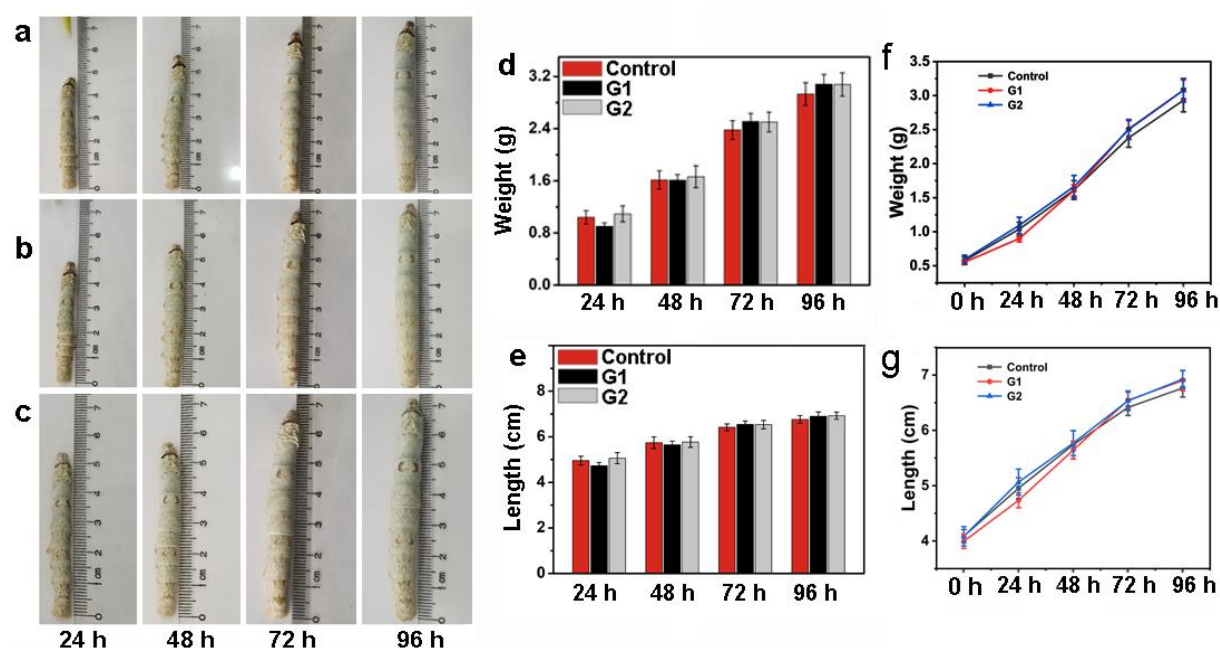


Figure 3: The effect of BN NSs on the growth of silkworms (qiufeng × baiyu). a, b, c)

The pictures of silkworm larvae after the intake of BN NSs for different times (a: control, b: G1, c: G2). d, e) The comparison of the average weight (AW, d) and length (AL, e) of silkworm larvae from different groups, the error bars stand for the standard deviation of AW and AL respectively. f, g) the growth trend of silkworm weight f) and length g) from different groups.

To study the concentration effect of BN NSs toxicity, dose dependency study was carried out, setting the BN NSs concentration as 2% and 8%, qiufeng × baiyu as example. Fig. 3-4 and S6-7 show that the intake of a certain amount (2%, 4%, 8%) of BN NSs-1 and BN NSs-2 causes no obvious adverse effects on the growth of silkworms. More studies on concentration effect of BN NSs should be carried out in the future.

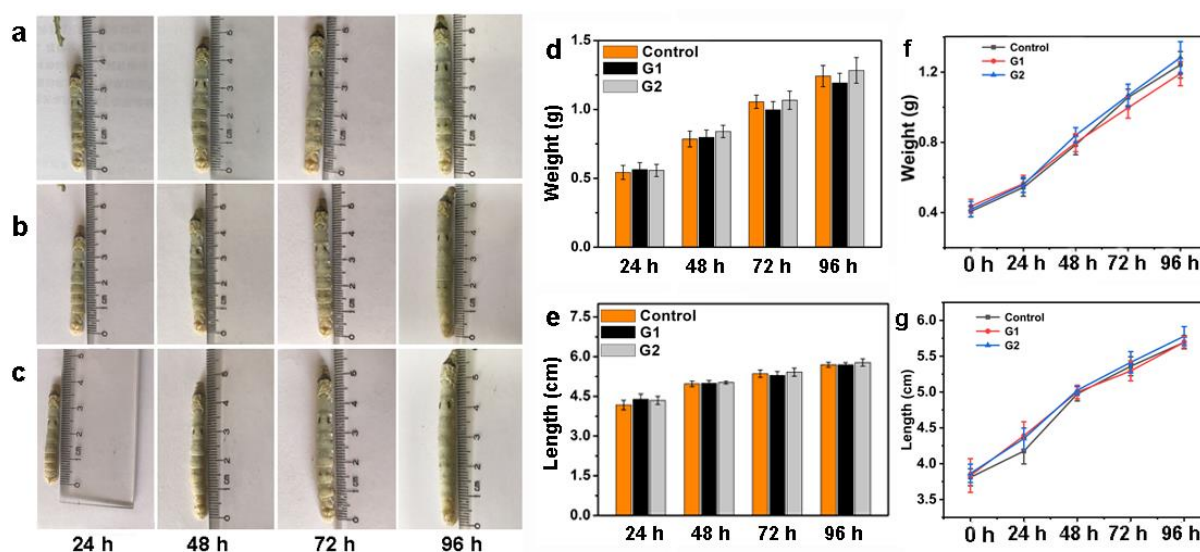


Figure 4: The effect of BN NSs on the growth of silkworms (Nistari 7019). a, b, c) The pictures of silkworm larvae after the intake of BN NSs for different times (a: control, b: G1, c: G2). d, e) The comparison of the average weight (AW, d) and length (AL, e) of silkworm larvae from different groups, the error bars stand for the standard deviation of AW and AL respectively. f, g) the growth trend of silkworm weight f) and length g) from different groups.

The influence of two sized BN NSs on the cocoons of silkworm larvae

In order to study the influence of two BN NSs on the cocoons of silkworm, the appearance, size and average weight (AW) of cocoons were observed every 24 hours. All the raw data are shown in Table S25-S26. The results in Fig. 5 show that cocoons (qiufeng × baiyu and Nistari 7019) from control, G1 and G2 have similar size and appearance. The data in Table S26 exhibit that the AW of cocoons (qiufeng × baiyu) from either G1 or G2 is a little larger than the one from control, suggesting that the intake of two BN NSs cause some positive effect on cocoon weight. Table S27 shows that the AW of cocoons (Nistari 7019) from three groups are almost the same, suggesting that the intake of BN NSs-1 and 2 do not cause adverse effect on cocoon weight. Skewness Kurtosis Test of the cocoons weight data was also carried out to verify whether they obey the normal distribution. The results are shown in Fig. S8, indicating that the cocoon weight data of silkworm larvae (qiufeng × baiyu) do not obey the normal distribution. Then Kruskal-Wallis Test was used to verify whether there are significant differences among cocoons treated with and without BN nanosheets. As we can see in Table S28 and S29 that there are significant differences among three groups of cocoon weight of qiufeng × baiyu, and there are no significant differences among three groups of cocoon weight of Nistari 7019.

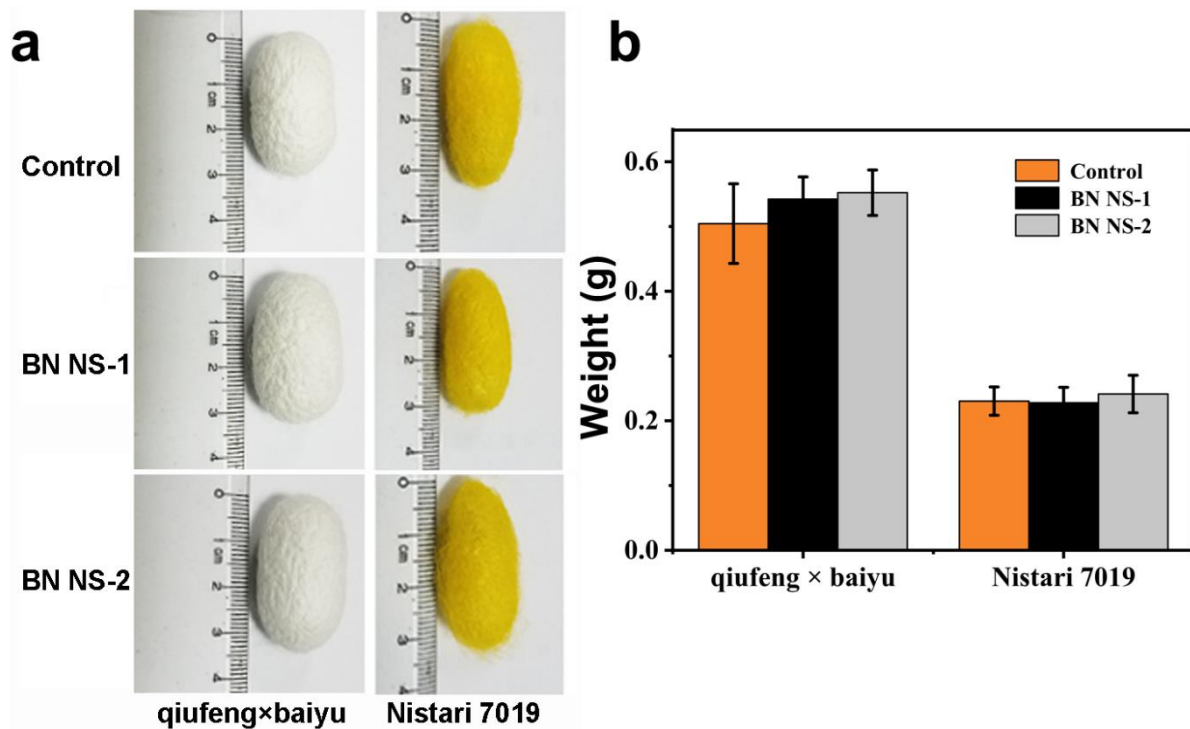


Figure 5: The pictures a) and histograms b) of cocoons from silkworms (qiufeng × baiyu and Nistari 7019) after the intake of BN NSs-1 and BN NSs-2.

The reasons for that phenomenon might be from the differences between Nistari 7019 and qiufeng × baiyu. First, Nistari 7019 is a yellow blood silkworm, whereas qiufeng × baiyu is a white blood silkworm, and their blood composition is rather different, such as protein, amino acid, mineral, vitamin, carotenoids, lutein and other pigments [31-32]. Second, the physiological and biochemical mechanism of silkworms spitting out colored cocoon silk is mainly reported in the process of carotenoids and lutein transport from hemolymph to the middle silk gland, which might be realized by pigment binding proteins. The cocoon silk color is not only derived from the pigment in cocoon sericin, such as carotenoids and lutein [33-35], but also is controlled by genes [36]. Third, some studies show that the cocoon silk from Nistari 7019 has more internal pores than that from qiufeng × baiyu, and the internal fibril surface structure is also tighter; the characteristic peaks of infrared absorption spectrum are similar, indicating that their molecular conformations are similar; X-ray diffraction curves show that there is no

obvious difference in their crystal structure. In summary, it is postulated that these differences between Nistari 7019 and qiufeng × baiyu have generated the variation in cocoons [37].

The influence of two sized BN NSs on the cell morphology of silkworm

Histophysiological study was performed to investigate the impact of the two BN NSs on the cell morphology of several tissues (midgut, fat body and posterior silk gland), the results are shown in Fig. 6 (qiufeng × baiyu) and 7 (Nistari 7019). It is achieved that all the samples from either control or G1, G2 groups have normal pathological microstructures, no obvious difference is observed among the samples, proving that the intake of two sized BN NSs causes no damage to the cell morphology of silkworms.

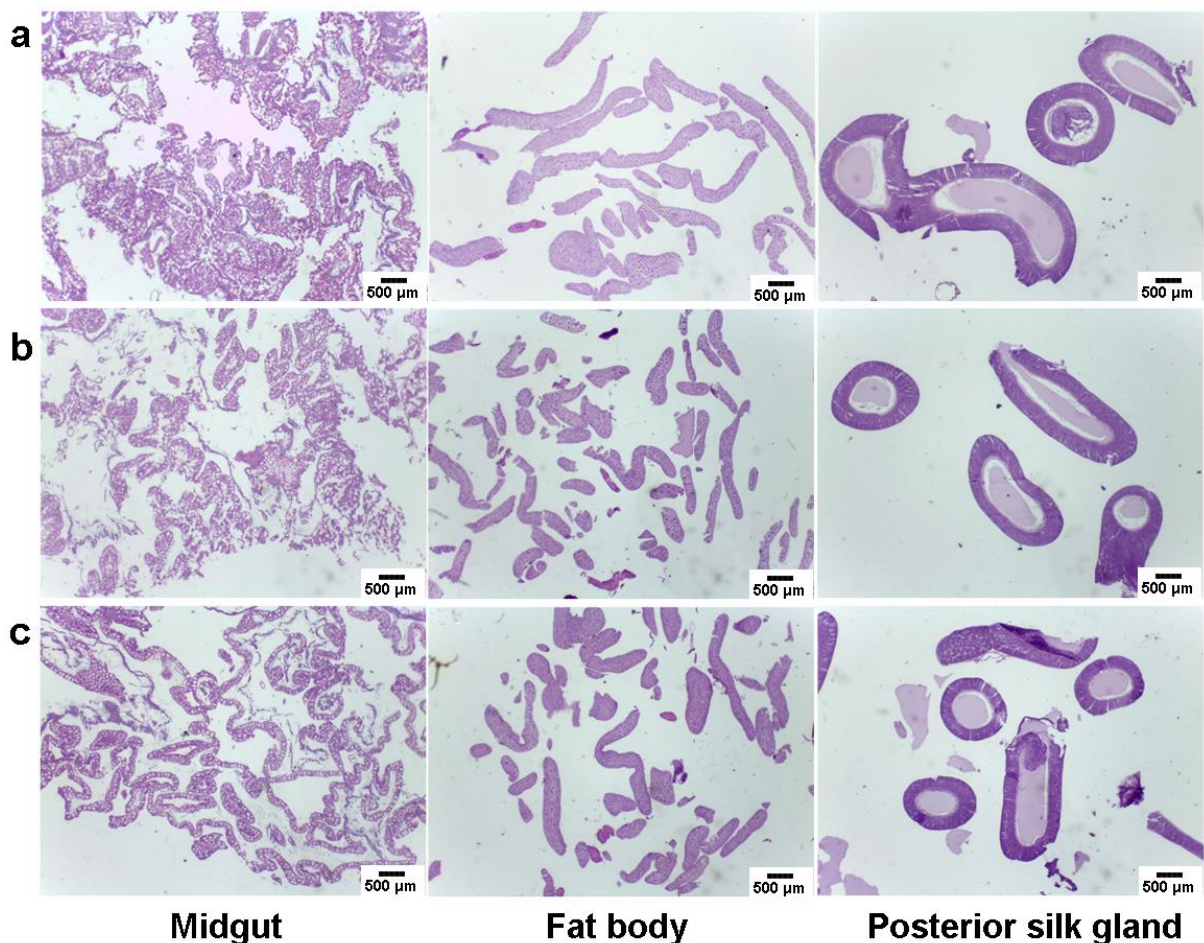


Figure 6: Histophysiological pictures of midgut, fat body and posterior silk gland of silkworms (qiufeng x baiyu) from control a), G1 b) and G2 c) after the intake of BN NSs for 96 hours.

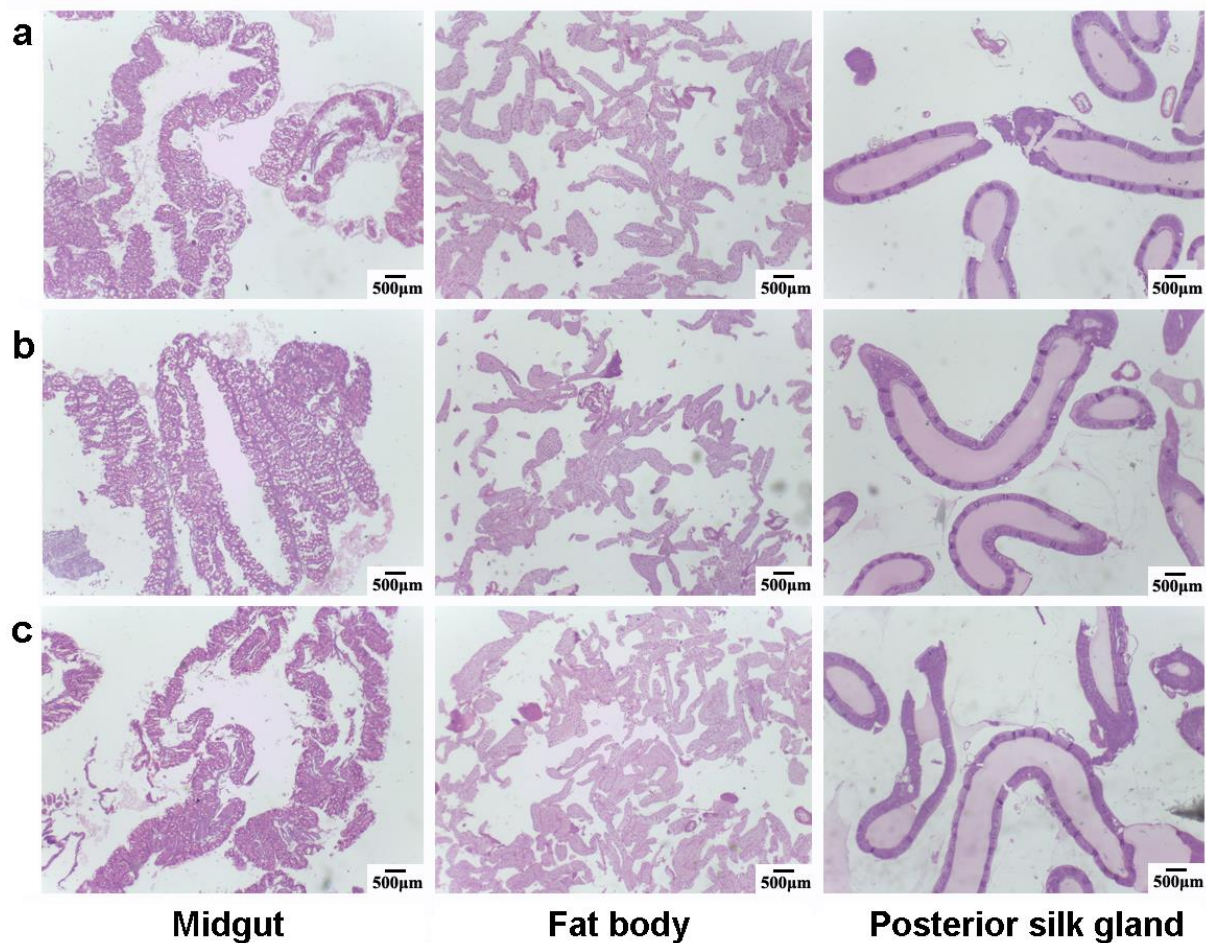


Figure 7: Histophysiological pictures of midgut, fat body and posterior silk gland of silkworms (Nistari 7019) from control a), G1 b) and G2 c) after the intake of BN NSs for 96 hours.

Conclusion

In this study, we present an efficient method to evaluate the size effect on the in vivo toxicity of BN NSs, by using two strains of silkworm (qiufeng × baiyu, Nistari 7019) as model animals. It is observed that different sized BN NSs display no toxicity or adverse effect on the growth status (silkworm larvae appearance, average weight and average length), the cocoon of silkworms (cocoon appearance and weight) or cell morphology of silkworms, which enrich the toxicology data of BN NSs. More diverse sizes and shapes need to be studied to get a general regularity of size and shape-dependent toxicity in the future.

Experimental

Materials

All reagents were of analytical reagent grade and used as received. Water was deionized and further purified with a thermo scientific water purification system (Lab Tower EDI 15, Sweden). Different sizes of boron nitride nanosheets (BN NSs) were purchased from Beijing Deke Daojin science and technology Co., Ltd. Bombyx mori silkworm eggs (qiufeng × baiyu, Nistari 7019) were from Shandong Guangtong silkworm egg Group Co., Ltd., which were supplied by Yong-Zhu Yi and Ai Zhang from College of Biotechnology at Jiangsu University of Science and Technology.

Characterizations

The morphology, diameter, thickness, EDS (energy-dispersive X-ray spectra) and size distribution of BN NSs were characterized by Shanghai Yuyi Analysis and Testing Center: A scanning electron microscopy (SEM, Zeiss Merlin Compact, Germany) was used to study the morphology, diameter, thickness and EDS of BN NSs; a Zetasizer

Nano ZS ZEN3600 particle size analyzer (Malvern, UK) was used to study the size distribution of BN NSs. After continuously eating BN NSs for 96 hours, the silkworm larvae were dissected to get three tissues (midgut, fat body and posterior silk gland), which were then made into histological sections by Zhenjiang first people's hospital, a microscope (Leica EZ4HD, Leica Microsystems GmbH, Germany) was used to observe the cell morphology. The transmission electron microscopy (TEM) characterization was recorded by a JEOL-2010 electron microscope from JEOL Ltd.

Preparation of BN NSs solution

Two kinds of BN NSs of different sizes were used in this assay, which were named as BN NSs-1 and BN NSs-2, respectively. Then, BN NSs-1 (4 g) and BN NSs-2 (4 g) were mixed with 100 mL distilled water, respectively, which were then ultrasound-treated for 30 min to make the solution dispersed evenly.

The intake of BN NSs by silkworms

40 healthy uniform silkworm larvae (qiufeng × baiyu) were chosen randomly on the 1st day of the 5th instar, which were divided into 2 groups evenly and fed with mulberry leaves sprayed by BN NSs solution until cocooning. The silkworm larvae were fed twice a day, once in early morning, once at dusk. The 2 groups of silkworms were named as G1 and G2 respectively according to the type of BN NSs they took (G1: BN NSs-1, G2: BN NSs-2). 20 silkworms (qiufeng × baiyu) fed with normal mulberry leaves were set as control. Do the same experiment on silkworm larvae (Nistari 7019). According to rough statistics, one silkworm larvae took approximately 2 g of mulberry leaves each time. The mass ratio of mulberry leaves to BN NSs each silkworm larva took in was about 1 g: 10.8 mg. The silkworms were fed twice a day, so each of them ate about 43.2 mg per day.

The digestive system of silkworm is a large tube that runs through the center of the body cavity from the mouth to the anus, which includes the foregut (mouth, throat, esophagus), midgut (the most developed part of the digestive system, accounting for 78% of the total length of the digestive tract, which exchanges substances in the blood), and hindgut (small intestine, colon, rectum) [21-22]. So the midgut was selected to study the influence of two sized BN NSs on the cell morphology of silkworm.

Statistical analysis

During the experiments, statistical analysis was carried out to make the conclusions of logical induction more persuasive and decide whether there is a statistically significant trend [23]. The data was expressed as means \pm relative stand deviation (RSD) and analyzed using SPSS software (version 22) and origin software (version 75). All the data were analyzed for the normal distribution [24-25] and nonparametric test [26] using SPSS software.

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