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Mechanism of fat emulsion-mediated PTEN-PI3K-AKT signaling pathway on neurotoxicity of Bupivacaine

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ABSTRACT

This study was to explore the application value of chromosome ten (PTEN) - phosphatidylinositol 3-kinase (PI3K) - protein kinase B (AKT) signaling pathway in the treatment of Bupivacaine toxicity to neuronal cells under the regulation of fat emulsion. Neurons in the hippocampus of newborn rats were treated with Bupivacaine and fat emulsion and divided into five groups. The activity and action potential of neurons in each group were measured and Nissl's staining was performed. The results showed that the neuron activity of Bupivacaine group (42.36 ± 5.48%), Bupivacaine + fat emulsion group (70.23 ± 3.66%), and Bupivacaine + fat emulsion + PTEN/PI3K/AKT inhibitor group (79.28 ± 5.14%) was lower than that of the blank group (99.95 ± 3.42%). The duration of action potential in Bupivacaine group was increased (5.19 ± 0.48ms) and the frequency of action potential was decreased (13.87 ± 1.95) compared with the blank group (2.44 ± 0.37ms, 19.59 ± 2.14). The duration of the fat emulsion group (2.39 ± 0.39ms, 19.76 ± 2.05), Bupivacaine + fat emulsion group (2.88 ± 0.52ms, 18.53 ± 1.66), and Bupivacaine + fat emulsion + PTEN/PI3K/AKT inhibitor group (3.43 ± 0.69ms, 17.57 ± 1.58) was decreased, but the number of times increased ($P < 0.05$). In short, the fat emulsion can reverse the toxic effects of Bupivacaine on rat hippocampal neurons by regulating the PTEN/PI3K/AKT signaling pathway. This study provided a reference for the clinical treatment of the neurotoxicity of Bupivacaine.

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Introduction

Bupivacaine, also known as marcaine, is a long-acting amide local anesthetic, which is often used in clinical surgery and can act in local infiltration anesthesia, peripheral nerve block, and spinal canal block (1,2). Bupivacaine is slightly bitter, odorless, and easily soluble in ethanol and water. It has a good effect of local anesthesia, short onset time, long duration of anesthesia, and low concentration in the blood (3,4). The mechanism of Bupivacaine is increasing the threshold of the neural action potential, and it can also reduce the rate of action potential increase and delay the expansion of nerve impulse, so it is a relatively safe local anesthetic (5,6). However, some adverse reactions may occur after the use of this anesthetic, such as dizziness, nausea, vomiting, slow heart rate, and hypotension (7). In addition, Bupivacaine is highly cardiotoxic and may cause serious toxic effects and cardiotoxicity when a large dosage is used (8,9). Its accidental entry into the blood may lead to the central nervous system and circulatory system complications, cardiac arrest, and death in severe cases (10,11). Therefore, it is necessary to deal with severe neurotoxic reactions timely and effective.

The central nervous system toxicity of local anesthetics is closely related to the hippocampus. Some scholars pointed out that Bupivacaine can inhibit the activity of hippocampal neurons in rats and accelerate the process of apoptosis (12). The fat emulsion is a uniform and stable fat emulsion agent composed of soybean oil and lecithin, which can provide high energy and essential fatty acids in parenteral feeding (13,14), and is mainly used for nutritional disorders, nutritional disorders, or nitrogen balance disorders before and after surgery (15). As early as the end of the last century, studies found that fat emulsion can significantly increase the probability of successful cardiac resuscitation in rats with cardiac arrest caused by excessive Bupivacaine dose (16). In recent years, the fat emulsion has played an important role in the treatment of poisoning caused by local anesthetics. At present, the mechanism of fat emulsion reversing the toxic reaction of local anesthetic drugs is not clear, but there are several common theories in clinical practice. One is that fat emulsion may use its adsorption effect to achieve torsional toxicity, because fat emulsion is a hydrated emulsion, and the fat formed by fat emulsion can purposefully move Bupivacaine to organs that are easy to bind to lipids (17). Second, there is the metabolic mechanism. The cardiac function of the heart can be directly affected by fat, and inhibiting the oxidative metabolism of fatty acids will inhibit the cardiotoxic reaction caused by Bupivacaine. The fat emulsion can increase the concentration of fatty acids in cardiomyocytes and improve cardiac muscle strength (18). The inhibition of Bupivacaine on sodium channel current can be inhibited by free fatty acids, and fat emulsion can improve the metabolism of cardioid mitochondria and reduce the inhibition of Bupivacaine on intima carnitine lipoyltransferase (19). This may be the mechanism by which fat emulsion reverses the toxic effects of local anesthetics.

Chromosome ten (PTEN) is a very important tumor suppressor protein, which can prevent tumor development

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by effectively antagonizing phosphatidylinositol 3-kinase (PI3K) -protein kinase B (AKT) signaling pathway. As a lipid phosphatase, PTEN can dephosphorylate PIP3 on the cell membrane to generate PIP2, and then antagonize the signal of cell growth, metabolism, proliferation, and survival mediated by PI3K (20). PI3K signaling is also involved in the regulation of a variety of cell functions, and the signaling pathway formed by PI3K and AKT regulates cell survival and proliferation through the presence of silver. Studies revealed that fat emulsion reduces hippocampal neuron apoptosis, improves the survival rate of nerve cells, and then reverses the neurotoxic reaction caused by Bupivacaine through the BDNF-TrKB pathway (21). The PTEN/PI3K/AKT signaling pathway is one of the downstream pathways.

Therefore, Bupivacaine and fat emulsion were applied to cultured hippocampal neurons of rats born within 24 hours in vitro. The role of fat emulsion-mediated PTEN/PI3K/AKT signaling pathway in the neurotoxicity of Bupivacaine was comprehensively evaluated by observing the results of cell counting reagent on the activity of neurons in each group, the indicators of hippocampal neuron action potential detected by electrophysiological patch-clamp technique and the results of Nissl's staining.

Materials and Methods

Main instruments, materials, and reagents

The carbon dioxide cell incubator was purchased from Dute Scientific Instruments Co., LTD., Shanghai, China. An inverted biological microscope was purchased from Ailaibao Instrument Equipment Co., LTD., Shandong, China. Enzyme-linked immunoassay was purchased from Gaoxin Biosensor Research Institute Co., LTD., Shandong, China. Ultra-low temperature refrigerator was purchased from Heli Ultra-low Temperature Technology Co., LTD., Zhejiang, China. Microelectrode needle puller was purchased from Gairdner Technology Co., LTD., Hubei, China. 96-well plate was purchased from Muchen Biotechnology Co., LTD., Shanghai, China. Centrifugal tube was purchased from Muchen Biotechnology Co., LTD., Shanghai, China. The pipette was purchased from Muchen Biotechnology Co., LTD., Shanghai, China. 0.75% Bupivacaine Hydrochloride Injection was purchased from Harvest (Hefeng) Pharmaceutical Co., LTD., Shanghai, China. 20% fat emulsion injection was purchased from Kelun Pharmaceutical Co., LTD., Sichuan, China. PI3K inhibitor was purchased from Huizhi Biotechnology Co., LTD., Jiangsu, China. AKT was purchased from Yiyan Biotechnology Co., LTD., Shanghai, China. Polylysine was purchased from Shangbao Biological Co., LTD., Shanghai, China. Phosphate buffer salt solution was purchased from Hewei Pharmaceutical Technology Co., LTD., Guangdong, China. Cell Counting reagent (CCK-8) kit was purchased from Shangbao Biological Co., LTD., Shanghai, China. B27 Additive was purchased from Jiheng Pharmaceutical Technology Co., LTD., Guangdong, China. Toluidine blue dye was purchased from Gefan Biotechnology Co., LTD., Shanghai, China.

Culture of rat hippocampal neurons

The slides were soaked in the sulfuric acid solution for 24 hours, cleaned and dried to ensure that they were sterile, and placed in a new 96-well plate. The cell culture plate was treated with 0.01% polylysine, and the coating solution covered the bottom of the culture plate. After 30 minutes, the excess coating solution was absorbed and removed, and it was placed overnight in a constant temperature incubator at 37℃. The balanced salt solution (BSS) was used for cleaning, and the plate was washed repeatedly about 3 times until clean and dried under control for later use.

Two new centrifuge tubes (15mL and 50mL) were filled with phosphate buffer salt solution and placed in a -20℃ refrigerator for 40 minutes to ensure that the tubes were mixed with ice water. After routine disinfection of surgical instruments, a newborn SD rat less than 24 hours old was selected. The skin was disinfected with 75% alcohol, and the rat's head was cut off. The skin was cut open, the layers were dissected until the brain tissue was fully exposed, and the brain tissue was removed and soaked in a balanced salt solution for about 5 minutes. The rat brain tissue was removed from the BSS and placed on the operating dish. The hippocampus (on both sides) in the rat brain tissue was separated, and the blood vessels and meninges were removed in the process. The BSS was utilized for cleaning, and the tissue was washed twice.

After separation, the excess buffer was absorbed and removed, and trypsin was added, and the incubator was placed in 37℃ constant temperature carbon dioxide incubator for 40 minutes, during which the incubator could be gently shaken to speed up digestion. After digestion, the rat brain tissue was removed, cleaned, and digested with a phosphoric acid buffer solution. The supernatant was discarded after about five minutes. Then, 1mL inoculation solution was added and blown to be mixed under gentle action. Then, it is placed for about five minutes, and the supernatant was absorbed and injected into the centrifugal tube. The above operation was repeated three times, and the supernatant obtained was put into the centrifuge tube to remove the remaining undigested tissue. The supernatant in the tube was fully inoculated into the culture plate.

After five hours, the culture plate was gently shaken and washed with an inoculation solution. The excess dead cells and cell fragments were absorbed and removed from the upper layer, and the original culture medium was replaced with the basic culture medium supplemented with B27. On day two, half of the old medium was discarded, and another half of the new one was supplemented. The culture was terminated on the 8th day. The purity of rat neuron cells was determined by using neuronal microtubule-associated protein 2. If the purity was greater than 90%, it was qualified and could be used in subsequent experiments.

Experimental grouping

Isolated and cultured rat hippocampal neurons were divided into five groups.

Group 1: Blank control group;

Group 2: Bupivacaine group (concentration 1mM);

Group 3: fat emulsion group (concentration 1%);

Group 4: Bupivacaine (1mM) + fat emulsion (1%);

Group 5: Bupivacaine (1mM) + fat emulsion (1%) + PTEN-PI3K-AKT inhibitor (20μM).

Cell counting reagent detection

The activity of rat hippocampal neurons was determined by CCK-8 reagent 24 hours after the reaction. The

old media was removed from the 96-well plates used in the culture process. 100μL new medium containing 10% CCK-8 was added to each well and left at 37°C for 3 hours. The absorbance was then measured at 450nm by enzyme-linked immunoassay. The above operation was repeated 3 times. Cell viability was calculated as follows: cell viability (%) = [*V* (administration) - *V* (none)] / [*V* (non-administration) -*V* (none)], where, *V* (administration) represented the absorbance value of the hole containing neurons, CCK-8 reagent, and drug solution, *V* (non-administration) represented the absorbance value of the well-containing neurons and CCK-8 reagent, and *V* (none) represented the absorbance value of the well-containing medium and CCK-8 reagent.

Recording of action potentials in neurons

Under an inverted biological microscope, the induced electrodes were combined with neuron cells using a microelectrode needle puller. Suction was performed under negative pressure to obtain a GΩ sealed connection. Power factor compensation was followed by negative pressure aspiration until the cell membrane of the neuron ruptured and whole-cell recording was achieved. Then, slow power factor compensation was carried out. After the operation, the total resistance of the film capacitor and the circuit in series was recorded. Neurons were stimulated by electric current, and peak potential and post potential were recorded.

All data during the experiment were collected using an Axon Instruments patch-clamp amplifier, uploaded and stored in the locking amplifier software developed by HEKA, and the final data were exported using patch-clamp analysis software.

Nissl's staining

Toluidine blue stain was diluted with an ethanol solution, and the glass slide with cells was immersed in it for about five minutes. The glass slide was rinsed with distilled water three times, five minutes each time. Then, they were placed in a constant temperature incubator at 60°C, and toluidine blue was used for dyeing for 30 seconds. The dyes were then washed with distilled water, dehydrated in 70%, 80%, 95%, and 100% ethanol, transparent with xylene, and finally sealed with neutral gum.

Statistical analysis

SPSS 24.0 was used for the statistical analysis of all experimental data. Mean ± standard deviation was used to represent the measurement data consistent with normal distribution. One-way ANOVA was used for comparison between groups, and $P < 0.05$ was considered statistically significant.

Results

Effect of bupivacaine on neuron cell viability at each time point

Bupivacaine concentration of 1mM was used in this study. After 1h, 3h, 6h, 12h, and 24h of reaction to hippocampal neuron cells, the activity of neuron cells was measured by CCK-8 assay. In Figure 1, with the Bupivacaine reaction time prolonged, the activity of hippocampal neurons decreased gradually, and the activity at 24h decreased by about half compared with the initial one. Thus, 24h was chosen as the time for the subsequent experiment.

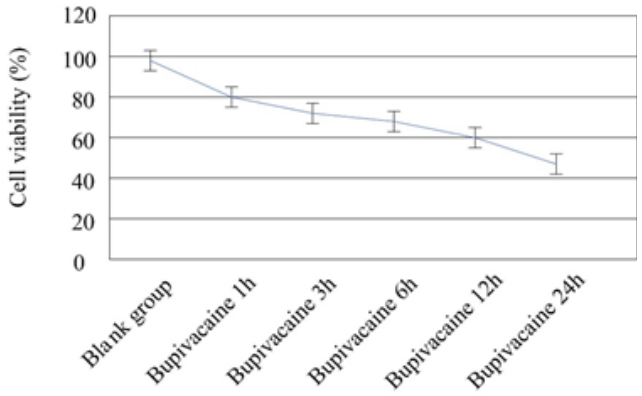

Figure 1. Effect of Bupivacaine on neuron activity at each time node.

Effects of PTEN/PI3K/AKT inhibitors on the activity of hippocampal neurons

After the addition of PTEN/PI3K/AKT inhibitor, compared with 99.95 ± 3.42% of the control group, the activity of hippocampal neurons in Bupivacaine group (42.36 ± 5.48%), Bupivacaine + fat emulsion group (70.23 ± 3.66%), and Bupivacaine + fat emulsion + PTEN/PI3K/AKT inhibitor group (79.28 ± 5.14%) were decreased, and the differences were statistically significant ($P < 0.05$). Compared with Bupivacaine group, the activity of hippocampal neurons in the fat emulsion group, Bupivacaine + fat emulsion group, and Bupivacaine + fat emulsion + PTEN/PI3K/AKT inhibitor group was increased, with statistical significance ($P < 0.05$). The cell viability of Bupivacaine + fat emulsion + PTEN/PI3K/AKT inhibitor group was higher than that of Bupivacaine + fat emulsion group, but the difference was not statistically significant ($P > 0.05$, Figure 2). Bupivacaine at the concentration of 1mM can reduce the cell viability of rat hippocampal neu-

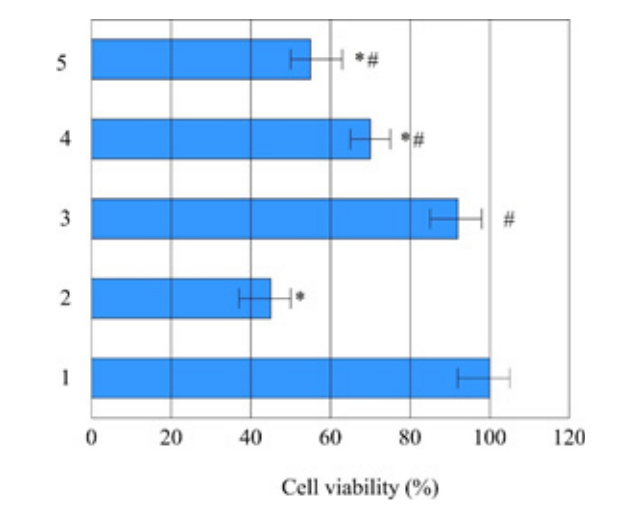

Figure 2. Effect of each group on the activity of hippocampal neurons. Note: 1 represented the blank group, 2 represented Bupivacaine group, 3 represented the fat emulsion group, 4 represented Bupivacaine + fat emulsion group, and 5 represented Bupivacaine + fat emulsion + PTEN/PI3K/AKT inhibitor group.
* represented a statistically significant difference in hippocampal neuron activity between the blank group and each group ($P < 0.05$), and # represented a statistically significant difference in hippocampal neuron activity between the Bupivacaine group and each group ($P < 0.05$).

rons, 1% fat emulsion can reverse the toxic effect of Bupivacaine on rat hippocampal neuron activity, and PTEN/PI3K/AKT can enhance the therapeutic effect of fat emulsion on Bupivacaine neurotoxicity.

Detection of action potential in rat hippocampal neurons

The electrophysiological patch clamp technique was used to detect the action potentials of hippocampal neurons in each group. Figure 3 and 4 showed that compared with the blank group (2.44 ± 0.37 ms and 19.59 ± 2.14), the action potential duration (APD) of rat hippocampal neurons in Bupivacaine group increased (5.19 ± 0.48 ms), and the number of action potential decreased (13.87 ± 1.95), with significant differences ($P < 0.05$). Compared to the Bupivacaine group, APD decreased in the fat emulsion group (2.39 ± 0.39 ms, 19.76 ± 2.05), Bupivacaine + fat emulsion group (2.88 ± 0.52 ms, 18.53 ± 1.66), and Bupivacaine + fat emulsion + PTEN/PI3K/AKT inhibitor group (3.43 ± 0.69 ms, 17.57 ± 1.58). The number of occurrences increased, and the difference was statistically significant ($P < 0.05$). In summary, the effect of Bupivacaine on the duration and frequency of action potential generation in rat hippocampal neurons can be attenuated by fat emulsion. Action potential amplitude (APA), the peak amplitude of the action potential, resting membrane potential (RP), and action potential depolarization thresholds were not statistically significantly different ($P > 0.05$).

Nissl's staining of hippocampal neurons of rats in each group

Nissl substance is a basophilic substance in the cytoplasm, which is widely distributed in various neurons. The shape, size, and quantity of Nissl substance vary from neuron to neuron. Nissl's staining can be used to observe the cell structure of neurons. It can also be used to understand the damage to neurons. In Figure 5, hippocampal neuron cells in the blank group were darker in color, with a large number of cells and complete morphology. In Figure 6, the staining color of neuron cells in Bupivacaine group became lighter and lighter, and the number also decreased significantly. In Figure 7-9, there was no significant dif-

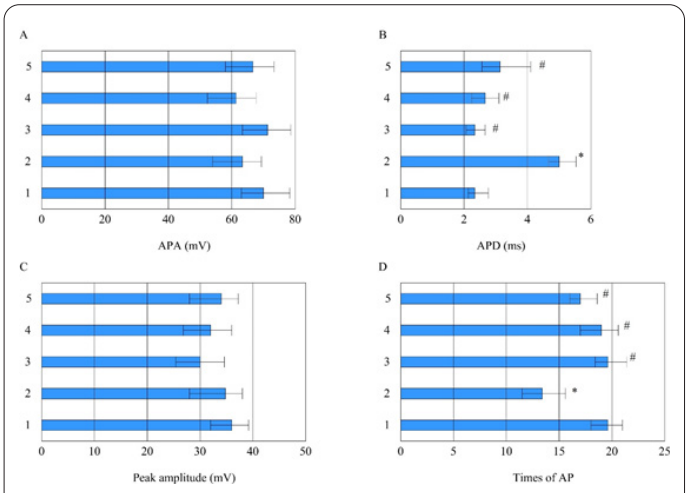

Figure 3. Comparison of hippocampal neuron action potential amplitude, duration, the corresponding peak of amplitude, and frequency of generation in each group. Note: 1 represented the blank group, 2 represented Bupivacaine group, 3 represented the fat emulsion group, 4 represented Bupivacaine + fat emulsion group, and 5 represented Bupivacaine + fat emulsion + PTEN/PI3K/AKT inhibitor group.* represented a statistically significant difference in hippocampal neuron activity between the blank group and each group ($P < 0.05$), and # represented a statistically significant difference in hippocampal neuron activity between the Bupivacaine group and each group ($P < 0.05$).

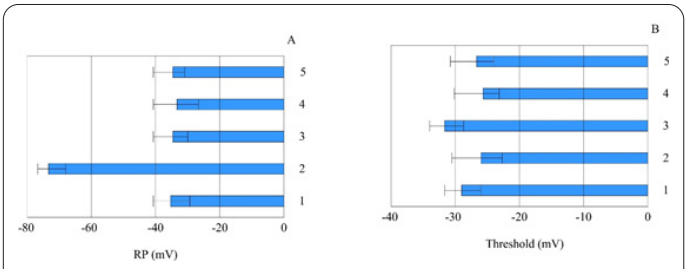

Figure 4. Comparison of hippocampal neuron action potential resting membrane potential and threshold of depolarization in each group. Note: 1 represented the blank group, 2 represented Bupivacaine group, 3 represented the fat emulsion group, 4 represented Bupivacaine + fat emulsion group, and 5 represented Bupivacaine + fat emulsion + PTEN/PI3K/AKT inhibitor group.

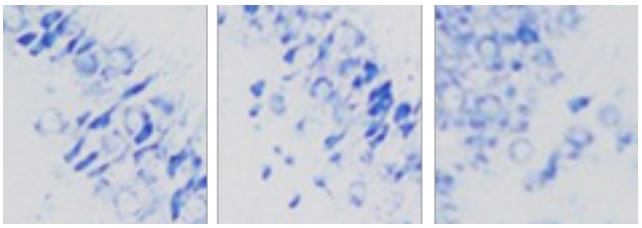

Figure 5. Nissl's staining of hippocampal neurons of rats in the blank group.

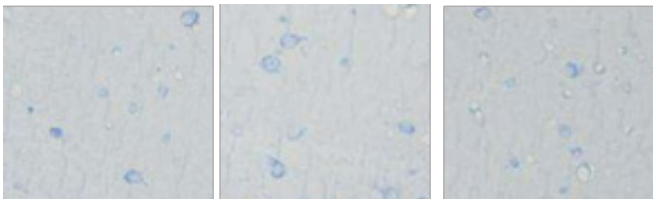

Figure 6. Nissl's staining of rat hippocampal neurons in Bupivacaine group.

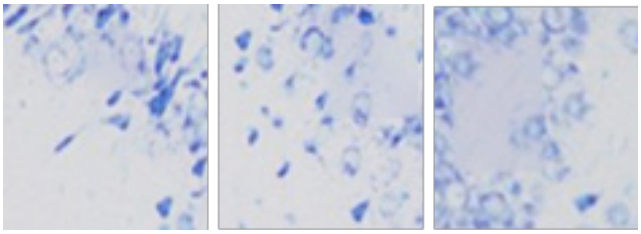

Figure 7. Nissl's staining of hippocampal neurons in the fat emulsion group.

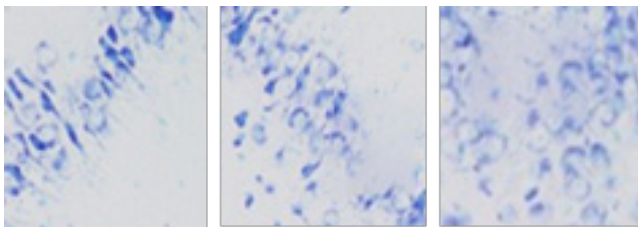

Figure 8. Nissl's staining of rat hippocampal neurons in Bupivacaine + fat emulsion group.

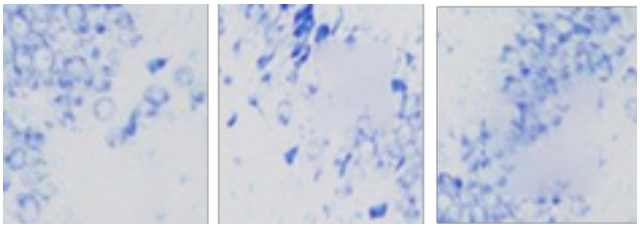

Figure 9. Nessl staining of rat hippocampal neurons in Bupivacaine + fat emulsion + PTEN/PI3K/AKT groups.

ference in Neuronal staining of the fat emulsion group, Bupivacaine + fat emulsion group, and Bupivacaine + fat emulsion + PTEN/PI3K/AKT group.

Discussion

Bupivacaine, an amide drug, is often used as a local anesthetic in clinical surgery and is a relatively safe long-term anesthetic (22). However, when the drug dose is too large or accidentally goes into the blood vessel, it is easy to cause neurotoxicity, and its toxic effects are commonly seen in the human body, such as convulsion, lower limb numbness, numbness, and chest and back pain, (23). The neurotoxicity of Bupivacaine can also cause abnormalities in the urinary system, such as urinary retention and incontinence. In recent years, there have been frequent reports of central nervous system complications caused by the neurotoxicity of Bupivacaine. Bupivacaine can also cause sinus bradycardia, atrioventricular, or indoor conduction block, ventricular tachycardia, cardiac arrest, etc., and even death in severe cases (24). The fat emulsion is an energy supplement, a component of intravenous nutrition that provides energy and essential fatty acids to the body. Kim et al. (2020) (25) indicated that fat emulsion has an important value in reversing the neurotoxic response of Bupivacaine.

In this experiment, 1mM Bupivacaine and 1% fat emulsion were utilized to culture rat hippocampal neurons born within 24 hours. The hippocampus is responsible for memory and learning and is where short-term memories of everyday life are stored. The neuron is the basic unit that constitutes the structure and function of the nervous system. Neuron has long protuberances and consists of cell bodies and protuberances. It is an effective cell model commonly used in clinical experiments. At the beginning of the experiment, it was observed that the activity of rat hippocampal neurons decreased gradually with the passage of Bupivacaine reaction time, and by 24h the activity had decreased by about 50%. Therefore, the reaction time node of the experiment was set at 24 hours. According to the experimental results, after the addition of PTEN/PI3K/AKT inhibitor, the activity of hippocampal neurons in Bupivacaine group, Bupivacaine + fat emulsion group, and Bupivacaine + fat emulsion + PTEN/PI3K/AKT inhibitor group was lower than that in the blank group. Compared with Bupivacaine group, the cell viability of the fat emulsion group, Bupivacaine + fat emulsion group, and Bupivacaine + fat emulsion + PTEN/PI3K/AKT inhibitor group increased, with statistical significance ($P < 0.05$). Ok et al. (2017) (26) concluded through experiments that fat emulsion had a therapeutic effect on the neurotoxicity caused by Bupivacaine, which was consistent with previous research results and found that the therapeutic effect could be enhanced through the PTEN/PI3K/AKT signaling pathway.

Chen et al. (2021) (27) pointed out that the therapeutic effect of fat emulsion on neurotoxicity may be realized through the PTEN/PI3K/AKT signaling pathway. It can antagonize the toxic reaction of excitatory amino acids and prevent neuronal apoptosis. This experiment also verified this statement, and PTEN/PI3K/AKT inhibitors can attenuate the toxic response of Bupivacaine to neuronal cells and play its protective effect. In addition, the action potentials of hippocampal neurons in each group were detected by the electrophysiological patch clamp technique. The results showed that compared with the blank group, the duration of action potential was increased and the number of action potential generations was decreased in Bupivacaine group. Compared with Bupivacaine group, the time duration of the other groups was shortened, and the number of occurrences increased, with statistical significance ($P < 0.05$), indicating that the neurotoxicity of Bupivacaine was weakened after fat emulsion supplementation, so the amplitude of action potential also decreased. As for the results of Nissl staining of neuron cells, it was indicated that after Bupivacaine was added, neuron cells changed from the initial number of large and complete structures to the state of the small number and light color. However, the number of neuron cells in the groups supplemented with fat emulsion increased gradually, indicating that the toxicity of Bupivacaine to neuron cells gradually improved under the effect of fat emulsion.

In this experiment, the hippocampal neurons of newborn rats were isolated and cultured, and the concentration of 1mM Bupivacaine and 1% fat emulsion were applied to the neurotoxicity test of Bupivacaine. The results showed that the toxicity of Bupivacaine to hippocampal neurons could be reversed by fat emulsion, and the reduction and damage of hippocampal neurons caused by Bupivacaine could also be improved by fat emulsion. By regulating the PTEN/PI3K/AKT signaling pathway, fat emulsion had a therapeutic effect on the toxicity of Bupivacaine-induced hippocampal neurons. However, there are still some shortcomings in this study. Since the hippocampus neurons of rats were cultured in vitro, the results of reactions in the body can't be copied. In addition, many signaling pathways can regulate cell functions in the body, and the PTEN/PI3K/AKT signaling pathway is only one of them, and the results may be interfered by other factors in the body during the reaction process. Therefore, it is expected to conduct more perfect and in-depth exploration of this research direction in the future, so as to solve the problem of the toxic effect of Bupivacaine on neurons. To sum up, this study provides a reference for the treatment of neurotoxic reaction of Bupivacaine in the clinic.

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