

RESEARCH PAPER

## Comparison of the cytotoxic effects of bupivacaine, lidocaine, and mepivacaine in equine articular chondrocytes

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### Abstract

**Objective** To compare the chondrotoxicity of bupivacaine, lidocaine, and mepivacaine in equine articular chondrocytes *in vitro*.

**Study design** Prospective, experimental study.

**Study material** Equine articular chondrocytes.

**Methods** Primary cultured equine chondrocytes were exposed to 0.5% bupivacaine, 2% lidocaine, or 2% mepivacaine for 30 or 60 minutes. After treatment, cell viability was evaluated by trypan blue exclusion and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay in a dose dependent manner. Apoptosis and necrosis of chondrocytes were analyzed with the double staining of Hoechst 33258 and propidium iodide using fluorescence microscopy, and the results were confirmed using flow cytometry.

**Results** After 30-minute exposure, trypan blue exclusion assay revealed that cell viability of 0.5% bupivacaine group was  $28.73 \pm 8.44\%$ , and those of 2% lidocaine and 2% mepivacaine were  $66.85 \pm 6.03\%$  and  $86.27 \pm 2.00\%$ , respectively. The viability of chondrocytes after saline treatment was  $95.95 \pm 2.75\%$ . The results of MTT assay and fluorescence microscopy had similar tendency with trypan blue assay. Each result showed that bupivacaine was the most toxic of the three local anaesthetics. Mepivacaine was less toxic than lidocaine.

The results of the viability test suggest that bupivacaine and lidocaine exhibit a marked chondrotoxicity, and that this is mainly due to necrosis rather than apoptosis.

**Conclusions and clinical relevance** Bupivacaine may induce detrimental chondrotoxicity when administered intra-articularly, especially in patients with joint disease, and we suggest that it should be used cautiously in equine practice. Mepivacaine may be an alternative to both bupivacaine and lidocaine.

**Keywords** bupivacaine, chondrotoxicity, equine chondrocyte, lidocaine, mepivacaine.

### Introduction

Intra-articular injections of local anaesthetics are common procedures in equine practice and are used both to provide diagnostic and therapeutic analgesia. Such injections, until recently, have been considered to have no directly mediated detrimental effects. However, in humans, recent cases of postoperative chondrolysis after arthroscopic procedures have been linked to intra-articular injection of bupivacaine, and it is now thought that use of this analgesic agent may be related to occasional but serious joint complications (Petty et al. 2004; Hansen et al. 2007; Webb & Ghosh 2009). As a result there have been many studies in species as diverse as bovine (Chu et al. 2006), lapine (Dogan et al. 2004; Gomoll et al. 2009), and human (Chu et al. 2008; Piper & Kim 2008) which have investigated the effects of

a number of local anaesthetic agents both on cartilage and on chondrocytes. Despite this literature on the subject of systemic and local tissue toxicity caused by local anaesthetic agents, little is known about the direct effects of these agents on equine chondrocytes (Bolt et al. 2008; Santos et al. 2009).

Mepivacaine is the most frequently used local anaesthetic agent for intra-articular injection in equine practice (Tranquilli et al. 2007), although the longer-acting agents, bupivacaine and ropivacaine have been recommended for post-operative analgesia (Taylor & Clarke 2007), and lidocaine is used in other situations. Intra-articular bupivacaine has also been used commonly in arthroscopic procedures in human medicine before the recent identification of its possible toxicity. We hypothesized that bupivacaine, mepivacaine and lidocaine may have different toxic effects on equine chondrocytes. The aim of this study, therefore, was to evaluate the cytotoxicity of these three local anaesthetic agents in equine chondrocytes.

## Materials and methods

### Primary culture of equine chondrocytes

Articular cartilage was aseptically harvested from the metacarpophalangeal joints of horse (Thoroughbred, female, 3 years old) sacrificed for reasons unrelated to this study. The cartilage was rinsed in Dulbecco's phosphate-buffered saline without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (D-PBS). After enzymatic digestion in 0.25% collagenase type IA (Sigma-Aldrich, MO, USA) in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated foetal bovine serum (FBS), the cell suspension was filtered to remove undigested cartilage fragments. The filtered fluid was centrifuged at 80–100 *g* for 5 minutes, and the supernatant discarded. The cell pellet was resuspended in normal growth medium (high-glucose DMEM containing 10% FBS and 1% penicillin/streptomycin). The cell suspension was cultured in a 5%  $\text{CO}_2$ , humidified atmosphere at 37 °C until it formed monolayers suitable for propagation. Cells were preserved with the cryoprotective agent (10% dimethyl sulfoxide) in liquid nitrogen. The equine chondrocytes of the first passage were used in the various assays.

### Experimental groups

Equine chondrocytes were divided into four treatment groups. Each group was treated with one of

the following experimental agents: 1) 0.9% normal saline solution (Daihan Sterile Saline; Daihan Pharm, Korea); 2) 0.5% bupivacaine (Bupivacaine HCl; Huons, Korea); 3) 2% lidocaine (Daihan Lidocaine HCl 2%; Daihan Pharm, Korea); and 4) 2% mepivacaine (Emcaine 2%; Reyon Pharm, Korea), for 30 minutes. Cells from each treatment group were then subjected to one of four different cell viability tests as methods of evaluating toxicity. For investigation by one method, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, each group was further subdivided, and each subdivision was treated with one of three different concentrations of each of the three local anaesthetic agents. The concentrations used were that of the commercial local anaesthetic solution, and its two-fold serially diluted solutions, with normal saline as the solvent. This resulted in the concentrations begin investigated being as follows: bupivacaine, 0.5%, 0.25%, 0.125%; lidocaine, 2%, 1%, 0.5%, and mepivacaine 2%, 1%, 0.5%.

### Cell viability tests

#### *Trypan blue exclusion assay*

After 30 minutes of exposure to the anaesthetics, chondrocyte viability was evaluated by trypan blue exclusion. This assay was used to determine the number of viable cells in a given cell population, and it is based on the principle that viable cells maintain the integrity of the cell membrane which prevents dye from penetrating. The cells from each group were stained with 0.4% trypan blue (Trypan blue solution 0.4%; Welgene, Korea) for 5 minutes. The number of dye-excluding (live) cells and positively stained (dead) cells was counted using a haemocytometer. The assays were performed in quadruplicate and the results were represented as the mean  $\pm$  standard deviation (SD).

#### *MTT assay*

Cell viability was determined using the MTT colorimetric assay in quintuplicate. This assay is founded on the measurement of activity of mitochondrial dehydrogenase that converts MTT into blue formazan in viable cells. The chondrocytes were seeded onto 96-well plates at a density of  $2 \times 10^4$  cells per well and incubated for 24 hours in a humidified 5%  $\text{CO}_2$  incubator at 37 °C. The

growth medium was then removed from each well, the wells washed with D-PBS, and 100  $\mu$ L of anaesthetic added to each well. After exposure for the designated time (30 or 60 minutes), the anaesthetics were removed by suction, and all of the wells filled with 100  $\mu$ L of DMEM and 10  $\mu$ L of MTT solution (final concentration of MTT: 0.5 mg mL<sup>-1</sup>). After a 3–4-hour incubation, the 96-well plate was emptied and 100  $\mu$ L of dimethyl sulfoxide (DMSO) added to each well to dissolve the formazan crystals. The optical density (OD) was measured at 570 nm using a spectrophotometer (Emax; Molecular Devices, CA, USA). The optical densities were converted into percentages using the following formula:

Cell viability (%) = OD sample/OD negative control  $\times$  100.

Negative control cells were treated with complete DMEM alone instead of anaesthetic.

### Fluorescence microscopy

Samples for fluorescence microscopy were stained with Hoechst 33258 (Sigma-Aldrich) and propidium iodide to distinguish between apoptotic and necrotic cells. The propidium iodide (PI) can only enter the affected plasma membrane just as trypan blue dye can. The Hoechst dye can display the remaining cells which are not stained with PI, and the morphologic changes of apoptosis can be distinguished from the normal nucleus because of this staining. Hoechst staining solution was added to the cell suspensions to a final concentration of 10  $\mu$ M (about 5  $\mu$ g mL<sup>-1</sup>), and the cell-dye mixture was incubated at 37 °C for 60 minutes. Propidium iodide solution (50  $\mu$ g mL<sup>-1</sup>) was added to the mixture shortly before examination by fluorescence microscopy.

The stained nuclei were observed using a fluorescence microscope (S46; Microscopes Inc., MO, USA) with ultraviolet filter (excitation spectrum: 360–400 nm, emission wavelength: 425 nm). Both red (PI) and blue (Hoechst) color could be visualized with this filter (Fig. 2), however, red nuclei were recounted more precisely using green filter (excitation spectrum: 460–550 nm, emission wavelength: 590 nm).

### Flow cytometry

Flow cytometry is an analytical technique for counting and distinguishing microparticles with

their sizes or labeled fluorescence. For the flow cytometric detection of cell damage, equine chondrocytes were labeled with fluorescein-conjugated annexin V and propidium iodide. Annexin V binds to phosphatidylserine (PS) exposed on the outer plasma membrane of apoptotic cells, or on to the inner plasma membrane of necrotic cells where the integrity of the membrane is breached. If the chondrocyte membrane loses its ability to exclude cationic dyes, such as propidium iodide, the dye can easily penetrate the membrane and stain the nucleus.

For flow cytometric analysis, cultured chondrocytes were detached by prewashing with D-PBS followed by trypsinization, and the cell pellet was obtained through centrifugation for 5 minutes at 80–100 *g*. The cells were resuspended in a solution containing one of the three anaesthetics and incubated for 30 minutes. Cells were again recovered by centrifugation, and labeling was performed using the Vybrant Apoptosis Assay Kit #3 (Molecular Probes, OR, USA). The treated cells were washed with cold PBS and the pellet was resuspended in 100  $\mu$ L of 1 $\times$  annexin-binding buffer. Fluorescein isothiocyanate (FITC), annexin V (5  $\mu$ L) and propidium iodide (1  $\mu$ L) were then added to the cell suspension according to the manufacturer's instructions. The cells were then incubated at room temperature for 15 minutes before 400  $\mu$ L of 1 $\times$  annexin-binding buffer was added. The samples were analyzed by flow cytometry (FACSCalibur-S System; Becton Dickinson, NJ, USA) using fluorescence excitation/emission wavelengths of 488/530 nm (FITC-annexin V) and 536/617 nm (PI) respectively. PI-positive and annexin V-positive/negative staining represents late apoptotic, or necrotic, cells. PI-negative and annexin V-positive staining represents apoptotic cells. Both PI- and annexin V-negative staining represents live, non-apoptotic cells.

### Statistical analysis

The MTT results are represented as mean  $\pm$  standard deviation. Significant differences in cell viability were determined using one-way ANOVA (analysis of variance) with respect to the exposure time and the concentration of each anaesthetic. The analysis was followed by the Tukey post-hoc test for multiple comparisons for assessment of the differences between the anaesthetic concentrations. A value of  $p < 0.01$  was considered significant.

## Results

### Trypan blue exclusion assay

The trypan blue exclusion test was used for preliminary evaluation of chondrotoxicity. After 30 minutes of exposure to the local anaesthetic solution, the viability of groups was as follows:  $95.95 \pm 2.75\%$  (saline),  $28.73 \pm 8.44\%$  (bupivacaine),  $66.85 \pm 6.03\%$  (lidocaine), and  $86.27 \pm 2.00\%$  (mepivacaine). After 60 minutes, the viability was further decreased and each viability was as below:  $97.09 \pm 2.06\%$  (saline),  $6.51 \pm 6.72\%$  (bupivacaine),  $31.83 \pm 7.49\%$  (lidocaine), and  $80.43 \pm 1.78\%$  (mepivacaine). Cell viabilities of bupivacaine and lidocaine were significantly lower than those of control and mepivacaine.

### MTT assay

The results of the MTT assay are shown in Fig. 1, which demonstrates both the differences between viability after the different anaesthetic agents, and in the case of bupivacaine, the improved viability when more dilute concentrations of agent were employed. The percentage of live cells in the group subjected to a 30-minute exposure to 0.5% bupivacaine was  $49.56 \pm 12.55\%$ . The viability in the groups subjected to a 30-minute exposure to 2% lidocaine, 2% mepivacaine and saline was  $62.69 \pm 12.37\%$ ,  $84.88 \pm 13.52\%$ , and  $99.31 \pm 4.88\%$  respectively. Each viability of the 60-minute treatment was  $26.55 \pm 16.76\%$  (bupivacaine),  $28.96 \pm 17.27\%$  (lidocaine),  $79.91 \pm 6.21\%$  (mepivacaine), and  $91.28 \pm 12.51\%$  (saline). Thus, with this assay,

bupivacaine appeared the most toxic anaesthetic and mepivacaine the least.

### Fluorescence microscopy

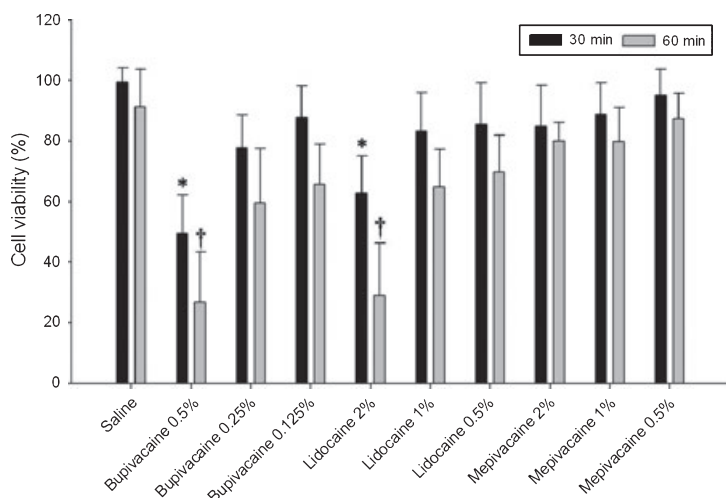
Apparent apoptotic morphologic changes, such as shrunken or fragmented nuclei, were not observed by fluorescence microscopy. However, results similar to those of the trypan blue exclusion assay (i.e., regarding the population of necrotic cells) were obtained using Hoechst-propidium iodide double staining (Fig. 2). The results of viability obtained by the fluorescent microscopic examination were  $36.16 \pm 10.70\%$  (bupivacaine),  $60.08 \pm 5.49\%$  (lidocaine),  $85.63 \pm 3.99\%$  (mepivacaine) and  $96.65 \pm 1.38\%$  (saline). Necrotic cell populations of bupivacaine and lidocaine groups were significantly higher than those of control and mepivacaine groups.

### Flow cytometry

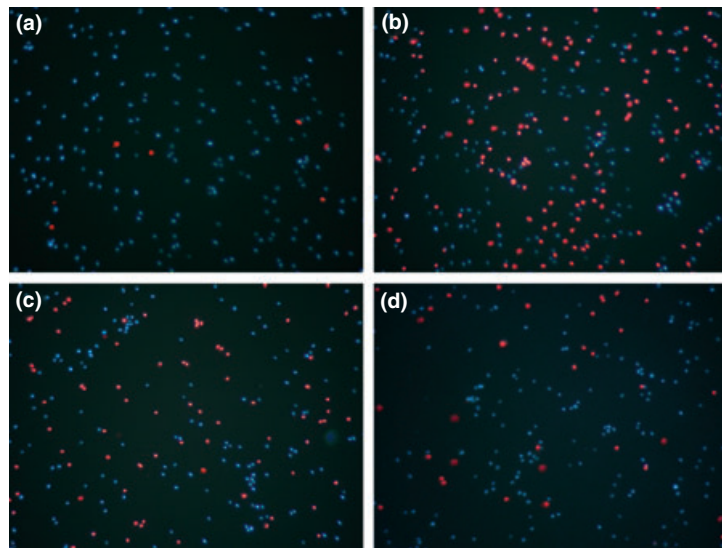
The result of the flow cytometry experiments are expressed as scatter plots (Fig. 3). The upper left and right quadrants represent PI-positive, necrotic populations. The lower right quadrant corresponds to the annexin V-positive/PI-negative, apoptotic cells. The lower left section represents negatively stained cells, which comprise the live cell population.

## Discussion

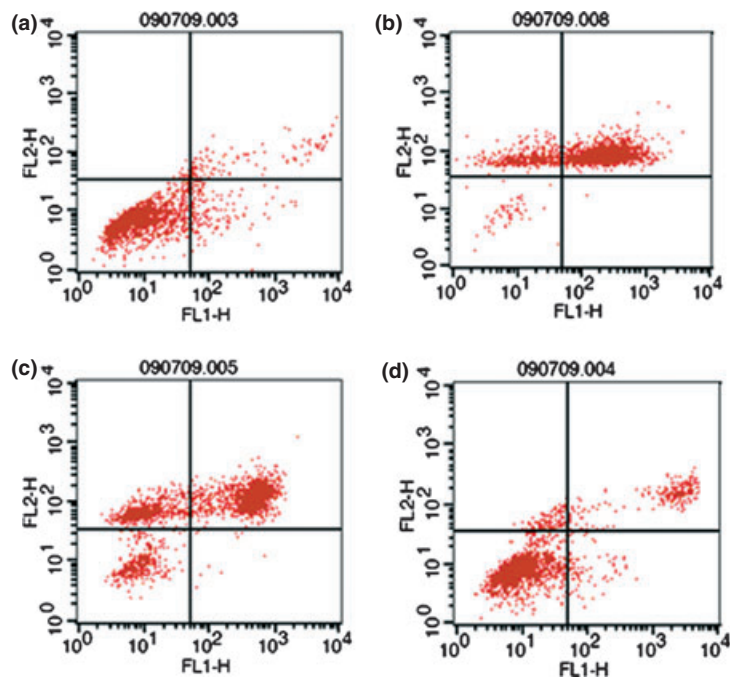
The type and number of agents which are injected into joints has increased steadily in recent years, and although most do not cause problems, some may have adverse side-effects. Local anaesthetics



**Figure 1** MTT assay. The cell viability is represented as a bar graph. The viability of the chondrocytes is significantly lower after a 30-minute treatment with 0.5% bupivacaine and 2% lidocaine compared with saline (\* $p < 0.01$ ). After 60 minutes, 0.5% bupivacaine and 2% lidocaine groups are significantly different from the saline group († $p < 0.01$ ). Black bars = 30-minute exposure, grey bars = 60-minute exposure.



**Figure 2** Fluorescent microscopic examination with Hoechst 33258 (blue) and propidium iodide (red) double staining. Each photograph shows a group of cells treated with saline (a), 0.5% bupivacaine (b), 2% lidocaine (c), or 2% mepivacaine (d). The necrotic chondrocytes are red because propidium iodide can only enter cells with loss of membrane integrity, and the nuclei of intact cells are blue because the intact cells can exclude propidium iodide from their plasma membrane, and can be counterstained with blue Hoechst. These two colors can be visualized with ultraviolet filter.



**Figure 3** Graphic representation of flow cytometry results. (a) saline, (b) 0.5% bupivacaine, (c) 2% lidocaine, and (d) 2% mepivacaine. Each scatter plot has four compartments that consist of necrotic (upper two quadrants), apoptotic (lower right quadrant), and live (lower left quadrant) cell populations.

have been considered to be relatively safe, especially when administered in the precise anatomic location and at the appropriate dose. Undesirable effects (either systemic or local toxicity) generally occur as

a consequence of unintentional intravascular injection, or administration of an excessive dose, when they may result in systemic or local tissue toxicity. However, even after accurate injection of



an appropriate dose, some complications can still occur unexpectedly.

Stimulation of cartilage does not cause pain directly, as there is a lack of neural innervations in these tissues, but intra-articular local anaesthetics provide analgesia through action at the synovial membrane. Thus in equine surgery they are used commonly for the diagnosis of lameness, and also for post-operative pain management after arthroscopy. For this latter use, intra-articular bupivacaine provides a simple and effective protocol for pain management. However, the recent reports concerning damage to joints in humans following the use of intra-articular bupivacaine (Webb & Ghosh 2009) are cause for concern, and even though there have been no reports of detrimental effects of the technique in veterinary use to date, there is need for the potential for the toxic effects of each of the analgesic agents used to be evaluated in the species concerned before clinical veterinary use.

In this study we investigated the difference in the effects of three local anaesthetics on equine chondrocytes. We could control three factors: 1) the local environment (composition of the culture media, CO<sub>2</sub> levels in the atmosphere, and temperature); 2) the subject, namely equine chondrocytes; and 3) the three local anaesthetics. We wanted to combine the local anaesthetics with normal growth media, but mixing the anaesthetics with DMEM resulted in an unexpected precipitation reaction when using bupivacaine. Therefore normal saline (which did not affect MTT results) was chosen as the solvent for each drug. The different concentrations of each drug were obtained by dilution with saline, and saline alone was used for the control group.

The cells could only be harvested for further experiments (e.g., FACS) by trypsinization, and we were concerned that this procedure could induce membrane damage during detachment from the flasks (Schutte et al. 1998). To avoid these problems, the cells were exposed to the experimental agents in suspension rather than as monolayers. Also, in suspension, the cells were completely exposed to the anaesthetics.

The exposure times in the present study were chosen on the basis of the pharmacokinetics of bupivacaine when injected into joints. The time to peak concentration varied depending on the species and the particular anaesthetic. In humans given bupivacaine, the time to peak blood concentration is approximately 43 minutes (Katz et al. 1988), and the peak plasma concentration time of lidocaine is

about 60 minutes during arthroscopic procedures. Experimental data from normal dogs given bupivacaine show a peak plasma concentration time of 10 minutes (Bubenik et al. 2007). Although several factors may influence these times, it is presumed that the anaesthetics may have their toxic effect on the joints <1 hour. Also, our chosen exposure period (30 minutes) was determined on the basis of previous studies.

The results of our cytotoxicity assays were consistent with each other, and the individual drugs showed differing levels of toxicity. The effects of bupivacaine on equine chondrocytes were similar to those on bovine and human chondrocytes (Chu et al. 2006, 2008). The most toxic agent was bupivacaine, followed by lidocaine. Mepivacaine was the least toxic local anaesthetic to equine articular chondrocytes, which is consistent with the opinion that mepivacaine causes less irritation than does lidocaine (Day & Skarda 1991).

The effects of bupivacaine have been investigated on numerous cell types including Schwann cells (Park et al. 2005), tenocytes (Scherb et al. 2009), neuronal cells (Perez-Castro et al. 2009), and corneal endothelial cells (Chang et al. 2006). However, bupivacaine showed different toxic effects on the different cell types. Some cells underwent apoptosis, whereas others became necrotic.

The mechanism of toxicity triggered by local anaesthetics has not been fully elucidated, and further studies are needed to explain the different mechanisms that operate in different cells. In the present study, necrosis may be considered to be the main mechanism of equine chondrotoxicity, according to flow cytometric analysis and Hoechst 33258 nuclear staining. However, we cannot completely rule out a correlation between apoptosis chondrocytes and local anaesthetics.

From a clinical point of view, normal chondrocytes are surrounded by the extracellular matrix. This matrix has a chondro-protective function, both mechanically and chemically. Regardless of this protective effect, a highly toxic agent such as bupivacaine can damage not only injured cartilage, but also intact cartilage (Chu et al. 2006).

In conclusion, our study shows that, of the three agents tested bupivacaine is the most toxic local anaesthetic to equine articular chondrocytes *in vitro*, followed by lidocaine (slightly less chondrotoxic than bupivacaine), and both are significantly more toxic than mepivacaine. The results are not necessarily indicative of what happens *in vivo*, as it is

possible that damage to the chondrocytes is limited by the fact they are surrounded by the abundant extracellular matrix. However, those using bupivacaine and lidocaine for clinical use intra-articularly should be aware of the risk for patients with cartilage disorders by reason that chondrocytes could be exposed to such local anaesthetics directly. In the future, ropivacaine, which has a similar duration of action to bupivacaine and is less toxic to human chondrocytes (Piper & Kim 2008), might become useful for prolonged intra-articular analgesia in horses but until the necessary toxicology studies have been performed, mepivacaine may be a better alternative for equine practice and carry less risk.

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