Editor's Announcement

Quantitative morphometric analysis of vimentin filaments in Sertoli cells of rats after *in utero* DBP exposure

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I have retracted the above paper as Editor-in-Chief of *Fundamental Toxicological Sciences* since I have serious concerns about it, primarily due to inappropriate authorship on a non-negligible scale.

Since the possibility of inappropriate authorship in this paper was raised, I contacted the co-authors to confirm this point. I found out that several of them considered their listing as co-authors to be inappropriate. In addition, more than half of the co-authors agreed to the retraction of this paper.

These facts raise concerns about the paper. From the standpoint of maintaining the integrity of the research community, I felt that such a paper should be retracted at once.

Accordingly, I sent a summary of my concerns about this paper to the corresponding author, Dr. Shin Wakui. I also had an online interview with him to discuss this matter. I told Dr. Wakui that inappropriate authorship on a non-negligible scale is a serious problem that raises concerns about the paper.

I prepared a draft of this Editor's Announcement and sent it to Dr. Wakui for review prior to revision and release. Although he did not agree to the retraction, I have decided to take this action from the standpoint of maintaining the integrity of the research community.

I coordinated my response to this issue with Dr. Toshiyuki Kaji, Editor-in-Chief of *The Journal of Toxicological Sciences*, a sister journal of *Fundamental Toxicological Sciences*.

Akira Naganuma, Ph.D.
Editor-in-Chief
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Original Article

Quantitative morphometric analysis of vimentin filaments in Sertoli cells of rats after *in utero* DBP exposure

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ABSTRACT — The intermediate filament of mature Sertoli cells is vimentin (Vim). One of the toxicological consequences of phthalate exposure is a selective decrease in Vim, an intermediate-sized (10 nm) cytoplasmic microfilament, in Sertoli cells. Vim in Sertoli cells of rats exposed *in utero* to 100 mg/kg/day di(*n*-butyl) phthalate (DBP) on gestation days 12-21 was quantified. Immunohistochemical analysis revealed that Vim aggregated in Sertoli cells, but desmin filaments did not. Vim images were extracted from electron microscopic images using the computer program Imaris (Bitplane Scientific, Zeiss) and analyzed using Image-Pro plus (Media Cybernetics, USA). The amount of perinuclear Vim located within 0.5 μm of the nuclear membrane, where most Vim is aggregated, and the Vim volume ratios of the DBP group were similar to those of the vehicle group at 7 and 9 weeks, but those of the DBP group had decreased 0.63-times at 14 weeks and 0.48-times at 17 weeks compared to those of the vehicle groups. The present study showed that the testicular toxicity of *in utero* exposure to DBP seemed to be delayed type toxicity, and showed that improved morphometric methods cold be used widely for quantitative analysis of cellular cytoplasmic filaments.

Key words: Electron microscopy, Rat testis, Sertoli cell, *In utero* DBP exposure

INTRODUCTION

Phthalates are present in a wide variety of products from personal care products to medical devices because they provide flexibility and other desirable characteristics. Di(*n*-butyl) phthalate (DBP) is one of the most commonly used phthalates (Rodrigeuez-Sosa *et al*., 2014). Sertoli cells appear to be a primary target of the toxic effect of these chemical phthalates in rats (Richburg and Boekelheide, 1996; Kleymenova *et al*., 2005; Boekelheide *et al*., 2009) .

Sertoli cells possess well-developed intermediate-sized microfilaments, called vimentin (Vim), that are 10 nm in diameter and have a molecular weight of 55-58 kDa (Franke *et al*., 1979). It has been reported that exposure of 3-week-old rats to di(2-ethlhexl)phthalate (DEHP) caused disruption and collapse of Vim microfilaments in Sertoli cells (Erkekoglu *et al*., 2012). Moreover, the volume of Vim microfilaments in Sertoli cells was reported to be reduced in young rats after exposure to other phthalates including DEHP, mono(2-ethylhexyl)phthalate (MEHP), and DBP (Richburg *et al*., 1999; Tay *et al*., 2007; Alam *et al*., 2010). Although one study concerning the alteration of Vim in Sertoli cells after *in utero* DBP exposure was reported, it was limited to five postnatal days (Kleymenova *et al*., 2005).

In the present study, rats *in utero* were exposed to DBP (intragastrically, 100 mg/kg/day) (Wakui *et al*., 2013; Motohashi *et al*., 2016), and the perinuclear volume of Vim microfilaments in Sertoli cells was quantified at 7, 9, 14, and 17 weeks using improved electron microscopy morphometric quantitative analysis techniques.

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MATERIALS AND METHODS

Animals, chemicals, and experiment design

DBP (99.8% pure) was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Sixteen 8-week-old time-mated female Sprague-Dawley rats were obtained from SRL Co. (Japan SLC, Shizuoka, Japan) on gestation day 0; the day of copulation was confirmed. Upon arrival, animals were allocated to dose groups using randomization of body weights to ensure equal weight distribution among groups. Animals were housed in a HEPA-filtered, mass-air-displacement room maintained on a 12-hr light-dark cycle at approximately 18-26°C with a relative humidity of 30-50% and fed a conventional diet (MF, Oriental Yeast). Pregnant dams were housed individually, and three offspring rats were housed per polycarbonate cage with wood chips as bedding after weaning.

All experimental procedures were conducted with the approval of the Animal Care and Use Committee of the Azabu University School of Veterinary Medicine and guidelines set by the National Institutes of Health and Public Health Service Policy on the Humane Use and Care of Laboratory Animals were followed at all times. Eight pregnant rats were treated intragastrically (i.g.) with 100 mg DBP (Tokyo Kasei Kogyo Co.)/kg in corn oil (Nacalai Tesque Inc.) or for other eight rats treated intragastrically (i.g.) with an equivalent volume of corn oil (~0.5 mL/animal, i.g.) on days 12 through 21 post-conception. This dose of phthalate was chosen because several previous studies described adverse effects on fetal rats at the level of 100 mg/kg/day (Mylchreest *et al*., 1999; Barlow and Foster, 2003). The offspring were sexed at birth, and litters were reduced so that each dam was left with ten offspring (five males and females/dam), then we investigated 40 DBP treated male rats and 40 vehicle treated male rats. Weaning was carried out on day 21 post-partum. At 7, 9, 14, and 17 weeks of age, one male offspring of each dam was randomly selected from each group, anesthetized with isoflurane via a CO_2 overdose, and the testes were removed. For immunohistochemical analysis, ten rats' testes of each DBP or vehicle treated group were frozen, cut into serial 5 mm transverse sections, and fixed in 10% phosphate-buffered formalin. For electron microscopy, before frozen, small testicle samples from rats' tests of each DBP or vehicle treated group were fixed in 1.2% glutaraldehyde buffered with 0.1 M phosphate for 2 hr and then post fixed in 1.0% osmium tetroxide for 2 hr. After dehydration in graded alcohols, the specimens were embedded in Epon 812 (TAAB, Berkshire, UK). One-µm sections were cut on a Porter-Blum MT-IIb ultramicrotome and stained with methylene blue. Further, for ultrastructural study, thin sections were cut on a Porter-Blum MT-IIb ultramicrotome and mounted on formvar-coated slit grids. After double staining with uranyl acetate and lead citrate, the sections were observed using a Hitachi H500H 7100 electron microscope.

Immunohistochemical analysis

Immunohistochemical expressions of vimentin or desmin was analyzed using the avidin-biotin complex (ABC) method. After deparaffinization and hydration, 4-µm thick sections were treated with 0.3% H_2O_2 for 10 min and blocked with 10% goat or horse serum in phosphate-buffered saline (PBS, 10 mM KPO_4, 150 mM NaCl, pH 7.4) for 20 min. Then, sections were rinsed in PBS and treated with vimentin or desmin antibodies (Sigma-Aldrich, St. Louis, MO, USA; vimentin antibody diluted x20, desmin antibody diluted x30), which were detected with biotinylated IgG (Vector Laboratories, Burlingame, CA, USA; diluted x100), followed by ABC-peroxidase staining (Vector Laboratories) and diaminobenzidine (Sigma-Aldrich). Sections were counterstained with Mayer's hematoxylin. As a negative control, non-immunized serum was substituted for the primary antibody. The number and diameter of seminiferous tubules and the number of Sertoli cells in all vimentin and desmin antibody-stained sections were counted using light microscopy (Nikon Coolscope, Tokyo, Japan).

Morphometric analysis

The nuclear margin of each Sertoli cell was observed using a transmission electron microscope and photographed at X24,000, requiring 10-14 photographs to acquire a target Sertoli cell nucleus by electron microscopy. Then each micrograph was converted to 50 mm x 50 mm (1,200 pixel/inch) (Adobe Photoshop, San Jose, CA, USA), and figures were converged using color gamut with a tolerance region of cluster color lenses (75-80%) to extract only 10-nm sized microfilaments (Imaris, Bitplane Scientific, Zeiss) (Fig. 1). After that, the accuracy of extracted microfilament sizes was checked (Image-pro Plus ver.7., CA, USA), and if the size of filaments was out of alignment, the tolerance region of the cluster color lenses was implemented again according to the recommendations (Imaris, V 6.1.0 Reference Manual). Then, whole Sertoli cell nuclei (Fig. 2) and the total volume of extracted intermediate-sized microfilaments in the converted micrographs were counted by Image-pro Plus ver.7. The volume of 10-nm microfilaments in the peripheral area (0.5 µm distance) surrounding the nucleus membrane (Figs. 3-1, 3-2) where most Vim was aggregated without any cellular organellae was calculated. The Vim

Table 1. Morpholomitrical analysis vimentin volume ratios at perinulear (0.5 um) of Sertoli cell *in utero* DBP exposed rats.

Group	No. of rats	Weeks of autopsy[a]	No. of Sertoli cells[b]	Vimentin occupancy ratio[c,d]
DBP	10	7	50	0.096 + 0.008[NS]
Vehicle	10	7	50	0.090 + 0.009
DBP	10	9	50	0.083 + 0.005[NS]
Vehicle	10	9	50	0.092 + 0.008
DBP	10	14	50	0.065 + 0.016*
Vehicle	10	14	50	0.102 + 0.009
DBP	10	17	50	0.052 + 0.018*
Vehicle	10	17	50	0.107 + 0.009

[a] Day of autopsy at postnatal weeks old.
[b] Total number of investigated cells number per each rat.
[c] Total area of Vim in the peripheral area (0.5 um) of the nucleus / Total area of the peripheral area (0.5 um) of the nucleus.
[d] Values are means ± S.D.
[NS] Student's *t*-test, non-significant (as compared with values of Vehicle group same postnatal weeks olds).
*Student's *t*-test, $p < 0.05$ (as compared with values of Vehicle group same postnatal weeks old).

volume ratio was identified as the (total area of Vim in the peripheral area of the nucleus) / (total area of the peripheral area of the nucleus).

Statistical analysis

For each set of mean values, standard deviation and standard error of means were calculated and compared by Student's *t*-test ($p < 0.05$) using the statistical computer analysis system Stat View-J 5.0 (Abacus Concepts Co. Ltd., Piscataway, NJ, USA).

RESULTS

The immunohistochemical analysis of vimentin (Vim) protein expression qualitatively revealed normal expression in Sertoli cells in the vehicle groups at 7 through 17 weeks, while that of the groups administered DBP revealed decreased Vim expression at 14 and 17 weeks (Fig. 3). On the other hand, desmin protein expression was not observed in Sertoli cells in rats 7 to 17 weeks old in both the DBP and vehicle groups (Fig. 4). Quantitative analysis using the improved electron microscopy morphometric analysis technique showed that Vim volume ratio at the amount of perinuclear in Sertoli cells, 0.5 um from the nuclear membrane, of the DBP group were similar to those of the vehicle group at 7 and 9 weeks, but those of the DBP group had decreased 0.63 times at 14 weeks and 0.48 times at 17 weeks compared to those of the vehicle groups. (Table 1, Figs. 1, 2).

DISCUSSION

In rat testis, vimentin (Vim) microfilaments are an important component of the Sertoli cell cytoskeleton and are thought to play a role in positioning the perinuclear region of Sertoli cells and projecting into the apical cytoplasm and Sertoli cells to anchor spermatogenic cells to the seminiferous epithelium. Further, they act as a mediator of cellular signal transduction between the plasma membrane and nucleus, and are involved in maintaining the integrity of adjacent spermatogenetic cell contacts (Richburg and Boekelheide, 1996; Amlani and Vog, 1988).

However, the direct quantitative analysis of Vim microfilaments was not possible by immunohistochemical and/or routine electron microscopy analysis, while the present study, the first quantitative analysis by the improved method applied computer programs, indicated for the first time the reduction of Vim microfilaments in Sertoli cells of rats at 14 and 17 weeks following *in utero* DBP exposure. The decrease of Vim in Sertoli cells seemed to be linked to testicular testosterone levels (Wakui *et al*., 2013; Motohashi *et al*., 2016). Moreover, the present study also shows that new morphometric methods can be used widely for quantitative analysis of cellular cytoplasmic filaments.

DBP exposure leads to reduced numbers of Vim microfilaments in Sertoli cells, as has previously been described, and suggests that the collapse of Vim is the first step toward cell degeneration (Alam *et al*., 2010; Zhang *et al*., 2015). Similarly, alteration of distributions of Sertoli cell Vim microfilaments has been shown after administration of 2,5-hexanedione (Hall *et al*., 1991) and the fungicide benomyl (Hess and Nakai, 2000) and hormone withdrawal (Show *et al*., 2003). However, the mechanism of the dysfunction of the Sertoli cells induced by phthalates is still unclear, and further study required.

The present study utilized an improved method of elec-

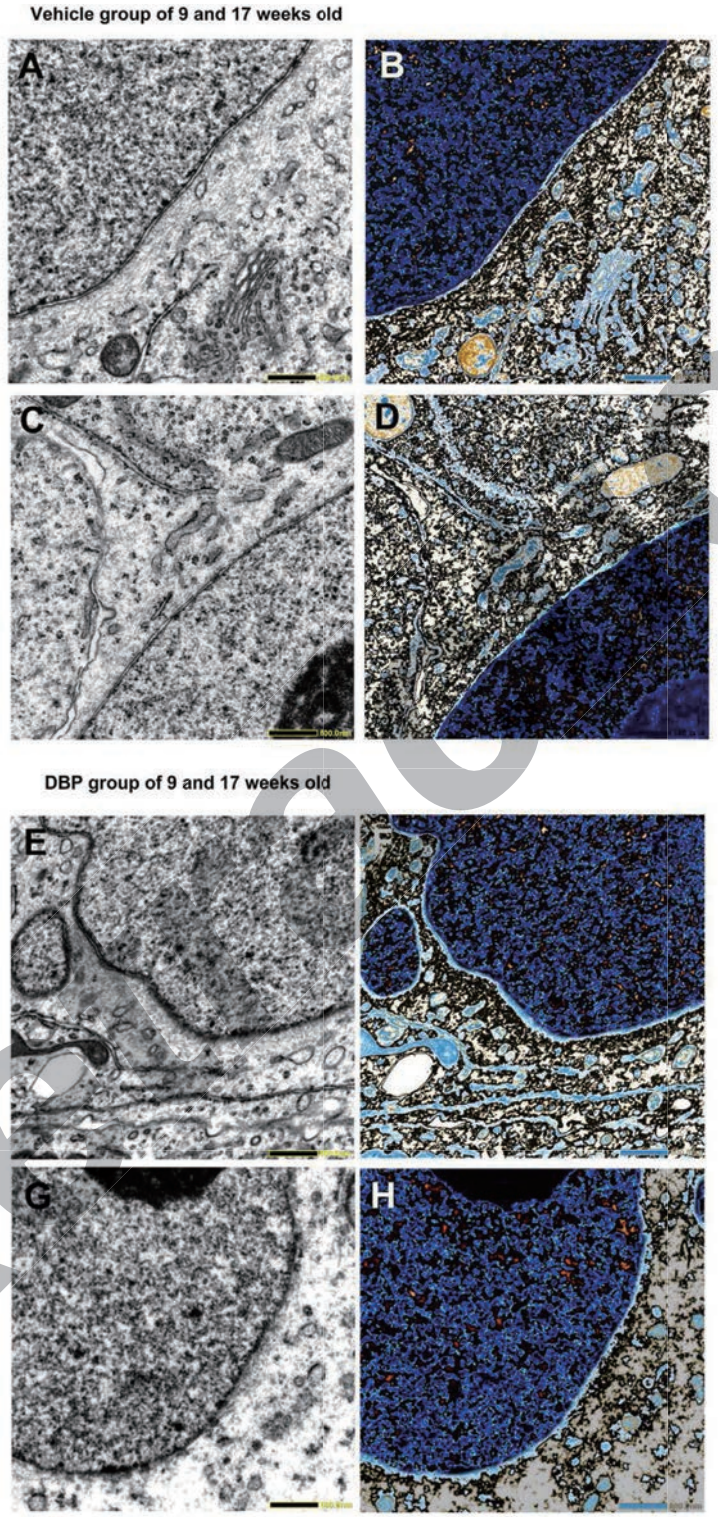

Fig. 1. High power view of routine electron micrographs (A, C, E, G), and vimentin (Black) extracted (B, D, F, H). Representative vimentin images of Sertoli cells in vehicle group at 9 weeks old (A, B) and 17 weeks old (C, D), and vimentin of Sertoli cells of DBP groups at 9 weeks old (E, F) and 17 weeks old (G, H). N: nucleus Bar = 500 nm.

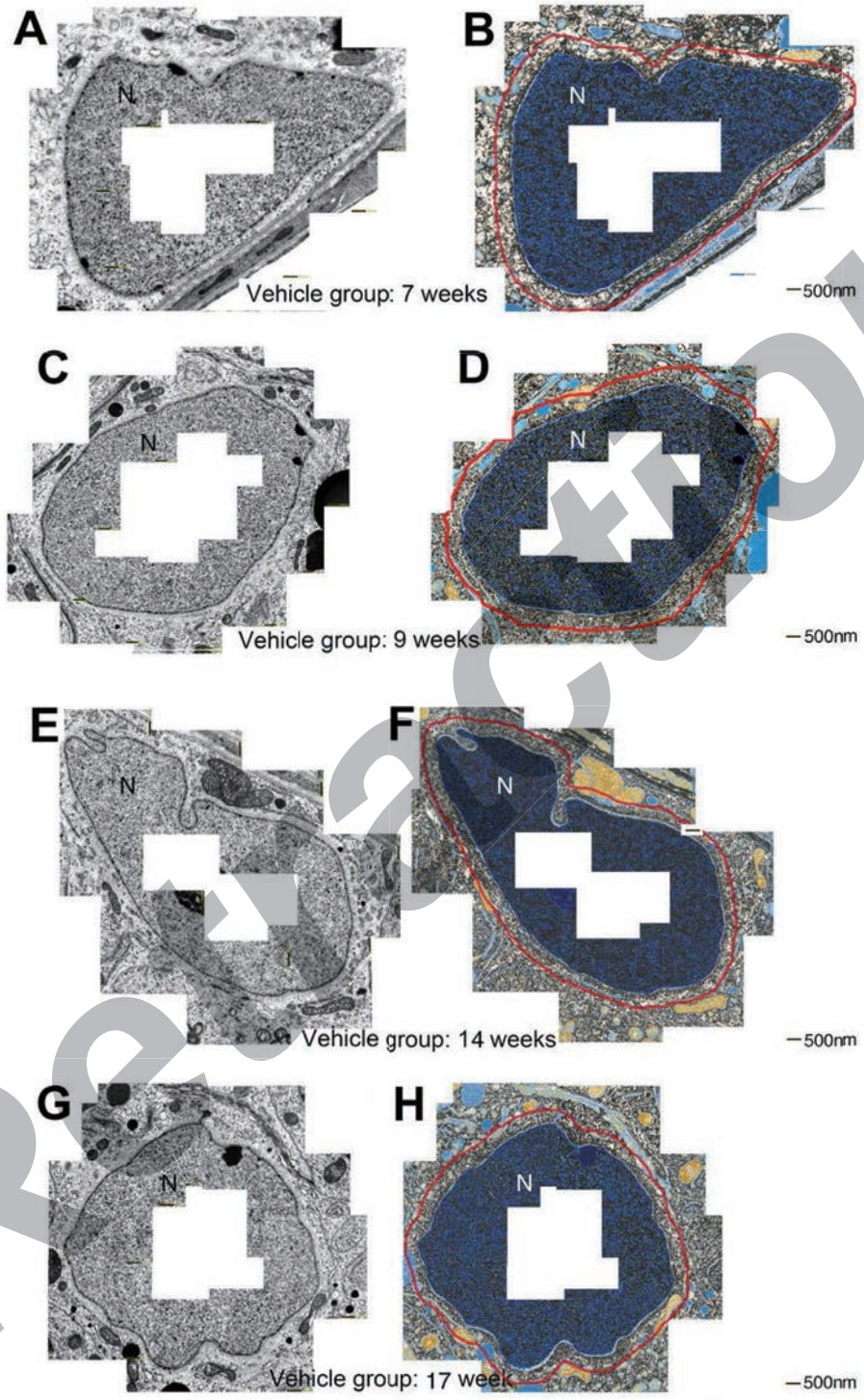

Fig. 2. Low power view of representative routinely stained electron micrographs (left side) and vimentin-extracted micrographs (right side). Vehicle group, A, B are at 7 weeks; C, D are at 9 weeks; E, F are at 14 weeks; and G, H are at 17 weeks. Red lines show the perimeter of the nucleus and 0.5 um from the nucleus membrane except no data area. N: nucleus Bar = 500 nm.

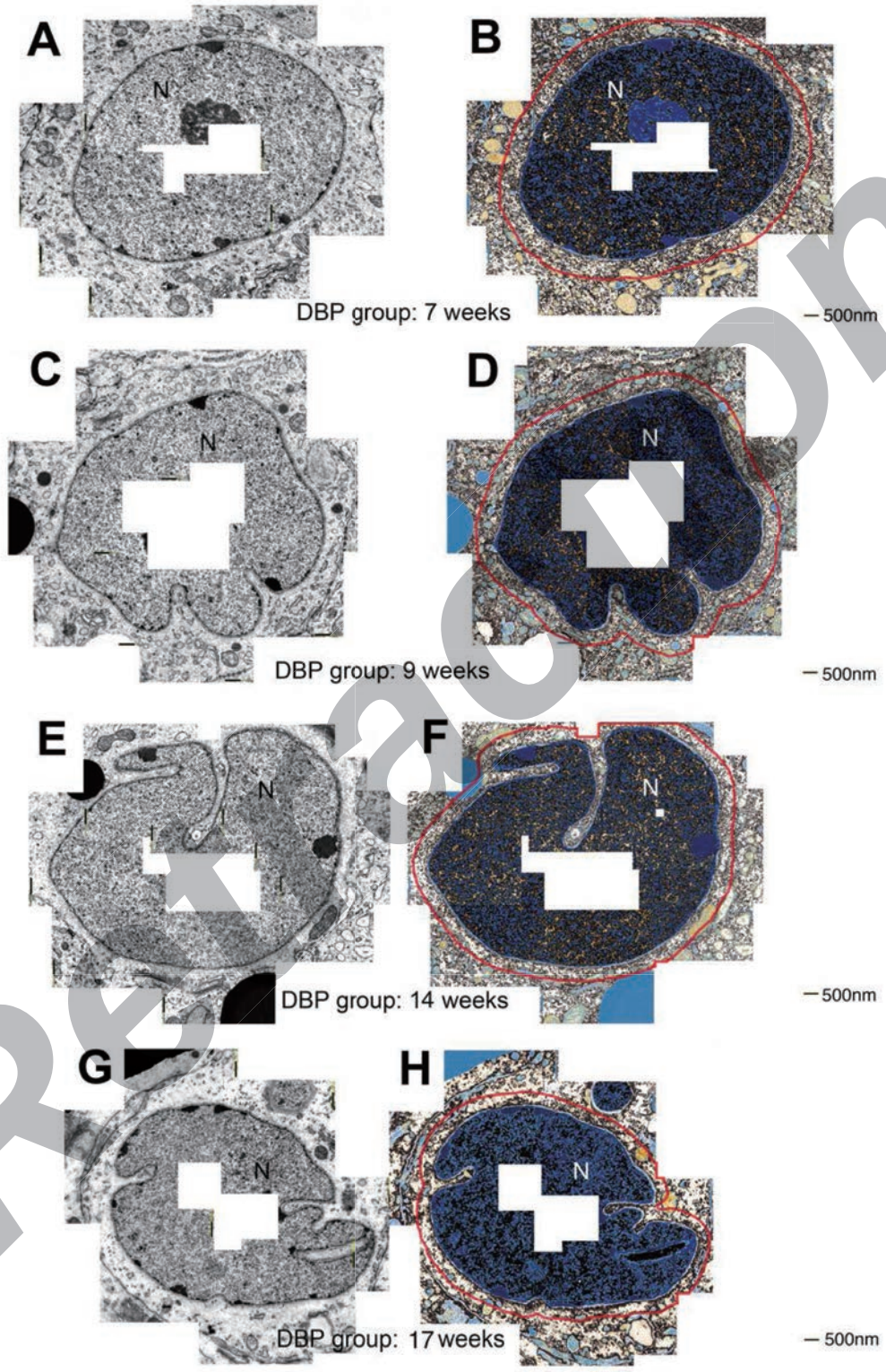

Fig. 3-1. Representative immunohistochemical analysis of vimentin. DBP group, A, B are at 7 weeks; C, D are at 9 weeks; E, F are at 14 weeks; and G, H are at 17 weeks. Red lines show the perimeter of the nucleus membrane except no data area. N: nucleus.

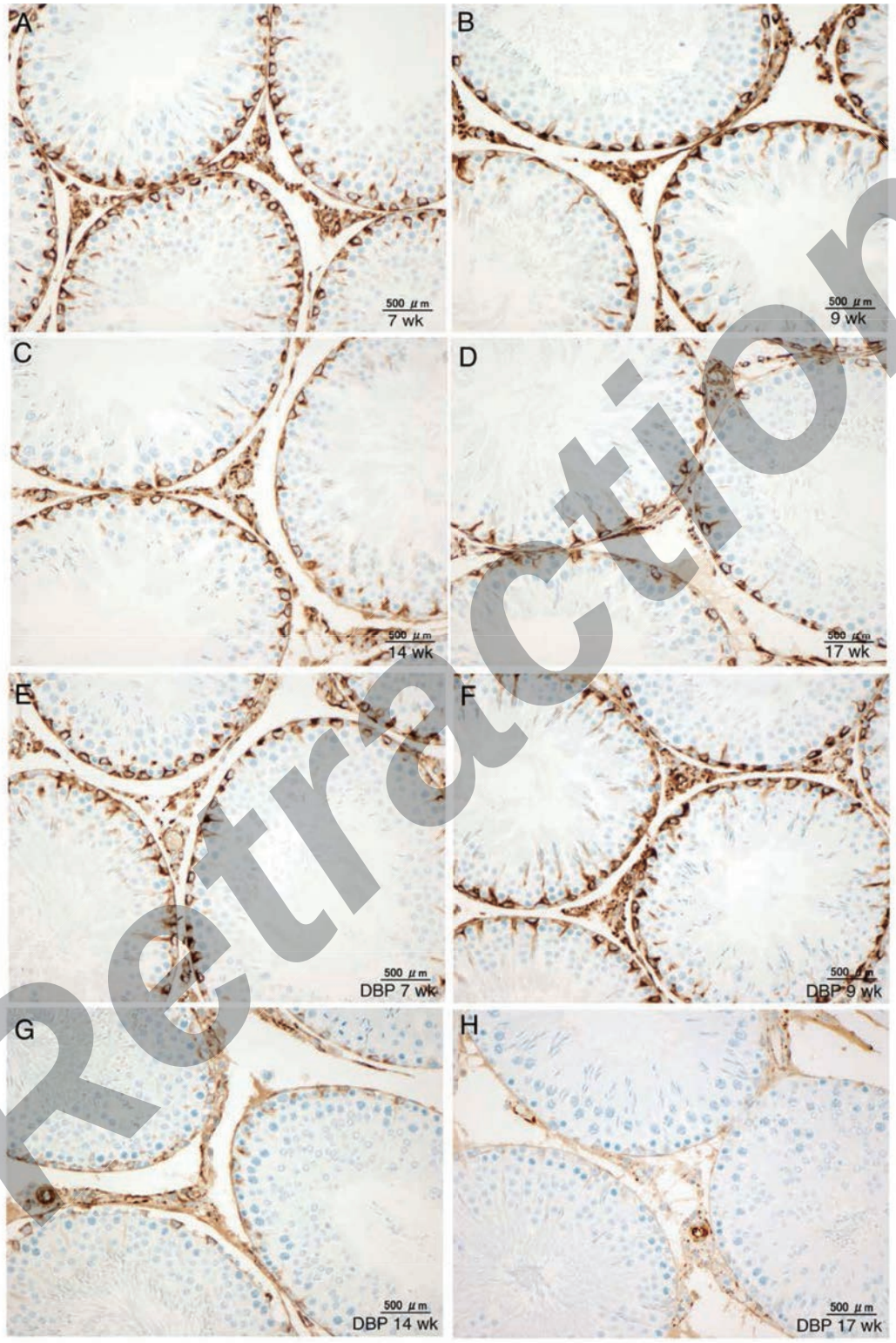

Fig. 3-2. Representative immunohistochemical analysis of vimentin. Vehicle group: A, 7 weeks old; B, 9 weeks old; C, 14 weeks old; D, 17 weeks old. Bar = 500 µm. DBP group: E, 7 weeks old; F, 9 weeks old; G, 14 weeks old; H, 17 weeks old.

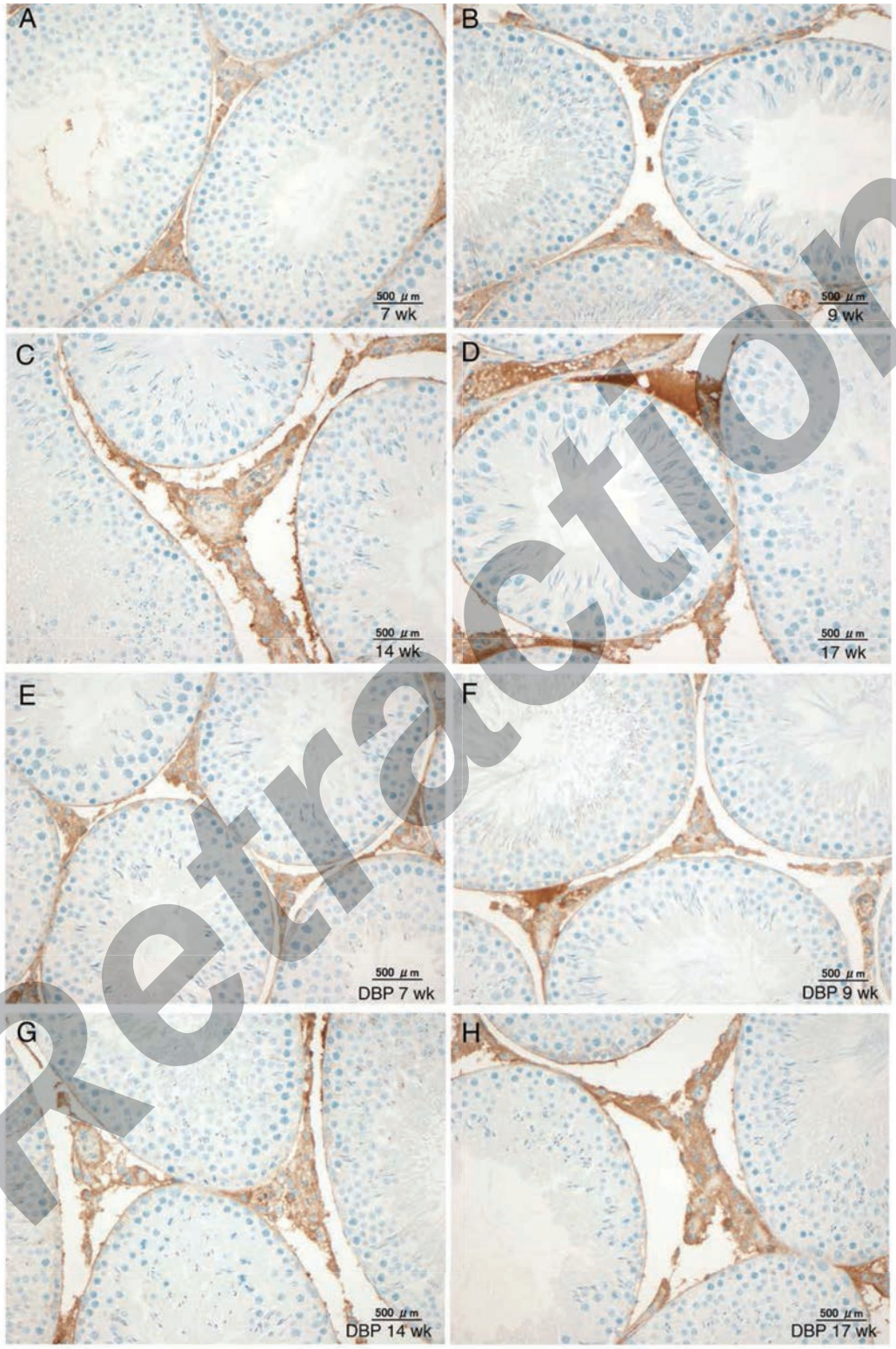

Fig. 4. Representative immunohistochemical analysis of desmin. Vehicle group: A, 7 weeks old; B, 9 weeks old; C, 14 weeks old; D, 17 weeks old. DBP group: E, 7 weeks old; F, 9 weeks old; G, 14 weeks old; H, 17 weeks old. Bar = 500 µm.

tron microscopy, morphometric analysis. The amounts of Vim in the DBP and vehicle groups were similar in juvenile to puberty periods, but that of the DBP group significantly decreased in adult periods. It shows that the testicular toxicity of *in utero* exposure to DBP seemed to be a delayed type of toxicity.

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

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