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RESEARCH ARTICLE

Juvenile toxicity of atropine sulfate eye drops in young rats

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ABSTRACT

Objectives: This study was to investigate the effects of atropine sulphate eye drops (ASED)on the development of partial systems in young rats and their toxic reactions following repeated eye-drop administration over a period of 40 days.

Methods: SD rats of 20 days old were randomly assigned to control group, 0.01, 0.02, and 0.04% ASED groups, with 60 females and 25 males per group. ASED was given by eye drops from PND_{21} onwards and normal saline was given in the control group at 10 µL/eye once a day for 40 days, in both right and left eyes. Rats of ASED groups were instilled with eye drops at the 10 µL/day per eye, from postnatal day 21 (PND_{21}) to PND_{60} for 40 consecutive days. The clinical observation, body weight, food intake, physical development, physiological development, reproductive development, ophthalmic examination, intraocular pressure, and axial length of the rats were examined during the study period.

Results: ASED at concentrations of 0.01, 0.02, 0.04%, dose levels of 0.002, 0.004, 0.008 mg/day per rat, had no toxicological effects on the clinical observation, body weight, food intake, physical development, physiological development, reproductive development, ophthalmic examination, intraocular pressure, and axial length in rats.

Conclusion: The no-observed-adverse-effect-level (NOAEL) of ASED in young SD rats equivalent to human over 2 years old was 0.008 mg/day at a concentration of 0.4 mg/mL.

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KEYWORDS
Atropine sulphate eye drops; repeated dose; young rats; developmental toxicity; ophthalmic examination

1. Background information

Myopia is one of the common eye diseases, and its incidence is increasing year by year. It is expected that myopia patients will account for nearly 50% of the global population with about 4.8 billion by 2050 [1]. Studies have shown that before the age of 6 years is a period of rapid visual development in children, from 7 to 18 years of age is a period of visual plasticity, and by the end of puberty visual acuity becomes basically stable [2]. The prevalence of myopia in children and adolescents in China in 2022 was 51.9%, with the prevalence of myopia among primary school students at 36.7%, junior high school students at 71.4%, and senior high school students as high as 81.2% [3]. When myopia develops in early childhood without control and finally develops into the high myopia in adulthood, it may cause serious ophthalmic complications, such as glaucoma, retinal detachment, and macular haemorrhage, which eventually lead to blindness [4,5]. Therefore, it is important to develop effective long-term prevention and treatment strategies to slow down myopia progression in children, and the safety of long-term medication use needs to be addressed simultaneously.

Several studies have demonstrated that atropine eye drops is effective in controlling myopia with slowing down the growth rate of refractive error and axis, may it as an adjunctive treatment for myopia prevention and control [6–11]. A 5-year clinical trial of atropine for the treatment of myopia conducted by the Singapore National Eye Centre confirmed that atropine drops at a concentration of 0.01% was effective in delaying the progression of myopia in children and had a better safety profile than atropine drops than at a higher concentration [7,8]. Another European study observed 184 myopic children treated with 0.01% atropine eye drops for 5 consecutive years, no adverse effects occurred during the study period [9]. It is also reported that some sensitive paediatric populations experience symptoms such as photophobia and blurred vision, and a very small percentage of patients experience allergic and irritant reactions [10,11]. ASED with concentration of 0.01%, was approved and marketed in China in March 2024

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for the purpose of slowing down the progression of myopia in children at aged 6–12 years old with spherical lens dioptres ranging from −1.00 D to −4.00 D (astigmatism ≤1.50 D and refractive error ≤1.50 D). In this study, PND_{21} rats were selected to systematically evaluate the developmental toxicity of ASED following repeated administration for 40 days, which provides a reference for the clinical application of ASED in the population of children over 2 years old.

2. Materials and methods

2.1. Test substances and reagents

ASED (concentration: 0.01%, batch No.: 20122; concentration:0.02%, batch No.: 20011; concentration:0.04%, batch No.: 20012), supplier: Shenyang Xingqi Pharmaceutical Co., Ltd. (Shenyang, China). Sodium chloride injection (batch No.: G20061803B), supplier: Guangdong Kelun Pharmaceutical Co., Ltd. (Guangdong, China). Fluorescein Sodium Injection (supplier: Guangzhou Baiyunshan Ming Xing Pharmaceutical Co., Ltd., batch No.: 191101). Isoflurane (supplier: RWD Life Science, batch No.: 20081001, 21022101). Giemsa's stain (supplier: Macklin, batch No.: C10891331). Glycerol and methanol (supplier: Chinasun Specialty Products Co., Ltd., batch No.: 20200415, 20200401). Disodium hydrogen phosphate (supplier: Taishan Yueqiao Reagent Plastics Co., Ltd., batch No.: 20170901, 20180501). Monopotassium phosphate (suppliers: Chinasun Specialty Products Co., Ltd., Guangzhou Xincheng Fine Chemicals Co., Ltd., batch No.: 20180901, 131231). 95% ethanol (suppliers: Chinasun Specialty Products Co., Ltd., batch No.: 20210308).

2.2. Laboratory animals

There were 240 female rats and 100 male rats, with body weight of 50–70 g, were naturally delivered from healthy SD rats at 20–23 days of gestation. Pregnant rats were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China, Licence No.: SCXK (Xiang) 2019–0004). The animals were kept in the specific-pathogen-free (SPF) grade animal room of Guangdong Lewwin Pharmaceutical Research Institute Co., Ltd., and the feed and drinking water were freely available to the animals. The room temperature was controlled at 20–26 °C, the relative humidity was kept at 40–70%. The ventilation rate was no less than 15 times per hour with a 12-h light/dark cycle. This study was reviewed and approved by the Animal Welfare and Ethics Committee of Guangdong Lewwin Pharmaceutical Research Institute Co., Ltd. (No.: IA-SE2021028-01).

2.3. Randomisation

At 2 days of age, whole litters with more than 10 live pups and more than 4 males and 4 females per litter were selected, and then 4 males and 4 females were kept for each litter, and pups from different litters were randomly assigned to form new litters. Three hundred and forty healthy postnatal 20-day-old (PND_{20}) SD rats were selected and randomly assigned to four groups according to body weight and sex, that is, normal control group, 0.01, 0.02, and 0.04% ASED groups, with 25 males and 60 females in each group.

2.4. Animal treatment

After randomisation, eye drop administration was given to PND_{21} rats of all groups at 10 μL/eye once a day. ASED was administered at concentrations of 0.1 mg/mL (0.01%), 0.2 mg/mL (0.02%), and 0.4 mg/mL (0.04%) (at doses of 0.002, 0.004, and 0.008 mg/rat/day, respectively) to the left and right eyes continuously for 40 days (PND_{21} ~ PND_{60}), and equal volume of 0.9% sodium chloride injection was given to the control group. During the administration, the test article or control article was aspirated with a pipette and dropped on the central surface of the cornea, and then the head of the animal was restrained to keep the eyeball upward and unclosed for at least 5 s.

2.5. Clinical observations

During the dosing period (PND_{21} ~ PND_{60}), the young rats (female rats: 60; male rats: 25) in each group were observed at least once a day, including animal appearance, physical signs, secretions, respiration, faecal characteristics, toxic reactions, and mortality.

2.6. Body weight

During the dosing period, body weight was measured once for each group of rats (female rats: 60; male rats: 25) on PND_{24}, PND_{27}, PND_{30}, PND_{33}, PND_{36}, PND_{42}, PND_{49}, PND_{56}, and PND_{60}, respectively.

2.7. Food intake

The amount of feed added to each cage was weighed and recorded weekly after administration, and the amount of feed remaining in each cage was weighed and recorded approximately 24 h later, and finally the average daily food intake of a single animal in each cage was calculated based on the equation. Feed addition weighing dates: PND_{22}, PND_{28}, PND_{34}, PND_{40}, PND_{48}, PND_{55}, and PND_{59}; Feed remaining weighing dates: PND_{23}, PND_{29}, PND_{35}, PND_{41}, PND_{49}, PND_{56}, and PND_{60}.

Calculation formula: Average daily food intake per animal = (amount of feed added per cage − amount of feed remaining per cage)/number of animals in the same cage.

2.8. Physical examination

Physical examination of the animals (female rats: 60; male rats: 25) in each group was carried out on PND_{21}, PND_{28}, PND_{35}, PND_{42}, PND_{49}, and PND_{56} using digital calliper (Shanghai Meinaite Industrial Co., Ltd., China), including measurements of crown-rump length, tail length, limbs length, and chest circumference.

2.9. Physiological development

Vaginal opening refers to the disappearance of the membrane in the vagina and the occurrence of a small hole. The vaginal opening of female animals (60 animals/group) in each

group was examined on PND_{35}, and the number of animals with normal vaginal opening in each group was recorded in detail.

Foreskin separation refers to the separation of the glans within the outer skin of the penis from the prepuce. The foreskin separation of male animals (25 animals/group) in each group was examined on PND_{42}, and the number of animals with normal foreskin separation in each group was recorded in detail.

2.10. Reproductive development

Examination of oestrus cycle in female animals: Vaginal smears were performed on female animals (60 animals/group) in all groups on PND_{49} ~ PND_{60}, once a day, for a total of 12 smears per animal. The smears were fixed with 95% ethanol for 10 min, then stained with Giemsa stain, washed, and dried, and finally examined for vaginal cytology under a biological microscope (LEICA, Germany). The average frequency of female rats in the preestrus, oestrus, postestrus, and anestrum periods, respectively, was observed in each group over a period of 12 days.

Semen analysis of male animals: The male rats (25 animals/group) in each group were euthanized on PND_{60}. One side of the epididymis of each rat was taken, sperms in the epididymis were counted by semen analyser (Xuzhou Guojian Electronic Equipment Technology Co., Ltd., China), the proportion distribution of sperms in grades A, B, and C was examined, and the total sperm count and sperm mobility in the epididymis were statistically analysed.

2.11. Ophthalmic examination

Ophthalmic examination was performed as the route of administration in this study was by eye drops. The eyelids, conjunctiva, appearance of the eyeballs, orbits, and lacrimal glands of young rats (female rats: 60; male rats: 25) in each group were observed on $PND_{20–21}$, $PND_{40–41}$, and $PND_{58–59}$, and the cornea, pupil, sclera, anterior chamber, lens, vitreous, retina, fundus vascular morphology, optic papillae, macular structure, and corneal damage of all rats in each group was examined byophthalmoscope. The corneal injury of all rats in each group was examined through fluorescein sodium examination.

2.12. Intraocular pressure

On PND_{21}, PND_{41}, and PND_{59}, 10 male and 10 female rats in each group were randomly selected to measure intraocular pressure. Examination method: The rats were anaesthetised by isoflurane-induced inhalation anaesthesia, and after anaesthesia, the intraocular pressure of both eyes of the rats was measured by ophthalmotonometer (ICARE, Netherlands). The tip of the probe of the metre was contacted to the central cornea of the rat's eyes for intraocular pressure measurement.

2.13. Axial length

On PND_{41}, 10 male and 10 female rats in each group were randomly selected to measure axial length measurement. Examination method: Isoflurane-induced inhalation anaesthesia was used to anaesthetise the rats, and after anaesthesia, the axial length of both eyes of the animals was measured using the ophthalmic ultrasound diagnostic instrument (Chongqing KangHua RuiMing S&T Co., Ltd., China), in which the probe was placed vertically in the middle of the pupil of the examined eyes, and the A-ultrasonic waveforms were automatically immobilised and calibrated to obtain the measurement data.

2.14. Statistical analysis

The statistical data were represented by $\bar{x}x \pm S$ and analysed by GraphPad Prism 8.0, and the experimental data were all evaluated by One-way Anova for the overall differences. When the variance was homogeneous, Least Significant Difference was adopted for intergroup difference test; otherwise, the Dunnett's T3 was used. Chi-squared (x^2) test or Fisher's exact test was used to compare the enumeration data between groups, and $P < 0.05$ indicated that the difference was statistically significant. The number of male and female rats used for each measurement is listed in Table 1.

Table 1. Distribution of the number of male and female young rats.

		n			
Indicators	Gender	Control	0.01% ASED	0.02% ASED	0.04% ASED
Clinical observation	♀	60	60	60	60
	♂	25	25	25	25
Body weight	♀	60	60	60	60
	♂	25	25	25	25
Food intake	♀	60	60	60	60
	♂	25	25	25	25
Physical examination	♀	60	60	60	60
	♂	25	25	25	25
Physiological development	♀	60	60	60	60
	♂	25	25	25	25
Reproductive development	♀	60	60	60	60
	♂	25	25	25	25
Ophthalmic examination	♀	60	60	60	60
	♂	25	25	25	25
Intraocular pressure	♀	10	10	10	10
	♂	10	10	10	10
Axial length	♀	10	10	10	10
	♂	10	10	10	10

3. Results

3.1. Clinical observation

During the dosing period (PND_{21} ~ PND_{60}), no abnormalities were noted in young rats of control group, 0.01, 0.02, and 0.04% ASED groups.

3.2. Body weight

Compared with the control group, no significant difference was noted in the body weight of young rats in the 0.01, 0.02, and 0.04% ASED groups ($P > 0.05$, Figure 1).

3.3. Food intake

Compared with the control group, the food intake of female young rats in the 0.02% ASED group was significantly higher ($P < 0.05$, Figure 2A) on PND_{48}, and male young rats in the 0.04% ASED group was significantly lower ($P < 0.05$, Figure 2B) on both PND_{48} and PND_{59}.

3.4. Physical examination

Compared with the control group, female young rats in 0.01% ASED group had a significantly lower tail length on PND_{49} ($P < 0.05$, Figure 3B[1]), female young rats in 0.01% ASED group

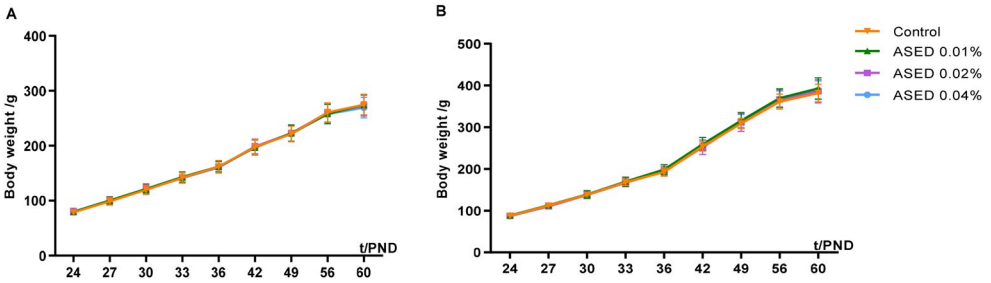

Figure 1. Effect of ASED on body weight of rats. (A) Body weight of female rats (B), Body weight of male rats. Female rats: $n = 60$; Male rats: $n = 25$.

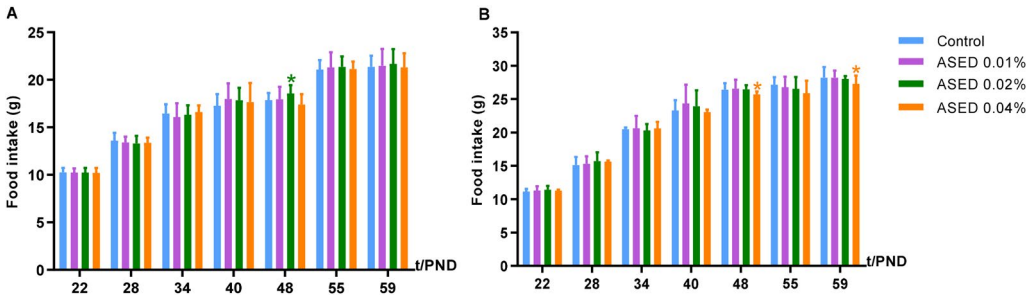

Figure 2. Effect of ASED on food intake of rats. (A) Food intake of female rats, (B) Food intake of male rats. *Compared with the control group, $P < 0.05$. Female rats: $n = 60$; Male rats: $n = 25$.

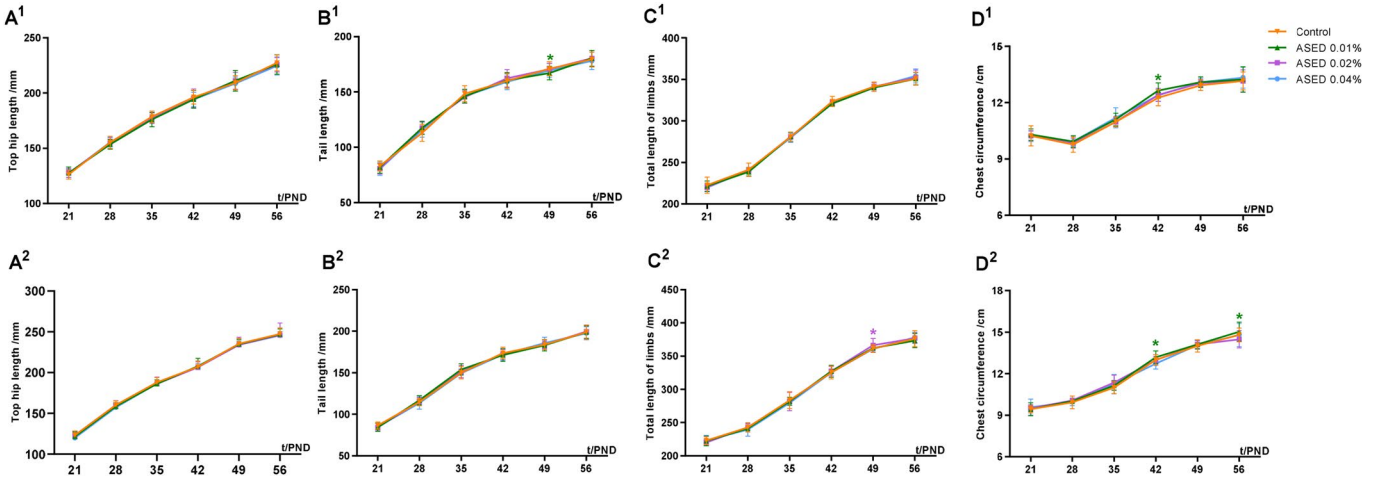

Figure 3. Effect of ASED on physical examination of rats. (A^1) Top hip length of female rats, (B^1) Tail length of female rats, (C^1) Total length of female rats' limbs, (D^1) Chest circumference of female rats, (A^2) Top hip length of male rats, (B^2) Tail length of male rats, (C^2) Total length of male rats' limbs, (D^2) Chest circumference of male rats. *Compared with the control group, $P < 0.05$. Female rats: $n = 60$; Male rats: $n = 25$.

had a significantly higher chest circumference on PND_{42} ($P<0.05$, Figure 3D[1]), male young rats in 0.02% ASED group had a significantly higher total limbslength on PND_{49} ($P<0.05$, Figure 3C[2]), and male young rats in 0.01% ASED group had a significantly higher chest circumference on PND_{42} and PND_{56} ($P<0.05$, Figure 3D[2]). In the 0.01, 0.02, and 0.04% ASED groups, no significant differences ($P>0.05$, Figure 3A[1], C[1]) were observed in top hip length and total limbs length of female young rats from PND_{21} to PND_{56}, nor in top hip length and tail length of male young rats during this period ($P>0.05$, Figure 3A[2], B [2]).

3.5. Physiological development

The physiological development indicators (vaginal opening and foreskin separation) in the 0.01, 0.02, and 0.04% ASED groups all reached the standard of the same period, and no statistical difference was noted between them and the control group ($P>0.05$, Table 2).

3.6. Reproductive development

Compared with the control group, no significant difference was found ($P>0.05$) in the frequency of preestrus, oestrus, postestrus, and anestrum of female rats in the 0.01, 0.02, and 0.04% ASED groups (Figure 4A); no significant difference was found ($P>0.05$) in the total sperm count (Figure 4B) and sperm motility (Figure 4C) (sperm in A, B, C class proportion distribution) of male rats in the 0.01, 0.02, and 0.04% ASED groups.

3.7. Ophthalmic examination

Ophthalmic examination showed no abnormalities in the eyelids, conjunctiva, ocular appearance, orbit, lacrimal glands, cornea, pupil, sclera, anterior chamber, lens, vitreous, retina, fundus vascular morphology, optic papillae, and macular structure in all rats of the control group, 0.01, 0.02, and 0.04% ASED groups. Sodium fluorescein staining examination revealed no injury to the cornea in all groups of animals.

3.8. Intraocular pressure

Compared with the control group, the intraocular pressure of the right eye of the female young rats in the 0.02 and 0.04% ASED groups was significantly increased on PND_{40} ($P<0.05$, Figure 5A). No significant differences were seen ($P>0.05$) in the intraocular pressures of the left eye of female young rats (Figure 5A), and of the left and right eyes of male young rats in the 0.01, 0.02, and 0.04% ASED groups on PND_{20} ~ PND_{58} (Figure 5B).

3.9. Axial length

Compared with the control group, no significant changes were noted in the axial lengths of the left and right eyes of female young rats, and of the left eye of male young rats in the 0.01, 0.02, and 0.04% ASED groups ($P>0.05$, Figure 6A), and the axial length of the right eye of male young rats in the 0.01 and 0.04% ASED groups was significantly decreased ($P<0.05$, Figure 6B).

4. Discussion

Atropine, an acetylcholine M receptor antagonist, slows myopia progression by inhibiting the synthesis and release of acetylcholine, inhibiting parasympathetic excitability, and competitively occupying muscarinic receptors on the cell membrane of the pupillary sphincter [12]. Studies have demonstrated that the myopia control effect of 0.01% atropine eye drops is negatively correlated with age, and the younger the age at baseline gets the better responsiveness. This may be related to the organic changes such as ocular axial lengthening have not yet occurred in the younger age group [4, 13]. Similarly in a prospective study, it was found

Table 2. Effects of ASED on physiological development of rats.

	Physiological development (number of normal-standard rats)	
Group	Vaginal opening (♀ PND_{35})	Foreskin separation (♂ PND_{42})
Control	60	25
0.01% ASED	59	25
0.02 % ASED	60	25
0.04 % ASED	58	25

Vaginal opening (♀ PND_{35}): $n=60$; Foreskin separation (♂ PND_{42}): $n=25$.

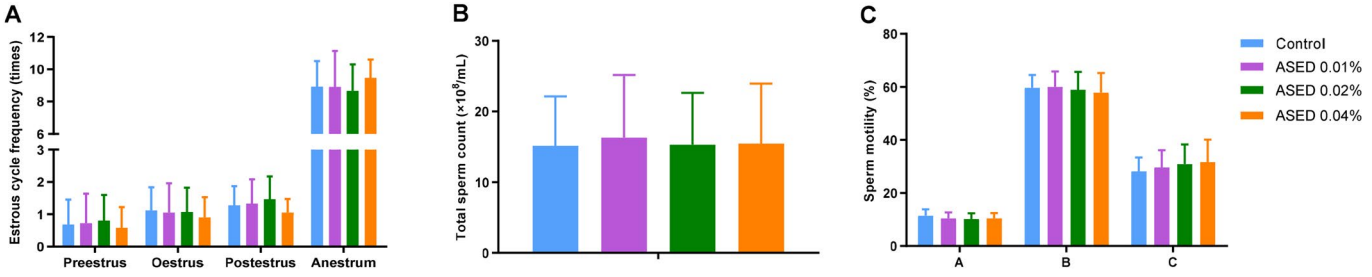

Figure 4. Effect of ASED on reproductive development of rats. (A) Oestrous cycle frequency of female rats, (B) Total sperm count of male rats, (C) Sperm motility of male rats. Female rats: $n=60$; Male rats: $n=25$.

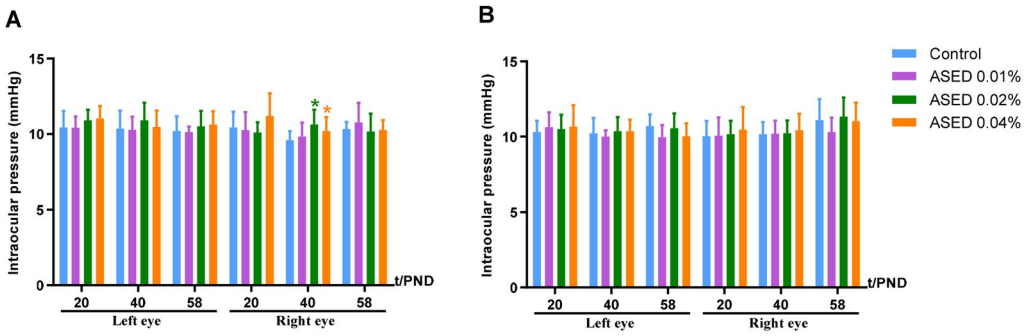

Figure 5. Effect of ASED on intraocular pressure of rats. (A) Intraocular pressure of female rats, (B) Intraocular pressure of male rats. *Compared with the control group, $P < 0.05$. Female rats: $n = 10$; Male rats: $n = 10$.

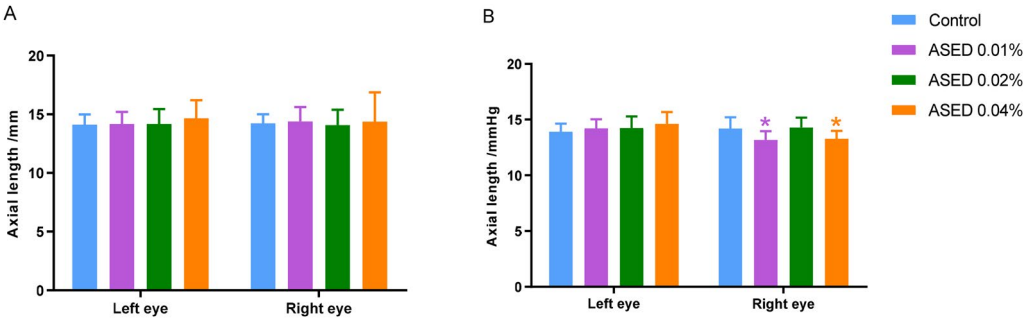

Figure 6. Effect of ASED on axial length of rats. (A) Axial length of female rats, (B) Axial length of male rats. *Compared with the control group, $P < 0.05$. Female rats: $n = 10$; Male rats: $n = 10$.

that the earlier treatment of atropine eye drops in paediatric patients with myopia, the less possibly they were to develop high myopia at a later stage [14]. Safety studies have demonstrated that myopic rebound occurs after discontinuation of short-term application of atropine eye drops at high concentrations (1, 0.5, and 0.1%), while long-term application causes significant adverse effects, such as photophobia, blurred vision, allergic reactions, eye irritation, and infections [6,15,16]. Low concentrations of atropine drops (0.01, 0.025, and 0.05%) are more acceptable to paediatric patients for its adequate myopia control and lower incidence of adverse effects [7,8,17,18]. The paediatric population is in a special period of development, affected by circadian rhythms, immature psychological and physiological functions as well as pathological factors, there are differences in drug absorption, distribution, metabolism and excretion between them and adults, making them a sensitive population in the risk assessment of clinical medication [19,20]. ASED is indicated for children aged 6–12 years, based on the short develop state of animal, in this study, young rats at PND_{21}–PND_{60} (equivalent to the childhood period of 2–14 years old) were selected for intervention [21]. Asystematic developmental evaluation, including ophthalmic development, was carried out after repeated administration of ASED.

Non-clinical toxicity studies of atropine formulations by other routes of administration are well reported [22–24], but there are almost no reports on eye-drop administration of atropine sulphate eye drops. With reference to the Harmonised Tripartite Guideline (ICH) guideline S11 [25], the juvenile animal study (JAS) of atropine sulphate eye drops was conducted in this study, and developmental indicators (physical examination, physiological development, and oestrous cycle) were added to the JAS study. In our study, repeated eye-drops of 0.01, 0.02, and 0.04% of ASED in young rats caused no significant abnormalities in the general condition and body weight of the animals. There were significant changes in food intake and physical examination parameters (crown-rump length, tail length, limb length, and chest circumference) in individual ASED dose groups of female or male rats. But the changes were irregular or in a small degree, so not judged as the toxicological change. The physiological developmental results suggested that the indicators (vaginal opening and foreskin separation) of male and female rats in the 0.01, 0.02, and 0.04% groups of ASED had reached the contemporaneous standard, and no significant difference was observed in the frequency of occurrence of the oestrous cycle (pre-menstrual, oestrous, post-menstrual and inter-menstrual phases) within 12 days in females in all the ASED dosage groups. Special attention should be paid to indicators reflecting changes in eye structure and function in the preclinical JAS safety evaluation of ophthalmic drugs. The examinations of eyes, intraocular pressure and ocular axial length were added in this study [26]. No irritating changes in the eye appearance were found in all groups of animals, and some significant statistical differences appeared on the intraocular pressure of the left and right eyes of female or male rats between the individual ASED dose groups and control group. The changes on the intraocular pressure were within the physiological range and without an overall trend, so they were not considered as the toxicological changes. The axial length of the right eye of male young rats in the 0.01 and 0.04% groups of ASED was significantly

reduced by about 7%, which was considered to be pharmacological effects of ASED.

Immediately after completion of the dosing period of this study, the animals were transferred to the study "Fertility and Early Embryonic Developmental Toxicity Study of ASED in SD Rats", where all examinations and measurements were considered as those conducted in recovery period for the developmental toxicity study in young SD rats. Results of the examinations and measurements will be published in a later reproductive toxicity study.

5. Conclusion

In conclusion, ASED at concentrations of 0.01, 0.02, 0.04%, dose levels of 0.002, 0.004, 0.008 mg/day per rat, had no toxicological effects on the clinical observation, body weight, food intake, physical development, physiological development, reproductive development, intraocular pressure and axial length in rats. The NOAEL of atropine sulphate eye drops in young SD rats equivalent to human >2 years old was 0.008 mg/animal/day at a concentration of 0.4 mg/mL (0.04%).

Disclosure statement

The authors declare that we have no declaration of interest.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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