Bupivacaine at clinically relevant concentrations induces toxicity in human intervertebral disc cells via the induction of autophagy *in vitro*

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Abstract. It has been reported that bupivacaine, the most widely used local anesthetic to relieve discogenic back pain in clinical settings, is cytotoxic to intervertebral disc (IVD) cells *in vitro*; however, the precise mechanisms of cytotoxicity induced by bupivacaine remain unclear. Autophagy is an intracellular lysosomal degradation process that is important for cellular survival. The present study investigated the role of autophagy in the survival of IVD cells subjected to bupivacaine treatment. Human nucleus pulposus (NP) cells isolated from IVD cells were exposed to various concentrations of bupivacaine for 2, 6 and 12 h, and analyzed for cellular viability using MTT assay and western blotting. Additionally, autophagosome formation and autophagy-associated biomarkers were evaluated by electron microscopy and western blotting to determine the autophagic activity and signaling alterations in NP cells under bupivacaine treatment. Furthermore, autophagic activity was inhibited *in vitro* using 3-methyladenine to further analyze the association between autophagy and apoptosis in bupivacaine-treated NP cells. Bupivacaine exhibited time- and dose-dependent cytotoxic effects on human IVD cells at clinically relevant concentrations. Bupivacaine increased autophagic activity by promoting autophagosome formation, and LC3-II and Beclin-1 production. Additionally, bupivacaine inhibited protein kinase B (Akt)/mammalian target of rapamycin (mTOR)/S6 kinase (S6K) signaling, which is a negative regulator of autophagic activity. Of note, pharmacological inhibition of autophagy alleviated bupivacaine-induced cytotoxicity of IVD cells. The findings indicated that application of clinically relevant concentrations of bupivacaine upregulated autophagic activity via inhibition of Akt/mTOR/S6K signaling. In addition, the inhibition of autophagic activation served as a protective mechanism against bupivacaine-induced cytotoxicity. Collectively, these findings may provide novel insight into the mechanisms underlying cytotoxicity induced by bupivacaine when controlling spine-associated pain.

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Introduction

Back pain-associated health care and psychosocial problems are costly, placing a major burden on modern societies (1); disc degeneration is regarded as one of the primary contributors to back pain (2). Due to convenience and efficiency, intradiscal injection of local anesthetics has been frequently used for the diagnostic block and treatment of discogenic back pain. Notably, the use of local anesthetics in managing patients who are unwilling to receive surgery has markedly increased over the past decade (3).

Clinically, bupivacaine has long been used in intradiscal injections to control back pain symptoms (4,5). Based on the mechanisms of relieving inflammation (6) and inhibiting the sensitization of nerve endings in degenerative discs (7), the therapeutic effects of bupivacaine on back pain have been well documented in clinical settings; however, certain negative effects of bupivacaine have been reported *in vitro*, particularly with regard to its cytotoxicity towards intervertebral disc (IVD) cells (8). This observation was first demonstrated by Quero *et al* (9), who reported that IVD cells exposed to bupivacaine at clinically administered concentrations exhibit decreased cellular viability. A subsequent study suggested that these negative effects are dose- and time-dependent (10); however, the exact mechanisms underlying the cytotoxicity of bupivacaine towards IVD cells remain unclear.

Autophagy is an evolutionarily conserved process that degrades cytoplasmic proteins and organelles. Under normal conditions, basal autophagy is essential to maintain cellular homeostasis by sequestrating damaged materials (11). Additionally, autophagy is activated by harmful cellular conditions. Studies have demonstrated that autophagic activation

may be protective or harmful to cells under stressful conditions (12,13); however, to the best of our knowledge, the role of autophagy in bupivacaine-treated IVD cells is yet to be determined.

Mammalian target of rapamycin (mTOR) protein is an important contributor to autophagic induction, and is involved in regulating the phosphorylation and activation of protein kinase B (Akt) (14). It has previously been illustrated that autophagy is negatively regulated by the Akt/mTOR/S6 kinase (S6K) signaling pathway (15). Additionally, it was reported that bupivacaine suppresses Akt and S6K activation in cells (13,16), suggesting a potential function for bupivacaine in mediating autophagy; however, the effects of bupivacaine on autophagic activity in IVD cells via Akt/mTOR/S6K signaling remain unclear.

In the present study, human IVD cells were treated with bupivacaine at clinically relevant concentrations, and the dose- and time-dependent cytotoxic effects of bupivacaine were characterized. Subsequently, the potential roles of autophagy and the Akt/mTOR/S6K signaling pathway were investigated. Furthermore, the association between autophagy and apoptosis in IVD cells was investigated to provide novel insight into the cytotoxic mechanisms of bupivacaine.

Materials and methods

Human nucleus pulposus (NP) isolation and culture. Human IVDs were collected from 10 patients (7 male and 3 female patients; age, 33-55 years old) at the First Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, China) during percutaneous lumbar discectomy between June 2016 and October 2017 (Table I). The inclusion criteria for the study were as follows: i) Patients were diagnosed with lumbar disc herniation on the basis of clinical symptoms and MRI findings; ii) the absence of a history of lumbar spine disease, such as lumbar trauma or infection; and iii) the absence of lumbar spondylolisthesis or scoliosis. Disc degeneration was graded in patients using the Pfirrmann classification system (17). This study was approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University. Written informed consent was obtained from all patients. The obtained IVDs were washed in normal saline and then minced with a scalpel. Freshly prepared digesting solution containing Dulbecco's Modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA), 5% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc.) and a combination of 0.05% collagenase type II and type IV (Sigma-Aldrich; Merck KGaA) was used for enzymatic digestion for 15 min at room temperature. Subsequently, IVDs were collected by centrifugation at 250 x g for 5 min, and suspended and seeded in DMEM supplemented with 10% FBS and 1% gentamicin at 37˚C in a humidified atmosphere containing 5% CO_2. Primary generation IVD cells at a confluence of 80-90% were used for all experiments.

IVD cellular viability assay. IVD cellular viability was determined using an MTT assay (Beyotime Institute of Biotechnology) according to a standard protocol. Briefly, IVD cells ($4x10^6$/ml) were seeded in 24-well plates and cultured with 0.9% saline (control group), and 0.25% (8 mM) and 0.5% (17.5 mM) bupivacaine for 2, 6 and 12 h at room temperature. As 3-methyladenine (3-MA; Sigma-Aldrich; Merck KGaA) inhibits phosphatidylinositol 3-kinase to suppress autophagy by inhibiting autophagosome formation (18), 3-MA (4 mM) was applied to IVD cells at room temperature (with or without 8 or 17.5 mM bupivacaine) to investigate the role of autophagy in IVD cellular viability. At the appropriate time points following treatment, cellular viability was monitored using MTT (0.5 mg/ml) at 37˚C for 4 h. The formed crystals were solubilized using dimethyl sulfoxide (Beyotime Institute of Biotechnology) and quantified by spectrophotometry at 570 nm wavelength.

Table I. Patient data.

Patient number	Sex	Age (years)	Grade	Disc level
1	M	54	IV	L4/L5
2	M	52	IV	L5/S1
3	F	43	III	L4/L5
4	F	49	III	L3/L4
5	M	51	IV	L3/L4
6	F	33	II	L4/L5
7	M	41	III	L3/L4
8	M	47	IV	L3/L4
9	M	55	IV	L4/L5
10	M	37	III	L4/L5

Intervertebral disc cells from patients were used for more than one method or time point. F, female; Grade, Pfirrmann grade for disc degeneration; M, male.

Transmission electron microscopy. For electron microscopy, IVD cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer overnight at room temperature, and then incubated in 1% osmium tetroxide and 2% uranyl acetate for an additional 1 h at room temperature. Following dehydration in a graded alcohol series, samples were embedded into Araldite (Sigma-Aldrich; Merck KGaA) and cut into 1-μm sections, which were stained with toluidine blue at room temperature for 1 min to locate cells. Finally, 70-nm sections were made and examined using a transmission electron microscope (JEM-1220; JEOL, Ltd.).

Western blot analysis. Total protein was extracted from IVD cells washed with ice-cold PBS and lysed with RIPA lysis buffer (Sigma-Aldrich; Merck KGaA). Cell lysates were extracted, and the protein concentration was determined using a Bicinchoninic Acid Protein Assay Reagent kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocols. Protein samples (20 μg) were separated by 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% fat-free milk for 1 h at room temperature, and then incubated overnight at 4˚C with LC3 (1:500; cat. no. ab51520; Abcam), cleaved caspase-3 (1:1,000; cat. no. ab32042; Abcam), cleaved caspase-9 (1:1,000; cat. no. ab2324; Abcam), Beclin-1 (1:2,000; cat. no. MAB5295; R&D Systems, Inc.), S6K (1:6,000; cat. no. ab9366; Abcam),

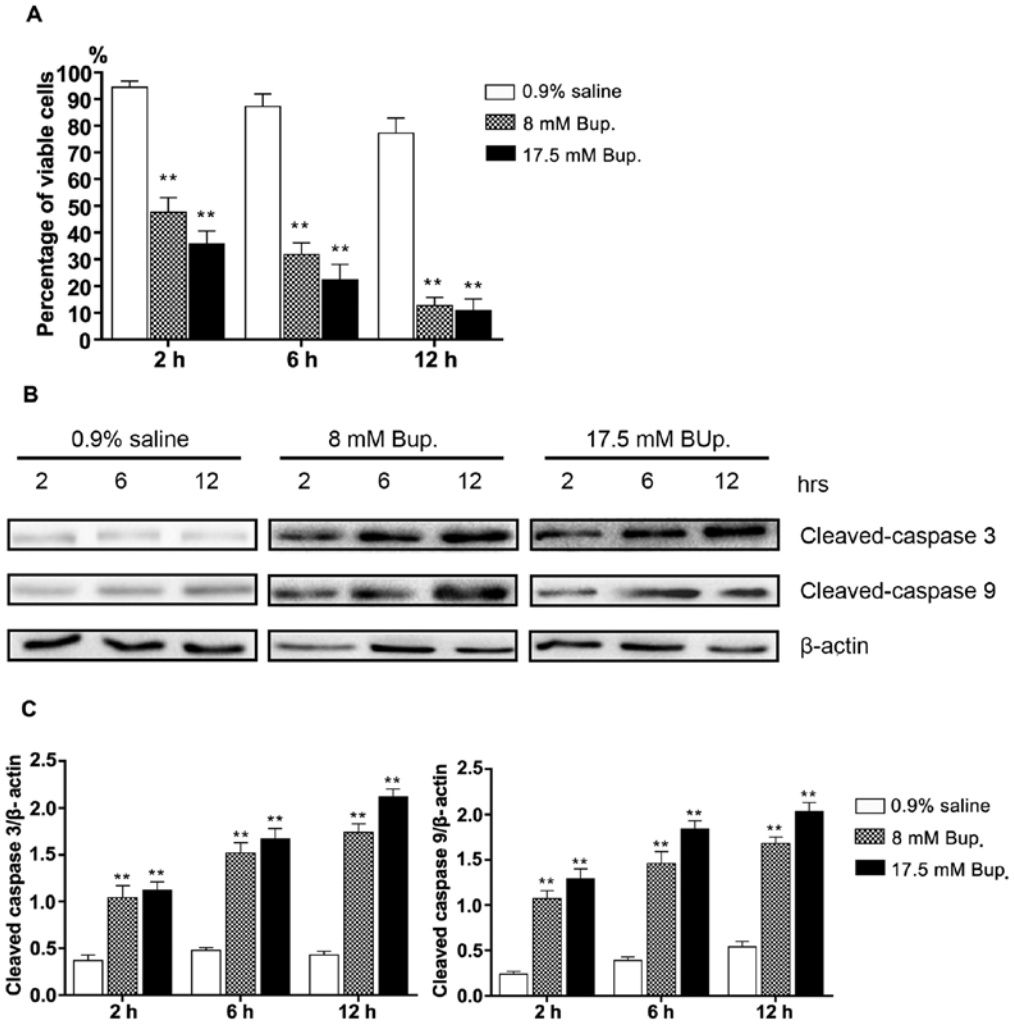

Figure 1. Cytotoxicity and apoptosis of IVD cells over time following bupivacaine treatment. (A) IVD cells were incubated with 0.9% saline, or 8 or 17.5 mM bupivacaine. Cellular viability was measured via an MTT assay following treatment for 2, 6 or 12 h. **P<0.01 vs. 0.9% saline; n=4/group. (B) Saline (0.9%), 8 and 17.5 mM bupivacaine were applied to observe the effects of bupivacaine on the apoptosis of IVD cells at 2, 6 and 12 h. (C) Semi-quantitative analysis of western blots, presenting the cleaved caspase-3/β-actin and cleaved caspase-9/β-actin ratios. **P<0.01 vs. 0.9% saline; n=6/group. Bup., bupivacaine; IVD, intervertebral disc.

phosphorylated (p)-S6K (1:1,000; cat. no. ab9973; Abcam), Akt (1:5,000; cat. no. ab179463; Abcam), p-Akt (1:2,000; cat. no. AF887; R&D Systems, Inc.) and β-actin antibodies (1:10,000; cat. no. A1978; Sigma-Aldrich; Merck KGaA). The membranes were then washed five times with TBS-0.05% Tween-20 buffer, followed by incubation for 30 min at 37˚C with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG; 1:5,000; cat. no. HAF007; R&D Systems, Inc.) and anti-rabbit IgG (1:5,000; cat. no. HAF008; R&D Systems, Inc.). β-actin served as a loading control. Target proteins were imaged using a FluorChem E Chemiluminescent Western Blot Imaging system (ProteinSimple) and semi-quantified by densitometric analysis using ImageJ software (version 1.48; National Institutes of Health).

Statistical analysis. The experimental results from between three and five independent experiments were quantified and presented as the mean ± standard error of the mean. For analysis of time- and dose-dependent effects of bupivacaine and signaling pathway proteins, one-way ANOVA was conducted followed by post hoc Bonferroni correction. For the inhibition of autophagic activity experiments, control vs. 3-MA groups at each bupivacaine dose were compared using independent-samples t-tests. The distribution of normality was determined for all variables using a Kolmogorov-Smirnov test. Statistical analysis was performed using STATA version 12.0 software (StataCorp LP). P<0.05 was considered to indicate a statistically significant difference.

Results

Evaluation of time- and dose-dependent effects of bupivacaine on IVD cellular viability and apoptosis. To test the time- and dose-dependent effects of bupivacaine on the viability of IVD cells, cells were exposed to a control (0.9% saline) group, and 8 and 17.5 mM bupivacaine treatment groups, for various periods of time.

Following treatment of IVD cells with 8 and 17.5 mM bupivacaine for 2 h, cellular viability was significantly reduced compared with the control group (P<0.01; Fig. 1A). Additionally, the difference in cellular viability between the control and bupivacaine treatment groups markedly increased over time. Following treatment with bupivacaine for 12 h, only 12.6±3.2 and 10.9±4.3% of the IVD cells in the 8 and 17.5 mM-treated

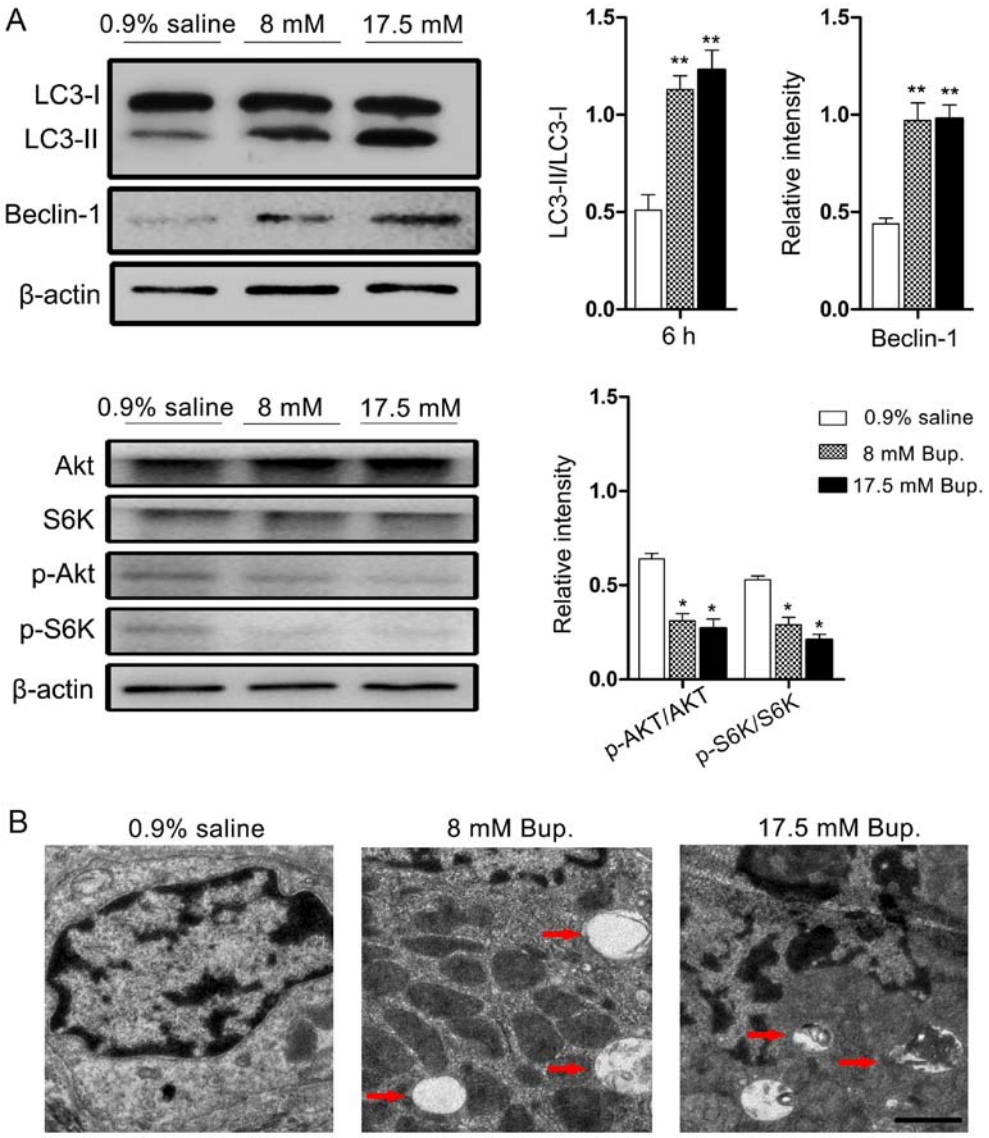

Figure 2. Bupivacaine induces autophagic responses in IVD cells via inhibition of the Akt/mTOR/S6K signaling pathway. (A) Cells were treated with 0.9% saline and bupivacaine for 6 h at the indicated concentrations. Western blot analysis revealed that bupivacaine significantly increased LC3-II and Beclin-1 levels, and decreased Akt and S6K phosphorylation in IVD cells compared with in the control group. Beclin-1, p-Akt/Akt and p-S6K/S6K were normalized to β-actin expression. *P<0.05, **P<0.01 vs. 0.9% saline, n=3-4/group. (B) Ultrastructural observations of autophagic vesicles deposited in IVD cells. Cells treated with 0.9% saline displayed normal cell morphology. Cells treated with bupivacaine for 6 h at the indicated concentrations presented double-membrane autophagosomes indicated by red arrowheads. Scale bar, 1 μm. Akt, protein kinase B; Bup., bupivacaine; IVD, intervertebral disc; mTOR, mammalian target of rapamycin; S6K, S6 kinase.

groups were viable, respectively, whereas there were 77.4±5.6% viable IVD cells in the control group after 12 h.

Subsequently, IVD cellular apoptosis was investigated via western blot analysis. During the 12-h treatment period, cells exposed to bupivacaine doses of 8 or 17.5 mM exhibited upregulated expression of apoptotic proteins across the 12-h period, as indicated by a significant increase in cleaved caspase-3 and cleaved caspase-9 expression compared with the control (P<0.01; Fig. 1B and C). The results indicated that bupivacaine induced apoptosis in IVD cells compared with control-treated cells.

Induction of autophagy in bupivacaine-treated IVD cells via inhibition of the Akt/mTOR/S6K signaling axis. Autophagy is important to cell survival and death under pathological conditions (12). To determine the effect of bupivacaine on autophagy in IVD cells, the autophagic activity of IVD cells was evaluated following exposure to 0.9% saline or bupivacaine for 6 h, at which point bupivacaine notably induced cell apoptosis.

Following treatment with reagents, autophagy was activated in the 8 and 17.5 mM treatment groups, as determined by an increase in Beclin-1 expression and the ratio of LC3-II to LC3-I, which are considered biomarkers of autophagy (Fig. 2A). The stimulatory effect of bupivacaine on autophagy was verified under an electron microscope. As presented in Fig. 2B, autophagosomes clearly accumulated in bupivacaine-treated cells, but not in the control group.

To assess the potential involvement of signaling pathways in bupivacaine-induced autophagy in IVD cells, the activation of Akt/mTOR/S6K signaling was investigated, as this signaling pathway serves an important role in the negative regulation of autophagy initiation. The results indicated that

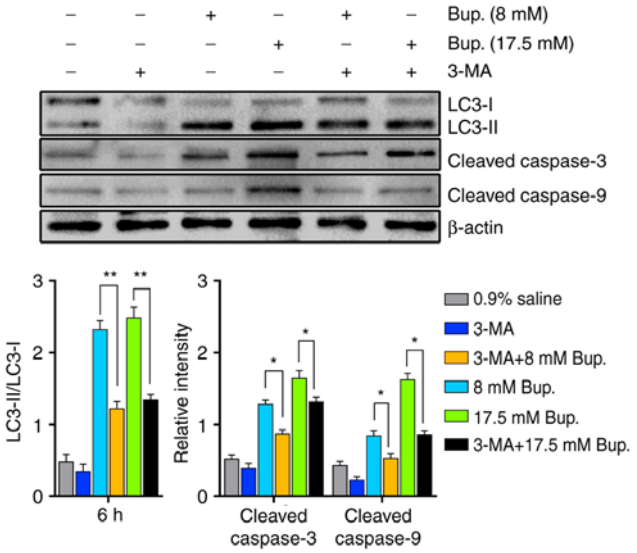

Figure 3. Inhibition of autophagy protects cells from bupivacaine-induced apoptosis. Intervertebral disc cells were treated for 6 h with control (0.9% saline), or 8 or 17.5 mM bupivacaine in the presence or absence of 3-MA. Western blot analysis indicated that 3-MA decreased bupivacaine-induced cellar autophagic and apoptotic protein expression. *P<0.05, **P<0.01; n=4/group. 3-MA, 3-methyladenine; Bup., bupivacaine.

bupivacaine treatment induced a significant reduction in the p-Akt/Akt ratio by 51.6 and 57.8% at the doses of 8 and 17.5 mM, respectively, in addition to a significant reduction in the phosphorylation of S6K compared with in the control group (P<0.01; Fig. 2A).

Suppression of bupivacaine-induced autophagic activity increases IVD cellular viability. To determine whether inhibition of autophagic activity affected viability and apoptotic processes in bupivacaine-treated IVD cells, IVD cells were co-treated with the autophagy inhibitor 3-MA and bupivacaine, and IVD cellular apoptosis and viability were determined via western blotting and MTT assay, respectively.

As shown in Fig. 3, the expression levels of apoptotic biomarkers in IVD cells 6 h after co-treatment with 3-MA and bupivacaine were significantly reduced compared with in the bupivacaine treatment groups (P<0.05). In addition, treatment with 3-MA alone resulted in a decrease in the expression levels of autophagic and apoptotic factors in IVD cells; however, there were no statistical significance compared with in the control group (P>0.05; Fig. 3). Additionally, cellular viability was evaluated over time using an MTT assay. Following co-treatment with 3-MA and bupivacaine, cellular viability was significantly increased compared with in the bupivacaine treatment groups at 2, 6 and 12 h (Fig. 4). This finding suggested that inhibition of autophagic activity decreased apoptotic cell death and improved viability in bupivacaine-treated IVD cells.

Discussion

Bupivacaine is widely used to manage patients with discogenic back pain; however, the toxic effects of bupivacaine on IVD cells have been documented in a number of studies (10,19). To the best of our knowledge, this is the first study to report the effects of

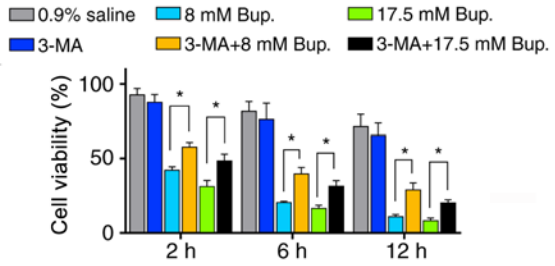

Figure 4. Inhibition of autophagy protects cells from bupivacaine-induced cytotoxicity. Intervertebral disc cells were treated for 2, 6 and 12 h with control (0.9% saline), or 8 or 17.5 mM bupivacaine in the presence or absence of 3-MA. An MTT assay indicated that 3-MA significantly reduced bupivacaine-induced cytotoxicity. *P<0.05, n=6/group.

bupivacaine at clinically relevant concentrations on autophagy and apoptosis in human IVD cells *in vitro*. The findings revealed that bupivacaine may activate autophagy in IVD cells by inhibiting the Akt/mTOR/S6K pathway. Additionally, 3-MA treatment inhibited autophagic activity and effectively protected IVD cells from bupivacaine-induced apoptosis. Collectively, these results suggested that bupivacaine exerted cytotoxic effects on IVD cells via activation of cellular autophagy.

Discogenic back pain is a common and burdensome problem in developed countries, the treatment options for which remain limited (20). With the advantages of minimal trauma and long duration, local anesthesia is accepted as an important method to alleviate the symptoms of patients with discogenic back pain (3). Previously, it was reported that bupivacaine at clinically relevant doses induces toxic effects on human NP cells (9,19). The present results revealed that bupivacaine exhibited cytotoxicity towards cultured human IVD cells in a time- and dose-dependent manner. In addition, the expression levels of apoptotic proteins, including cleaved caspase-3 and cleaved caspase-9, in human IVD cells treated with bupivacaine were increased compared with in the control group, suggesting that the cytotoxic effects of bupivacaine may be mediated by apoptosis in human IVD cells.

Apoptosis and autophagy frequently occur together in response to cellular stress, and autophagic activity acts as both a protector and promoter of cell death (21); however, the role served by autophagy in the cytotoxicity of local anesthetic drugs and the specific underlying mechanisms are yet to be determined. Since LC3-II and Beclin-1 are important in the formation of autophagosomes, the expression levels of Beclin-1 and the LC3-II/I ratio are widely used as biomarkers to evaluate the activation of autophagy (22-24). In the present study, the results revealed that the levels of LC3-II/I and Beclin-1 in human IVD cells treated with 8 and 17.5 mM bupivacaine were significantly upregulated compared with in the control group. Electron microscopy revealed that the formation of autophagosomes was significantly increased in the bupivacaine groups. These results suggested that autophagy was activated and closely associated with the cytotoxicity of bupivacaine, which promoted autophagic marker expression and autophagosome formation in human IVD cells.

It has been reported that activation of the Akt/mTOR/S6K pathway serves as a negative regulator of autophagy (25,26), whereas bupivacaine upregulates autophagy by inhibiting Akt/mTOR/S6K signaling in cardiac and nerve cells (27,28). In the

present study, it was observed that phosphorylation of Akt and S6K in human IVD cells treated with bupivacaine was significantly decreased compared with in the control group. These findings suggested that the Akt/mTOR/S6K signaling pathway may be associated with bupivacaine-induced autophagy. Furthermore, 3-MA, a selective autophagy inhibitor, was applied to human NP cells in combination with bupivacaine, revealing that the expression of autophagic proteins decreased following the addition of 3-MA. Consistent with previous studies, when the autophagy inhibitor 3-MA was applied, the levels of the apoptosis effectors cleaved caspases-3 and -9 were significantly decreased (12,29). Subsequently, the activity of human IVD cells co-treated with 3-MA and bupivacaine was evaluated, and changes in the levels of the apoptosis-associated proteins cleaved caspase-3 and cleaved caspase-9 were determined. It was demonstrated that the expression levels of cleaved caspase-3 and cleaved caspase-9 were significantly decreased, whereas the viability of IVD cells was significantly increased following inhibition of autophagy by 3-MA in the 8 and 17.5 mM bupivacaine-treated groups. These findings suggested that autophagy serves an important role in mediating bupivacaine-induced apoptosis in IVD cells.

The present study should be interpreted in the context of certain limitations. For example, increased autophagy is not only associated with the induction of autophagosome formation, but also the inhibition of autophagosome degradation (30). In this study, only the activation of important pathways in autophagy formation were explored, with the mechanisms of autophagy clearance not investigated. Additionally, bupivacaine can induce autophagy and inhibition of the Akt/mTOR/S6K pathway; however, only 3-MA was used to determine the effects of bupivacaine on the apoptosis of NP cells. In the future, the specific protein expression of mTOR pathway components should be regulated using gene knockout techniques, and alterations in the levels of autophagy should be further demonstrated in disc tissues, to provide further insight into cellular responses to the effects of bupivacaine on the autophagy and apoptosis of IVD cells.

In conclusion, the present study reported that the levels of autophagic activity in human NP cells were significantly increased following bupivacaine treatment, potentially via inhibition of the Akt/mTOR/S6K signaling pathway. Additionally, the expression of apoptotic effectors was decreased following co-treatment with the autophagy inhibitor 3-MA. To the best of our knowledge, this study is the first to report that autophagy induced by bupivacaine is an upstream mechanism underlying apoptosis; however, the specific pathways via which bupivacaine-induced autophagy affects apoptosis remain unclear and require further investigation. Collectively, the findings from this study may provide novel insight to improve understanding of the specific mechanisms underlying the cytotoxicity of bupivacaine.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GY, ZYL and QT designed the experiments. GY, ZYL, HBM, WHY, SXH and KL performed the experiments and analyzed the data. GY, ZYL and QT drafted and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University. Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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