

## Original Article

# Parthanatos is involved in bupivacaine induced injury in SH-SY5Y cells

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**Abstract:** Bupivacaine is one of the most toxic local anesthetics. An increasing number of studies have shown that bupivacaine could induce intracellular oxidative stress and cause cell death. However, the mechanisms underlying its neurotoxicity are still unclear. In this study, we evaluate the neurotoxicity effect and the underlying mechanisms of bupivacaine in SH-SY5Y cells. First, we monitored the intracellular reactive oxygen species content after bupivacaine exposure. We also measured the expression level of PARP-1 via western blotting and Quantitative Real-Time PCR methods. Changes in intracellular NAD<sup>+</sup> and adenylate depletion were analyzed. We further assessed cell viability and nuclear morphological changes after bupivacaine treatment. Finally, mitochondrial-associated protein expression was examined to verify the pathways of bupivacaine-induced cell death. In addition, mitochondrial membrane potential was also measured by JC-1 staining of SH-SY5Y cells treated with bupivacaine. Results showed that bupivacaine induced reactive oxygen species production and activate PARP-1 expression and PAR polymer formation in SH-SY5Y cells. Bupivacaine-induced PARP-1 activation was associated with intracellular NAD<sup>+</sup> utilization and adenylate depletion. Furthermore, bupivacaine was neurotoxic to cells and this toxicity was mitochondrial-associated protein dependent and involved in changes of mitochondrial membrane potential. Collectively, our results suggested that the PARP-1 dependent programmed cell death parthanatos, which characterized by PARP-1 activation and intracellular NAD<sup>+</sup> depletion, was involved in bupivacaine-induced neurotoxicity, and inhibition of PARP-1 attenuated this neuronal injury.

**Keywords:** Bupivacaine, PARP-1, AIF dependent, parthanatos

## Introduction

Local anesthetics (LAs) are extensively used and generally accepted as being safe in clinic during regional anesthesia and pain control [1, 2]. However, increasing neurological complications, such as transient neurological symptoms and persistent lumbosacral neuropathy, have been reported following wide clinical LAs administration [3, 4]. The mechanisms underlie the LAs induced neuronal injury remains unclear. Bupivacaine (bup), an amide type local anesthetic, was widely used for epidural anesthesia, nerve blockade, and postoperative analgesia [5]. Several clinical observations have suggested that the administration of bupivacaine in close proximity to nerves causes critical dysfunction, such as radiculopathy and paresthesia [6-8]. Bupivacaine also appears to have higher neurotoxicity than other LAs in vitro

[9]. An increasing number of studies have shown that bupivacaine could induce intracellular oxidative stress and cause DNA damage and then result in cell death through apoptosis, necrosis and parthanatos [10, 11]. However, the mechanisms by which bupivacaine triggers neurotoxicity have not been elucidated precisely.

Poly(ADP-ribose) (PAR) polymerase-1 (PARP-1) is an important nuclear enzyme that responds to DNA damage and is required for DNA repair [12, 13]. In some cases, however, PARP-1 is also involved in death program. PARP-1-dependent cell death is different from classical subtypes of cell death, such as apoptosis, necrosis, and autophagy [14, 15], which is defined as parthanatos. Parthanatos is a new form of programmed cell death and characterized by PARP-1 activation, NAD<sup>+</sup> depletion,

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**Table 1.** Primers used for Quantitative Real-Time PCR analysis

Gene	Name	Primers (5' to 3')
PARP-1	PARP-1-F	GTAGCTGATGGCATGGTGTTCC
	PARP-1-R	GTGTCTGTGTCTTGACCATAC
Actin	Actin-F	GTCCACACCCGCCACCAGTTC
	Actin-R	TCCCACCATCACACCTGGTG

Primers were designed using Primer Express version 2.0 software. Primer specificity was confirmed using Primer-BLAST web software (National Centre for Biotechnology Information).

and Poly(ADP-ribose) (PAR) accumulation. In addition, the mitochondrial-associated protein (AIF), a death-effector molecule, is also released from the mitochondria and translocated to the nucleus to initiate this cell death [16-18].

Our previous work showed that bupivacaine could induce neurotoxicity and intracellular  $\text{NAD}^+$  depletion. Intracellular  $\text{NAD}^+$  depletion contributes to bupivacaine-induced neuronal injury and  $\text{NAD}^+$  repletion attenuates this injury [19]. Here, we hypothesized that parthanatos was involved in bupivacaine-induced injury. Therefore, we employed an in vitro model of cytotoxicity using SH-SY5Y cells treated with bupivacaine. In addition, we monitored the reactive oxygen species (ROS) production, PARP-1 activation, cell death, concentration of adenylate, mitochondrial-associated protein (AIF), and mitochondrial depolarization, following bupivacaine treatment.

### Materials and methods

#### Materials

The SH-SY5Y cell line was purchased from Shanghai Institutes for Biological Sciences. Bupivacaine (99.9% purity) were purchased from Sigma (USA). DMEM medium and fetal bovine serum were purchased from Gibco (USA). 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole-carbocyanideiodine (JC-1) and Hoechst 33258 were obtained from KeyGEN (China). N-(6-Oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino)acetamide hydrochloride (PJ34) and N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD.fmk) were obtained from Sigma (USA). 2,7-dichlorofluorescein diacetate (DCFH-DA) was purchased from Beyotime (China). Cell viability assay kit was from Dojindo (Japan).

#### Cell culture and experimental classification

SH-SY5Y cells were cultured in DMEM/F12 media supplemented with 15% fetal bovine serum, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin in a humidified 5%  $\text{CO}_2$  incubator at 37°C. The media was replenished every two days. Cells were grown in 100 mm dishes and were sub-cultured in 6-well (seeding density  $5.0 \times 10^5$  cells), 12-well (seeding density  $1.0 \times 10^5$  cells), or 96-well ( $1.0 \times 10^4$  cells). Experiments were conducted when cells reached 85% confluence. Bupivacaine hydrochloride, z-VAD and PJ34 in pulverized form were dissolved in the media.

#### RNA isolation and quantitative real-time PCR analysis

Total RNA were extracted from the cultured SH-SY5Y cells, which exposed to different concentrations of bupivacaine (1 mM, 2 mM, 5 mM, 10 mM) for 2 hr or 5 mM bupivacaine at different times (0.5 hr, 1 hr, 2 hr, 4 hr, 6 hr), using TRIZOL Regent (Invitrogen). The cDNA Synthesis Kit (Takara) was used for the synthesis of cDNA according to the manufacturer's instructions. Quantitative PCR was accomplished using the iCycleriQ Detection System (Bio-Rad, Richmond, CA) and interaction dye SYBR Green. Primer sequences are listed in **Table 1**.

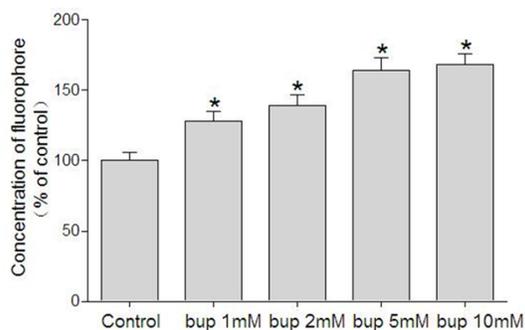
#### Western-blotting

Total proteins were prepared from the cultured SH-SY5Y cells that were exposed to different concentrations of bupivacaine (1 mM, 2 mM, 5 mM, 10 mM) for 2 hr or 5 mM bupivacaine at different times (0.5 hr, 1 hr, 2hr, 4 hr, 6 hr) of each group. The protein concentration was determined using Bio-Rad protein assay system. Proteins were analyzed with SDS polyacrylamide gel electrophoresis. After electrophoresis, they were electro-transferred to the PVDF membrane. The membrane containing the proteins was used for immunoblotting with appropriate antibodies. The protein bands were scanned and quantified.

#### ROS measurements

The SH-SY5Y cells were seeded into 12-well plates at a concentration of  $5.0 \times 10^5$  cells/well with 500  $\mu\text{l}$  culture media per well. After exposure to different concentrations of bupivacaine

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**Figure 1.** Bupivacaine Induces reactive oxygen species (ROS) production in SH-SY5Y cells. Intracellular ROS levels were measured after exposure to various concentrations of bupivacaine (1 mM, 2 mM, 5 mM, 10 mM) for 2 hr. Data are the mean  $\pm$  SEM (n=3), each bar represents the mean of three independent experiments carried out in triplicate. \*Compared with control group,  $P < 0.05$ ; #Compared with bup group,  $P < 0.05$ .

(1 mM, 2 mM, 5 mM, 10 mM) for 2 hr, intracellular ROS levels were evaluated by staining cells with DCFH-DA. Cells were incubated at 37°C with 10  $\mu$ M DCFH-DA during the last 20 minutes of treatment. The cells were washed three times, collected, and re-suspended in PBS. Subsequently, the fluorescent signal intensity of DCF was determined using flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

### NAD<sup>+</sup> determination

The intracellular NAD<sup>+</sup> concentration was measured using an NAD<sup>+</sup> assay kit (EnzyChrom, BioAssay Systems, USA), which is based on a lactate dehydrogenase cycling reaction. In brief, cells were seeded into the 12-well plates at a concentration of  $5.0 \times 10^5$  cells/well with 500  $\mu$ l culture media per well. After 5 mM bupivacaine treatment, cells were washed with cold PBS and collected in Eppendorf tube. Each pellet was re-suspended in 100  $\mu$ l extraction buffer. After heating the extracts for 5 minutes at 60°C, 20  $\mu$ l assay buffer and 100  $\mu$ l opposite extraction buffer were added to neutralize the extracts. Samples were centrifuged at 14,000 rpm for 5 minutes, and the supernatants were prepared for NAD<sup>+</sup> assays. After adding the working reagent (a mixture of 60  $\mu$ l assay buffer, 1  $\mu$ l enzyme A, 1  $\mu$ l enzyme B, 14  $\mu$ l lactate, and 14  $\mu$ l MTT), the absorbance of the standards and samples was measured immediately

at 565 nm ( $OD_0$ ) as well as 15 minutes later ( $OD_{15}$ ). We used the DOD values ( $OD_{15} - OD_0$ ) to determine the NAD<sup>+</sup> concentration in samples from the standard curve.

### Adenylate assay

The adenine nucleotide from SH-SY5Y cells, treated with 5 mM bupivacaine, 5 mM bupivacaine together with 100 mM PJ34 or 200 mM PJ34, were determined using high performance liquid chromatography (HPLC) methods. The wavelength of HPLC was 254 nm, and sensitivity was 0.01 AUFS. Chromatographic column was 4.6 mm  $\times$  250 nm, YWG-ODS C18 10  $\mu$ m. The mobile phase was 2 mmol/L PBS (pH 5.5). The speed was 1 mL/min.

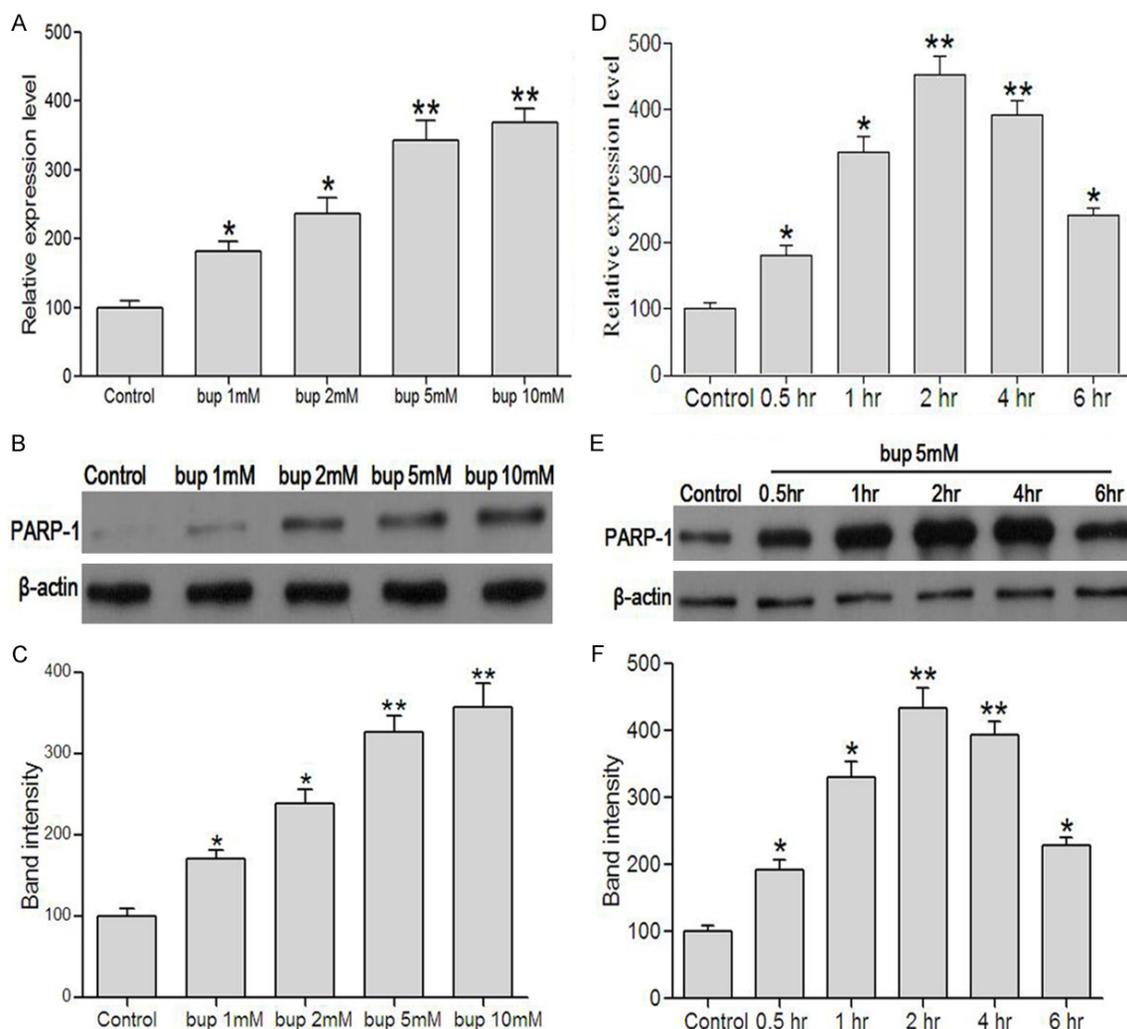
### Cell viability assay

Cells were seeded into 96-well plates at a concentration of  $1.0 \times 10^4$  cells/well with 100  $\mu$ l culture media per well. After treatment with culture media alone or equal amounts of bupivacaine with or without PJ34, 10  $\mu$ l of the cell counting kit solution was added to each well, followed by incubation of the plates for 3 hours in 5% CO<sub>2</sub> at 37°C. Absorbance was measured at 450 nm using a spectrophotometer (Bio-Tek, USA) with reference wavelength of 650 nm. The control group (without any treatment) was set at 100%, and the other groups were normalized to the corresponding control values.

### Hoechst 33258 nuclear staining

After treatment with culture media alone or equal amounts of bupivacaine with or without PJ34, cells in 12-well plates were rinsed 3 times with PBS and stained with Hoechst 33258. Subsequently, cells were examined and photographed using a fluorescence microscope equipped with a camera (Nikon Eclipse TE2000-u, Japan) and UV excitation at 300-500 nm. Damaged nuclei were defined based on changes in nuclear morphology such as chromatin condensation and fragmentation. Apoptotic and normal cells were counted manually by researchers blinded to the treatments. Three fields in each well were examined randomly at a magnification of 200. Nuclear changes were expressed as a percentage of total nuclei examined (n=6 in each condition).

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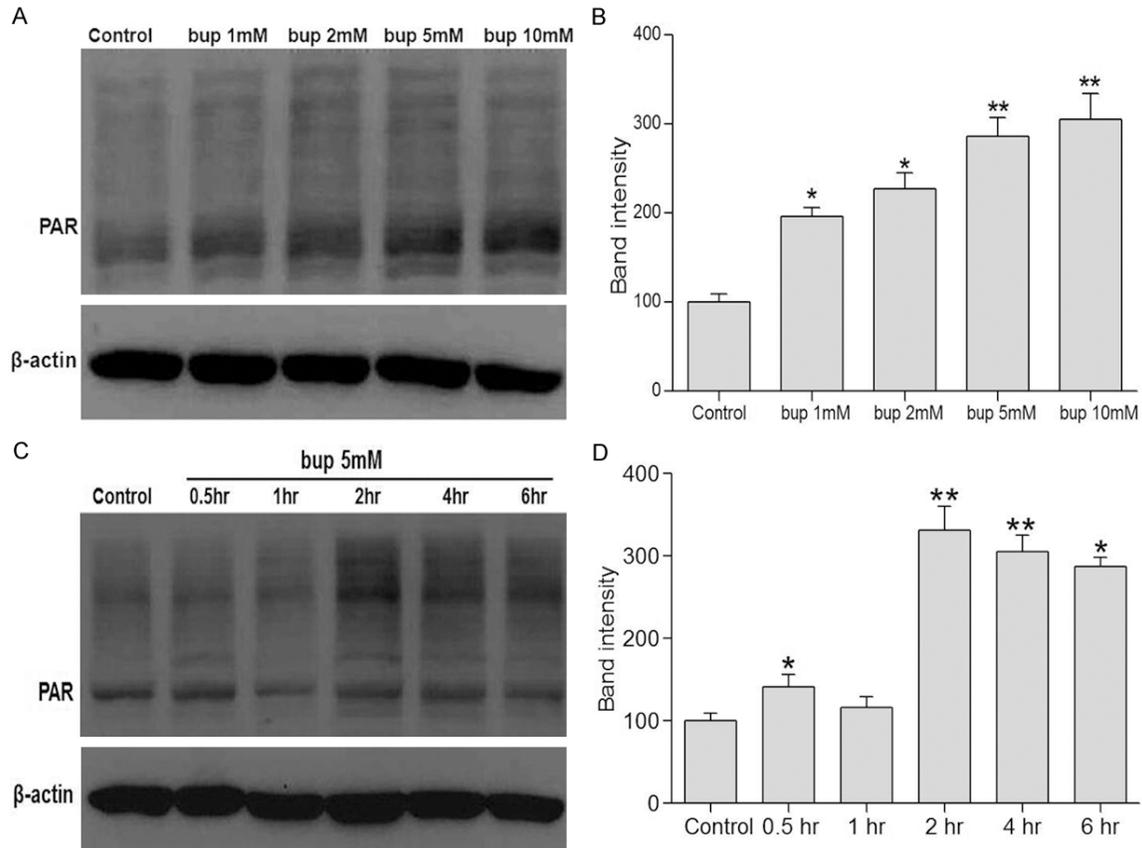
**Figure 2.** Bupivacaine induces PARP-1 activation in a time and concentration dependent manner. A. qRT-PCR analysis of PARP-1 mRNA in SH-SY5Y cells exposed to various concentrations of bupivacaine (1 mM, 2 mM, 5 mM, 10 mM) for 2 hr compared with control group. B, C. Western blots analysis of PARP-1 expression in SH-SY5Y cells exposed to various concentrations of bupivacaine (1 mM, 2 mM, 5 mM, 10 mM) for 2 hr compared with control group. D. qRT-PCR analysis of PARP-1 mRNA in SH-SY5Y cells exposed to 5 mM bupivacaine at the indicated time points compared with control group. E, F. Western blots analysis PARP-1 expression in SH-SY5Y cells exposed to 5 mM bupivacaine at the indicated time points compared with control group. Data are the mean  $\pm$  SEM (n=3), each bar represents the mean of three independent experiments carried out in triplicate. \*Compared with control group, P<0.05; \*\*Compared with control group, P<0.01.

### Mitochondrial membrane potentials ( $\Delta\psi_m$ ) assay

We employed JC-1 to measure mitochondrial depolarization in SH-SY5Y cells. JC-1 is a lipophilic, cationic dye that can selectively enter the mitochondria and reversibly change color from green to red as the membrane potential increases. In healthy cells with high  $\Delta\psi_m$ , JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. However, in injured cells with low  $\Delta\psi_m$ , JC-1

remains in the monomeric form and shows only green fluorescence. After exposure to different concentrations of bupivacaine (1 mM, 2 mM, 5 mM, 10 mM) for 2 hr, cells were incubated for 20 minutes with an equal volume of JC-1 staining solution (5  $\mu$ g/ml) at 37°C and rinsed twice with PBS.  $\Delta\psi_m$  was monitored by determining the relative amount of dual emissions from mitochondrial JC-1 monomers or aggregates using flow cytometry. Mitochondrial depolarization is indicated by a decrease in the polymer/monomer fluorescence intensity ratio.

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**Figure 3.** Bupivacaine induces PAR polymer formation. A, B. Western blots analysis of PAR polymer formation in SH-SY5Y cells exposed to various concentrations of bupivacaine (1 mM, 2 mM, 5 mM, 10 mM) for 2 hr compared with control group. C, D. Western blots analysis PAR polymer formation in SH-SY5Y cells exposed to 5 mM bupivacaine at the indicated time points compared with control group. Data are the mean  $\pm$  SEM (n=3), each bar represents the mean of three independent experiments carried out in triplicate. \*Compared with control group,  $P < 0.05$ ; \*\*Compared with control group,  $P < 0.01$ .

### Apoptosis-inducing factor (AIF) release assay

The SH-SY5Y cells, exposed to 5 mM of bupivacaine for 2 hr, were washed with ice-cold PBS, left on ice for 10 minutes, and then re-suspended in isotonic homogenization buffer [250 mM sucrose, 10 mM KCl, 1.5 mM  $MgCl_2$ , 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM Tris-HCl (pH 7.4)] containing a proteinase inhibitor mixture (Roche, Basel, Switzerland). After 80 strokes in a Dounce homogenizer, the unbroken cells were spun down at  $30 \times g$  for 5 minutes. The nuclei and heavy mitochondria fractions were fractionated at  $750 \times g$  for 10 minutes and  $14,000 \times g$  for 20 minutes, respectively, from the supernatant. The nuclei fraction was washed three times with homogenization buffer containing 0.01% NP40. Then, western

blot was performed to analyze the AIF release from mitochondria to nuclear.

### Statistical analyses

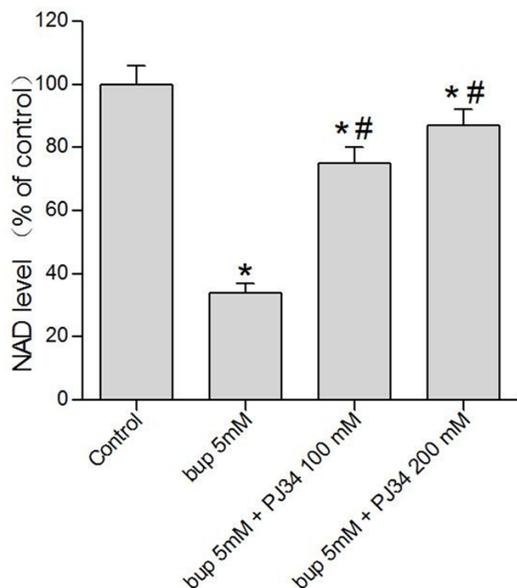
All the original experimental data were analyzed by SPSS17.0. Comparison among the groups was carried out by One-way ANOVA. The comparison between two groups was carried out by LSD.  $P < 0.05$  was considered as statistically significant differences.

## Results

### Bupivacaine induces reactive oxygen species (ROS) production

Since application of bupivacaine was often associated with ROS generation [1], we assessed the intracellular redox state by con-

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**Figure 4.** PARP-1 activation induced by bupivacaine results in intracellular NAD<sup>+</sup> utilization and depletion. Cellular NAD<sup>+</sup> levels were measured following treatment with 5 mM bupivacaine after 2 hr in the presence or absence of PARP-1 inhibitor PJ34 in SH-SY5Y cells. Data are the mean  $\pm$  SEM (n=3), each bar represents the mean of three independent experiments carried out in triplicate. \*Compared with control group, P<0.05; #Compared with bup group, P<0.05.

focal microscopy using the fluorescent hydroperoxide probe dichlorofluorescein (DCF). As shown in **Figure 1**, we observed more ROS accumulation in bupivacaine treated groups than control group in SH-SY5Y cells. Moreover, ROS accumulation was in a concentration-dependent manner after bupivacaine induced. These data suggest that bupivacaine, widely used for local anesthetics in clinic, could cause intracellular oxidative stress in SH-SY5Y cells.

### *Bupivacaine induces excessive activation of PARP-1 and PAR polymer formation*

It is well known that intracellular oxidative stress might causes DNA damage and then results in excessive activation of PARPs, especially PARP-1 [10, 12]. To identify whether PARP-1 was induced in SH-SY5Y cells after bupivacaine treatment, we firstly examined the PARP-1 expression using q-PCR and western blot methods after 2 hr treatment with bupivacaine at various concentrations including 1 mM, 2 mM, 5 mM and 10 mM. Results showed that PARP-1 expression increased dramatically in

bupivacaine induced groups and significantly higher than control group (**Figure 2A-C**). Then, we measured the expression level of PARP-1 after 5 mM bupivacaine induced for the indicated times (ranging from 0.5 hr to 6 hr). An significantly increased mRNA and protein expression of PARP-1 were detected in bupivacaine treated groups at the various induced-times (**Figure 2D-F**).

PARP-1 could catalyzes the polymerization of ADP-ribose (PAR) units from donor NAD<sup>+</sup> molecules and lead to an extensive amount of PAR polymer formation [21]. Therefore, we determined the levels of PAR polymer through western blot analysis after bupivacaine treatment, and we found that PAR formation significantly increased after bupivacaine treatment (**Figure 3**).

### *Excessive activation of PARP-1 induced by bupivacaine results in intracellular NAD<sup>+</sup> utilization and depletion*

Our previous results indicated that bupivacaine could induced intracellular NAD<sup>+</sup> depletion [19], and the NAD<sup>+</sup> is required for the synthesis of PAR when PARP-1 is activated, therefore, there is excessive utilization and depletion of cytosolic and nuclear pools of NAD<sup>+</sup> [18]. Intracellular NAD<sup>+</sup> depletion was determined in SH-SY5Y cells treated with 5 mM bupivacaine in the presence or absence of PARP-1 inhibitor PJ34. We detected that PJ34 could attenuated intracellular NAD<sup>+</sup> depletion in a concentration-dependent manner (**Figure 4**). This data indicate that PARP-1 activation induced by bupivacaine involved in the intracellular NAD<sup>+</sup> consumption.

### *PARP-1 activation induced by bupivacaine depletes intracellular ATP pool*

We determined intracellular adenine nucleotide level in SH-SY5Y cells using HPLC method because NAD<sup>+</sup> and ATP participate in intracellular energy production and maintain energy balance. Results established that compared with control group, adenine were deduced significantly (P<0.05) (**Table 2**) in bup group and bup + PJ34 groups. Moreover, PJ34 treatment group notably increased ATP, ADP and AMP contents compared with bup group. These results demonstrated that PARP-1 activation induced by bupivacaine involved in the intracellular ATP pool regulation.

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**Table 2.** The concentration of adenylate in different groups

Group	ATP (nmol/mg)	ADP (nmol/mg)	AMP (nmol/mg)
Control	2.81±0.18	4.42±0.31	4.91±0.32
bup 5 mM	1.10±0.11*	1.71±0.18*	3.93±0.28*
PJ34 100 mM	1.16±0.11*	1.58±0.12*	3.91±0.29*
PJ34 200 mM	1.13±0.09*	1.66±0.16*	3.97±0.33*
bup 5 mM + PJ34 100 mM	2.16±0.20*,#	3.50±0.29*,#	1.92±0.11*,#
bup 5 mM + PJ34 200 mM	2.36±0.21*,#	3.76±0.32*,#	4.17±0.35*,#

The adenine nucleotide in SH-SY5Y cells were determined using HPLC method. Data are the mean ± SEM, \*compared with control group, P<0.05; #compared with bup group, P<0.05.

### *Bupivacaine-induced PARP-1 activation contributes to neurotoxicity*

It is well established that intracellular oxidative stress induced by bupivacaine was toxic and would cause cell injury and neurotoxicity [22]. In response to this toxicity, PARP-1 was activated in a variety of model systems and cell types [20]. In order to determine the relationship between PARP-1 expression and neuronal death, we assessed cell viability and nuclear morphological changes after bupivacaine treatment with or without PJ34 treatment.

First, MTT method was used to detect cell viability in different groups. Experiments exhibited that cell viability decrease significantly in bup group without PJ34 treatment, compared with control group (**Figure 5A**). On the contrary, cell viability increased significantly in bup group combined with PJ34 treatment. These results indicated that inhibited PARP expression could improve cell viability.

Nuclear morphological changes such as nuclear condensation or fragmentation is another representative phenomenon marker suggesting neuronal damage during bupivacaine exposure [23]. We subsequently examined the effect of PJ34 co-incubation on bupivacaine-induced nuclear injury by Hoechst 33258 staining. As shown in **Figure 5B**, the typical sign of nuclear injury, such as coalesced, condensed, and segmented nuclei with a brighter blue fluorescence, were much lower in bupivacaine treated with PJ34 than in bupivacaine alone. No significant nuclear morphological changes were observed in cells treated with PJ34 alone at both concentrations employed in this study. Quantitatively analyzed of condensation of nuclei were demonstrated in **Figure 5C**.

Taken together, these data suggest that PARP-1 was a death regulatory molecule in neural injury induced by bupivacaine and PARP-1 inhibitor attenuates this neurotoxicity.

### *AIF release occurs in bupivacaine-induced neurotoxicity*

The apoptosis-inducing factor (AIF), a death-effector molecule, is released from the mitochondria and translocated to the nucleus to initiate cell death [24, 25]. We further explored the potential role of AIF and examined the relationship between AIF release and PARP-1 activation through western-blot analysis. Analysis by western-blot clearly revealed that treatment of cells with bupivacaine dramatically redistributed AIF from the mitochondria to the nucleus (**Figure 6**). This result suggest that AIF translocation into the nucleus is required in bupivacaine-induced cell death of SH-SY5Y cells and bupivacaine induces nuclear condensation and cell death in an AIF-dependent manner.

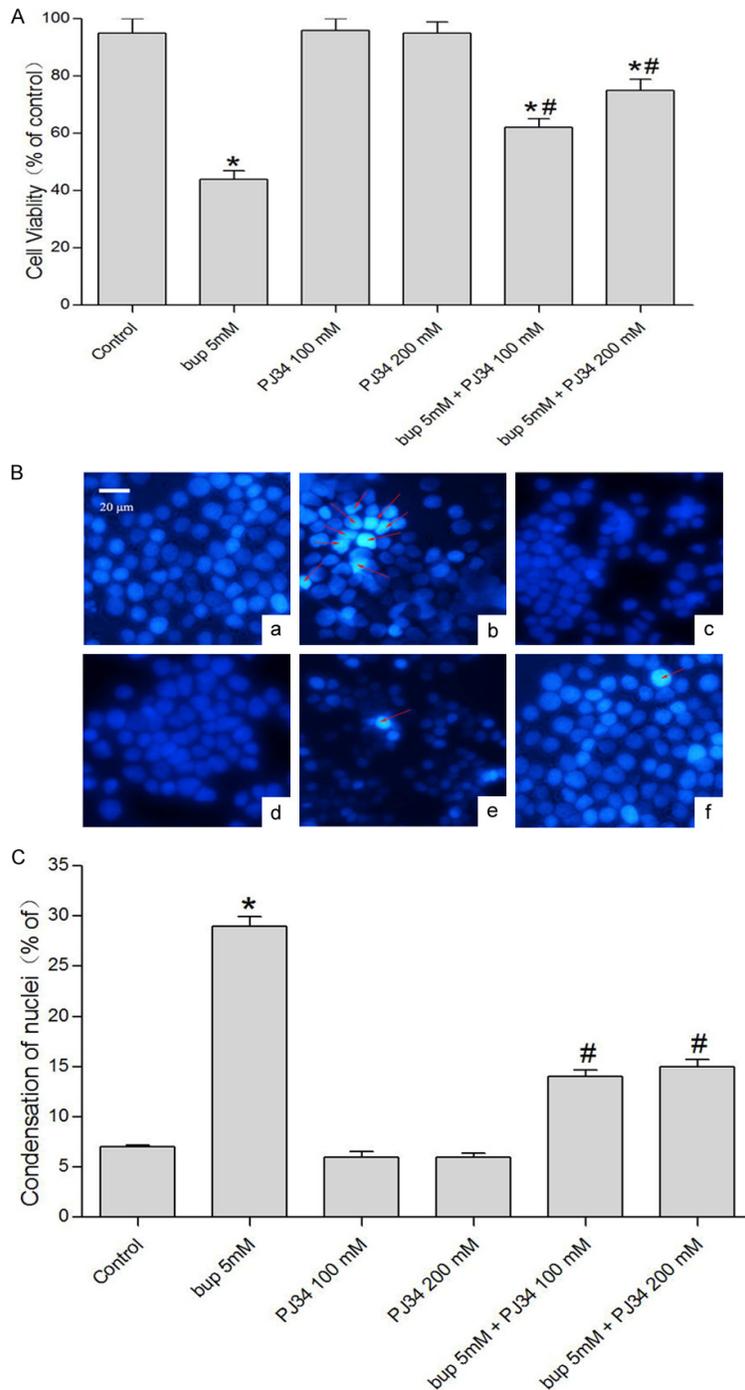
### *Bupivacaine-induced neurotoxicity was associated with mitochondrial depolarization*

Growing evidences demonstrated that bupivacaine could induce ROS production and ROS was relevant to mitochondrial depolarization [26]. We measured mitochondrial membrane potential ( $\Delta\psi_m$ ) by JC-1 staining of SH-SY5Y cells treated with various concentrations of bupivacaine. The result demonstrated that mitochondrial membrane potential significantly reduced in bupivacaine treated cells (**Figure 7**).

## Discussion

Bupivacaine has proven neurotoxic to cause neuronal injury including nuclear damage, mitochondrial depolarization, which subsequently induces cell death [11, 20]. Numerous studies concentrate on revealing the mechanisms underlying its neurotoxicity [21, 22]. Most of them pay close attention to bupivacaine-induced apoptosis, characterized by eventual caspases activation, and reveal some apoptotic signal pathways, such as threonine-serine kinase B (AKT) pathway and AMP-activated

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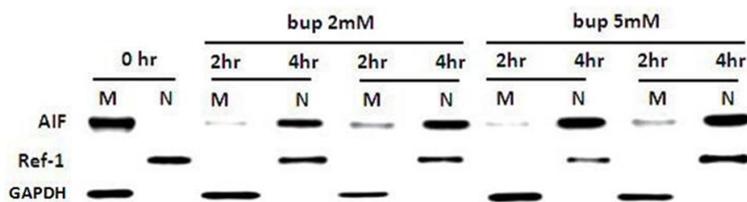
**Figure 5.** Bupivacaine-induced PARP-1 activation contributes to neurotoxicity. (A) Cell viability was measured by the MTT colorimetric assay in different groups. (B) Nuclear morphological changes were occurred in different groups. Arrows indicate bupivacaine-induced, condensed, coalesced, and segmented nuclei (200 $\times$ ). Scale Bar=20  $\mu$ m. (a) Control group; (b) 5 mM bupivacaine treatment group; (c) 100 mM PJ34 treatment group; (d) 200 mM PJ34 treatment group; (e) 5 mM bupivacaine and 100 mM PJ34 treatment group; (f) 5 mM bupivacaine and 200 mM PJ34 treatment group. (C) Condensation of nuclei were analyzed in different groups. Data are the mean  $\pm$  SEM (n=3), each bar represents the mean of three independent experiments carried out in triplicate. \*Compared with control group, P<0.05; #Compared with bup group, P<0.05.

protein kinase (AMPK) pathway [1, 2, 20, 21]. Apoptosis and necrosis, classic cell death nomenclatures based on morphological appearance, are now considered less accuracy for cell death modalities definition. With cell death nomenclature development, some of cell-death modalities defined as necrosis initially, now are considered to be programmed cell death using biochemical/functional criteria [23].

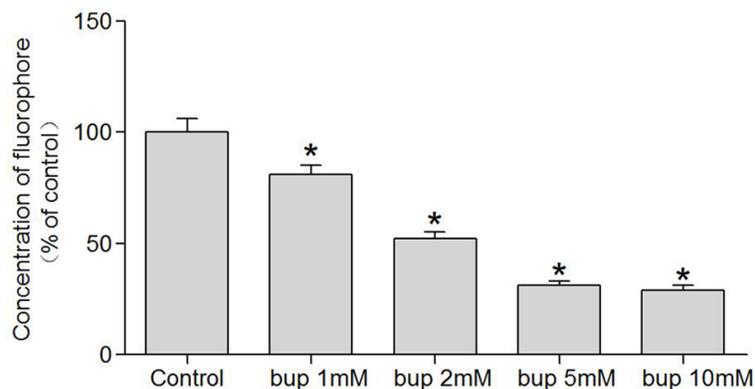
Parthanatos is one of new-found cell death modalities, characterized by its unique cascade including PARP-1 activation, subsequently PAR polymer production and intracellular NAD<sup>+</sup> depletion, as well as non-obligatory AIF release at late stage [27-29]. Parthanatos is involved in neuronal death and injury such as stroke and other neurodegenerative disorders, great efforts had been made to explore the underlying mechanisms of this PARP-1-dependent programmed cell death [17, 18]. To date, energy depletion, PAR accumulation and cytotoxicity, and AIF release are now recognized as key death mediators in parthanatos cascade [30, 31]. In this study, we employed an in vitro model of cytotoxicity and found that parthanatos was involved in bupivacaine induced injury in SH-SY5Y cells.

PARP-1 is seemingly the most famous and important of PARP family and play the most essential role in parthanatos [32-35]. Previous studies have revealed that PARP-1 can be activated by oxidative stress and this activation resulting in PAR polymer pro-

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**Figure 6.** AIF release occurs in bupivacaine-induced neurotoxicity. Western blots analysis of AIF release from the mitochondria (M) to the nucleus (N) in SH-SY5Y cells with bupivacaine treatment.



**Figure 7.** Bupivacaine-induced neurotoxicity was associated with mitochondrial depolarization. Data are the mean  $\pm$  SEM ( $n=3$ ), each bar represents the mean of three independent experiments carried out in triplicate. \*Compared with control group,  $P<0.05$ .

duction [36, 37]. PAR is a key mediator of AIF translocation and may be cytotoxic itself. In addition, serving as substrate for PAR polymer synthesis, intracellular  $NAD^+$  and ATP is also exhausted and leads to subsequently energy failure when PARP-1 was activated. All of these molecular events eventual lead to cell death [18, 38]. Bupivacaine has also proven to be neurotoxicity by increasing intracellular ROS production and oxidative stress [19, 20]. However, little is known regarding the link between bupivacaine-induced neurotoxicity and these molecular events above.

Our previous work showed that bupivacaine was toxic and could induces intracellular  $NAD^+$  depletion. Intracellular  $NAD^+$  depletion contributes to bupivacaine-induced neuronal injury and  $NAD^+$  repletion attenuates the injury in SHSY5Y cells. In this study, we found that bupivacaine could induced ROS production and AIF release from mitochondria to nucleus and then triggers DNA damage. DNA damage might lead to PARP-1 activation, then it result in  $NAD^+$  and ATP depletion, and finally it probably cause cell

death. In addition, bupivacaine-induced cell death was closely relevant to mitochondrial depolarization. These data indicated that the bupivacaine-induced cell death (parthanatos), which characterized by PARP-1 activation, intracellular  $NAD^+$  depletion and AIF release, was involved in neurotoxicity in SHSY5Y cells, and inhibition of PARP-1 activation attenuates this neuronal injury.

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### Disclosure of conflict of interest

None.

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