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Letter

K16ApoE inhibits the activity of acetylcholinesterase but is not the primary toxicological mechanism in mice

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ABSTRACT — The blood-brain barrier restricts the administration of drugs for neurological diseases. K16ApoE is an effective drug delivery carrier to deliver drugs across the blood-brain barrier, but it contains acute and high toxicity. The toxicity mechanism of K16ApoE must be revealed for clinical uses. Previous studies hypothesized that the toxicity mechanism was acetylcholinesterase inhibition in the brain. However, these studies used improper buffers in the AChE assay, further leading to anomalous results. Meanwhile, previous studies have not investigated the effects of K16ApoE on all the AChE-containing tissues and organs throughout the body. The previous dose design was also too narrow. Herein, we designed a more comprehensive and rational dose interval for K16ApoE, observed and recorded the mouse responses after receiving K16ApoE, and collected the brain, diaphragm, and serum to investigate the systemic K16ApoE effects on AChE. We also incubated purified AChE with K16ApoE *in vitro*. It could reveal the direct effect of K16ApoE on AChE without the influence of absorption and metabolism *in vivo*. The *in vitro* results demonstrated that K16ApoE inhibits rhAChE activity as the dose increases. However, the *in vivo* results demonstrated that K16ApoE does not affect tissue AChE activity in female mice. Therefore, we confirmed the AChE inhibitory effect of K16ApoE, but contrary to our hypothesis, AChE inhibition is not the toxicological mechanism of K16ApoE. We also recorded toxicological responses after the mice received K16ApoE, which would promote further toxicological investigation on K16ApoE.

Key words: CNS-targeted drug carrier, K16ApoE, Toxicity, Acetylcholinesterase

INTRODUCTION

Neurological diseases are the top causes of disability and mortality worldwide (Feigin *et al.*, 2019). However, the blood-brain barrier prevents all the macromolecules and 98% of the small molecules from entering the central nervous system (CNS) (Pardridge, 2005) and therefore presents difficulties for the treatment of neurological diseases. K16ApoE is a CNS-targeted drug carrier that contains 16 lysine residues and apolipoprotein E. It can deliver the CNS-targeted drugs into the brain and distribute the drugs evenly in the brain (Sarkar *et al.*, 2011; Sarkar *et al.*, 2014). It also maintains the effectiveness of drugs during delivery (Meng *et al.*, 2017). However, K16ApoE has acute and high toxicity in a dose-dependent manner. A related study (Lu *et al.*, 2018) demonstrates that all mice die after receiving 160 nmol and higher doses of K16ApoE.

The studies (Lu *et al.*, 2018; Michelena *et al.*, 2018) hypothesized that K16ApoE toxicity is induced by acetylcholinesterase (AChE) inhibition in the brain. From molecular docking, the authors found that K16ApoE

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could bind to AChE and reduce its activity. However, they discovered that K16ApoE randomly reduced or increased AChE activity in the brain during animal experiments, and their data were extremely inconsistent. To date, there are no detailed and rational dose tests for K16ApoE, either.

We also hypothesized that the toxicological mechanism of K16ApoE was AChE inhibition. First, the previous study (Lu *et al.*, 2018) proved it through molecular docking. Second, another related study (Lin *et al.*, 1977) discovered that polylysine reduces AChE activity as concentration increases. Because K16ApoE contains 16 lysine residues, it should also reduce AChE activity in the same way.

We think the current conflictive effects and inconsistency are caused by the wrong homogenization buffer. Previous research (Wilson, 2014) revealed that AChE contains collagen tails and binds to the plasma membrane. Detergent should be used to promote the release of AChE from membranes. However, no detergent was used in the traditional AChE assay (Ellman *et al.*, 1961) in previous K16ApoE effect research (Lu *et al.*, 2018; Michelena *et al.*, 2018), which means the AChE was not released from the membranes. Therefore, we prepared a homogenization buffer containing 0.1% Triton X-100 to promote the release of AChE. AChE is distributed in nervous tissue, muscles, and blood (Wilson, 2014). However, hemoglobin reduces the sensitivity of the AChE assay (Wilson, 2010). Neuromuscular dysfunction is also observed when AChE is inhibited in the diaphragm (Eyer, 2003). Therefore, we collected brain and diaphragm and separated the serum to conduct the AChE assay in animal experiments. Since the diaphragm is too small for homogenization, we used the histoenzymological method (Paul and Borah, 2017) to determine the AChE activity in the diaphragm. We also investigated the more comprehensive doses of K16ApoE and recorded the mouse responses. To investigate the direct effect of K16ApoE on AChE, we additionally incubated the recombinant human acetylcholinesterase (rhAChE) *in vitro* with the same doses of K16ApoE as in animal experiments and then conducted the rhAChE activity assay.

MATERIALS AND METHODS

Reagents

K16ApoE (KKKKKKKKKKKKKKKKLRVRLASHLRKLRKRLLRDA, > 98% purity) was synthesized in GenScript (Nanjing, China) and stored at -80°C. Zoletil® 50 was purchased from Virbac (Carros, France). Acetylthiocholine iodide, 5,5'-dithio-bis-[2-nitrobenzoic acid] (DTNB), rhAChE, and Brij® 35 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium phosphate dibasic anhydrous (AR, 98%), potassium phosphate monobasic (99.5%), sodium bicarbonate (≥ 99.5%), sodium dihydrogen phoshate anhydrous (AR, 99.0%), sodium phosphate dibasic (99%), and Triton X-100 (AR) were purchased from Macklin (Shanghai, China).

In vitro rhAChE activity assay

The rhAChE (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in Milli-Q water (0.1 mg/mL) as stock and stored at -20°C. The rhAChE was multi-serially diluted in 0.1 M pH 8.0 phosphate buffer with Brij® 35 and incubated with sequential doses (Control: 0 nmol; 60 nmol, 120 nmol, 180 nmol, 240 nmol, 300 nmol, 360 nmol, and 420 nmol) of K16ApoE in 200 μL of the same buffer at 220 rpm and 37°C for 10 min, 20 min, and 30 min, respectively. The working concentration for rhAChE was 0.002 μg/mL as the protocol recommends. Then, the rhAChE activity assay was conducted in the 96-well plate ratio as previously described (Ellman *et al.*, 1961) as follows: 50 μL of the rhAChE-K16ApoE mixtures were mixed with 50 μL of the substrate mixture (Acetylthiocholine iodide: 200 μmol/L; DTNB: 128 μmol/L) per well. The plates were measured at 412 nm in the kinetic mode for 6 min by the Varioskan® Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific, Waltham, MA, USA).

Animals and treatments

Female ICR mice (10 weeks of age) were purchased from Zhejiang Provincial Laboratory Animal Center (Hangzhou, China). The mice were housed in barrier facilities with a temperature of 25 ± 2°C, humidity of 50%−60%, and a 12-hr light/dark schedule for one week to recover their physiological indicators from transportation. They had free access to food and water.

The mice (n = 24) were injected with sequential doses (Control: 0 nmol; 60 nmol, 120 nmol, 180 nmol, 240 nmol, 300 nmol, 360 nmol, and 420 nmol) of K16ApoE in 200 μL PBS via the tail vein. Each dose was repeated in three mice as a replication. After receiving K16ApoE, all the tissue samples were collected following immediate cardiac perfusion. Dead mice were sampled directly, and surviving mice were sampled after being overanesthetized and euthanized by intraperitoneal injection of Zoletil 50 (50 mg/kg) 30 minutes later. Whole blood was collected from the heart and mixed with ethylenediaminetetraacetic acid (EDTA) to prevent clotting (1.2 mg EDTA per 1 mL blood) (Banfi *et al.*, 2007). Cardiac perfusion was performed using saline at a flow

rate of 5.0 mL/min by the peristaltic pump (BT100-2J, Rongbai, Baoding, China). The brain was snap-frozen in liquid nitrogen, and the diaphragm was fixed in 4% paraformaldehyde on ice.

The animal work was performed at Wenzhou Institute, University of Chinese Academy of Sciences. All procedures were approved by the Experimental Animal Ethics Committee of Wenzhou Research Institute of State Science and Technology (Ethical Approval Number: WIUCAS22092901).

Brain AChE activity assay

The whole brain was pulverized on dry ice, and the tissue powder was dissolved in a 0.1 M pH 8.0 phosphate buffer with 0.1% Triton X-100 (20 mg tissue per milliliter of buffer). Next, the tissue solution was homogenized by a Polytron PT1300 D disperser (Kinematica AG, Malters, Switzerland) at 30,000 rpm for 30 sec. Then, the brain AChE activity analysis was conducted in the cuvette as previously described (Ellman *et al.*, 1961) as follows: 400 µL of the tissue homogenization was mixed with 2.6 mL of the abovementioned phosphate buffer and 100 µL of the DTNB (0.1 M). After blanking the machine, triggered the reaction by adding 20 µL of acetylthiocholine iodide (0.075 M). Then, measured the cuvettes at 412 nm in the kinetic mode for 6 min by the Evolution™ 260 Bio UV-Visible Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Tested three times per sample as a replication.

Serum AChE activity assay

The serum was separated from the blood-EDTA mixture by centrifuging it at 2,000 rpm for 10 min at 4°C. Next, it was diluted in a 0.1 M pH 8.0 phosphate buffer with 0.1% Triton X-100 (10 µL serum in 300 µL buffer). Then, the serum AChE activity analysis was conducted in the 96-well plate ratio as previously described (Ellman *et al.*, 1961) as follows: 8.5 µL of the serum was mixed with 246.5 µL of the 0.1 M pH 8.0 phosphate buffer with 0.1% Triton X-100 and 8.5 µL of the DTNB (0.1 M). The reaction was triggered after adding 1.7 µL of acetylthiocholine iodide to each well. Then, the plates were measured at 412 nm in the kinetic mode for 6 min by the Varioskan® Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific, Waltham, MA, USA).

Histoenzymology of diaphragm AChE

The AChE histoenzymological analysis of the diaphragm was conducted as previously described (Paul and Borah, 2017) as follows: Each diaphragm piece was washed with ice-cold PBS (Gibco, Waltham, MA, USA) three times and incubated in 10 mL of reaction mixture in MaxQ™ 8000 incubated stackable shaker (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C and 220 rpm for 45 min. The reaction mixture was freshly prepared as previously described (Paul and Borah, 2017) as well, and the detailed recipe was described in the supplementary material (Supplementary Table 1). After being washed with Milli-Q water, the results were photographed with a camera-attached microscope (Eclipse CiL, Nikon, Tokyo, Japan).

Statistical analysis

All statistical analysis was conducted using GraphPad Prism 9.4.1 (GraphPad Software, Inc., San Diego, California, USA). One-way ANOVA was used to evaluate the significance among all the groups, whereas Tukey's test was used to compare individual groups. All data were displayed as mean ± standard deviation (SD), and the statistically significant difference was set at $p < 0.05$.

RESULTS

K16ApoE inhibits rhAChE activity *in vitro*

The rhAChE activity analysis demonstrates that K16ApoE inhibits rhAChE activity in a dose-dependent manner. At the highest dose (420 nmol), about 30% of the rhAChE activity is inhibited by K16ApoE. The analysis demonstrates a significant reduction ($p < 0.0002$ for 10-min incubation, $p < 0.0001$ for 20-min and 30-min incubation) in rhAChE activity *in vitro* incubated with sequential doses of K16ApoE (Fig. 1).

Mouse responses to K16ApoE

The mice started to die after receiving 120 nmol of K16ApoE, and all the mice died after receiving 240 nmol and higher doses of K16ApoE (Fig. 2). Dyspnea and spasms were observed after the injection of K16ApoE. Interestingly, the mouse ears gradually shrank after exposure to middle doses of K16ApoE (Fig. 3A-3C). Detailed mouse responses and death times were described in the supplementary material (Supplementary Table 2).

The K16ApoE effect on tissue AChE activity in the brain, diaphragm, and serum

The brain AChE activity analysis demonstrates that K16ApoE has no effect on AChE activity. Except for a 11% reduction in brain AChE activity in mice after receiving 120 nmol of K16ApoE, the AChE activity difference is within 5% in the mice after receiving different doses of K16ApoE. Statistical analysis also suggests no significance ($p = 0.4329$) in brain AChE activity in

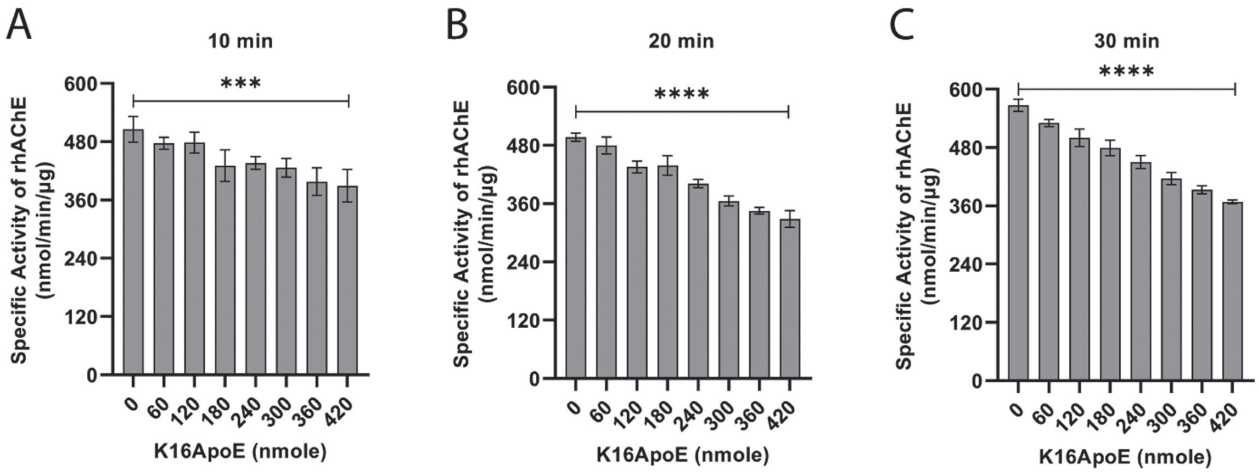

Fig. 1. Effects of K16ApoE on rhAChE activity *in vitro*. The rhAChE was incubated with sequential doses of K16ApoE at 37°C and 220 rpm. The incubation time corresponded to the mouse death time in animal experiments. A: The incubation duration was 10 min. B: The incubation duration was 20 min. C: The incubation duration was 30 min. ***, **** Significant difference at $p < 0.0002$ and 0.0001, respectively.

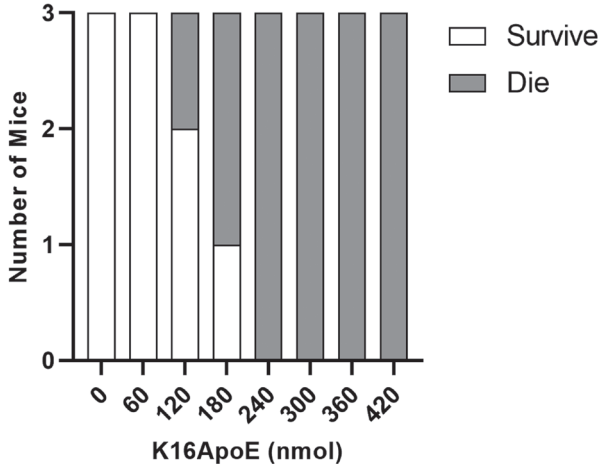

Fig. 2. Mouse survival results. Mouse survival rate after receiving sequential doses of K16ApoE. The mice start to die after receiving 120 nmol or higher doses of K16ApoE.

mice exposed to different concentrations of K16ApoE (Fig. 4A).

The diaphragm AChE histoenzymological result also demonstrates that K16ApoE does not affect AChE activity. After staining, the diaphragms showed the iconic yellow of AChE activity in all the mice, and there was no visible depth difference in the color (Fig. 5).

The serum AChE activity analysis indicates a complex dose-response effect. Several doses of K16ApoE randomly reduce the AChE activity, but others keep it the same as the control (Fig. 4B). A large standard deviation demonstrates the results are inconsistent and ineffective. Statistical analysis also suggests no significance ($p = 0.4206$).

DISCUSSION

This project aimed to investigate whether the toxicological mechanism of K16ApoE is AChE inhibition. It also aimed to investigate how K16ApoE inhibits AChE activity in the brain, diaphragm, and serum. *In vitro* results support our hypothesis that K16ApoE significantly inhibits rhAChE activity when rhAChE is incubated with sequential doses of K16ApoE. However, contrary to our hypothesis, *in vivo* results demonstrate that K16ApoE does not affect tissue AChE activity in both the brain and diaphragm. Moreover, the serum AChE activity results are anomalous and inaccurate and cannot be used to evaluate the K16ApoE effect.

In vitro results confirm that K16ApoE has an inhibitory effect on AChE activity. It is the direct interaction between K16ApoE and rhAChE without the *in vivo* interference in the absorption and metabolism. The 10-, 20-, and 30-min incubations are used to simulate the death times of mice responding to different doses of K16ApoE. Just after 10 min, K16ApoE starts to show its significant

Fig. 3. Ear shrinkage after receiving K16ApoE. A: Control. Normal ear after receiving the same volume of PBS. B: Ear shrinkage after receiving 120 nmol of K16ApoE. C: Ear shrinkage after receiving 180 nmol of K16ApoE.

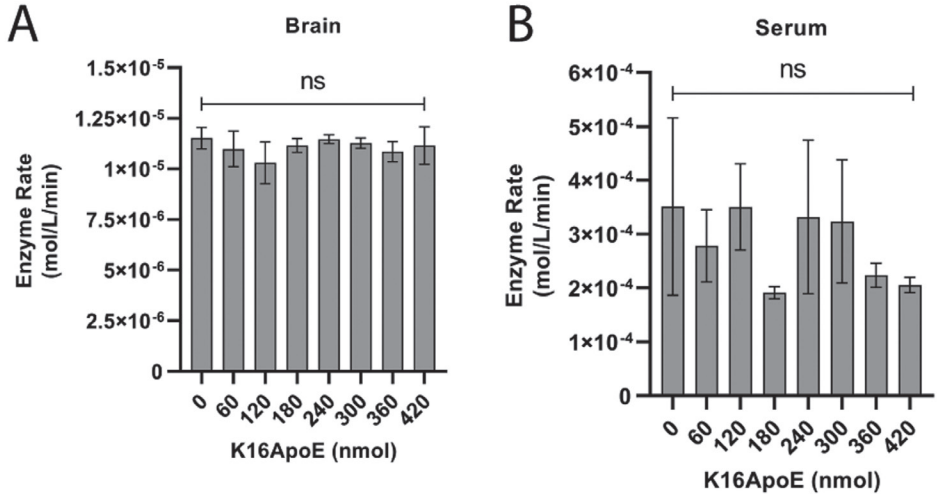

Fig. 4. Effects of K16ApoE on tissue AChE activity in animal experiments. The tissue AChE activity was assayed after the administration of sequential doses of AChE. A: AChE activity results in the brain. B: AChE activity results in the serum.

inhibitory effect. After 30 min, the inhibitory effect reaches its peak (Fig. 1).

AChE is mainly distributed in nervous tissue and muscles (Wilson, 2014). Previous research (Michelena *et al.*, 2018) hypothesized that K16ApoE inhibits AChE activity in the neuro-muscular junction and further leads to muscle spasms and respiration failure. The diaphragm is directly associated with respiration, and the brain can regulate respiration. Therefore, we tested tissue AChE activity in the brain and diaphragm. After observing the mouse ears shrink and the muscles spasm, we confirmed that the muscles must be affected by K16ApoE. However, the results suggest no significant tissue AChE activity difference in either the brain or diaphragm after the mice received different doses of K16ApoE. Even though there is an 11% reduction in brain AChE activity after the mice receive 120 nmol of K16ApoE (Fig. 4A), the reduction is not significant ($p = 0.3597$) compared with the control. We think K16ApoE does have an inhibitory effect on tissue AChE activity, but AChE inhibition is not the toxicological mechanism of K16ApoE. We confirmed that K16ApoE significantly inhibits rhAChE activity *in vitro*. We also found that 120 nmol of K16ApoE inhibits 11% of the brain's AChE activity *in vivo*. However, the mice died immediately after receiving 180 nmol or higher doses of K16ApoE. That means K16ApoE cannot be absorbed and effectively delivered into the brain and muscles in such a short time. Therefore, we cannot effectively evaluate the K16ApoE effect on tissue AChE activity after the mice receive the lethal doses of K16ApoE.

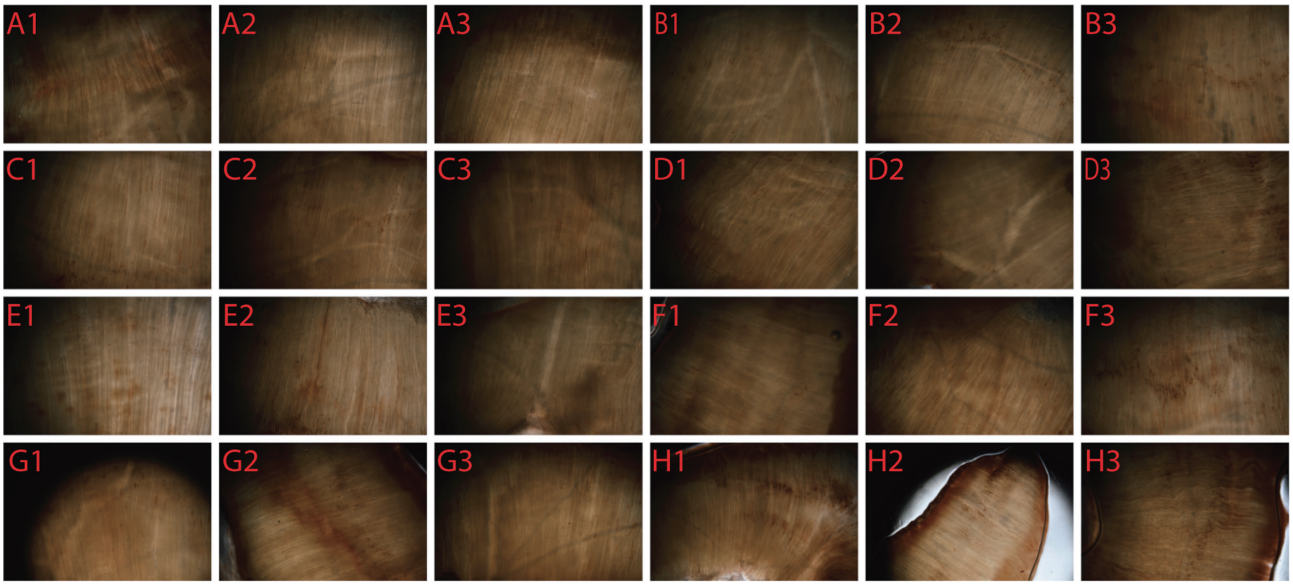

Fig. 5. Effects of K16ApoE on diaphragm AChE activity in animal experiments. The diaphragm was stained at 220 rpm and 37°C for 45 min and observed at the microscope (Eclipse CiL, Nikon, Tokyo, Japan) at a magnification of 4 ×. A: 0 nmol (control). B: 60 nmol. C: 120 nmol. D: 180 nmol. E: 240 nmol. F: 300 nmol. G: 360 nmol. H: 420 nmol.

That's why we found that K16ApoE reduced rhAChE *in vitro* but did not affect AChE activity *in vivo*.

AChE is also distributed in the blood (Wilson, 2014). We separated the serum to test its AChE activity because the color of hemoglobin can interfere with the AChE activity assay (Wilson, 2010). We think that K16ApoE can be directly exposed to the blood immediately after intravenous injection. Therefore, the serum AChE analysis should clearly and directly demonstrate the effect of K16ApoE on tissue AChE. However, the results are anomalous and extremely inconsistent. When we separated the serum, we mentioned that the supernatant of some samples was red. However, the color of the serum should be an extremely slight yellow. Thus, we think the red blood cells were broken when they were exposed to K16ApoE, and the hemoglobin was released from the red blood cell into the serum. The hemoglobin contamination interfered with the AChE activity assay and led to the anomalous results. A related study (Aasen *et al.*, 2019) also discovered abnormal morphological changes in the erythrocytes after the nontoxic doses of K16ApoE were administered. Because we focused on the toxicological mechanism of K16ApoE and mainly used toxic doses of K16ApoE, the erythrocytes were broken after exposure to K16ApoE in our trials. That is the reason that hemoglobin contaminated our serum samples and our serum AChE activity assay failed.

Although the serum AChE results are insufficient, we found that K16ApoE affects the state of red blood cells when we separate serum. Therefore, further research should investigate the erythrocyte damage response to toxic doses of K16ApoE. Whether erythrocyte damage can lead to death in mice is also an important topic. We also observed ear shrinkage and muscle spasm. Thus, we encourage further research to investigate the relationship between muscle contraction and K16ApoE.

In conclusion, we discovered the inhibitory effect of K16ApoE on AChE activity. However, we confirmed that AChE activity inhibition is not the toxicological mechanism of K16ApoE. We recorded the mouse responses to toxic doses of K16ApoE and suggested further research to explore muscle contraction and erythrocyte morphology.

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

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