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Original Article

Anatomical and histological sex differences in the eye and its accessory tissues in Dutch belted rabbits

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ABSTRACT — Rabbits are commonly used as laboratory animals in ocular toxicity studies. During development of ophthalmic drugs and medical devices, knowledge regarding sex differences of ocular anatomy and histology is important in the interpretation of toxicity data. However, limited information is available regarding ocular sex differences in rabbit eyes. Information on sex differences of rabbit eyes supports the justification of using single sex in the design of toxicity studies. We therefore investigated anatomical and histological ocular sex differences in Dutch belted rabbits aged 6 to 20 weeks which are frequently used in toxicity studies. Anatomical parameters of the eyeball (axial length, diameter, weight, volume), cornea (height, diameter), lens (thickness, diameter, weight, volume), and vitreous humor (weight, volume) were measured. Paraffin blocks of eye, meibomian glands, and lacrimal glands from males and females were sectioned and stained using hematoxylin and eosin, and compared. As the results, the anatomical parameters and their growth ratios showed no difference between males and females. In the histological comparison, although the development of glandular tissues was observed in both sexes according to aging, no sex differences were found. We concluded that there is no anatomical or histological sex differences in the eyes of Dutch belted rabbits from the period of post-weaning to sexual maturation.

Key words: Dutch belted rabbit, Sex difference, Eyeball, Meibomian gland, Lacrimal gland

INTRODUCTION

Rabbits are commonly used as laboratory animals in ocular toxicity studies, since their eyes are of an appropriate size for examinations (Kurata *et al*., 2016), and have relatively higher sensitivity against irritants than human eyes (Roggeband *et al*., 2000). Knowledge of ocular anatomy and histology is important in the interpretation of toxicity of drugs, especially locally applied ophthalmic drugs such as eye drops, and in advanced ocular therapeutics, including medical devices. So far, some basic information is available on the ocular anatomy of laboratory rabbits; e.g., inter-breed differences among Dutch belted (Dutch), Japanese white (JW), and New Zealand white (NZW) male rabbits (Atsumi *et al*., 2015); and postnatal growth until 8 weeks of age in NZW rabbits (Barathi *et al*., 2002).

Dutch rabbit is a major breed used in ocular toxicity studies (Kurata *et al*., 2017). In rabbits, sexual maturity occurs earlier in females than in males, and it has been reported that smaller breeds mature earlier than large ones; Dutch rabbits mature at 4 to 5 months of age and NZW rabbits mature at 5 to 7 months of age (Hillyer, 1994). As mentioned above, some information regarding ocular anatomy and histology has been reported in rabbits. However, the information regarding ocular sex differences is limited to axial length of albino rabbits (Barathi *et al*., 2002; Bozkir *et al*., 1997). Because inter-breed differ-

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ences in anatomical parameters of eyeball between pigmented and albino rabbits have been reported (Atsumi *et al*., 2013), it is difficult to extrapolate the results for NZW rabbits directly to Dutch rabbits. No investigation on sex differences in ocular tissues of Dutch rabbits has been made throughout the period from post-weaning to sexual maturation, the ages frequently employed in the toxicity studies, even though both male and female animals are commonly used in toxicity studies. Information regarding ocular sex differences of Dutch rabbits would strengthen the rationale for using single sex in the design of toxicity studies.In the present study, we therefore investigated differences in anatomical parameters and histological structure between male and female Dutch rabbits aged from 6 to 20 weeks.

MATERIALS AND METHODS

Animals and ocular tissues

The eyes and ocular accessory tissues (eyelids and lacrimal glands) of Dutch rabbits (species, *Oryctolagus cuniculus*; strain/breed, Kbl:Dutch) were obtained from Kitayama Labes Co., Ltd. (Nagano, Japan), in which the eyes were collected from healthy purpose-bred rabbits (both sexes; at 6, 13, or 20 weeks of age; n = 5/sex/age) ethically euthanized for other purpose. Since we needed to collect the ocular tissues from Dutch rabbits at as close to the same age as possible to prevent variation, we used 5 animals at each age and sex. The purpose and all the experimental procedures of this study complied with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research were approved by the Institutional Animal Care and Use Committee (IACUC).

Measurement of anatomical parameters of the eyeballs

The right eyes were used for measurement of anatomical parameters. The eyeballs were frozen at the time they were collected. The frozen eyeballs were thawed completely before measurement. Axial length and diameter of eyeballs and height and diameter of corneas were measured using an electronic digital caliper (NSK MAXCAL, Nihon Sokutei Kogu Co., Ltd., Hyogo, Japan), weight of eyeballs was measured using an electronic balance (AC210S, Sartorius AG, Göttingen, Germany), and volume of eyeballs was measured using a measuring cylinder. Prior to dissection, the eyeballs were completely frozen in a deep-freezer at approximately -80°C for at least one day. Immediately after moving the frozen eyeballs to the laboratory at room temperature, the eyeballs were gradually half-thawed by means of touching the surface of the eyeballs gently with operator's fingers (Robbins and Galin, 1969). The half-thawed eyeballs were dissected using surgical scissors and tweezers. The lens and vitreous humor were carefully separated from the half-thawed eyeballs, and then lens diameter and lens thickness were measured using the electronic digital caliper. Weight of lens and vitreous humor were measured using the electronic balance. Volume of lens and vitreous humor were measured using the measuring cylinder. The instruments used for measuring parameters are summarized in Table 1.

Tissue preparation

The left eyeballs and periocular gland tissues were fixed using 1% neutral-buffered formalin/2.5% glutaraldehyde solution. Following fixation, the tissues were embedded in paraffin and sectioned at 5-µm thickness. The slices were stained with hematoxylin and eosin (HE).

Statistical analysis

The statistical differences in anatomical parameters of eyeballs were determined by Student's *t*-test using Excel 2010 software (Ver. 14.0.7015.1000; Microsoft Corporation, Redmond, WA, USA). Differences were considered significant at $p < 0.05$.

RESULTS

Anatomical parameters of the eyes

Measurement values of body weight and the anatomical parameters of eyeballs, corneas, lens, and vitreous humor in males and females at each age examined are summarized in Table 2. Statistically significant differences between sexes were noted in horizontal diameter of eyeballs (males < females), vertical diameter of corneas (males < females), and vitreous humor weight (males > females) at 6 weeks of age. At 13 weeks of age, significant differences between sexes were shown in the vertical and horizontal diameters of cornea (males > females). No significant difference was shown at 20 weeks of age. The differences presented at 6 and 13 weeks of age were very slight, and ranges of values were overlapped or close in both sexes. Table 3 shows growth ratios of anatomical parameters. Growth ratios were calculated for each of the following periods: 6 to 20 weeks of age (whole period), 6 to 13 weeks of age (early period), and 13 to 20 weeks of age (later period). Although weight and volume of eyeball, lens, and vitreous humor showed higher growth ratios during the whole period and the early period, no sex differences were noted. Lens weight showed especial-

Table 1. The instruments for measuring parameters and units.

Tissue	Parameter	Instrument/container for measurement	Unit
Eyeball	Axial length	Electronic digital calipers	mm
	Vertical diameter	Electronic digital calipers	mm
	Horizontal diameter	Electronic digital calipers	mm
	Weight	Electronic balance	g
	Volume	Measuring cylinder	mL
Cornea	Height[a)]	Electronic digital calipers	mm
	Vertical diameter	Electronic digital calipers	mm
	Horizontal diameter	Electronic digital calipers	mm
Lens	Thickness	Electronic digital calipers	mm
	Diameter[b)]	Electronic digital calipers	mm
	Weight	Electronic balance	g
	Volume	Measuring cylinder	mL
Vitreous	Weight	Electronic balance	g
	Volume	Measuring cylinder	mL

a) The length measured from limbus to the anterior pole of the eyeball.
b) The lens diameter measured three times and adopted the average value.

Table 2. The ocular anatomical parameters of male and female Dutch eyes at each age.

Age (Week-old)		6		13		20	
Sex		Male	Female	Male	Female	Male	Female
Body weight (kg)		0.98 ± 0.04	0.92 ± 0.03	1.62 ± 0.06	1.71 ± 0.04	2.01 ± 0.11	2.17 ± 0.13
		0.86 - 1.08	0.84 - 0.98	1.48 - 1.82	1.58 - 1.82	1.74 - 2.34	1.94 - 2.66
Eyeball	Axial length (mm)	13.17 ± 0.12	13.31 ± 0.17	14.91 ± 0.23	14.83 ± 0.11	15.51 ± 0.13	15.45 ± 0.09
		12.86 - 13.46	12.89 - 13.76	14.41 - 15.45	14.61 - 15.21	15.27 - 15.95	15.16 - 15.74
	Vertical diameter (mm)	13.30 ± 0.25	13.65 ± 0.19	15.16 ± 0.25	15.19 ± 0.23	15.34 ± 0.14	15.41 ± 0.10
		12.42 - 13.93	13.24 - 14.26	14.38 - 15.89	14.45 - 15.76	15.03 - 15.75	15.18 - 15.74
	Horizontal diameter (mm)	14.39 ± 0.17*	14.83 ± 0.24	16.16 ± 0.25	16.25 ± 0.28	16.41 ± 0.13	16.57 ± 0.13
		13.86 - 14.80	14.10 - 15.35	15.32 - 16.75	15.34 - 16.85	16.12 - 16.89	16.25 - 16.88
	Weight (g)	1.43 ± 0.03	1.46 ± 0.04	2.00 ± 0.07	2.04 ± 0.02	2.32 ± 0.06	2.31 ± 0.04
		1.37 - 1.49	1.35 - 1.56	1.84 - 2.18	1.99 - 2.11	2.14 - 2.50	2.18 - 2.36
	Volume (mL)	1.40 ± 0.00	1.44 ± 0.04	2.02 ± 0.06	2.00 ± 0.00	2.32 ± 0.07	2.34 ± 0.04
		1.40 - 1.40	1.30 - 1.50	1.90 - 2.20	2.00 - 2.00	2.10 - 2.50	2.20 - 2.40
Cornea	Height (mm)	3.24 ± 0.07	3.34 ± 0.18	3.86 ± 0.17	3.94 ± 0.21	4.25 ± 0.04	4.35 ± 0.03
		3.02 - 3.44	3.02 - 3.91	3.54 - 4.49	3.51 - 4.65	4.10 - 4.36	4.25 - 4.41
	Vertical diameter (mm)	9.93 ± 0.14*	10.16 ± 0.15	11.84 ± 0.13*	11.36 ± 0.07	12.67 ± 0.07	12.52 ± 0.05
		9.42 - 10.23	9.72 - 10.47	11.58 - 12.35	11.18 - 11.58	12.52 - 12.86	12.39 - 12.70
	Horizontal diameter (mm)	10.99 ± 0.22	10.98 ± 0.17	12.82 ± 0.13*	12.40 ± 0.12	13.74 ± 0.08	13.52 ± 0.08
		10.33 - 11.45	10.37 - 11.37	12.56 - 13.24	12.10 - 12.82	13.55 - 13.98	13.34 - 13.78
Lens	Thickness (mm)	3.80 ± 0.11	3.93 ± 0.03	5.01 ± 0.15	4.77 ± 0.12	5.91 ± 0.14	5.82 ± 0.19
		3.43 - 4.09	3.86 - 3.99	4.60 - 5.38	4.33 - 4.99	5.54 - 6.17	5.28 - 6.23
	Diameter (mm)	7.81 ± 0.19	6.44 ± 0.48	8.39 ± 0.28	8.29 ± 0.24	8.60 ± 0.09	8.52 ± 0.09
		7.19 - 8.26	5.06 - 7.34	7.75 - 9.45	7.45 - 8.77	8.35 - 8.77	8.33 - 8.78
	Weight (g)	0.13 ± 0.01	0.11 ± 0.02	0.22 ± 0.02	0.23 ± 0.01	0.32 ± 0.01	0.30 ± 0.02
		0.11 - 0.16	0.05 - 0.16	0.17 - 0.26	0.21 - 0.26	0.29 - 0.34	0.24 - 0.35
	Volume (mL)	0.14 ± 0.02	0.14 ± 0.02	0.22 ± 0.02	0.20 ± 0.00	0.30 ± 0.00	0.28 ± 0.02
		0.10 - 0.20	0.10 - 0.20	0.20 - 0.30	0.20 - 0.20	0.30 - 0.30	0.20 - 0.30
Vitreous humor	Weight (g)	0.61 ± 0.02*	0.51 ± 0.04	0.80 ± 0.02	0.85 ± 0.03	1.12 ± 0.03	1.10 ± 0.04
		0.57 - 0.69	0.44 - 0.66	0.76 - 0.89	0.79 - 0.94	1.06 - 1.22	0.95 - 1.21
	Volume (mL)	0.58 ± 0.04	0.51 ± 0.03	0.78 ± 0.02	0.84 ± 0.02	1.06 ± 0.04	1.06 ± 0.06
		0.50 - 0.70	0.40 - 0.60	0.70 - 0.80	0.80 - 0.90	1.00 - 1.20	0.90 - 1.20

Upper: Average ± SEM (n = 5) Lower: Minimum value - Maximum value *: $p < 0.05$ vs. female average value at the same age (Student's t-test).

Table 3. The growth ratios of ocular anatomical parameters of male and female Dutch rabbits.

Period		Whole (6 to 20 weeks of age)		Early (6 to 13 weeks of age)		Later (13 to 20 weeks of age)	
Sex		Male	Female	Male	Female	Male	Female
Body weight		2.05	2.36	1.65	1.86	1.24	1.27
Eyeball	Axial length	1.18	1.16	1.13	1.11	1.04	1.04
	Vertical diameter	1.15	1.13	1.14	1.11	1.01	1.01
	Horizontal diameter	1.14	1.12	1.12	1.10	1.02	1.02
	Weight	1.62	1.59	1.40	1.41	1.16	1.13
	Volume	1.66	1.63	1.44	1.39	1.15	1.17
Cornea	Height	1.31	1.30	1.19	1.18	1.10	1.10
	Vertical diameter	1.28	1.23	1.19	1.12	1.07	1.10
	Horizontal diameter	1.25	1.23	1.17	1.13	1.07	1.09
Lens	Thickness	1.56	1.48	1.32	1.21	1.18	1.22
	Diameter	1.10	1.32	1.07	1.29	1.03	1.03
	Weight	2.46	2.73	1.77	2.09	1.39	1.30
	Volume	2.14	2.00	1.57	1.43	1.36	1.40
Vitreoushumor	Weight	1.84	2.12	1.31	1.63	1.40	1.29
	Volume	1.83	2.12	1.34	1.68	1.36	1.26

ly high growth ratio at each period. On the other hand, growth ratios of length parameters such as axial length and diameters of eyeball and cornea were small, especially during the later period, at which the ratios were near to 1.00. These data indicate that the anatomical parameters of eyeball, cornea, lens, and vitreous humor are comparable between males and females at each age examined.

Histological observation for the ocular tissues

Representative histological findings at 6 and 20 weeks of age are shown in Fig. 1 and Fig. 2, respectively. Images at 13 weeks of age are not shown. No apparent difference of eyeball histology, including the retina, choroid, and sclera, was observed between the sexes, nor at each age examined. We also found no histological difference at cornea, iris, ciliary body, and lens (data not shown). Meibomian glands and central ducts in upper and lower eyelids appeared to be undeveloped in both sexes at 6 and 13 weeks of age. However, they developed markedly in both males and females at 20 weeks of age. In lacrimal glands, although the sizes of acini were almost the same in all ages, the boundary of each acini group become clearer at 20 weeks of age compared to 6 weeks of age. In these glandular tissues, no clear histological sex differences were found at any age examined.

DISCUSSION

Regarding ocular sex differences, it is known that axial length of eyes differs between sexes in humans (Midelfart, 1996), rhesus monkeys (Fernandes *et al*., 2003), and NZW rabbits (Barathi *et al*., 2002; Bozkir *et al*., 1997). In humans, axial length of elders is shorter in females than in males, since a shortening of axial length occurs in elder women (Midelfart and Asamo, 1994). Isenberg *et al*. reported that the growth speed of neonate eyes is faster in males than in females (Isenberg *et al*., 1995). In rhesus monkeys, axial length in males is longer than that in females at 25 years of age or older (Fernandes *et al*., 2003), which indicates that the sex difference occurs long after sexual maturity. In NZW rabbit, axial length is longer in males than in females at 1 and 56 days after birth (Barathi *et al*., 2002). In the present study, we found no apparent ocular sex differences in Dutch rabbits, including axial length at the ages frequently used in toxicity studies. These findings also suggest that a pattern of sex differences is not common among the animal species or breeds.

In regards to growth of the eye, the growth ratios of ocular anatomical parameters were higher in the early period than in the later period in Dutch rabbits. The decline of ocular growth ratio with aging has been reported in several breeds of rabbit at different growth stages (Atsumi *et al*., 2015; Barathi *et al*., 2002). A similar tendency of the change in growth ratio has been also reported in humans (Fledelius *et al*., 2014) and rhesus monkeys (Smith *et al*., 2005). In the present study, the lens weight showed high growth ratio at each age examined. It has been reported that the growth ratio of lens weight is maintained from 6 to 96 weeks of age in rabbits (Atsumi *et al*., 2015), and in humans, the lens weight increases continuously (1.38 mg/year) during the lifetime

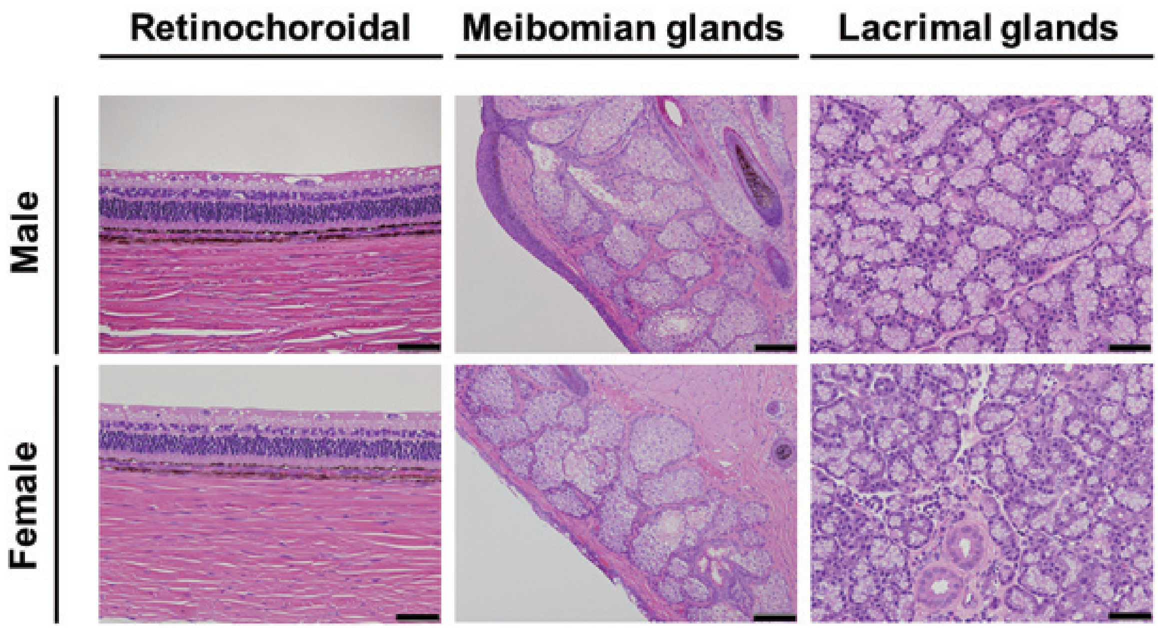

Fig. 1. HE-stained specimen of the retinochoroidal and sclera, meibomian glands with palpebras, and lacrimal glands of male and female Dutch rabbits aged 6 weeks. Representative images of males (upper) and females (lower) are shown. Scale bars represent 50 µm (retinochoroidal and lacrimal glands) and 100 µm (meibomian glands).

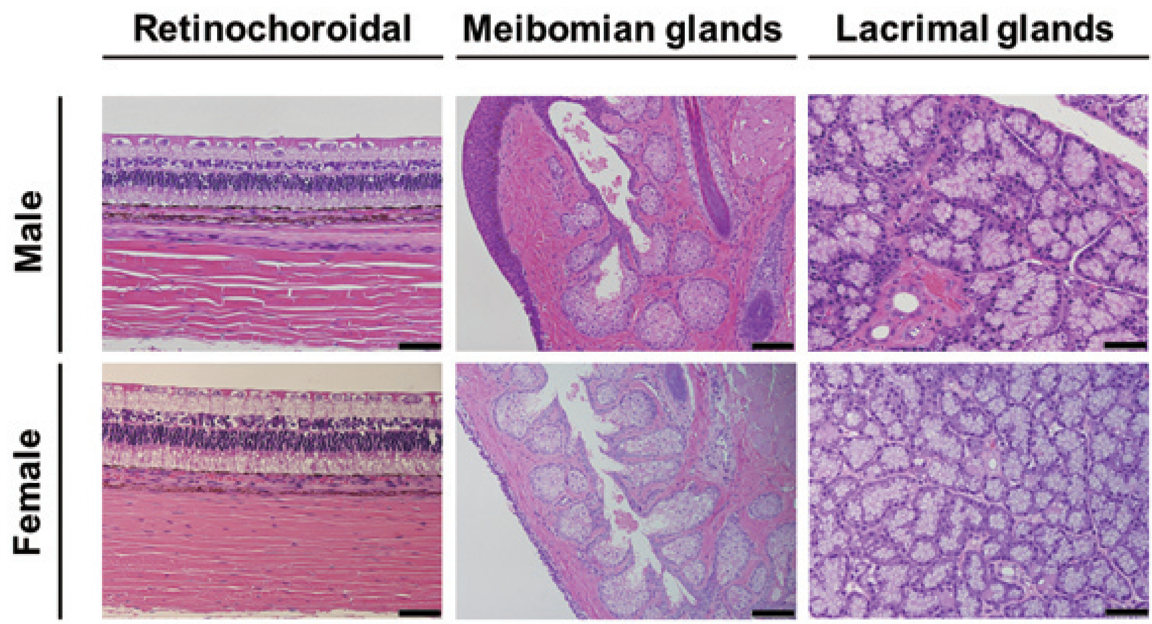

Fig. 2. HE-stained specimen of the retinochoroidal and sclera, meibomian glands with palpebras, and lacrimal glands of male and female Dutch rabbits aged 20 weeks. Representative images of males (upper) and females (lower) are shown. Scale bars represent 50 µm (retinochoroidal and lacrimal glands) and 100 µm (meibomian glands).

(Augusteyn, 2007). Our present results are consistent with the previous reports, and indicate that the tendency for the growth of the eye is relatively common among the species.

The present study indicates that no histological sex differences exist in Dutch rabbits, at least during the ages of 6 to 20 weeks, except for the development of glandular tissues according to maturation. Meanwhile, functional sex differences of lacrimal glands are reported in mature NZW rabbits, e.g., activity of enzymes, total amount of glandular protein, and expression of several receptors (Azzarolo *et al*., 1993). In humans, it has also been reported that lacrimal fluid peroxidase activity is different between males and females (Marcozzi *et al*., 2003). Regarding meibomian glands, expression pattern of sex hormone receptors were different between the sexes of rats and humans, although no apparent histological differences were observed (Sullivan *et al*., 2000; Wickham *et al*., 2000). It is likely that further investigation on functional sex differences of eye is needed to clarify the ocular character of rabbits.

Currently, in drug development, design of toxicity studies using juvenile animals is argued for supporting human clinical trials in a pediatric cohort (ICH S11 Step 1, Nonclinical Safety Testing in Support of Development of Pediatric Medicines). As described above, in the ophthalmologic field, rabbits under 20 weeks of age are a frequently employed species in toxicological studies as well as pharmacological studies. The weaning period of Dutch rabbits is around 6 weeks of age, and experimental treatments can be started after that. Hence, the information on ocular anatomical/histological characteristics at the ages examined in this study is valuable for development of ophthalmic drugs. The data will support the conduction of toxicity studies using single sex in which the study aim is limited to the toxicity of eyes, leading to reduction of animal use in accordance with of the philosophy of 3Rs.

In conclusion, the present results clarify anatomical parameters and histological characteristics of Dutch rabbits from the period of post-weaning to sexual maturation. We have provided background data on ocular sex differences of Dutch rabbits, which are frequently used in non-clinical studies for development of ophthalmic drugs.

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

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