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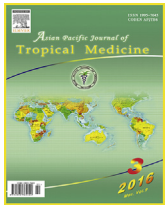

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Effect of TRPV1 combined with lidocaine on cell state and apoptosis of U87-MG glioma cell lines

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ABSTRACT

Objective: To study the effects of Transient receptor potential cation channel, subfamily V, member 1 (TRPV1) combined with lidocaine on status and apoptosis of U87-MG glioma cell line, and explore whether local anesthetic produces neurotoxicity by TRPV1.
Methods: U87-MG cells were divided into control group, gene silencing group, empty vector group and *TRPV* gene up-regulation group. For cells in each group, flow cytometry was employed to detect the intracellular calcium ion concentration and mitochondrial membrane potential at different time point from cellular perspective. Cell apoptosis of U87-MG was assayed by flow cytometry and MTT from a holistic perspective.
Results: Calcium ion concentration increased along with time. The concentration in *TRPV1* gene up-regulation group was significantly higher than those in other groups at each time point ($P < 0.05$). After adding lidocaine, mitochondrial membrane potential in U87-MG significantly increased ($P < 0.05$). This increasing trend in *TRPV1* gene up-regulation group was more significant than other groups ($P < 0.05$), while in *TRPV1* gene silencing group, the trend significantly decreased ($P < 0.05$). Flow cytometry result and MTT result both showed that cell apoptosis in each group significantly increased after lidocaine was added ($P < 0.05$). This increasing trend in *TRPV1* gene up-regulation group was more significant than other groups ($P < 0.05$), while in *TRPV1* gene silencing group, the trend significantly decreased ($P < 0.05$). Moreover, apoptosis was more severe along with the increasing concentration of lidocaine ($P < 0.05$).
Conclusions: In this study, it was proved that lidocaine could dose-dependently induce the increase of intracellular calcium ion concentration, mitochondrial membrane potential and apoptosis in U87-MG glioma cell line. The up-regulation of *TRPV1* enhanced cytotoxicity of lidocaine, which revealed the correlations between them. Lidocaine might have increased intracellular calcium ion concentration by activating *TRPV1* gene and induced apoptosis of U87-GM glioma cell line by up-regulating mitochondrial membrane potential.

1. Introduction

Nerve block is often used in clinical anesthesia, which can keep patients awake, recover anesthesia smoothly, be easy of postoperative analgesia, and save medical costs. However, with the extensive application of nerve block anesthesia, the reports of local anesthetics (LA) causing neurotoxic effects gradually increased, and caused great concern [1]. A multi-center prospective study investigated 41 251 cases of spinal anesthesia patients, 35 379 cases of epidural anesthesia patients and 1 474 cases of patients with combination of epidural anesthesia, showing serious neurological complication rate of 0.018% [2,3]. Capdevila *et al.* [4] reported that in 1 416 cases of patients treated with peripheral nerve block anesthesia, the occurrence of neuropathy was 0.21%. LA-caused nerve cell damage has correlations with dose, concentration and time, showing swelling of nerve cells, nerve demyelination, degeneration, mitochondrial swelling dissolution and apoptosis [5]. Currently, the ultrastructure of LA cause nerve tissue cells, metabolism, electrical physiological damage and how to prevent neurotoxic LA has become a hot topic in current clinical related disciplines.

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Transient receptor potential cation channel, subfamily V, member 1 (TRPV1), one of the transient voltage receptor family members, is protein encoded by the *TRPV1* gene [6]. TRPV1 is a ligand-gated non-selective cation channel protein mainly expressed in the dorsal root ganglion and trigeminal ganglion neurons. It is also present in sensory neurons, the afferent nerve fibers and the central nervous system, which can be activated by capsaicin and inflammatory mediators, also known as the capsaicin receptor [7–9]. When TRPV1 channel proteins are activated, they mainly cause the calcium ion influx, adjusting corresponding physiological function or pathological mechanisms in the form of increasing the intracellular calcium ion concentration. Studies have shown that TRPV1 plays an important role in the pathological process of the generation of pain and enhancement of pain sensitivity [10]. Additionally, Leffler *et al.* [11,12] demonstrated that on behalf of local anesthesia drugs-lidocaine, activates TRPV1, and results in calcium ion concentration increasing in dorsal root ganglion cells and releasing of excitatory amino acids by interacting with TRPV1 capsaicin binding sites. However, in LA-induced nerve cell toxic damage, what factors started intracellular calcium overload? What is the role of TRPV1 channel protein in the apoptosis induced by LA? Whether inhibiting the expression of TRPV1 channel protein can prevent LA-induced nerve cell toxic damage? It is unclear.

Therefore, this study attempted to detect intracellular calcium ion concentration and other indicators when U87-MG glioma cell line was damaged by LA by using gene transfection and RNAi technique to up-regulate and silence *TRPV1* gene, and then investigated its effect on mitochondrial membrane potential (JC-1) and apoptosis situation combining lidocaine, in order to explore whether LA producing neurotoxicity via TRPV1, aiming to discuss the molecular mechanism of LA neurotoxicity for providing theoretical basis to LA neurotoxicity prevention in depth.

2. Material and methods

2.1. Cell obtaining and culture conditions

The glioma U87-MG cell lines were kindly purchased from Hanbio Company (Shanghai, China). The cell lines were cultured and maintained in RPMI 1640 medium (Gibco, Life Technologies, UK) with 10% fetal bovine serum (FBS, Gibco) and containing 10% calf serum, 100 U/mL, 100 g/mL of penicillin streptomycin selection in the culture solution. The culture condition was set in 37 °C with 5% CO_2 in a humidified atmosphere and the melanoma stem cell lines were routinely checked in case of mycoplasma infection.

2.2. Grouping and treatment

Glioma U87-MG cells were cultured in DMEM medium containing 10% fetal bovine serum at 37 °C, 5% CO_2 and in saturated humidity condition. The plasmid was established and preliminarily identified according to previous articles. Cells in logarithmic growth phase were seeded in 24-well culture plate. The cells was grouped and treated when the bottom were covered 70%.

Then glioma U87-MG cells were randomized into 8 groups: (1) U87-MG-scramble: control group; (2) U87-MG-shTRPV1 group: the cells transfected with shTRPV1; (3) U87-MG-Vector group: the cells transfected with empty vector; (4) U87-MG-TRPV1 group: the cells transfected with TRPV1; (5) U87-MG-scramble-lidocaine: control group along with lidocaine treatment; (6) U87-MG-shTRPV1-lidocaine group: the cells transfected with shTRPV1 along with lidocaine treatment; (7) U87-MG-Vector-lidocaine group: the cells transfected with empty vector along with lidocaine treatment; (8) U87-MG-TRPV1-lidocaine group: the cells transfected with TRPV1 along with lidocaine treatment. The specific transfecting steps refer to Lipofectamine 2000 instructions.

2.3. Measurement of intracellular calcium ions by flow cytometry

Fluo-3 is a fluorescein-based calcium probe which allows flow cytometric measurement of calcium on instruments that are not equipped with a UV light source. Glioma U87-MG cells collected from every group at time point of 0, 1, 2, 3, 4 and 5 h were centrifuged at 180 × *g* (950 rpm in Beckman TJ-6) in 12 mm × 75 mm polypropylene tube for 6 min at room temperature, and resuspended in cell loading medium of 10^6–10^7 cells/mL. Then 10 mg/mL fluo-3 AM at 3–4 μg/mL final was added. Cells were centrifuged for 6 min at 180 × *g*, room temperature. Pellet was gently resuspended in cell loading medium at cell concentration (about 3×10^6/mL). Cells were stored at room temperature and protected from light until analysis. Flow cytometer was set up according to previous protocols.

2.4. Detection of mitochondrial transmembrane potential

JC-1 is an ideal cationic lipid fluorescent dye which can detect mitochondrial membrane potential. JC-1 can accumulate in cell at the state of monosome through normal cell membrane. Healthy mitochondrial membrane potential has polarity, so JC-1 is taken up rapidly into the mitochondria, and polymer formed in the mitochondria because of increasing concentration. The emitted light of the polymer is red fluorescence that can be detected by red channel of flow cytometry. When apoptosis occurs to cell, mitochondrial membrane potential is depolarized. JC-1 is released from the mitochondria, lumining green fluorescence in the form of monosome in the cytoplasm. And red light intensity decreased. Flow cytometry can be used to detect changes in mitochondrial membrane potential in U87-MG glioma cells of different groups based on this feature.

2.5. Apoptosis calculation by flow cytometry with PI staining

PI staining was employed to quantify the apoptosis of glioma U87-MG cells in each group. Briefly, cells were seeded in 6-well plates (2×10^5 cells/mL) then stained using PI (Kaiji Co., Ltd. Nanjing, China) fluorescence apoptosis detection kit following the manufacturer's instruction. Samples were analyzed using a FACSCalibur flow cytometer within 1 h after the staining.

2.6. MTT assay

Glioma U87-MG cells from each group were seeded in a 96-well tissue culture plate at a density of 1×10^4 cells per

well. Cell proliferation of each treatment group was determined by MTT assay performed for 72 h. Absorbance was read by using an ELx800 absorbance microplate reader (Bio-Tek Instruments, Winooski, VT) at 570 nm. The values of the treated cells were calculated as percentages of the untreated control.

2.7. Statistical analysis

All data are presented as the mean ± SD. SPSS 16.0 software (SPSS, Chicago, IL, USA) was employed to determine the statistical significance between samples by using analysis of variance and Dunnett's *t*-tests.

3. Results

3.1. Enhanced TRPV1 gene caused increase of intracellular calcium ion concentration in U87MG glioma cells

After group 1–4 were treated, intracellular calcium ion concentration in U87MG glioma cells was detected in different period of time. There were varying degrees of increasing calcium ion concentration in each group along with the time. In *TRPV1* gene up-regulation group, the calcium ion concentration in U87MG glioma cells was significantly higher than those in other groups at the same time point ($P < 0.05$). While in *TRPV1* gene silencing group, the calcium ion concentration in U87MG glioma cells was significantly lower than those in other groups at the same time point ($P < 0.05$) (Figures 1–4).

3.2. TRPV1 gene up-regulation combined with lidocaine strengthened the mitochondrial membrane potential in U87-MG glioma cells

Besides groups 1–4, groups5-8 were added to detect the mitochondrial membrane potential in U87-MG glioma cells. The ratio of cells with reduced mitochondrial membrane potential in group 1–8 was respectively 12.4%, 11.5%, 13.9%, 13.0%, 26.0%, 19.6%, 17.8%, and 38.3%. The results showed that the mitochondrial membrane potential in TRPV1 gene up-regulation group was significantly higher lower than in other groups ($P < 0.05$), while in gene silencing group, it was significantly lower higher than that in other groups ($P < 0.05$). Furthermore, after lidocaine was added, the mitochondrial membrane potential significantly increased decreased ($P < 0.05$). The mitochondrial membrane potential TRPV1 gene up-regulation combined with lidocaine group was significantly higher lower than that in other groups ($P < 0.05$).

3.3. TRPV1 gene up-regulation combined with lidocaine increased degree of apoptosis in U87-MG glioma cells

Besides groups 1–4, groups 5–8 were added to detect the effect of different treatment on cell apoptosis. In flow cytometry results, the ratio of apoptotic cell in group 1–8 was respectively 0.5%, 0.3%, 0.3%, 1.4%, 1.5%, 0.9%, 0.5% and 3.1%. Flow cytometry results and MTT results (Figure 5) showed that after the addition of lidocaine, apoptosis significantly increased in each group ($P < 0.05$). This increasing trend in *TRPV1* gene up-regulation group was more significant than that in other groups

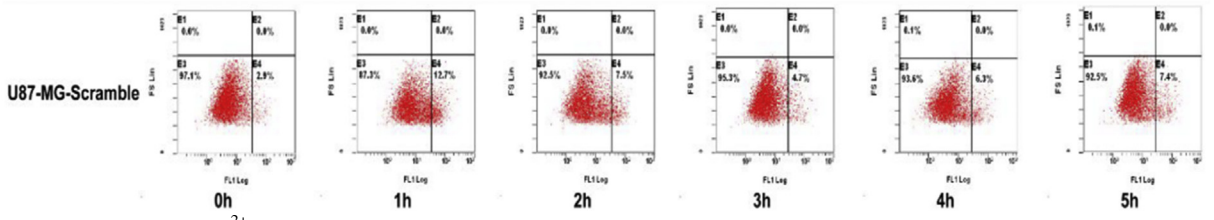

Figure 1. Detection result of Ca^{2+} in U87-MG-Scramble group.

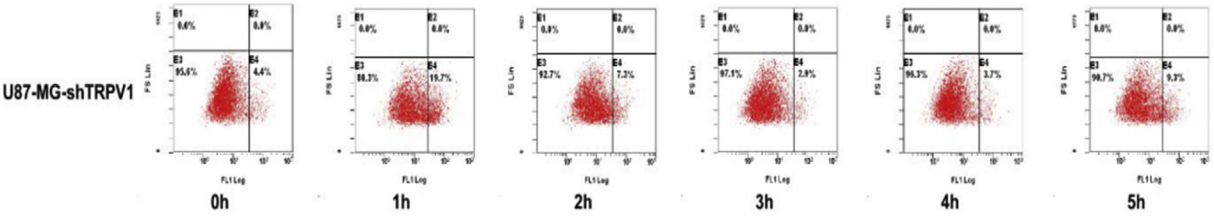

Figure 2. Detection result of Ca^{2+} in U87-MG-shTRPV1 group.

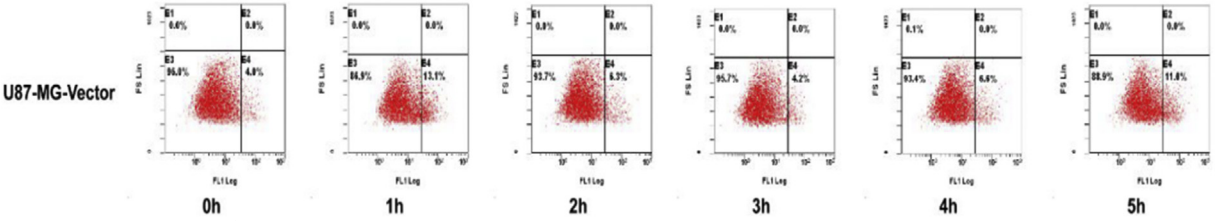

Figure 3. Detection result of Ca^{2+} in U87-MG-Vector group.

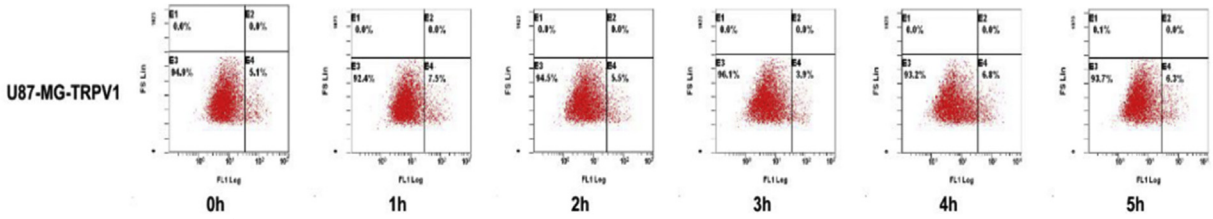

Figure 4. Detection result of Ca^{2+} in U87-MG-TRPV1 group.

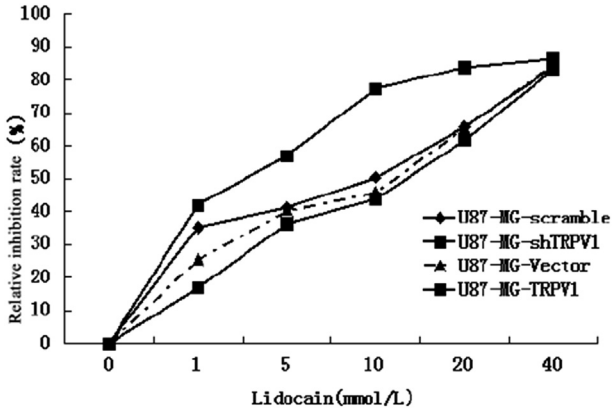

Figure 5. MTT results in each group.

($P < 0.05$). While it significantly decreased in *TRVP1* gene silencing group ($P < 0.05$). Moreover, with the increasing concentration of lidocaine, the apoptosis was more severe ($P < 0.05$).

4. Discussion

This experiment demonstrated that lidocaine could dose-dependently result in increasing of intracellular calcium concentration, the mitochondrial membrane potential and apoptosis of U87-MG glioma cell line. At the same time, up-regulation of *TRPV1* gene increased the cytotoxic effect of lidocaine, which revealed their correlation. Lidocaine probably increased the intracellular calcium concentration and mitochondrial membrane potential by activating *TRPV1* gene to cause glioma cell line U87-MG apoptosis.

Studies found that intracellular calcium overload was an important mechanism of LA causing neuronal apoptosis [13,14]. Calcium overload mediates cell damage and apoptosis by the following pathways. Calcium combines phosphate compounds in mitochondria to form insoluble calcium phosphate, interfering oxidative phosphorylation, decreasing production of ATP and causing mitochondrial permeability transition pore open; increasing of calcium ion concentration can activate a variety of phospholipase, promote membrane phospholipids decomposition, thus damaging the cell membrane and organelle membrane structure, increasing membrane phospholipid degradation products, arachidonic acid and lysophosphatidic, increasing cell dysfunction; it can activate calcium-dependent protease, promoting an increase in oxygen free radicals, activating calcium/calmodulin-dependent protein kinase (CaMK Ⅱ), mediated cell apoptosis [15]. LA induced intracellular calcium overload in neurons by calcium-induced calcium release mechanism, that is, after the extracellular calcium ions entering cells, it will induce the release of large amounts of calcium in intracellular calcium stores, suddenly increasing intracellular calcium ion concentration, causing calcium overload [16]. On the other hand, LA can promote the release of a large number of excitatory amino acids in nerve cells, excessively activating NMDA receptors, resulting in large amounts of calcium ions entering the cells through the NMDA receptor. After neuronal excitability increased, extracellular calcium ions can also enter the cells via the membrane voltage-dependent calcium channels, causing intracellular calcium overload [17].

Cell apoptosis is a common physiological phenomenon. Embryonic development, endometrial cyclical shedding, cleaning process immune cells are all accompanied by cell apoptosis. Bacterial toxins, heat shock, radiation and oxidative stress also can affect cell apoptosis by external factors [18–20]. Mitochondria-mediated Caspase-8 precursor activation pathway plays an important role in the induction of cell apoptosis. The main mechanisms are as follows. Cytochrome C released by mitochondria can activate Caspase-8 precursor, generating death stimulus signals including DNA damage and apoptotic precursor protein activation, starting the cascade reaction, inducing cell apoptosis [21–24]. In this study, lidocaine concentration and increased *TRPV1* gene plays a synergistic effect on mitochondrial membrane potential. With the gradual increase of mitochondrial membrane potential, apoptosis rate of U87-MG glioma cell line was more pronounced, which was consistent with previous studies [25].

In summary, LA drug, lidocaine, could produce neurotoxicity via *TRPV1* gene, and promote apoptosis generation. Its mechanism was associated with the intracellular calcium ion concentration and mitochondrial membrane potential, which provided some references for LA neurotoxicity prevention and application.

Conflict of interest statement

We declare that we have no conflict of interest.

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