

Effects of apolipoprotein E gene polymorphism on the intracellular Ca²⁺ concentration of astrocytes in the early stages post injury

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Abstract. The present study aimed to investigate the correlation between apolipoprotein E (APOE) polymorphisms and the intracellular concentration of Ca²⁺ in astrocytes in the early stages after an injury. The chondroitin sulfate region of three APOE alleles ($\epsilon 2$, $\epsilon 3$ and $\epsilon 4$) was obtained by reverse transcription-polymerase chain reaction (RT-PCR). A recombinant plasmid, pEGFP-N1-APOE, was constructed and identified by sequencing, while astrocytes were isolated from APOE gene-knockout mice and examined using immunocytochemistry. The recombinant plasmid was transfected into the astrocytes using the liposome-mediated method and cell injury models were constructed by a scratch assay. Laser confocal scanning microscopy (LCSM) was used to detect dynamic alterations in intracellular Ca²⁺ concentration at 12, 24, 48 and 72 h after injury. Compared with the control group, cells transfected with any of the three alleles demonstrated significant increases in the fluorescence intensity of Ca²⁺ ($P < 0.05$). The fluorescence intensity of Ca²⁺ was weak at 12 h after injury, with no statistically significant difference detected between any two groups at this time point ($P > 0.05$). However, the fluorescence intensity increased in a time-dependent manner and at 24, 48 and 72 h post injury, the fluorescence intensity of the $\epsilon 4$ allele-containing cells was significantly higher when compared with that of cells harboring the other two alleles ($P < 0.05$). These results indicate that intracellular Ca²⁺ overloading may contribute to the deterioration of brain cells and poor outcome subsequent to traumatic brain injury in APOE $\epsilon 4$ carriers.

Introduction

Traumatic brain injury (TBI) is the leading cause of mortality and disability among young people and the incidence rate of TBI has steadily risen around the world. Previous studies have demonstrated that genetic susceptibility may serve an important role in the clinical outcome of individuals with TBI (1-4). Apolipoprotein E (APOE) polymorphisms are the most extensively studied genetic factor in neurotrauma research. Several previous studies have identified that APOE gene polymorphisms are associated with the acute condition and outcome of TBI (1-5). The presence of the APOE $\epsilon 4$ allele has been revealed to predispose an individual to clinical deterioration in the acute phase of TBI and is indicative of a poor long-term outcome. However, the underlying mechanism of this association has not been investigated thus far (6,7). Furthermore, calcium is an important secondary messenger within cells. Cytoplasmic calcium, has extensive physiological effects as a cellular messenger following injury. An increased intracellular calcium concentration is an important cause of cell injury. It has recently been demonstrated that Ca²⁺ overload and disruption of the intracellular Ca²⁺ homeostasis are the final events in the process of cell death (8-10).

The aim of the current study was to investigate the effects of APOE polymorphism on the early intracellular Ca²⁺ concentration in astrocytes following scratch injury using laser confocal scanning microscopy (LCSM). In addition, the study explored the underlying molecular mechanism of the effects of APOE polymorphism in a cell injury model.

Materials and methods

Construction of recombinant plasmids. The present study was approved by the Ethics Committee of the Affiliated Hospital of Zunyi Medical College (Guizhou, China) and written informed consent was obtained from all participants. The chondroitin sulfate (CDS) domain of the APOE $\epsilon 3$ allele was amplified by polymerase chain reaction (PCR) exactly according to the previously described method (11). Briefly, total RNA was extracted from human fetal brain tissue obtained from

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aborted fetuses at the Affiliated Hospital of Zunyi Medical College (Guizhou, China), using an RNA Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). A High Fidelity PrimeSript™ RT-PCR kit (Takara Biotechnology Co., Ltd.) was used with primers designed by Takara Biotechnology, Co., Ltd. to synthesize total DNA. The reverse transcription reaction was amplified by PCR using PrimeSTAR™ HS DNA polymerase (Takara Biotechnology Co., Ltd.) to get APOE $\epsilon 3$. The gene was ligated with PMD-19 vector and then was excised from PMD19-T-APOE $\epsilon 3$ plasmid and inserted into pEGFP-N1 to construct eukaryotic expression vector. The expression of APOE $\epsilon 3$ gene was identified by western-blot (11). The *EcoRI* and *BamHI* restriction enzyme cleavage sites were then introduced at the 5'-ends in the upstream and downstream primers used for the amplification of the CDS domain of APOE $\epsilon 3$, by site-directed mutagenesis. All the primers used in the present study were synthesized by Takara Biotechnology Co., Ltd. Furthermore, the CDS domain of $\epsilon 2$ and $\epsilon 4$ alleles was amplified by reverse transcription-PCR (Table I). The target gene was excised by double enzyme cleavage, purified and subcloned into the eukaryotic expression vector pEGFP-N1 (Takara Biotechnology Co., Ltd.). The expression vector was inserted into *Escherichia coli* JM109 by thermal transformation as previously described (11,12). The transformed *E. coli* were plated (1×10^6 /well) in six-well plates and incubated overnight at 37°C (12). A co-digestion assay was performed by *EcoRI* and *NotI* to get 470 kbp and 950 bp fragments, and single colonies positive for the target genes (APOE $\epsilon 2$ and $\epsilon 4$) were cultured, and the recombinant plasmids containing the alleles were purified (11,12).

Cell transfection. Astrocytes were isolated from four 2-day-old APOE-gene-knockout suckling mice (Department of Zoology, Peking University, Beijing, China) using the method described by McCarthy and de Vellis (13). Then cells were washed with 0.1 mol/l PBS for 2 min three times and then incubated for 10 min at 37°C with 3% deionized H_2O_2 to block endogenous peroxidase. The cells were washed with PBS and incubated with anti-glia fibrillary acidic protein (GFAP) antibodies (1:100; cat. no. Q0287R; Bioengineering Co., Ltd., Shanghai, China) in a wet box overnight at 4°C. The cells were subsequently washed with PBS for 2 min three times and then incubated with poly-horseradish peroxidase anti-rabbit immunoglobulin G (1:4,000; cat. no. C030213; Bioengineering Co., Ltd.). Following identification, the astrocytes were transfected using Lipofectamine™ 2000 (Bioengineering Co., Ltd.), with the recombinant plasmids pEGFP-N1-APOE in order to obtain three groups of astrocytes with humanized APOE $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$. Following transfection with pEGFP-N1-APOE plasmids for 24 h the astrocytes were selected with 200 $\mu g/ml$ G418 (Ameresco, Inc., Framingham, MA, USA). Fresh medium containing 200 $\mu g/ml$ G418 was replenished every other day for 15 days. The positive clones of pEGFP-N1-APOE cells were cultured and proliferated in Roswell Park Memorial Institute 1640 medium with 10% fetal bovine serum (Boster Biological Technology, Pleasanton, CA, USA) and 200 $\mu g/ml$ G418, at 37°C under 5% CO_2 for screening stable cell lines expressing APOE.

The recombinant plasmids, pEGFP-N1-APOE, were sent to Takara Biotechnology Co., Ltd. (Dalian, China)

for sequencing. The sequences were compared against the sequence provided in the NCBI Databank (cat. no. ID:348) to confirm the successful formation of the recombinant plasmids.

The present study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (14). The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Chongqing Medical University (Chongqing, China).

Construction of cell scratch wound model. Cell strains with stable expression of each of the APOE alleles were cultured in LCSM-exclusive culture dishes at 37°C for 60 min, following the procedure described in an earlier study (15). Subsequently, astrocyte cell layers in each culture dish were scratched with the plastic tip of a fine micropipette. The scratch length was 10 mm and the width was 1 mm, while the scratches were spaced 2-3 cm apart. A total of 8 scratch lines (4 transverse scratch lines and 4 longitudinal scratch lines) were made in each culture dish. The scratch layout in all the dishes was kept consistent to the extent possible. Non-scratch groups for each of the alleles were established as pre-wound and post-wound controls.

Determination of intracellular Ca^{2+} levels. A working solution (4.4 $\mu mol/l$) of Fluo-3/acetoxymethyl ester dry powder (DingGuo Biotech Co., Ltd., Beijing, China) in anhydrous dimethyl sulfoxide was prepared, according to the method described in a previous study (16). Briefly, at 12, 24, 48 and 72 h after introducing the scratches, astrocytes in the dishes were rinsed with Hank's solution (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), treated with ~1 ml Fluo-3 working solution and placed in an incubator at 37°C, ensuring that the cells were protected from light, in order to load the cells with fluorescent probe for 45 min. The cells were then rinsed with phosphate-buffered saline to remove free dye and placed on the specimen stage of the microscope (Leica Microsystems GmbH, Wetzlar, Germany). Fluorescence was then measured using an excitation wavelength of 488 nm and an emission wavelength of 520 nm. The laser power and scanning parameters were kept constant throughout the experiment as previously described (17). Visual fields with even fluorescence intensity were selected using Leica LCSM matching analytical software (version 2.0; Leica Microsystems GmbH, Wetzlar, Germany), and 5 cells were randomly selected for measuring the intensity. The relative fluorescence intensity was considered to represent the relative concentration of Ca^{2+} .

Statistical analysis. Ca^{2+} fluorescence intensity is expressed as the mean \pm standard deviation. Statistical analysis was conducted using SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA). Comparison between two groups was performed using Student's t-test, while comparison between multiple groups was examined using one-way analysis of variance and a Student-Newman-Keuls post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Table I. Amplification primer sequences of APOE alleles ε2 and ε4.

Template	Primer sequence (5'→3')	Annealing temperature (°C)	Cycle times	Product
APOEε3	Forward: TGGAGGACGTGCGCGGCCCGCCTGGTGCAG Reverse: TGTCCGCGCCCAGCCGGGCCTG	64	30	APOE ε2
APOEε3	Forward: TGACCTGCAGAAGTGCCTGGCAGTGTAC Reverse: TCGGCATCGCGGAGGAGCCGCTTA	61	30	APOE ε4

APOE, apolipoprotein E.

Results

Sequencing of recombinant plasmids. The recombinant plasmids, pEGFP-N1-APOE, were sent to Takara Biotechnology Co., Ltd. for sequencing. The sequences were compared against the sequence provided in the NCBI Databank (cat. no. ID:348) and successful construction of the recombinant plasmids, (pEGFP-N1-APOEε3, pEGFP-N1-APOEε2 and pEGFP-N1-APOEε4) as well as that it complied with the requirements of the subsequent experimental procedures, was verified. By sequencing, we can see that APOEε2 and APOEε4 differ from APOEε3 by single amino acid substitutions at position 112 or 158, which corresponds with the gene bank data (Fig. 1A-C).

Construction of cell wound model. The purity of the isolated primary astrocytes was 95% and the yield was 3-6x10⁶/ml. The cell morphology was observed under an inverted microscope. It was observed that the cells fused with each other and their cell processes were distinct. The cells intersected and formed a cobblestone mass around the bottom of the flask and a small number of cells began to grow on the astrocyte layer (Fig. 2A). Immunofluorescent labeling of the astrocyte specific marker, GFAP, confirmed the identity of the cells (Fig. 2B). Astrocytes were then transfected with recombinant plasmids, and successful transfection was confirmed by the appearance of green fluorescein at 24 h after transfection, as observed under a fluorescence microscope (Fig. 2C). Successful establishment of the cell scratch wound model was also confirmed by fluorescence microscopy (Fig. 2D).

Alterations in the intensity of Ca²⁺ fluorescence. Prior to the scratch wounding, the three groups of ε2, ε3 and ε4 type astrocytes exhibited weak fluorescence intensities. The difference in the fluorescence intensities between any two types of cells was not statistically significant (P>0.05; Table II). However, subsequent to scratch, the Ca²⁺ fluorescence intensity of each type of astrocytes increased gradually until 72 h after the scratch (Fig. 3). The Ca²⁺ fluorescence intensity of each type of astrocytes at each time point following the scratch was significantly higher compared with that prior to the scratch (P<0.05; Table II).

Comparison of the fluorescence intensities among the different types of astrocytes at the three time points revealed that the fluorescence intensities in ε4 type astrocytes at 24, 48 and 72 h were significantly stronger in comparison with those

in the ε2 and ε3 type astrocytes at the corresponding time points (P<0.05). However, there were no statistically significant differences between the intensities in the different types of astrocytes at 12 h, while the difference in the fluorescence intensities between the ε2 and ε3 type astrocytes were not statistically significant at any point (P>0.05; Table II).

Discussion

APOE is an important apolipoprotein, synthesized in the liver, brain, spleen and kidney. It is distributed extensively over the entire body via the blood circulation, and serves an important role in the transport and metabolism of plasma cholesterol and triglycerides (18). APOE has three alleles, including APOE ε2, APOE ε3 and APOE ε4, which have base substitutions at two sites in the DNA sequence (19). The brain is the second leading organ that synthesizes APOE, mainly by the astrocytes and oligodendrocytes (20). The most important function of APOE in the central nervous system is to protect and repair the neural tissues (21,22). In recent years, APOE polymorphism has been associated with central nervous system diseases, particularly Alzheimer's disease and brain trauma (23).

Clinical studies have identified that, among Caucasians, patients with APOE ε4 presented decreased tolerance to brain injury and were likely to have a poor prognosis following brain injury (24,25). Compared with patients of the non-ε4 genotypes, those with the ε4 genotype exhibited longer duration of coma and high in-hospital mortality rate (26,27). The authors of the present study previously investigated the relevance of APOE polymorphism and TBI among the Asian population in the Chinese mainland, and observed that the presence of APOE ε4 was associated with exacerbation of the acute-ness (<7 days) and poor prognosis following a TBI (27-30). Furthermore, our group also investigated the association between APOE gene polymorphism and the occurrence of any acute alterations in the neural electrophysiology subsequent to TBI (31). It was observed that the ε4 genotype was a risk factor for acute changes in the electroencephalogram following mild to moderate brain injuries (31). Based on these results, preliminary experimental investigation of the mechanism underlying the effects of APOE from the perspective of ion channels was conducted in a previous study. The results revealed that, following scratching, the number of ε4 type neurons/gliocytes, which are considered as the early prognostic cells, was evidently higher compared with that of the ε2 and ε3 type (32). Furthermore, APOE ε4 cells have been demonstrated to exhibit

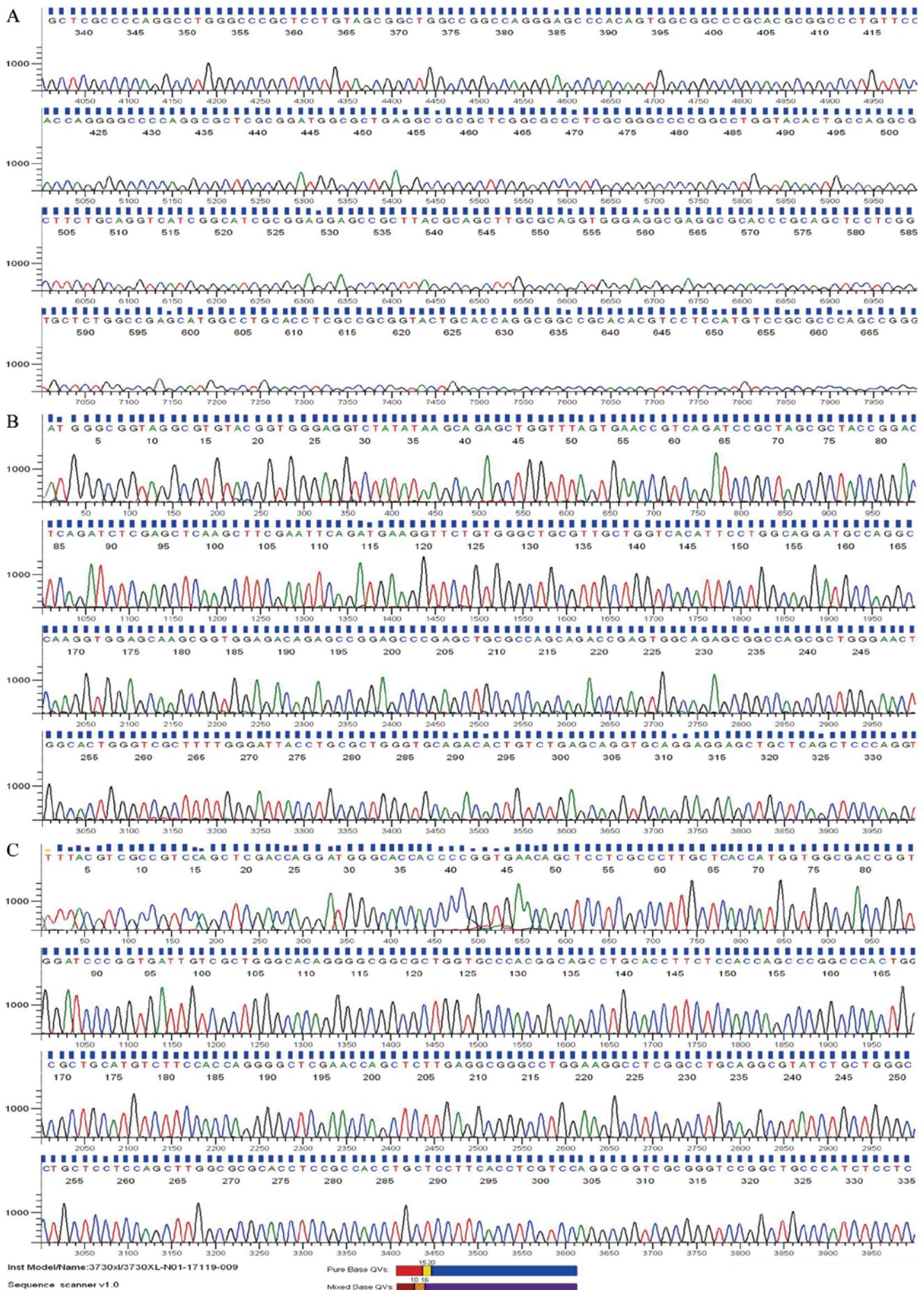


Figure 1. Sequencing maps of (A) pEGFP-N1-APOE ε3, (B) pEGFP-N1-APOE ε2 and (C) pEGFP-N1-APOE ε4. APOE, apolipoprotein E.

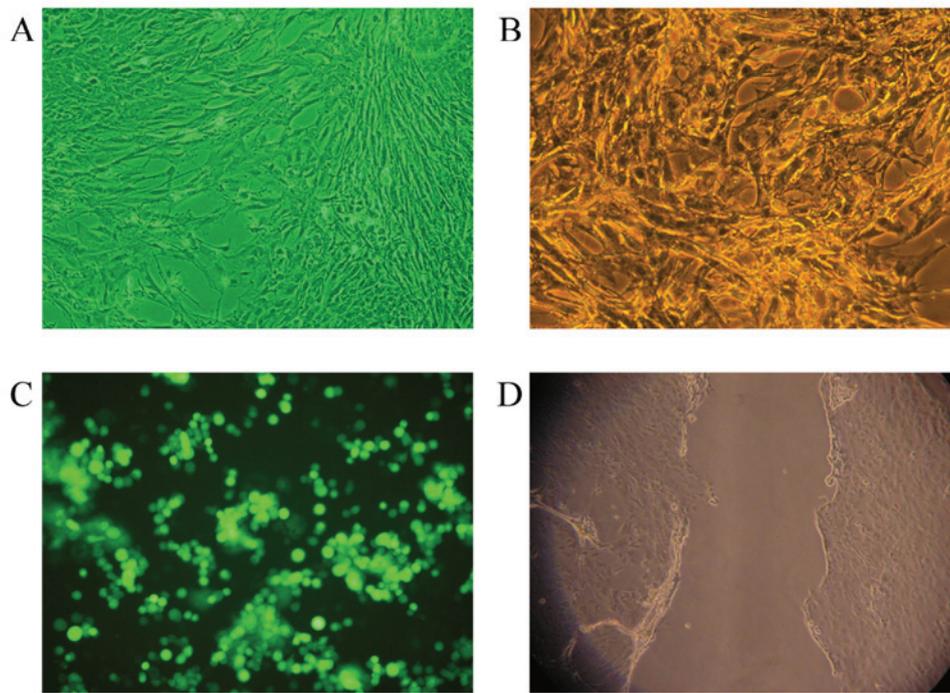


Figure 2. (A) Primary culture of astrocytes in APOE^{-/-} mice (magnification, x200). (B) Astrocyte glia fibrillary acidic protein immunostaining (magnification, x200). (C) Expression of pEGFP-N1-APOE was observed under a fluorescence microscope. (D) At 12 h after the cell scratch, cells were observed under an inverted microscope (magnification, x200). APOE, apolipoprotein E.

a greater inhibition of delayed rectifier potassium channels in neurons following scratching (32). The observations of these aforementioned studies suggested that, the effects of APOE on the pathophysiological alterations occurring following brain injury are subtype-specific, and the molecular mechanism of these effects possibly involves ion channels.

It has been demonstrated that Ca²⁺ overload and disruption of Ca²⁺ homeostasis in neurons are the final events in the death of neurons (9). The shear stress and tension associated with traumatic injury may stretch the cell membranes of injured neurons, increase the permeability of the membranes and result in an increased influx of Ca²⁺ (33). A series of secondary neurophysiological alterations in the microenvironment around the injury area, particularly the excess production of excitatory amino acids, may activate the ionotropic N-methyl-D-aspartate receptor (NMDAR), leading to further increase in the influx of Ca²⁺ (34). Increased Ca²⁺ subsequently triggers a cascade of events. It increases the activities of L-type voltage gated calcium channels and results in the release of Ca²⁺ from the endoplasmic reticulum, further increasing the intracellular Ca²⁺ levels. Interactions of several other factors finally lead to Ca²⁺ overload in neurons and axons through several channels via a cycle, and ultimately result in cell death around the injury area (29). However, the impact of APOE on the intracellular calcium concentration in neurons following an injury has not been reported thus far.

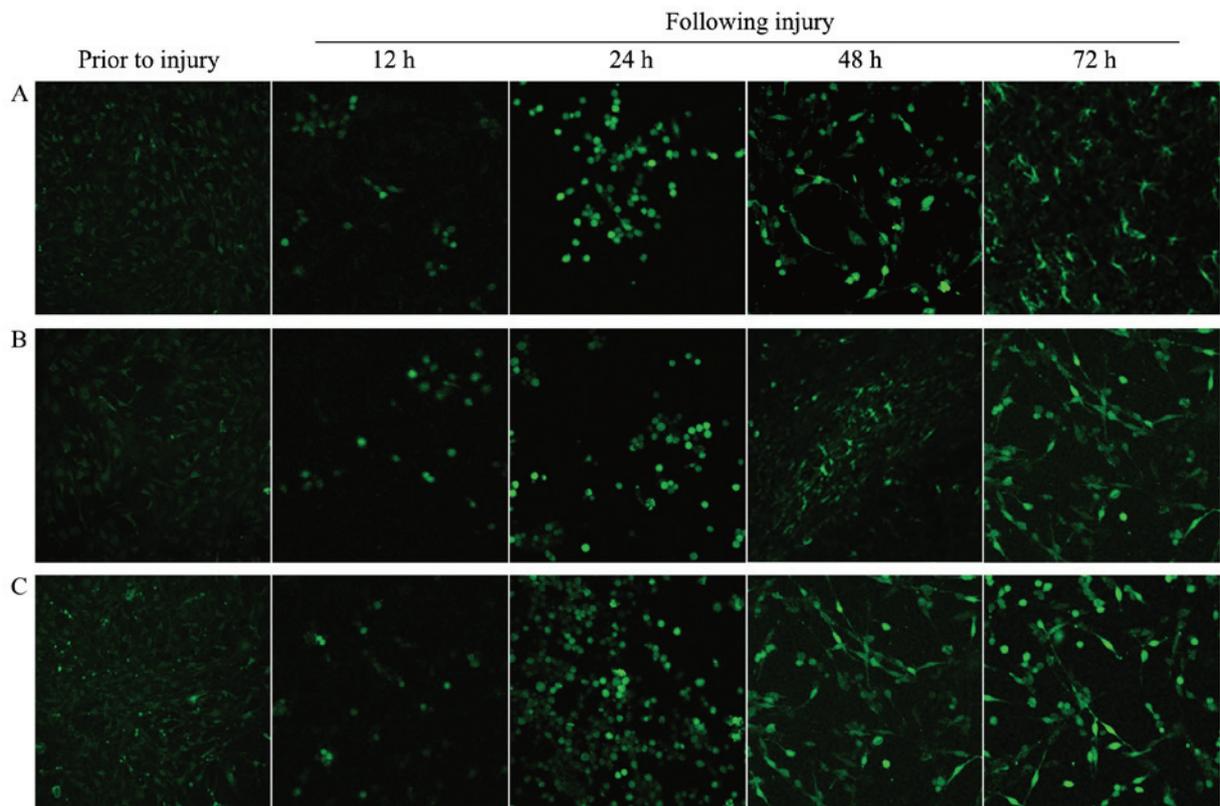
Astrocytes are the main cells producing APOE in the central nervous system. In the past, astrocytes were considered to mainly serve a nutritional and supporting role for neurons (34-36). Accumulating evidence in more recent studies has demonstrated that astrocytes serve an important role in maintaining the normal physiological activities of the nervous system, and are involved in brain development

and in the neuropathological processes (31). In the present study, astrocytes transfected with three humanized APOE alleles were used as experimental models to investigate the alterations in intracellular Ca²⁺ in the initial 72 h after the introduction of a scratch injury in cells. The present study also aimed to investigate the potential molecular mechanism underlying the effects of APOE polymorphism. The results demonstrated that Ca²⁺ in astrocytes increased progressively until 72 h after scratching, while the Ca²⁺ fluorescence intensity of all astrocyte types at any time point following the scratch was significantly different from that prior to the scratch. It was also observed that the early changes in the intracellular Ca²⁺ concentration in astrocytes after the scratch were APOE-allele-specific. The Ca²⁺ concentration in ε4 type cells was significantly higher at 24 h after the scratch as compared with that in ε2 and ε3 type cells. An earlier study (29) reported that, subsequent to scratching, the early apoptosis rate in neurons/gliocytes transfected with APOE increased in a time-dependent manner. Similarly, the apoptosis rate of ε4 type neurons/gliocytes in the current study was significantly higher in comparison with that of the ε2 and ε3 type neurons/gliocytes at 24, 48 and 72 h after the scratch. However, in the previous study there was no significant difference in the apoptosis rates among the three types of cells at 12 h after scratch (29). The results of the present and previous studies indicated that early apoptosis in neurons/gliocytes following scratching corresponded temporally with changes in the early intracellular Ca²⁺ concentrations in astrocytes subsequent to the scratch. Therefore, it is concluded that trauma results in Ca²⁺ overload in astrocytes and early apoptosis of neurons. This pathological process is APOE-subtype-specific, and the ε4 subtype is clearly more potent compared with the other two genotypes.

Table II. Fluorescence intensity of Ca^{2+} of three groups before and after injury (n=5; mean \pm standard deviation).

Group	Prior to injury	Following injury			
		12 h	24 h	48 h	72 h
APOE ϵ 2	34.70 \pm 12.04	80.28 \pm 24.62 ^a	88.47 \pm 23.82 ^a	106.04 \pm 31.37 ^a	129.72 \pm 38.24 ^a
APOE ϵ 3	30.58 \pm 13.61	72.75 \pm 20.57 ^a	78.29 \pm 35.20 ^a	87.33 \pm 34.80 ^a	98.16 \pm 30.90 ^a
APOE ϵ 4	40.39 \pm 8.41	90.68 \pm 29.71 ^a	152.29 \pm 46.63 ^{b,c}	178.82 \pm 32.67 ^{b,c}	208.00 \pm 35.49 ^{b,c}

^aP<0.05 vs. prior to injury; ^bP<0.05 vs. the ϵ 2 group; ^cP<0.05 vs. the ϵ 3 group. APOE, apolipoprotein E.

Figure 3. Under confocal laser scanning microscope, the fluorescence intensity of Ca^{2+} in the (A) APOE ϵ 2, (B) APOE ϵ 3 and (C) APOE ϵ 4 type astrocytes at different time points subsequent to injury was investigated (magnification, x200). APOE, apolipoprotein E.

The signaling and/or receptor pathway through which APOE influences the intracellular Ca^{2+} aggregation in wounded astrocytes remains unknown. The mechanism underlying the subtype specificity is also unclear. Certain studies have postulated that APOE may interfere with the Ca^{2+} influx mediated by NMDAR by activating extracellular signal-regulated kinase 1/2, an extracellular signal-adjusting kinase, in the neurons (37). Further investigations identified that APOE receptors and NMDAR may interact under certain conditions by forming a polyprotein complex (38). Future research needs to focus on the possible influence of the APOE gene on NMDAR in a subtype-specific way following brain injury and its role in altering the functioning of Ca^{2+} channels, which results in different Ca^{2+} levels in neurons around the injury area and different degrees of cell death, ultimately affecting the prognosis of cerebral trauma patients.

In conclusion, the present study demonstrated that APOE ϵ 4 may worsen the outcomes of TBI by secondary brain injury mediated by the calcium overload signaling pathway. Moreover, the results of the present study also provided evidence that there may be the potential to develop precise medications for TBI according to individual genotypes. Further studies are required to explore the therapeutic aspect of the mechanism and to identify a means to increase the protection by APOE ϵ 3 and decrease the impairment by APOE ϵ 4.

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