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SCIENTIFIC ARTICLE

Comparative effects of vitamin C on the effects of local anesthetics ropivacaine, bupivacaine, and lidocaine on human chondrocytes

Jun Tian*, **Yan Li**

Department of Anesthesiology, Xinxiang Central Hospital of Henan Province, Xinxiang, China

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KEYWORDS
Chondrocytes;
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Local anesthetics;
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Lidocaine

Abstract
Background: Intra-articular injections of local anesthetics are commonly used to enhance post-operative analgesia following orthopedic surgery as arthroscopic surgeries. Nevertheless, recent reports of severe complications due to the use of intra-articular local anesthetic have raised concerns.
Objectives: The study aims to assess use of vitamin C in reducing adverse effects of the most commonly employed anesthetics – ropivacaine, bupivacaine and lidocaine – on human chondrocytes.
Methods: The chondrocyte viability following exposure to 0.5% bupivacaine or 0.75% ropivacaine or 1.0% lidocaine and/or vitamin C at doses 125, 250 and 500 μM was determined by LIVE/DEAD assay and annexin V staining. Expression levels of caspases 3 and 9 were assessed using antibodies by Western blotting. Flow cytometry was performed to analyze the generation of reactive oxygen species.
Results: On exposure to the local anesthetics, chondrotoxicity was found in the order ropivacaine < bupivacaine < lidocaine. Vitamin C effectively improved the reduced chondrocyte viability and decreased the raised apoptosis levels following exposure to anesthesia. At higher doses, vitamin C was found efficient in reducing the generation of reactive oxygen species and as well down-regulate the expressions of caspases 3 and 9.
Conclusions: Vitamin C was observed to effectively protect chondrocytes against the toxic insult of local anesthetics ropivacaine, bupivacaine and lidocaine.

* Corresponding author.
E-mail: juntian2004@gmail.com (J. Tian).

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PALAVRAS-CHAVE
Condrócitos;
Vitamina C;
Anestésicos locais;
Ropivacaína;
Bupivacaína;
Lidocaína

Efeitos comparativos de vitamina C sobre os efeitos dos anestésicos locais ropivacaína, bupivacaína e lidocaína em condrócitos humanos

Resumo
Justificaiva: Injeções de anestésicos locais por via intra-articular são comumente usadas para melhorar a analgesia no período pós-operatório de cirurgia ortopédica como artroscopias. No entanto, relatos recentes de complicações graves devido ao uso de anestésico local por via intra-articular causou preocupações.
Objetivos: O objetivo do estudo foi avaliar o uso de vitamina C para reduzir os efeitos adversos dos anestésicos mais comumente usados (ropivocaína, bupivacaína e lidocaína) sobre condrócitos humanos.
Métodos: A viabilidade dos condrócitos após a exposição à bupivacaína a 0,5% ou ropivacaína a 0,75% ou lidocaína a 1,0% e/ou vitamina C em doses de 125, 250 e 500 μM foi determinada pelo ensaio VIVO/MORTO e coloração com anexina V. Os níveis de expressão das caspases 3 e 9 foram avaliados usando anticorpos pela técnica *Western blot*. Citometria de fluxo foi realizada para analisar a geração de espécies reativas ao oxigênio.
Resultados: Na exposição aos anestésicos locais, condrotoxicidade foi encontrada na seguinte ordem: ropivacaína < bupivacaína < lidocaína. A vitamina C efetivamente melhorou a redução da viabilidade dos condrócitos e diminuiu os níveis elevados de apoptose após a exposição à anestesia. Em doses mais altas, a vitamina C foi eficiente para reduzir a geração de espécies reativas ao oxigênio e assim regular negativamente a expressão das caspases 3 e 9.
Conclusões: Observamos que a vitamina C foi eficaz na proteção dos condrócitos contra a agressão tóxica dos anestésicos locais ropivacaína, bupivacaína e lidocaína.

Introduction

Local anesthetics are commonly employed in various clinical settings for either prevention or for symptomatic pain relief. During orthopedic practice, they are administered by many routes as spinal, epidural or intra-articular for post-operative pain relief, or as a modality in the treatment in osteoarthritis. Multiple clinical studies have shown that intra-articular injections of local anesthetics have high success rates when used for post-operative analgesia.[1–3]

Concern about the detrimental effects of local anesthetics came to note when multiple case studies demonstrated severe toxicity to cartilage and chondrolysis after local anesthetic infusions into the glenohumeral joint post arthroscopy.[4–8] As the evidence of chondrotoxicity due to the local anesthetics has been increasing, investigations on the use of other substances to either inhibit or reduce the toxicity are being done. In orthopedic surgeries, adding to the effects of local anesthetics limited healing potential of hyaline articular cartilage is also a well-known problem.[9]

Among local anesthetics that are often used for pain relief, lidocaine and bupivacaine are members of amide group, but the duration of action of lidocaine is about one-half as that of bupivacaine.[10] Ropivacaine is a long acting amino amide member of the pipecoloxylidide group of local anesthetics and is a promising substitute for bupivacaine for spine anesthesia.[1] However, recent studies have presented the deleterious effects of these anesthetics on chondrocytes.[11–14] Lo et al.[14] demonstrated that bupivacaine, ropivacaine, and lidocaine have a negative effect in a dose- and duration-dependent manner on the viability of chondrocytes.

In vitro studies have shown that lidocaine and bupivacaine can have cytotoxic effects on neurons and myocytes, with cell death occurring by both necrosis and apoptosis.[15,16] In chondrocytes, exposure to these anesthetics also leads to cell death by both necrosis and apoptosis in a dose- and duration-dependent manner.[1,17] Fedder et al.[18] reported that exposure to ropivacaine, lidocaine and bupivacaine reduced the viability of fibroblasts which is linked to generation of reactive oxygen species (ROS). Apoptosis is a highly regulated process distinct from necrosis that occurs in response to trauma, toxins, cytokines, and pathogens.[19,20] Thus, inhibition of chondrocyte apoptosis has been described as a prospective means to reduce chondrocyte loss.[20,21]

Vitamins have been considered to be vital for living. Vitamin C, an exogenous water-soluble substance,[22] is a cofactor in building blood vessels and takes the role of an antioxidant,[23] by acting as an electron donor and reducing agent and thus preventing lipid, protein and DNA oxidation.[24] There is growing recognition of the importance of nutritional factors in the maintenance of bone and joint health.[25] Bone matrix contains over 90% of protein as collagen[26] and vitamin C is an essential cofactor for collagen formation and synthesis of hydroxyproline and hydroxylysine.[27] Therefore, vitamin C may help in strengthening bone and prevent fractures. Experimentally induced deficiency of vitamin C in animals leads to impaired bone mass, cartilage and connective tissue.[28] A 17-year

follow-up study by Sahni et al.[29] demonstrated that vitamin C supplementation decreases risk of hip fracture and osteoporosis. Thus considering the effects of vitamin C, the present study investigates if vitamin C inhibits chrondocyte loss and protects cartilage against the adverse effects of local anesthetics.

Methods

Chemicals and cells

Normal human chondrocytes were procured from Promocell, Germany and were cultured in chondrocyte growth medium (Promocell, Germany) under standard laboratory conditions (humidified atmosphere of 95% air and 5% CO_2, 37 °C). Monolayer culture of chondrocytes has long been used as a method for assessing *in vitro* cell response to treatment. The cells at passage 5 were seeded at a density of 10^3 cells/cm^2 into 96-well plates and cultured until 75–80% confluence. Upon reaching the desired confluence, the cells were exposed to local anesthetics. All chemicals used in the study were obtained from Sigma-Aldrich, MO, USA; otherwise, they are mentioned.

Anesthetic exposure

Chondrocytes were exposed for 1 h to 1 mL of local anesthetic solutions: 0.5% bupivacaine or 0.75% ropivacaine or 1.0% lidocaine,[21,30] obtained from Sigma–Aldrich, MO, USA. All samples were treated using the same protocol. Specifically, culture medium was aspirated; 1 mL of 0.9% normal saline solution or anesthetic were added to each well; samples were incubated in 5% CO_2 at 37 °C for 60 min; and the treatment solution was aspirated and fresh culture medium was added. Samples were returned to the incubator and chondrocyte viability was measured 24 h later. In separate experiments, following exposure to anesthesia, the cells were incubated in fresh medium supplemented with vitamin C (125 or 250 or 500 μM) for 24 h, following which the cells were assessed for viability.

Apoptosis analysis

Chondrocytes exposed to anesthetics and/or vitamin C were rinsed with PBS and used for cell viability analysis. To measure apoptosis, the LIVE/DEAD cell viability (LIVE/DEAD cell viability kit, Invitrogen) assay was performed. The assay determines the intracellular esterase activity and plasma membrane integrity to assess the viability of cells. Chondrocytes, treated or untreated cells with anesthesia and/or vitamin C, were stained with 5 μmol/L ethidium homodimer and 5 μmol/L calcein-AM and incubated at 37 °C for 30 min. The cells were then analyzed under a Nikon labophot-2 fluorescence microscope. Live chondrocytes retain calcein-AM, a non-fluorescent polyanionic dye, and produce a green fluorescence through enzymatic (esterase) conversion. Further, ethidium homodimer enters damaged membranes of the dead cells and binds to nucleic acids thereby yielding a red fluorescence.

Apoptosis was also detected by measuring the loss of membrane asymmetry by assessing the binding properties of annexin V. The binding property of annexin V was evaluated using annexin V staining kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Annexin V antibody was conjugated with a Fluorescein isothiocyanate (FITC) dye. Briefly, 1×10^6 cells were exposed to bupivacine or ropivacaine or lidocaine for 1 h following treatment with vitamin C, or only those exposed to anesthetics were subjected to annexin V staining. The cells were washed in PBS, resuspended in 100 μL of binding buffer containing a FITC-conjugated anti-annexin V antibody, and then analyzed with flow cytometer (FACS Calibur, BD Biosciences).

Western blotting

To further analyze as to whether caspase activation was involved in the initiation of apoptosis following exposure to local anesthetics, western blot analysis was performed to determine the expression of caspase-3 and caspase-9 using respective antibodies (Cell Signaling Technology, Danvers, MA, USA). For isolation of total cellular proteins, cells were lysed in cell lysing buffer (Cell Signaling Technology, Danvers, MA, USA) and processed according to the manufacturer's instructions. Proteins were then fractionated by SDS-PAGE, electrotransferred to nitrocellulose membranes, blotted with respective antibodies and detected by enhanced chemiluminescence (GE Healthcare). The band signals of other proteins were normalized to those of β-actin using anti-β-actin at 1:2000 dilution (Cell Signaling Technology, Danvers, MA, USA).

Determination of reactive oxygen species (ROS)

The generation of intracellular ROS was measured by flow cytometry using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) staining. DCFH-DA is a non-fluorescent compound that can be enzymatically converted to DCF, a highly fluorescent compound, in the presence of ROS. Cells following exposure to anesthetic and/or vitamin C were further incubated with DCFH-DA (10 μM) for 30 min at 37 °C in dark. The cells were then washed twice with PBS and intensity of fluorescence was measured by flow cytometry.[31]

Statistical analysis

The results are represented as mean ± SD. Values presenting $p < 0.05$ are considered significant as determined by one way analysis of variance (ANOVA). The analyses were performed using version 17.0 SPPSS package.

Results

Vitamin C improves viability of chondrocytes following anesthetic exposure

Chondrocyte viability in response to anesthetic exposure was analyzed by LIVE/DEAD assay. After exposure of chondrocyte cultures to local anesthetics for 24 h, chondrotoxicity observed with 1% lidocaine was almost twice greater than

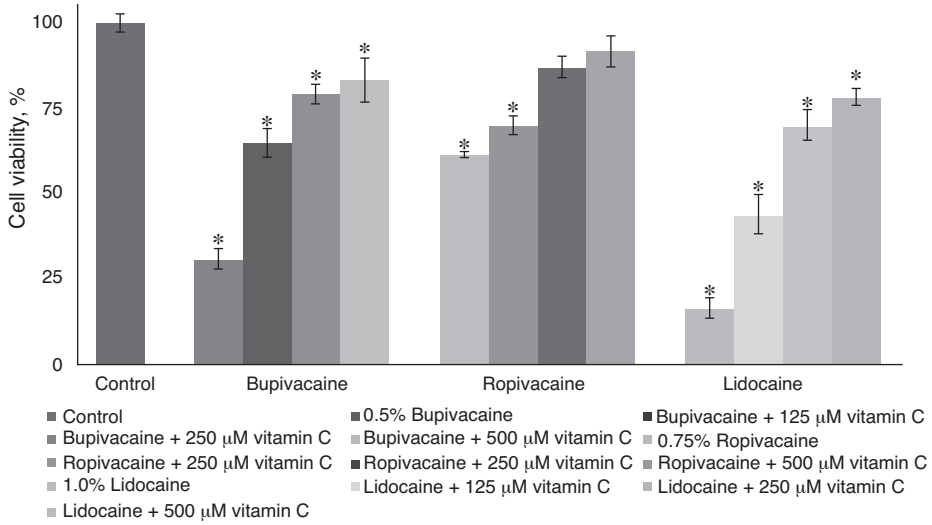

Figure 1 Influence of vitamin C on the cell viability of chondrocytes. Values are represented as mean ± SD; $n = 6$. *Represents $p < 0.05$ compared with control as determined by one way ANOVA.

0.75% ropivacaine (Fig. 1). There was a marked decrease in cell viability percentage after exposure to lidocaine, ropivacaine or bupivacaine. Comparing equipotent concentrations of ropivacaine (0.75%) and bupivacaine (0.5%), the cell viability rates were significantly higher 24 h after treatment with 0.75% ropivacaine than with 0.5% bupivacaine. Chondrotoxicity was more pronounced on exposure to lidocaine followed by bupivacaine. Exposure to vitamin C following anesthesia resulted in a significant ($p < 0.05$) improvement in cell viability percentage. The viable chondrocyte counts increased with increasing concentrations of vitamin C. The 500 μM concentration caused a marked raise in chondrocyte viability when compared to lower doses (125 and 250 μM).

Apoptosis of the chondrocytes following anesthesia exposure was also detected by measuring the loss of membrane asymmetry by annexin V staining. The apoptotic cell counts were markedly higher ($p < 0.05$) following anesthesia exposure. Ropivacaine at 0.75% caused considerably lower apoptosis as compared to 1% lidocaine and 0.5% bupivacaine. Incubation with vitamin C resulted in raised viability percentage with decreased apoptotic cell counts. Vitamin C at 500 μM concentration was more potent than lower doses in reducing apoptosis after exposure to local anesthetics (Fig. 2).

Vitamin C suppresses apoptosis by down-regulating expression of caspase-3 and caspase-9

To evaluate the possible involvement of caspase activation in anesthesia induced chondrotoxicity, expression of caspase-3 and caspase-9 was determined 24 h following exposure to anesthesia. One hour exposure to anesthetics caused up-regulation of caspase-3 and caspase-9 in the order, lidocaine > bupivacaine > ropivacaine (Fig. 3). Thought ropivacaine at 0.75% caused raised expressions of caspases, it was found to be a non-significant raise as compared to control chondrocytes not exposed to anesthetics. Nevertheless, 1% lidocaine resulted in marked enhancement in the level of expressions indicating caspase cascade activation in apoptosis. Incubation with vitamin C for 24 h markedly reduced the expression of caspases in a dose-dependent manner that was in line with the results of the LIVE/DEAD assay and annexin V staining. Vitamin C at 250 and 500 μM were more effective in down-regulating the expressions of caspases 3 and 9.

Influence of vitamin C on ROS generation

We determined the levels of ROS by flow cytometry using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) staining. Lidocaine, bupivacaine and ropivacaine caused a marked increase in ROS production. Lidocaine caused twice as much raised ROS generation as compared to ropivacaine and almost nearly 10% more than bupivacaine. This raise however was suppressed in chondrocytes incubated with vitamin C as against cells without vitamin C. Treatment with vitamin C significantly reduced ROS production in the order 125 < 250 < 500 μM (Fig. 4). The antioxidant capacity of vitamin C could have been responsible for the marked suppression of ROS.

Discussion

Intra-articular injections of local anesthetics are frequently employed in peri-operative and ambulatory settings.[32] Intra-articular injections of local anesthetics enhance post-operative analgesia.[1,2,33] Lidocaine, bupivacaine and ropivacaine are amide-type local anesthetics. Recent publications have suggested potential adverse effects of these three local anesthetics on articular chondrocytes *in vitro*.[1,11]

Several studies have described the detrimental effects of local anesthetics on chondrocyte viability. Chu et al.[34] demonstrated severe toxicity of bupivacaine. The study evidenced on exposure to 0.5% bupivacaine and >99% of the bovine chondrocytes were found to be killed in all exposed cultures. Gomoll et al.[35] reported marked histopathologic and metabolic changes in rabbit shoulders with continuous infusion of 0.25% bupivacaine with and without epinephrine.

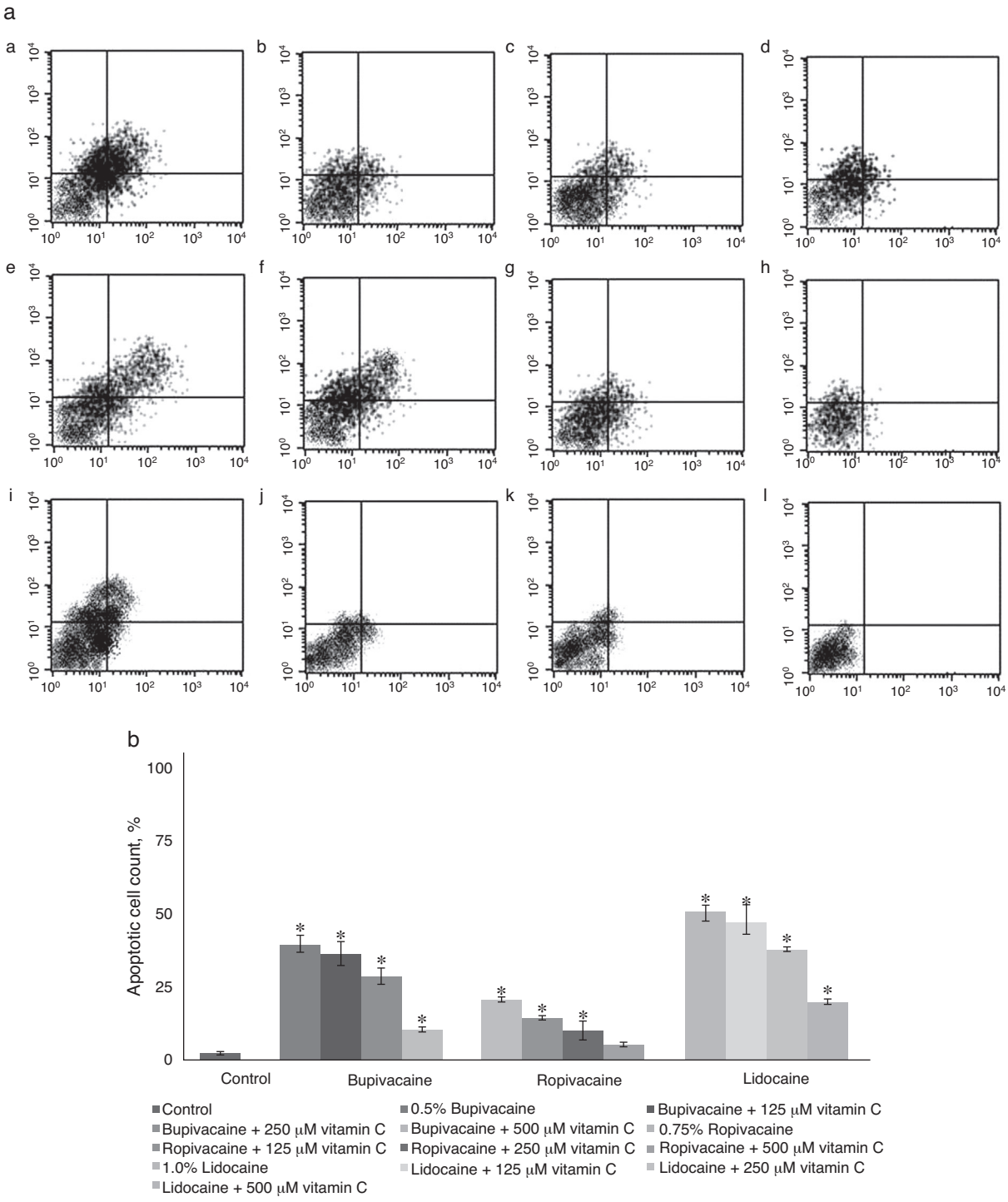

Figure 2 Chondrotoxicity induced by anesthetic exposure: (a) 0.5% bupivacaine; (e) 1% lidocaine; (i) 0.75% ropivacaine. Vitamin C on effect of anesthetics: (b) 0.5% bupivacaine + 125 μM vitamin C; (c) 0.5% bupivacaine + 250 μM vitamin C; (d) 0.5% bupivacaine + 500 μM vitamin C; (f) 1% lidocaine + 125 μM vitamin C; (g) 1% lidocaine + 250 μM vitamin C; (h) 1% lidocaine + 500 μM vitamin C; (j) 0.75% ropivacaine + 125 μM vitamin C; (k) 0.75% ropivacaine + 250 μM vitamin C; (l) 0.75% ropivacaine + 500 μM vitamin C. Apoptotic cell counts. Values are represented as mean ± SD; $n = 6$. *Represents $p < 0.05$ compared with control as determined by one way ANOVA.

Lidocaine at 1% and 2% doses was reported to exhibit chondrocyte toxicity.[11] Further, chondrocyte toxicity was reported by Piper and Kim[1] following exposure of human cartilage explants and chondrocytes to local anesthetics, bupivacaine and ropivacaine. In line with the previous reports, in our study, 0.5% bupivacaine, 0.75% ropivacaine and 1% lidocaine induced apoptosis and reduced the viability of chondrocytes. Lidocaine was observed to be potentially

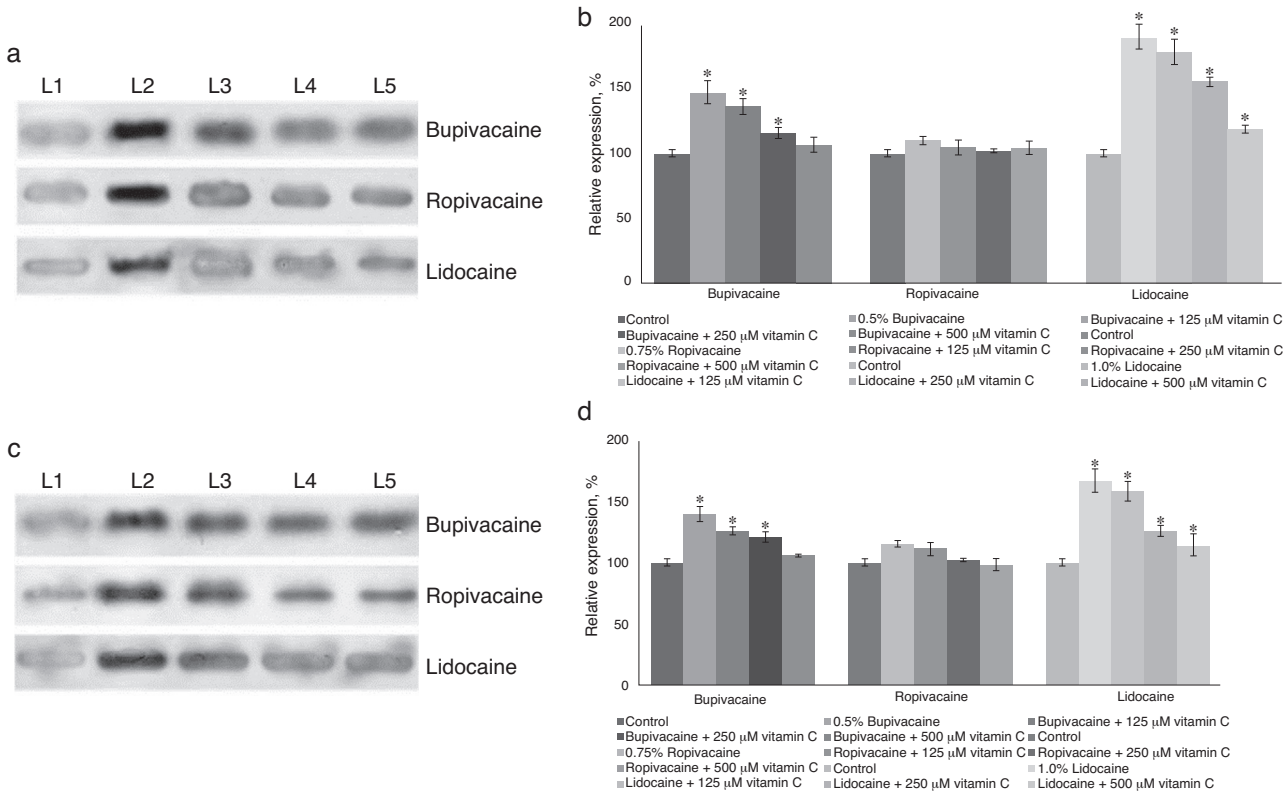

Figure 3 Effect of vitamin C on caspase-3 (a and b) and caspase-9 (c and d) expressions in chondrocytes following anesthetic exposure. L1, control; L2, anesthetic alone; L3, anesthesia + 125 μM vitamin C; L4, anesthesia + 250 μM vitamin C; L5, anesthesia + 500 μM vitamin C. (b and d) Values are represented as mean ± SD; $n = 6$ (b and d). *Represents $p < 0.05$ compared with control as determined by one way ANOVA.

more toxic that bupivacaine and ropivacaine. Similar to earlier studies, ropivacaine was found to be less chondrotoxic than bupivacaine in human chondrocytes.[1,36] Vitamin C significantly improved cell viability of the chondrocytes. Studies have demonstrated the positive effect of vitamin C on bone health.[29]

Apoptosis can be induced through intrinsic and extrinsic pathways, both involving the activation of cellular caspases. Caspases are key enzymes involved in regulating the highly specific proteolytic cleavage of cellular proteins leading to cell death. Evidence suggests that caspase-3 may also be involved in DNA fragmentation.[21] In our study,

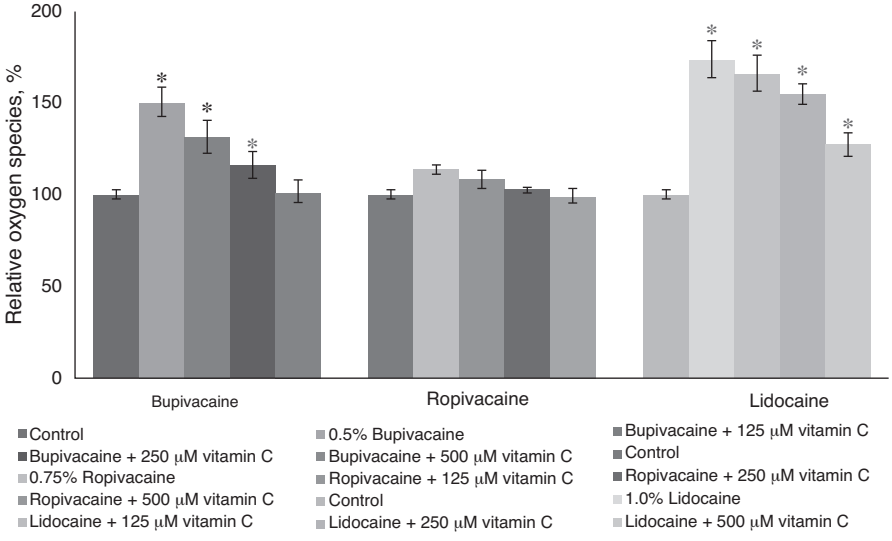

Figure 4 Influence of vitamin C on ROS generation by flow cytometry. Values are represented as mean ± SD; $n = 6$. *Represents $p < 0.05$ compared with control as determined by one way ANOVA.

local anesthetics – lidocaine, ropivacaine and bupivacaine were observed to induce caspase expression levels which is indicative of induction of apoptosis in the chondrocytes *via* caspase activation. Perez-Castro et al.[16] demonstrated activation of caspase-3/-7 in human neuroblastoma cells following incubation with lidocaine, ropivacaine and bupivacaine. Nevertheless, vitamin C suppressed the expression of caspases 3 and 9, markedly suggesting its anti-apoptotic efficacy.

The mechanisms that lead to the chondrotoxicity of local anesthetics are not completely understood. To analyze the involvement of ROS-mediated apoptosis, ROS generation in the chondrocytes was assessed. Marked raise in ROS generation following exposure to anesthetics was considerably normalized to almost normal levels on incubation of chondrocytes with various concentrations of vitamin C. This could be due to the potent antioxidant property of vitamin C. Vitamin C would have effectively prevented generation of ROS and/or neutralized ROS. However, we observed a striking correlation between cell viability, caspase expression and the levels of ROS production. Park et al.[37] have shown increased ROS concentration correlating with cell death of Schwann cells after incubation with bupivacaine. In our study, ROS concentrations increased upon exposure to lidocaine and bupivacaine, but non-significant levels were observed following ropivacaine exposure. Similar raise in ROS generation following exposure to local anesthetics was reported by Grishko et al.[38]

To summarize, we have shown that 0.75% ropivacaine, though toxic to human chondrocyte cells following 60 min exposure, is considerably on the lower toxic range as against 0.5% bupivacaine and 1% lidocaine. Lidocaine at 1% concentration presents more chondrotoxicity among the anesthetics studied. Enhanced ROS production evidenced in our study suggests that chondrotoxicity could be possibly due to ROS and ROS-mediated apoptosis. However, incubation with vitamin C significantly offered chondrocyte protection, which we could possibly be attributed to its antioxidant capacity.

Conclusion

Vitamin C was able to markedly protect the condrocytes against the toxic effects of local anesthetics – ropivacaine, bupivacaine and lidocaine.

Conflicts of interest

The authors declare no conflicts of interest.

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