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Original Article

Normal ovarian aging, but modified T-cell differentiation, in female mice following neonatal exposure to bisphenol A

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ABSTRACT — In a previous study, we found that early life exposure to low-dose diethylstilbestrol accelerated onset of abnormal estrous cycles in female C57BL/6J mice. In order to observe the ovarian aging of mice exposed to substances on the candidate list for endocrine-disrupting properties, neonates of C57BL/6J mice were orally administered bisphenol A (BPA) at doses of 5 and 50 μg/kg/day. As a result, normal ovarian aging was observed in females treated with BPA. However, females treated with 5 and 50 μg/kg/day of BPA showed modified T-cell differentiation in the thymus. These results suggested that early life exposure to low-dose BPA did not induce premature ovarian failure but modified T-cell differentiation in female mice.

Key words: Bisphenol A, Ovarian aging, Immunotoxicology, Mouse, Endocrine disruptor

INTRODUCTION

In humans, premature (early onset) ovarian failure is considered to be where menopause occurs before 40 years of age (Nelson, 2009; Shelling, 2010). It can occur in very young women, even in teenagers. There are several known causes of premature ovarian failure, although sometimes the cause remains unknown. Previously, we demonstrated that neonatal exposure to diethylstilbestrol (DES) accelerated onset of abnormal estrous cycles in female Sprague-Dawley (SD) rats (Ohta *et al.*, 2012a) and female C57BL/6J mice (Ohta *et al.*, 2014) as they aged, suggesting the importance of observing estrous cycles over a long period in research on endocrine disrupting chemicals (EDCs). Similarly, other investigators suggested that neonatal exposure to DES in Donryu rats (Yoshida *et al.*, 2011) and to 17alpha-ethynylestradiol in SD rats (Shirota *et al.*, 2012; Shirota *et al.*, 2015) or Wistar Hannover rats (Takahashi *et al.*, 2013) induced delayed effects on their estrous cycles. It was proposed that abnormal development of kisspeptin neurons plays a key role in early onset of abnormal estrous cycles in rats (Ichimura *et al.*, 2015; Takahashi *et al.*, 2016). Furthermore, we demonstrated that accelerated occurrence of pituitary tumors and shortened survival were found in both, female rats (Ohta *et al.*, 2012a) and female mice (Ohta *et al.*, 2014), following neonatal DES exposure. Therefore, early onset of abnormal estrous cycles is an important parameter indicating adverse effects of EDCs at low doses.

Immune system dysfunction also occurs in postmenopausal women and is associated with sex hormonal changes (Gameiro *et al.*, 2010; Ghosh *et al.*, 2014). Postmenopausal women, relative to premenopausal women, showed decreased immune functions related to high incidences of chronic diseases. Immune alterations also presented in women with premature menopause (Shuster *et al.*, 2010). Therefore, it is important to examine immune functions as well as observing estrous cycles in research on EDCs.

Although bisphenol A (BPA) is widely evaluated in endocrine disruptor studies, the ovarian aging (Adewale *et al.*, 2009; Rubin *et al.*, 2001) and immune function (Yoshino *et al.*, 2004) of the animals exposed to low doses of BPA are not well known. Therefore, the observation of estrous cycles during the aging period was examined in C57BL/6J mice neonatally treated with BPA. In addition, a study of immunotoxicology was conducted in these animals.

MATERIAL AND METHODS

Animals and administration

Pregnant C57BL/6J mice were purchased from Charles River Japan Laboratories at day 14 of pregnancy. We used this strain because it is reported to respond well

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to estrogens (Spearow *et al.*, 1999; Ashby *et al.*, 2003) and we have background data on the uterotrophic assay (Ohta *et al.*, 2012b) in addition to data on estrous cycles over a long period (Ohta *et al.*, 2014).

The pregnant females were kept individually in TPX synthetic resin cages (143W x 293D x 148H mm) with bedding, PAPER CLEAN (Japan SLC, Shizuoka, Japan), in an animal room maintained at a room temperature of 21-25°C, a relative humidity of 40-75%, and 12-hr lighting (7:00-19:00 lighting). Phytoestrogen-low diet (PLD) (Oriental Yeast Co., Tokyo, Japan) and tap water were available *ad libitum*. Because estrogenic chemicals at very low concentrations have been administered to animals, we used PLD (Kanno *et al.*, 2002) in this experiment. Animal protocols used in this study were reviewed and approved by the Animal Care and Use Committee of the Food and Drug Safety Center (FDSC), and the study was carried out in compliance with the Guideline for Animal Experiment in Hatano Research Institute, FDSC.

The pregnant females were divided into 3 groups of 12 animals per group. Their neonates delivered spontaneously and were checked for sex and external abnormalities on postnatal day 0 (PD 0). All pups without abnormalities were reared by their biological dams and subjected to the following experiment. All the neonates were orally administered BPA daily from PD 1 to PD 5 using a micro-syringe connected to a peripherally inserted Argyle catheter (Nippon Sherwood Medical Industries Ltd., Tokyo, Japan), as previously described (Ohta *et al.*, 2014). The doses of BPA were set at 5 and 50 μg/kg/day in this study, based on the U.S. Environmental Protection Agency (EPA) reference dose (50 μg/kg/day) at the time the study was undertaken. An oral route instead of a subcutaneous route was selected for this study because an exact amount of chemicals can be administered and the loss of dosing caused by maternal licking can be avoided. It is known that there are no differences in the pharmacokinetics of BPA between oral and non-oral dosing (Taylor *et al.*, 2008).

An amount, 20 mg, of BPA (Lot No. 009X1493, Kanto Chemical Industry, Tokyo, Japan) was dissolved in 1 mL of ethanol and then diluted with olive oil to prepare the doses. The dosing volume was set at 10 mL/kg body weight, and the control group received a similar treatment but with olive oil only. These dosing conditions were similar to those in the previous study using C57BL/6J mice (Ohta *et al.*, 2014). The number of neonates was not adjusted because the litter sizes were smaller in C57BL/6J mice, not exceeding 10 pups. The neonates were weaned on PD 21, and housed in larger cages (235W x 325D x 170H mm) with same-sex littermates.

Body weight, sexual maturation and estrous cycle

Body weights of the neonates were individually measured on PDs 1-5, and on PDs 7, 14 and 21. After weaning, body weights of the offspring were measured once a week from 3 to 10 weeks of age, every two weeks from 10 to 24 weeks, and then, every four weeks up to 48 weeks of age.

As an index of sexual maturation, the vaginal openings of all females and the preputial separations of all males were checked daily from PD 25 and PD 27, respectively.

Vaginal smears were collected from all females every two weeks from 24 to 49 weeks of age, as it was expected that abnormal cycles would appear in C57BL/6J mice at this time on the basis of a previous study (Ohta *et al.*, 2014). The estrous cycles were categorized as normal cycles (regular 4- or 5-day cycles) or abnormal cycles (long cycles, persistent estrus, or constant diestrus), based on the cell types observed in the vaginal smears.

Immunological examination I

At 14 and 50 weeks of age, more than six animals per group of both sexes, avoiding littermates, were subjected to an immune challenge against sheep red blood cells (SRBC). Prior to blood sampling, these animals were given a single intravenous tail vein injection of 0.2 mL SRBC (5 x 10^8/mL). At 5 days after SRBC injection, blood samples were collected by cardiac puncture through the anterior thoracic aperture under sevoflurane anesthesia, and then the spleens were removed and weighed. The sera were separated and stored at -80°C until determination of anti-SRBC-IgM (Temple *et al.*, 1993). The titer of anti-SRBC-IgM was determined by the ELISA method.

Immunological examination II

At 14 weeks of age, six animals per group of both sexes and that had not been given a SRBC injection were subjected to the following examinations. The animals were weighed and anesthetized using sevoflurane, and then blood samples for lymphocyte counts were collected by cardiac puncture. Then, the thymus and spleen were removed immediately after weighing. The cells were expelled from the thymus and spleen, stained with antibodies to CD4 and CD8, and analyzed by flow cytometry (FACS, Becton Dickinson) for immunophenotyping. The spleen cells (5 x 10^6/mL) were cultured for 48 hr in the presence of mitogens [Concanavalin A (Con A, 1 or 2.5 μg/mL) and Lipopolysaccharide (LPS, 2.5 or 5 μg/mL)], and measured by ELISA, after BrdU incorporation, for lymphoproliferation. The spleen cells (5 x 10^6/mL) were cultured with Con A (2.5 or 5 μg/mL) for

48 hr, and then supernatant cytokine (IFN-γ and IL-4) concentrations were determined by ELISA kit (R&D Systems, Minneapolis, MN, USA). All samples were blindly analyzed in the *in vitro* assay.

Statistical analyses

The data used the litter average as the statistical unit before weaning. Individual data were used as the statistical unit after weaning. Data collected on body weights, organ weights, and immunological examination II were analyzed by one-way ANOVA among the control and BPA groups for each sex. When the ANOVA was significant, Dunnett's test was applied. Data collected on sexual maturation and immunological examination I were analyzed by Kruskal-Wallis analysis of ranks. When significant differences were detected between the groups, a Dunnett-type, multiple comparison test was applied.

RESULTS

Body weight, sexual maturation and estrous cycle

The results of neonatal BPA exposure on body weight changes in male and female offspring are shown in Fig. 1. During pre- and post-weaning up to 10 weeks of age, there were no significant differences in body weights between the control and BPA groups for either sex, except for significant decreases at 6 and 7 weeks of age in females treated with 5 μg/kg BPA. After 12 weeks of age, males treated with 50 μg/kg BPA exhibited statistically significant suppression of body weight gain compared to the control group (Fig. 1A). However, females in these groups exhibited a similar body weight gain compared to the control group up to 48 weeks of age (Fig. 1B).

The results of neonatal BPA exposure on the completion days of preputial separation and vaginal opening in offspring are shown in Fig. 2. There were no significant differences between groups in the mean completion day of preputial separation (Fig. 2A) and vaginal opening (Fig. 2B).

The results of neonatal BPA exposure on estrous cycles in female offspring from 24 to 49 weeks of age are shown in Fig. 3. The percentage of females showing abnormal estrous cycles increased with age. There were, however, no effects of BPA on transition of the rates of females showing abnormal cycles during the aging period when compared to the control group. Most of the abnormal cycles observed at 36-37 weeks of age were long cycles, while most of the abnormal cycles observed at 48-49 weeks of age were persistent estrus.

Immunological examination I

The results of neonatal BPA exposure on the immune response to SRBC at 14 and 50 weeks of age are shown in Fig. 4. No significant effects of BPA were found in the levels of anti-SRBC IgM after SRBC injection in either sex. In addition, there were no significant effects of BPA on the weights of the spleen after SRBC injection at 14 and 50 weeks of age in either sex (data not shown).

Immunological examination II

The results of neonatal BPA exposure on the CD4/CD8 immunophenotyping at 14 weeks of age are shown in Fig. 5. Significant decreases in the percentage of CD4+/

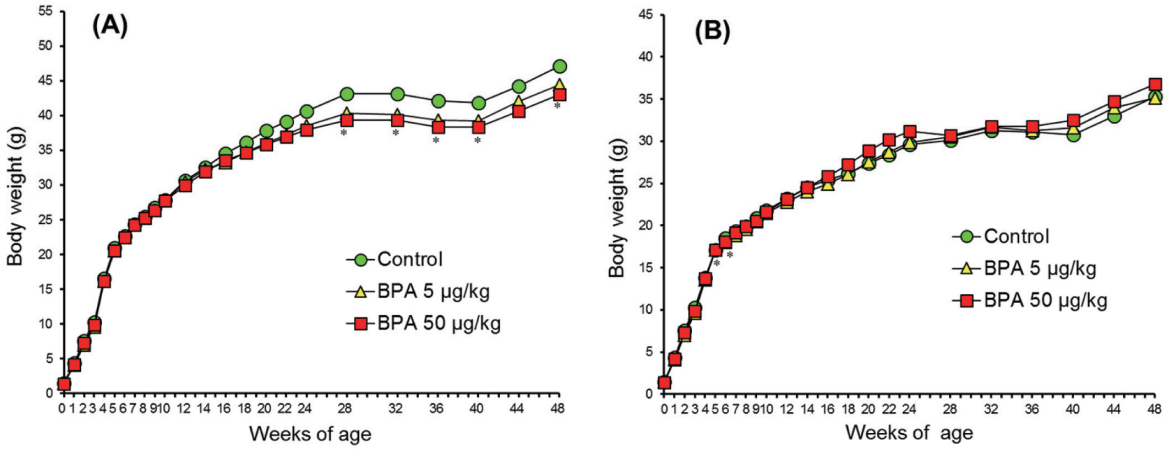

Fig. 1. Effects of neonatal BPA exposure on body weight changes in male (A) and female (B) C57BL/6J mice. Values are expressed as means. Asterisks indicate significant differences when compared to the control group. Statistical significance: *$p < 0.05$.

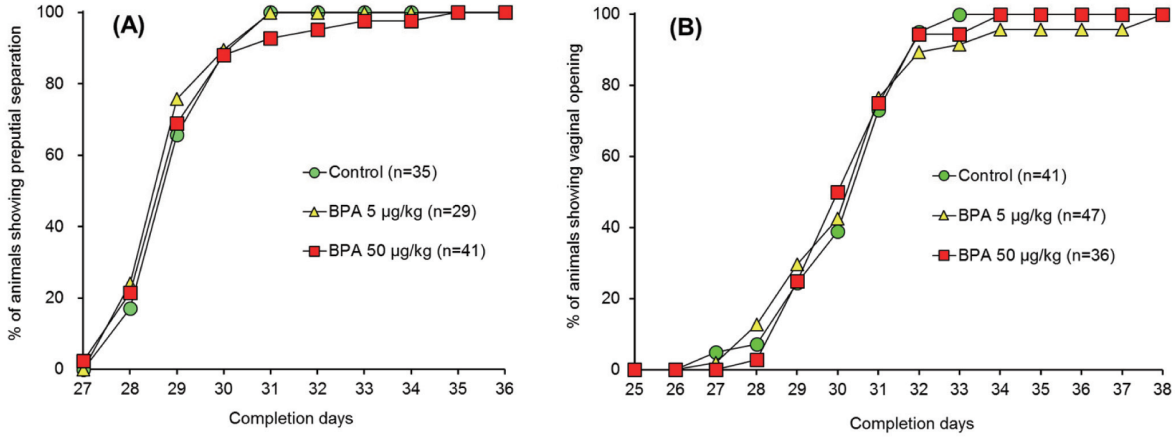

Fig. 2. Effects of neonatal BPA exposure on completion days of preputial separation (A) and vaginal opening (B) in C57BL/6J mice. Values are expressed as the percentage of animals with completion.

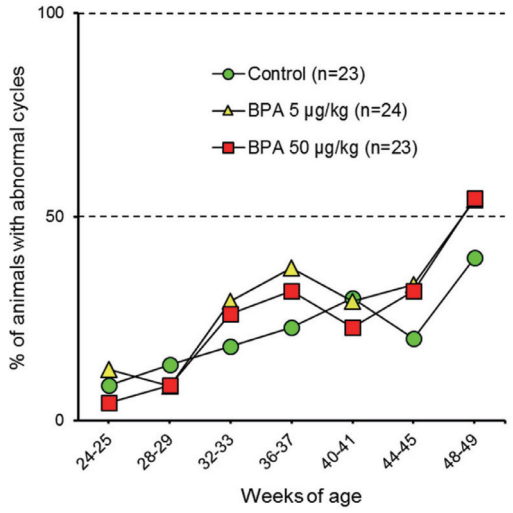

Fig. 3. Effects of neonatal BPA exposure on estrous cycles in female C57BL/6J mice. Values are expressed as the percentage of females with abnormal estrous cycles.

CD8+ lymphocytes (Fig. 5A) and significant increases in the percentage of CD4+/CD8- lymphocytes (Fig. 5B) were observed in the female thymus of the 5 and 50 μg/kg BPA groups. Significant decreases in the percentage of CD4+/CD8+ lymphocytes were also observed in the female spleens of the 50 μg/kg BPA group (Fig. 5C). There were, however, no significant effects of BPA in males on the CD4/CD8 immunophenotyping. There were no significant effects of BPA on the thymus and spleen weights or lymphocyte counts in either sex (data not shown). In addition, there were no effects of neonatal BPA exposure on the lymphoproliferative responses to Con A or LPS and on the concentrations of cytokines (IFN-γ and IL-4) in either sex at 14 weeks of age (data not shown).

DISCUSSION

Previously, we demonstrated that neonatal exposure to 0.5 μg/kg of DES accelerated onset of abnormal estrous cycles in female mice and increased the frequency of pituitary tumors, causing lifespan shortening (Ohta *et al.*, 2014). Therefore, we examined estrous cycles during the aging period in mice neonatally treated with 5 or 50 μg/kg of BPA, one of substances on the candidate list for endocrine-disrupting properties. As a result, the percentage of animals showing abnormal estrous cycles increased with age in the BPA groups, but the rate was similar to that in the control group. It was demonstrated that abnormal ovarian cycles occurred in rats exposed perinatally to high doses (1.2 mg/kg or 50 mg/kg) of BPA (Adewale *et al.*, 2009; Rubin *et al.*, 2001). However, our data suggest that low doses of BPA, based on the U.S. EPA reference dose or less, do not induce abnormal estrous cycles in mice, even when the estrous cycles were observed during the aging period.

Additionally, we examined the immunotoxicology in mice exposed neonatally to low doses of BPA. In immunophenotyping of lymphocytes in the thymus, an increased percentage of CD4+/CD8- and a decreased percentage of CD4+/CD8+ were observed in female mice treated with 5 and 50 μg/kg of BPA. These results sug-

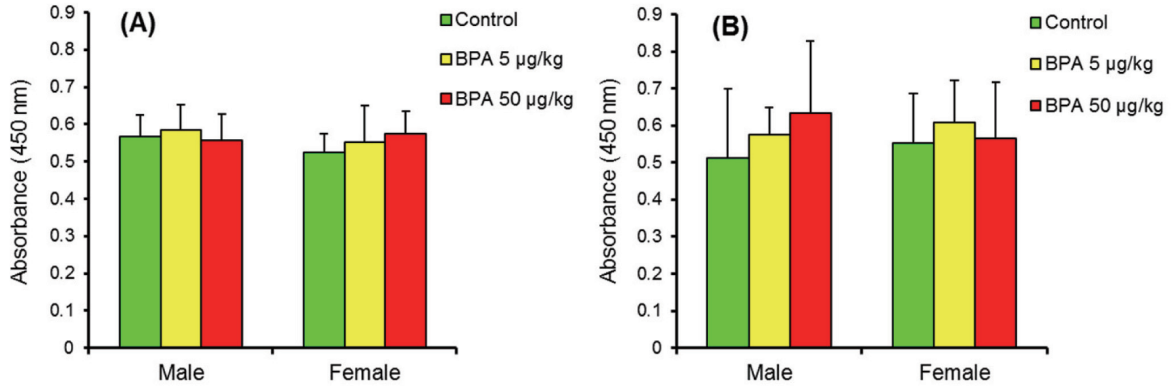

Fig. 4. Effects of neonatal BPA exposure on antibody response to SRBC in C57BL/6J mice at 14 weeks (A) and 50 weeks (B) of age. Values are expressed as the mean ± S.D. of 6 to 7 animals.

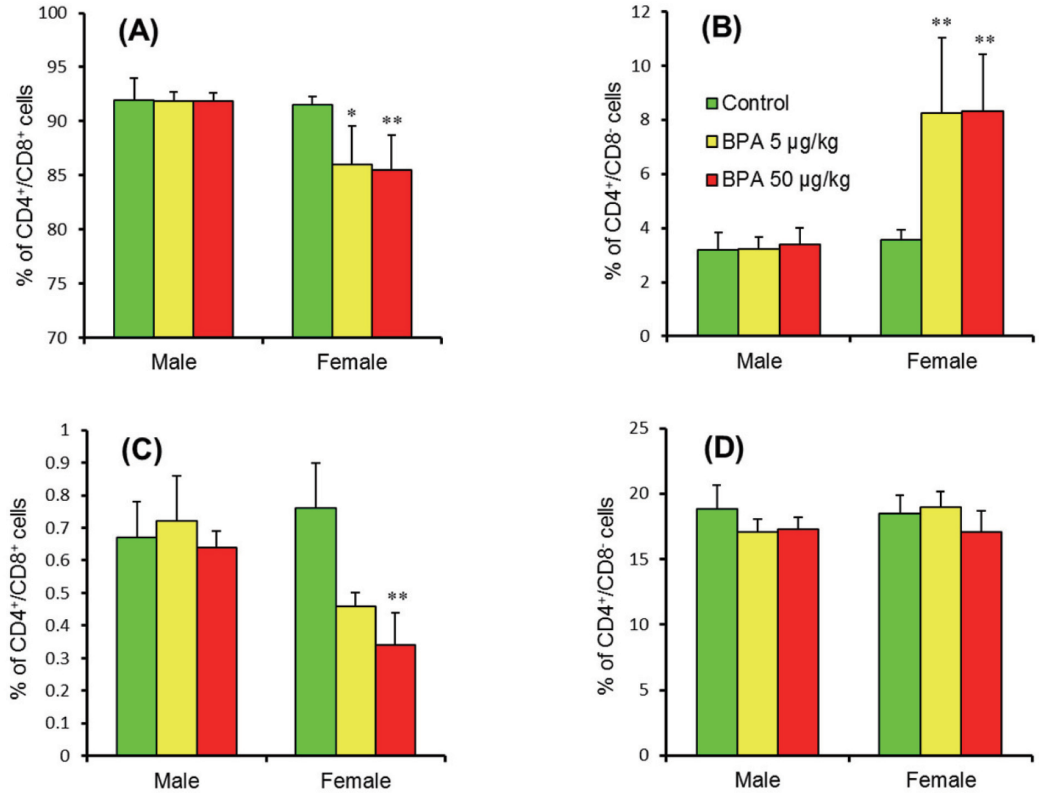

Fig. 5. Effects of neonatal BPA exposure on immunophenotyping in C57BL/6J mice at 14 weeks of age. (A) Percentage of CD4+/CD8+ lymphocytes in the thymus. (B) Percentage of CD4+/CD8- lymphocytes in the thymus. (C) Percentage of CD4+/CD8+ lymphocytes in the spleen. (D) Percentage of CD4+/CD8- lymphocytes in the spleen. Values are expressed as the mean ± S.D. of 6 animals. Asterisks indicate significant differences when compared to the control group. Statistical significance: *$p < 0.05$, **$p < 0.01$.

gest that T-cell differentiation is modified by low doses of BPA in the female thymus. To our knowledge, this is the first demonstration showing that neonatal exposure to low doses of BPA resulted in modified T-cell differentiation in mice. Maret *et al*. (2003) demonstrated that a physiological level of 17β-estradiol promoted antigen-specific CD4 T cells in ovariectomized mice without an adjuvant. Yoshino *et al.* (2004) demonstrated that mice exposed prenatally to 300 and 3000 μg/kg of BPA showed increases in anti-hen egg lysozyme (HEL) IgG, as well as antigen-specific cell proliferation, and that prenatal exposure to BPA resulted in increased secretion of IFN-γ and IL-4 and an increased rate of CD4+ T cells in the mouse spleen. In the present study, however, no effects of BPA were observed on anti-SRBC IgM levels, lymphoproliferative responses, cytokine (IFN-γ or IL-4) concentrations, or weights of thymus or spleen. Therefore, the doses of BPA in the present study did not have any effects on the immune functions in mice, though T-cell differentiation in the female thymus was modified. These results suggest that attention should also be paid to T-cell differentiation in the female thymus in research on EDCs.

Early onset of sexual maturation was reported in female mice delivered from dams treated with low doses of BPA (20 μg/kg/day) during gestation (Newbold *et al.*, 2007; Honma *et al.*, 2002). However, early onset of sexual maturation was not observed in mice neonatally treated with BPA (5 and 50 μg/kg) in the present study.

Although increased body weight in mice after exposure to low doses of BPA (Rubin *et al.*, 2001) or DES (Newbold *et al.*, 2007) has been reported, decreased body weight was observed in male mice after neonatal exposure to low doses of BPA in this study. When the body weights of the male mice were compared to the background data we have on the same strain, the data of the control group in the present study were relatively high and those of the BPA groups were similar to the background data.

In conclusion, early life exposure to low doses of BPA did not accelerate onset of abnormal estrous cycles in mice. However, T-cell differentiation in the female thymus was modified by low doses of BPA, though the doses of BPA did not have any effect on the immune functions in either sex.

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

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