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NNC55-0396 Reduces SH-SY5Y Cell Damage Induced by Bupivacaine Hydrochloride

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Abstract

Background: Local anesthetic neurotoxicity is a common complication in clinical anesthesia, which can cause permanent nerve damage in severe cases. The T-type calcium channel is an important channel for regulating the excitability of neurons. Normally, extracellular calcium ions enter the cell through the T-type calcium channel to change the excitability of neurons. When the intracellular calcium is overloaded, it can cause cell damage.

Aims: To investigated the roles of T-type calcium channel in the SH-SY5Y cells injury induced by the bupivacaine.

Methods: The SH-SY5Y cell culture model was used to observe the effect of T-type calcium channel blocker NNC55-0396 on the neurotoxicity of bupivacaine hydrochloride by MTT methold,flow cytometry, Western blotting and other methods.

Results: The results show that NNC55-0396 can block the T-type calcium channel of SH-SY5Y cells, improve the decrease of cell viability caused by bupivacaine hydrochloride, reduce the level of intracellular calcium ion, reduce the expression of Cleavedcaspase-3, and reduce cell apoptosis.

Conclusion: The above results indicate that the T-type calcium channel is involved in the SH-SY5Y cell damage caused by bupivacaine hydrochloride, and blocking the T-type calcium channel can reduce the neurotoxicity of bupivacaine hydrochloride.

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Introduction

Local anesthetics, with good analgesic effects, are commonly used in clinical analgesia and regional blockade [1, 2]. As well as, the central nervous system toxicity and cardiotoxicity injury induced by local anesthetics have attracted people's attention [3-6]. Standardized operation process of nerve block, visualization technology guided by ultrasound and strengthening the monitoring can reduce the occurrence of local anesthetic toxicity [7]. Studies have reported that the incidence of transient neurological syndrome caused by intra-spinal block is as high as 8.1%, manifested as lower limb pain or sensation abnormality after intra-spinal block several hours to several days later[8]. The neurological complications of intra-spinal block are also higher in obstetric anesthesia [9]. The exact mechanism of peripheral nerve injury induced by local anesthetics is not clear, which may be related to apoptosis, reactive oxygen species (ROS) and lactate dehydrogenase (LDH) release and increased intracellular calcium levels[10-13]. Some studies have shown that lidocaine, bupivacaine, tetracaine and dibucaine can induce cell apoptosis[14]. As well as, the increase of intracellular calcium level is closely related to the extracellular calcium influx and the release of intracellular calcium pool[12].

Voltage dependent calcium channel (VDCC) is an important channel for extracellular calcium ions to enter the cell[15]. It can be divided into high voltage dependent calcium channel and low voltage dependent calcium channel. T-type calcium channel is one low voltage dependent calcium channel[16]. It can be activated at the resting potential, and it is the initiator of regulating the excitability of neurons, participating in the regulation of various physiological and pathological processes [17-21]. In resting state, T-type calcium channel is activated, extracellular calcium enters the cell through T-type calcium channel, further activating high-voltage dependent calcium channel, increasing intracellular calcium concentration. Meanwhile, T-type calcium channel can also trigger intracellular calcium induced calcium release, further improving intracellular calcium level. The certain stable state of the intracellular and extracellular calcium is very important to the cells normal function. Under pathological conditions, this stable state is destroyed, which may lead to the change of cell function.

Bupivacaine hydrochloride is a kind of local anesthetics commonly used in clinic. Previous studies often focused on the cardiotoxicity of bupivacaine hydrochloride, especially the treatment of cardiac toxicity of bupivacaine [22, 23]. At the same time, bupivacaine, as a commonly used drug in spinal anesthesia, its peripheral neurotoxic injury also cannot be ignored [24, 25]. In this study, SH-SY5Y cells were cultured in vitro and NNC55-0396, one T-type calcium channel blocker, was used to investigate the role of T-type calcium channel in the neurotoxicity of bupivacaine hydrochloride.

Materials and Methods

Materials

SH-SY5Y cell line is a passage cell line of our laboratory. The antibody of procaspase-3, Cleavedcaspase-3 and β-actin were purchased from Abcam company, Cambridge, England. Bupivacaine hydrochloride was purchased from; NNC55-0396, Annexin V-FITC / PI cell apoptosis detection kit were purchased from Este Biotechnology Co., Ltd, Wuhan, China. Fluo-3 AM and Methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Beyotian Company, Shanghai, China. LDH test kit was from Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China. Trypan blue was from Tianjin Haoyang biology Co. Ltd.TianjinL China.

Experimental Protocols

The protocols use were approved by the ethics committee of the Second People`s Hospital of Foshan City, Foshan, Guangdong Province, China. SH-SY5Y cells were cultured in vitro at 5% CO2 and 37 °C. The cells were divided into 6 groups: Cells cultured with normal medium (S group); Cells cultured with 100 μM final concentration of NNC55-0396 medium(S+NNC100 group); Cells cultured with 1mM final concentration of bupivacaine hydrochloride medium (S+ B group); Cells cultured with 1mM final concentration of bupivacaine hydrochloride and 10μM final concentration of NNC55-0396 medium (S + B +NNC10 group); Cells cultured with 1mM final concentration of bupivacaine hydrochloride and 50μM final concentration of

NNC55-0396 medium (S + B +NNC50 group); Cells cultured with 1mM final concentration of bupivacaine hydrochloride and 100μM final concentration of NNC55-0396 medium (S + B +NNC100 group);

MTT Assay for Cell Viability

SH-SY5Y cells were treated with bupivacaine hydrochloride (0.1,0.5,0.75,1,2,5,10 mM) for 24 hours, and the cell viability were detected by MTT method. At the same time, cells in every group were treated as the experiment protocol for 6h, 12h, 24h, and the cell viability of the cells were detected by MTT method. The cells in every group were inoculated on 96 well plate. After treatment as the experiment protocol, 10 μl MTT was added into every well and the cells cultured at 37 °C for 4 hours, the medium was sucked out, and 150μl DMSO was added to shake for 10 minutes; the absorbance value of OD568 of every wells were measured by Microplate Reader (Thermo, Multiskan MK3,MA, USA). The OD568 value of the SH-SY5Y cells in normal group was regarded as 100%, the OD568 value of the cells in other group were normalized with the cells in normal group.

LDH Activity Detection

The cells in every group were treated as the experiment protocol for 6h, 12h, 24h, and then the supernatant was taken for LDH detection. In short, every sample is set with blank hole, standard hole, measuring hole and control hole respectively to detect OD450 value. Blank hole: 25μl double distilled water, 25μl matrix buffer, 15 min of 37 °C, 25μl 2,4-dinitrophenylhydrazine, take a bath at 37 °C for 15min, 250μl NaOH solution(0.4mol/l), and 5 min of room temperature for OD450 detection; Standard hole: 5μl double distilled water, 20μlstandard solution (0.2μmol / L), 25μl matrix buffer, take a bath at 37 °C for 15min, 25μl 2,4-dinitrophenylhydrazine, take a bath at 37 °C for 15min, 250μl NaOH solution(0.4mol/l), place it at room temperature for 5min, and then test the OD450 value; Test hole: 20μl sample to be tested, 25μl matrix buffer, 5μl coenzymeI, 15 minutes of bath at 37 °C, 25μl 2,4-dinitrophenylhydrazine, and take a bath at 37 °C for 15min, Add 250μlNaOH solution (0.4mol/l), place it at room temperature for 5min, and then test OD450 value; Control hole: 20 μ l of sample to be tested, 25 μ l of matrix buffer solution, 15 minutes of bath at 37 °C, 25 μl 2,4-dinitrophenylhydrazine, 15 minutes of bath at 37 °C, 250 μl of NaOH solution (0.4mol/l), place it at room temperature for 5min, and then test the OD450 value; LDH activity (U / L) = (Test hole OD450 - Control hole OD450) / (Standard hole OD450 - Blank hole OD450) × 0.2 * 1000.

Apoptosis Rate Detection

After treated as the experiment protocol, the cells in every group were digested with 0.25% pancreatin without EDTA . Cells were collected after digestion, centrifuged at 1500 rpm for 5 minutes, supernatant was removed, and PBS was added for resuspension. PBS rinses the cells twice, 1500rpm, 5min; According to the operation instructions of Annexin V-FITC / PI cell apoptosis detection kit: add 500 μl binding buffer, and suspend the cells again; 5 μl Annexin V-FITC is evenly mixed, and then add 5 μl PI, and evenly mixed; After 5-15min response at room temperature in dark room, flow cytometer is used to detect the apoptosis rate of every group.

Detection of Intracellular Calcium Level

After treated as the experiment protocol, the cells in every group were collected, centrifuged at 1200rpm for 3min, removed supernatant, and re-suspended with PBS. The cells were rinsed once with PBS, replaced with serum-free DMEM medium, added with fluo-3AM to the final concentration of 5 μmol / L, incubated in 37 °C and 5% CO_2 incubator for 30min in dark room. The cells were rinsed twice with PBS at 1200rpm for 3min; After PBS was re-suspended to 500 μl, the fluorescence intensity of normal cells was regarded as 1, and the ratio of other cells to normal cells was the intracellular calcium level.

Caspase-3 Expression by Western Blot

After treated as the experiment protocol, the cells in every group were collected, 120 μ l PMSF lysate was added into every pore, and then lysed on ice for 30min. In order to make the cell fully lysed, the culture dish should be shaken back and forth frequently. After the lysate, the cell fragments and lysate were moved into 1.5ml centrifuge tube and centrifuged at 12000rpm at 4 °C for 5min. The centrifuged supernatant was transferred into a 0.5ml centrifuge tube and stored at - 20 °C. The protein concentration of the sample was

calculated according to the standard protein concentration and the corresponding OD568 value. Put the extracted protein supernatant into boiling water for 10 min denaturation. Prepare 5% concentrated gel and 10% separation gel respectively, fix the prepared gel on the electrophoresis tank, and pour the electrophoresis solution into the storage tank. The sample quantity of every sample is 40 μg. The sample were under vertical electrophoresis with 120V voltage for 1.5h. Then Transfer the target strip to PVDF membrane with 200mA for 90min. The membrane was blocked with TBST (blocking solution) containing 5% skimmed milk powder, and seal it in a shaker at room temperature for 2h. The PVDF membrane was immersed in the antibody of procaspase-3, Cleavedcaspase-3 and β-actin incubation solution and incubated overnight at 4 °C. After the PVDF membrane washed by TBST for 5 times, the second antibody was added to incubate in shaker at 37 °C for 2h. The ECL reagent was used to as the substrate solution and the band was developed on the X-ray film. The ratio of the gray value of every sample to the gray value of β-actin was used to express the protein expression.

Dead Cells Stained with Trypan Blue

After treated as the experiment protocol, trypan blue staining solution was added to make the final concentration of the staining solution 0.04%. After 3 minutes of staining, the dead cells were dyed blue obviously, while the living cells were refused to be stained colorless and transparent. Take three fields in every group, and calculate the average of the percentage of dead cells in total cells.

Results

Cell Viability of the Cells

The viability of the cells treated with 0.1, 0.5, 0.75, 1, 2, 5, 10mM bupivacaine hydrochloride for 24h were (57.2±2.8)%, (49.3±2.3)%, (39.9±3.5)%, (26.9±2.7)%, (17.3±1.6)%, (6.6±1.6)%, (5.2±1.0)%, respectively, Fig 3. There were no differences of the cell viability between the cells in S group and S+NNC100 group at 6h,12h and 24h. The cells in S+B group were treated with 1 mM bupivacaine hydrochloride for 6h, 12h and 24h, the viability were (50±5)%, (44±4)%,and (23±3)%, respectively. Compared with the cells in S group and S+NNC group, the viability of the cells in S+B group, S+B+NNC10 group, S+B+NNC50 group and S+B+NNC100 group decreased. Compared with S+B group, the viability of the cells in S+B+NNC10 group, S+B+NNC50 group and S+B+NNC100 group increased. Compared with S+B+NNC10 group, the viability of the cells in S+B+NNC50 group and S+B+NNC100 group increased. There was no difference between the viability of the cells in S+B+NNC50 group and S+B+NNC100 group, See Tab 1.

LDH Activity of the Cells in Every Group

There were no differences of the cells LDH activity between those in S group and S+NNC100 group at 6h, 12h, 24 h. Compared with the cells in S group and S+NNC100 group, the activity of the cells LDH activity in S+B group rapidly increased and were (203.2±10.2)U/L, (258.8±24.9) U/L, (292.3±11.9) U/L at 6h, 12h, 24h, respectively. Although the LDH activity of the cells treated with bupivacaine hydrochloride increased, NNC55-0396, with different dosage, can inhibit the LDH activity increase induced by bupivacaine hydrochloride. As well as, the LDH activity of the cells in the median (50 μM) or high dosage (100 μM) NNC55-0396 group were lower than that in small dosage (10 μM) group. However, there were no differences between the cells in median NNC-55-0396 group (S+B+NNC50)and high dosage NNC55-0396 group (S+B+NNC100), Tab 2.

Cell Apoptosis Rate

The result showed that there were no significant differences in apoptosis rate of the cells in S group and S + NNC100 group. Compared with the cells in S group and S+NNC 100 group, the apoptosis rate of the cells in S+B group, S + B + NNC10 group, S + B +NNC 50 group, S + B + NNC 100 group significantly increased. Compared with the cells in S +B group, the apoptosis rate of the cells in S + B + NNC10 group, S + B + NNC 50 group, S + B + NNC 100 group significantly decreased. Compared with S + B + NNC10 group, the apoptosis rate of the cells in S + B + NNC 50 group, S + B + NNC 100 group significantly decreased. There were no significant difference in apoptosis rate between S + B + NNC50 group and S + B + nnc100 group. See Fig 1.

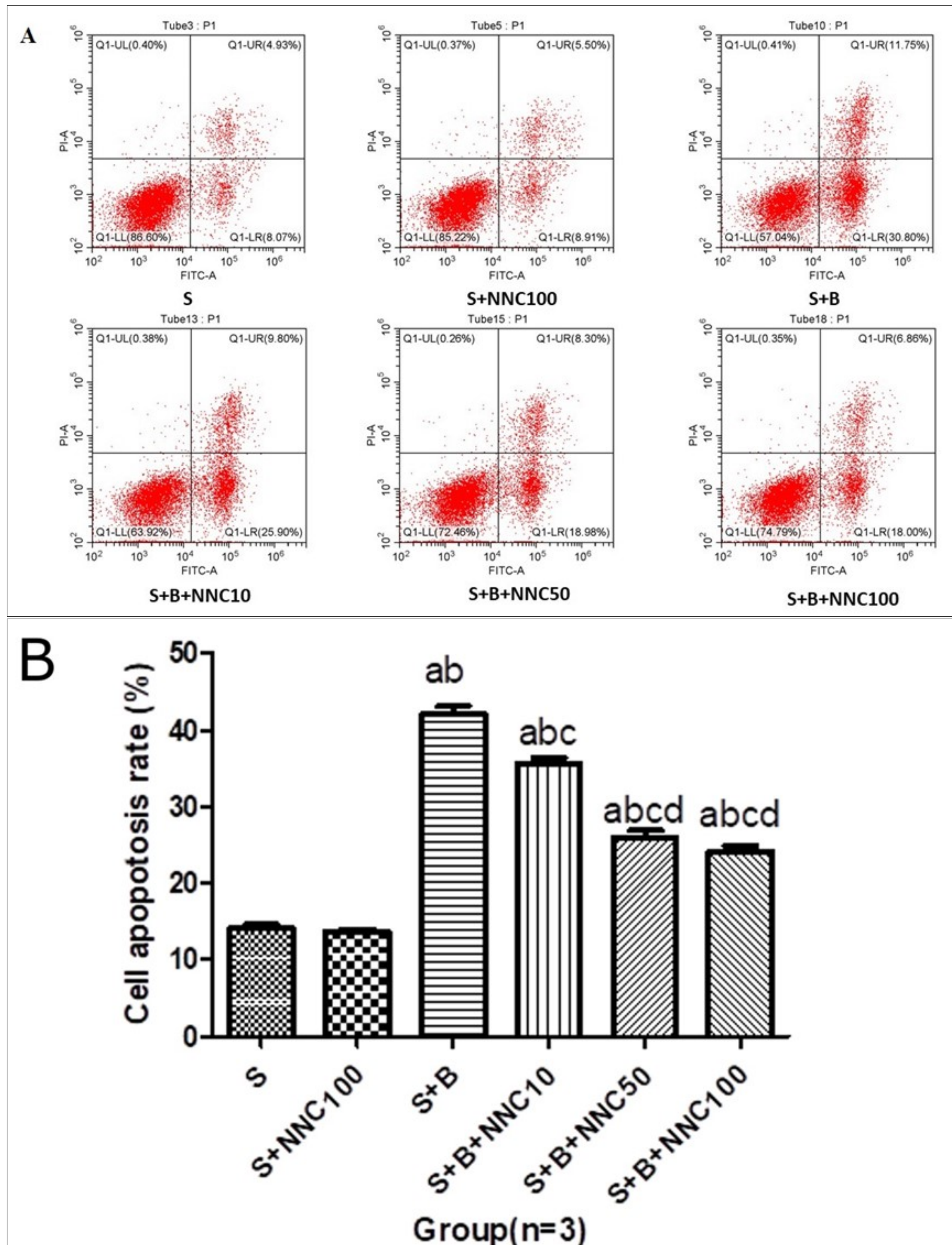

Figure 1. The apoptosis rate of the cells in every group. A: The represent sample apoptosis rate that detected with Flow cytometry. B: Rates of apoptosis in the different treatment groups (%, Mean±SD, n = 3). [a] *P*<0.05 vs. S group; [b] *P*<0.05 vs. S+NNC 100 group; [c] *P*<0.05 vs. S+B group; [d] *P*<0.05 vs. S+B+NNC 10 group.

Table 1. The viability of the cells in every group(mean±sd,%,n=6)

Group	6h	12h	24h
S	100±0	100±0	100±0
S+NNC100	100±4	100±5	100±4
S+B	50±5[c]	44±4[ac]	23±3[abc]
S+B+NNC10	61±3[cd]	50±3[acd]	40±2[abcd]
S+B+NNC50	70±6[cde]	62±4[acde]	54±5[abcde]
S+B+NNC100	69±2[cde]	62±5[acde]	54±4[abcde]

[a]P<0.05 VS 6h, [b]P<0.05 VS 12h, [c]P<0.05 VS S group, [d]P<0.05 VS S +B group, [e]P<0.05 VS S +B+NNC10 group.

Table 2. LDH activity of the cells in every group (Mean±SD, n=3, U/L)

Group	6h	12h	24h
S	95.9±2.7	99.8±21.0	102.7±10.5
S+NNC100	99.1±16.0	100.2±8.9	96.6±13.3
S+B	203.2±10.2[c]	258.8±24.9[ac]	292.3±11.9[abc]
S+B+NNC10	162.2±5.9[cd]	207.5±14.5[acd]	231.7±11.2[abcd]
S+B+NNC50	131.2±13.6[cde]	162.2±10.7[acde]	184.7±8.9[abcde]
S+B+NNC100	136.5±13.9[cde]	167.6±14.2[acde]	189.3±3.2[abcde]

[a]P<0.05 VS 6h, [b]P<0.05 VS 12h, [c]P<0.05 VS S group, [d]P<0.05 VS S +B group, [e]P<0.05 VS S +B+NNC10 group.

Expression of Caspase-3

There were no differences of the Cleaved caspase-3 of the cells in S group and S+NNC100 group. Compared with the cells in S group and S+NNC100 group, the expression of the Cleaved caspase-3 of the cells in S+B group, S+B+NNC10 group, S+B+NNC50 group, S+B+NNC100 group increased. Compared with the cells in S+B group, the expression of the Cleaved caspase-3 of the cells in S+B+NNC10 group, S+B+NNC50 group, S+B+NNC100 group decreased. Compared with the cells in S+B+NNC10 group, the expression of the Cleaved caspase-3 of the cells in S+B+NNC50 group, S+B+NNC100 group decreased. There were no differences between the cells in S+B+NNC50 group and S+B+NNC100 group, see Fig 2.

There were no differences of the procaspase-3 of the cells in S group and S+NNC100 group. Compared with the cells in S group and S+NNC100 group, the expression of the procaspase-3 of the cells in S+B group, S+B+NNC10 group, S+B+NNC50 group, S+B+NNC100 group decreased. Compared with the cells in S+B group, the expression of the procaspase-3 of the cells in S+B+NNC10 group, S+B+NNC50 group, S+B+NNC100 group increased. Compared with the cells in S+B+NNC10 group, the expression of the procaspase -3 of the cells in S+B+NNC50 group, S+B+NNC100 group increased. There were no differences between the cells in S+B+NNC50 group and S+B+NNC100 group, see Fig 2.

The Calcium ion Level of the Cells in Every Group

The results showed that there were no differences of the calcium level of the cells between S group and S+NNC100 group. After treated with 1mM bupivacaine hydrochloride, the calcium ion level of the cells in S+B group sharp increased. Compared the cells in S+B group, the calcium ion level of the cells in S+B+NNC10 group, S+B+NNC50 group and S+B+NNC100 group decreased. Compared the cells in S+B +NNC10 group, the calcium ion level of the cells in S+B+NNC50 group and S+B+NNC10 group decreased. There were no differences between the cells in S+B+NNC50 group and S+B+NNC100 group, Fig 3.

Dead Cells Detected by Trypan Blue

Trypan blue staining solution was added to detect the dead cells, the final concentration of the staining solution was 0.04%. After 3 minutes of staining, photos were taken, and the dead cells were dyed blue obviously, while the living cells were refused to be stained in a colorless and transparent state. The cells in S+B group showed an obviously cell dead, however, NNC55-0396 can reduced the cells dead. Compared with the cells in S+B group, the dead cells percent in S+B+NNC10, S+B+NNC50 and S+B+NNC100 were lower, and were10.9%±3.1, 11.2%±1.9%, respectively. There were no differences of the dead cells percent between the median and high dosage NNC55-0396 group, Tab 3, Fig 4.

Discussion

The peripheral nerve injury caused by local anesthetics is often manifested as numbness, alloesthesia, tingling and muscle weakness, which is related to the concentration, dose and block time of local anesthetics[26]. The exact mechanism is not clear, which may be related to reactive oxygen species release, cell apoptosis, p38 MAPK activation and intracellular calcium overload. Intracellular calcium is an important signal molecule regulating cell function, and the imbalance of intracellular calcium is an important cause of cell function damage[27, 28]. The results of this study showed that the concentration of intracellular calcium increased in the nerve injury induced by local anesthetics.

T-type calcium channel is a kind of low-voltage dependent calcium channel, which can be activated at the resting potential, and it is the promoter to regulate the excitability of neurons. Our previous studies have shown that T-type calcium channel participates in the formation of chronic neuropathic pain, and intrathecal injection of T-type calcium channel blocker can alleviate chronic neuropathic pain[29]. The expression of T-type calcium channel in SH-SY5Y cells is related to the neurotoxic injury of lidocaine hydrochloride[30].

Bupivacaine is commonly used in spinal canal block. In this study, it was found that after treatment with 1 mM bupivacaien hydrochloride for 24 hours, the cell apoptosis rate increased, the cell viability decreased, and the intracellular calcium level increased, suggesting that bupivacaine hydrochloride can cause SH-SY5Y cell damage. The results showed that NNC55-0396, a T-type calcium channel blocker, could partially reverse the

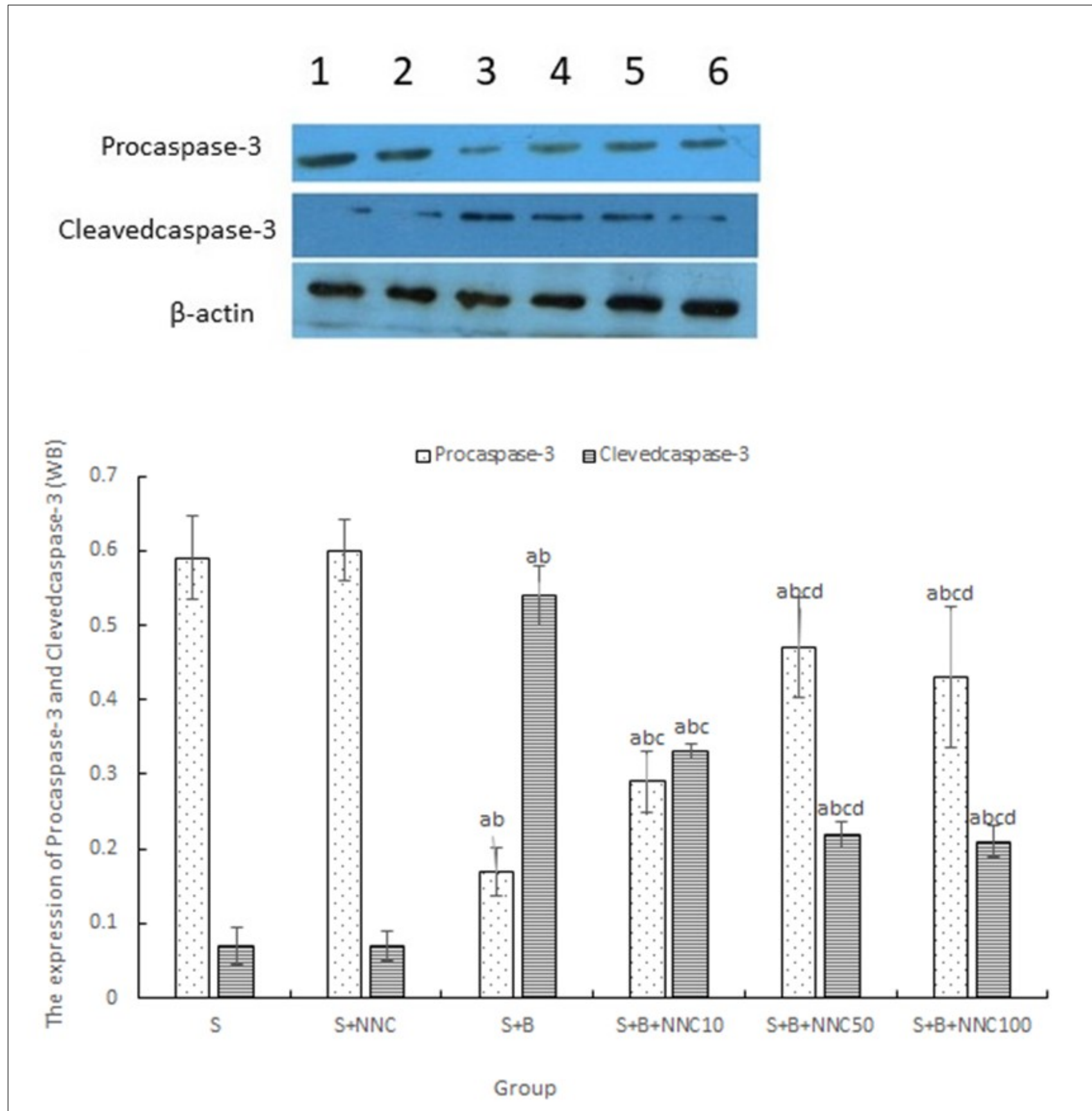

Figure 2. The expression of Caspase-3(Procase-3 and Cleavedcaspase-3) (Mean+S.D, n=3). Procaspase-3 (inactive form) and cleaved caspase-3 (active form) expression was measured by western blot analysis Lane 1=S group; Lane 2=S+NNC 100 group; Lane 3=S+B group; Lane 4=S+B+ NNC 10 group; Lane 5=S+B+NNC 50 group; Lane 6=S+B+NNC 100 group. [a]*P*<0.05 vs. S group; [b]*P*<0.05 vs. S+NNC 100 group; [c]*P*<0.05 vs. S+B group; [d]*P*<0.05 vs. S+B+NNC 10 group.

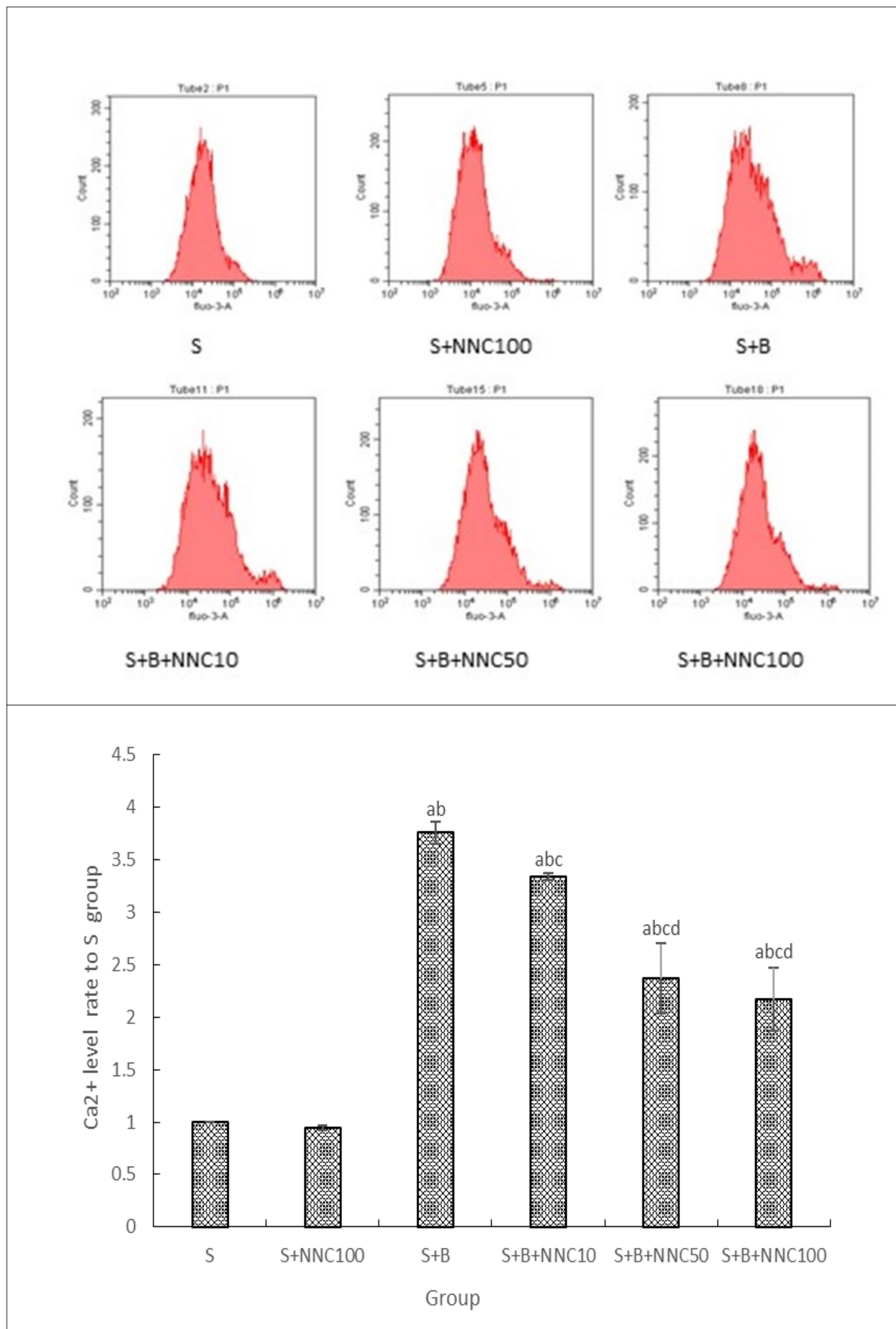

Figure 3. The Ca2+ level rated to the cells calcium fluorescence level in S group (Mean±SD,n=3). ^{a}P<0.05 VS S group, ^{b}P<0.05 VS S+NNC100 group, ^{c}P<0.05 VS S +B group, ^{d}P<0.05 VS S +B +NNC10 group.

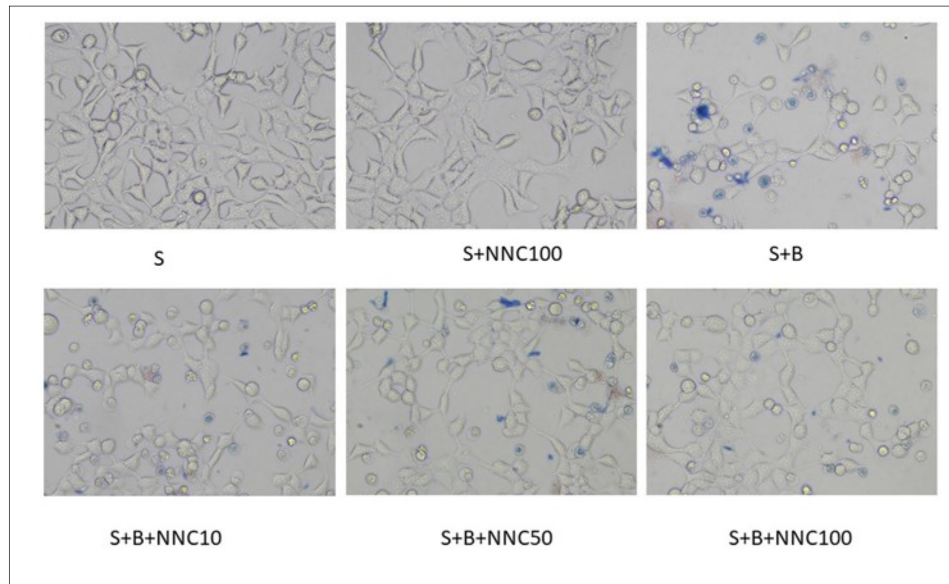

Figure 4. The dead cells in every group.（Trypan blue staining，200×）

Table 3. The dead cells in every group.（Mean±SD,n=3, Trypan blue staining，200×）

Group	Dead cell percent
S	1.6%±0.7%
S+NNC100	1.3%±0.5%
S+B	30.1%±2.2% [ab]
S+B+NNC10	19.2%±0.4%[abc]
S+B+NNC50	10.9%±3.1%[abcd]
S+B+NNC100	11.2%±1.9%[abcd]

[a]$P<0.05$ VS S group, [b]$P<0.05$ VS S+NNC100 group, [c]$P<0.05$ VS S +B group, [d]$P<0.05$ VS S +B +NNC10 group.

above changes. It suggested that blocking of the T-type calcium channel could improve the cell damage induced by bupivacaine hydrochloride.

T-type calcium channel participates in SH-SY5Y injury induced by bupivacaine, which may be related to the regulation of intracellular calcium level by T-type calcium channel. Under the effect of local anesthesia, the expression of T-type calcium channel increased, the extracellular calcium ions entering the cells through T-type calcium channel increased, further activating the high voltage dependent calcium channel, further increasing the intracellular calcium concentration, resulting in cell damage. It was found that NNC55-0396 could partially reverse the apoptosis of SH-SY5Y cells induced by bupivacaine. Interestingly, medium and high doses of NNC55-0396 (S+B+NNC50 group and S+B+NNC100 group) showed no differences in the improve of the cell damage induced by bupivacaine hydrochloride. This may be related to the dose selected in this study, 50μM final concentration of NNC55-0396 may reach the maximum effects.

As the blocker of T type calcium, NNC55-0396 is not subtype specific to the Cav3.1, Cav3.2 and Cav3.3. As well as, all the three subtypes T-type calcium channel were detected in SH-SY5Y cells. The limitation of this study was that there were no Cav3.1, Cav3.2 or Cav3.3 specific antagonists to testify which subtype of T type calcium channel induced the cell damage. It is necessary to construct plasmid by genetic engineering to inhibit the subtype expression of the T-type calcium to study the roles of the subtype of T-type calcium channel in local anesthetic toxicity.

In brief, NNC55-0396 reduces SH-SY5Y cell damage induced by bupivacaine hydrochloride. The mechanism maybe involved with the decrease of intracellular calcium level and cell apoptosis rate.

Author Contributions

Wen XJ designed and implemented the experiments and wrote the paper; Wu Zhaoxia and Xu Shiyuan collected the data and performed the statistical analysis.

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