

Lidocaine cytotoxicity to the bovine articular chondrocytes in vitro: changes in cell viability and proteoglycan metabolism

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Abstract

Purpose A lot of studies on the effect of intra-articular injections are clinical, but many questions on the effect of lidocaine to articular chondrocytes remain unanswered. This study was performed to determine the effects of varying concentrations and exposure times of lidocaine on the viability and proteoglycan metabolism of chondrocytes in vitro. **Method** Cartilage was obtained from metatarsal joints of adult bovines. Chondrocytes in alginate beads were cultured in medium containing 6% fetal calf serum at 370 mOsmol at cell densities of 4 million cells/ml. They were then cultured for 24 h under 21% oxygen with 0.125, 0.25, 0.5, and 1% lidocaine and without lidocaine as control. The cell viability profile across intact beads was determined by manual counting using fluorescent probes and transmission electron microscopy.

Result Lactate production was measured enzymatically as a marker of energy metabolism. Glycosaminoglycan (GAG) accumulation was measured using a modified dimethylmethylene blue assay. Cell viability decreased in a time- and dose-dependent manner in the concentration range of 0.125–1.0% lidocaine under the confocal microscope. Under the electron microscope, apoptosis increased as the concentration of lidocaine increased. GAG accumulation/tissue volume decreases as the concentration of lidocaine

increased. However, GAG produced per million cells and the rate of lactate production per live cell were significantly higher for cells cultured at 0.5 and 1% lidocaine than the control group. Bovine chondrocytes cultured in alginate beads under high oxygen pressure are negatively influenced by increasing concentrations of lidocaine.

Conclusion Cell viability and proteoglycan production (GAG accumulation/tissue volume) decreased as the concentration of lidocaine increased. These data suggest caution in prolonged exposure of cartilage to high concentration lidocaine. Repeated joint injection of lidocaine potentially worsens osteoarthritis by accelerating cartilage degradation.

Level of evidence Therapeutic studies—investigating the results of treatment, Level III.

Keywords Articular chondrocyte · Lidocaine · Cytotoxicity · Proteoglycan · In vitro

Introduction

Pain during activities of daily living is a common presenting complaint of individuals with osteoarthritis and is also associated with a decrease in quality of life for people with osteoarthritis. Intra-articular single bolus injections containing lidocaine are frequently used clinically for symptomatic treatment of peripheral osteoarthritis joint, especially the knee joint. Lidocaine can itself have some mild anti-inflammatory effect during its relatively short half-life of 1.5 h [33]. Recently, however, chondrolysis has been reported with the use of continuous intra-articular anesthetics infusion pumps following arthroscopy [1, 14, 27, 32]. Several papers reported toxicity of lidocaine [6, 16], bupivacaine [3, 5, 28], and ropivacaine [28] to

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articular cartilage in the diarthrodial joints of the lower extremity in humans [5, 28], bovine [3, 6, 19, 34], and rabbits [5]. So far, however, a lot of studies on the effect of joint injections are clinical, but many questions on the effect of lidocaine to articular chondrocytes remain unanswered. And also, there were some recent morphological evaluations of the cell viability to the anesthetics in the literature [3, 5, 6, 16, 28], but the effect of proteoglycan metabolism following exposure to lidocaine has not been extensively reported. Proteoglycan (PG) accounts for about 7–10% of cartilage tissues, and aggrecan, which is a member of PG representing macromolecules, plays a key role for mitigation of mechanical stress imposed on the cartilage tissues. A decrease in proteoglycans, in particular glycosaminoglycans (GAGs), is seen from early changes in osteoarthritis. The goals of this study are to examine how lidocaine concentration influences the GAG accumulation of articular chondrocytes in a three-dimensional cell culture system.

Materials and methods

The experiment was carried out under the control of the local animal ethics committee in accordance with the guidelines on animal experiments in Fukui University (Fukui, Japan), Japanese government animal protection and management law, and Japanese government notification on feeding and safekeeping of animals. Bovine metacarpal phalangeal joints from 18- to 24-month-old steers were obtained from a local abattoir within 2–3 h of slaughter and dissected aseptically. Cartilage from 21 feet was used for the experiments described here. The articular cartilage was removed from the joints using a scalpel, and the pooled tissue digested enzymatically for 18–20 h at 37°C in an incubator under 95% air and 5% CO₂. The digestion medium consisted of Dulbecco's modified Eagle's medium (DMEM: cat. no 22320-022 with 25 mM Hepes, 1 mM sodium pyruvate, 1,000 mg/l glucose, Invitrogen, Carlsbad, CA) containing 1 mg/ml collagenase-1 (Sigma–Aldrich, Poole, Dorset, UK), antibiotics/antimycotics (500 units ml⁻¹ penicillin G, 500 µg ml⁻¹ streptomycin sulfate and 25 µg ml⁻¹ amphotericin B, Life Technologies Ltd., Paisley, UK) made to 370 mOsm with NaCl. After incubation, the digested tissue suspensions were filtered initially through a coarse filter to remove undigested tissue and then through a 20-µm-pore cell strainer (Sigma–Aldrich). The cells in the filtrate were then washed three times by repeated centrifugation (2,500 rpm for 5 min) and resuspended in DMEM. The cell suspension was assessed manually using a hemocytometer and trypan blue exclusion for cell viability and cell number. Only cell preparations with cell viabilities >95% were then used.

Cell culture

The cells were encapsulated in alginate beads [13]. Briefly, the washed articular chondrocytes were resuspended uniformly into 1.2% low viscosity alginate (Fluka Biochemika, Kent, UK) at controlled cell densities by gentle pipetting. Microspheres were then formed by expressing the cell suspension through a 21-G needle attached to a 5-ml syringe into a 102 mM CaCl₂ solution (Nakarai Tesque Inc., Kyoto, Japan). The microspheres were then washed twice with 25 ml of 0.9% NaCl solution and washed twice again with 25 ml of DMEM. The beads were carefully placed into 48-well culture plates (5 beads per well); in each separate experiment, under each condition tested, 3 wells were used for biochemical analysis, 3 for measurement of sulfate incorporation rate, and 5 wells for histological examination of the beads.

The multi-well plates were then cultured for 1, 12, and 24 h at 37°C in DMEM containing 6% fetal bovine serum (Life Technologies Ltd.), 0.5% antibiotic/antimycotic, and 0.5% gentamycin (Life Technologies Ltd.) at 370 mOsm under 5% CO₂/95% air with 0.125, 0.25, 0.5, and 1% lidocaine (AstraZeneca, Osaka, Japan) and without lidocaine as control. Medium osmolality was controlled at 370 mOsm by the addition of NaCl and was monitored using a freezing point osmometer (Semi-micro osmometer, Knauer, Germany).

Measurement of cell viability and metabolism

At each time point, the cell viability in the beads examined by Live/Dead assay (Cambridge Biosciences, Cambridge, UK) under confocal microscope (Leica TCS SP2, Wetzlar, Germany). Ethidium homodimer-1 cannot penetrate live cells, but stains the DNA of dead cells red. Calcein-AM penetrates live cells where an esterase cleaves the molecule, which then fluoresces green—dead cells contain no esterase. In order to visualize cells, the beads were cut into half using a blade and soaked in the Live/Dead solution for 1 h. The number of live (green) and dead (red) cells was counted manually in the beads. At least 4 fields each containing at least 15 cells was counted in each area. The proportion of live and dead cells was then calculated from pooled results.

The beads ($n = 12$) in 3 separate experiments under 5 different concentrations of lidocaine were examined under the electron microscope (Hitachi H-7000, Tokyo Japan) after 24 h. After the beads were fixed by immersion in 2.5% glutaraldehyde with 0.15 M cacodylate buffer (Nakarai Tesque Inc.) at 4°C for 4 h, the specimens were postfixed in 2% OsO₄ (Nakarai Tesque Inc.), impregnated with 2% uranyl acetate (Nakarai Tesque Inc.), dehydrated in graded ethanol, and embedded in Epoxy resin. Ultrathin

sections contrasted with uranyl acetate and lead citrate were examined under an electron microscope.

Lactic acid production was used as a marker for cell metabolism as glycolysis is the major ATP-generating pathway for chondrocytes [18]. After 24 h, lactic acid production was measured using a commercial kit (Product no. 735-10, Sigma) using lactate dissolved in DMEM as a standard. Ten-microliter aliquots of culture medium and 100 μ l of the lactate reagent were added to each well into a 96 multi-well plate. The plate was left at room temperature for 10–15 min, and the absorbance recorded at 540 nm on a microplate spectrophotometer (Spectra MAX 250, Molecular Devices, CA). The beads from 3 wells at each condition were labeled with ^{35}S -sulfate. Beads were resuspended in 0.5 ml DMEM, supplemented with 5 $\mu\text{Ci ml}^{-1}$ of $^{35}\text{SO}_4^{2-}$ (Amersham Biosciences, Bucks, UK), and incubated for further 4 h at 37°C and 5% CO_2 . Radiolabeling was stopped by washing the beads twice in ice-cold PBS supplemented with 2 mM CaCl_2 , 5 mM Na_2SO_4 at 4°C, and then freezing at -20°C . Labeled alginate beads were defrosted and digested in 0.5 ml of PBS and 0.5 ml of citrate buffer. The solution was then exhaustively dialyzed to separate the bound and free $^{35}\text{SO}_4^{2-}$. The dialysate and tubing were then placed into a scintillation vial, and 4 ml of scintillation (Liquidscint, LSC-5100, Aloka, Japan) was added. The activity of the dialysate and of 20 μ l samples of the incubation medium was measured on a beta scintillation counter. Rates of sulfated GAG synthesis were calculated assuming that the specific activity of the $^{35}\text{SO}_4^{2-}$ sulfate was the same in the medium and in the incorporated GAGs.

GAG accumulation was measured using a modified dimethylmethylene blue (DMB) assay (Serva Feinbiochemica, Heidelberg, Germany) [7]. Beads from another 3 wells were removed from the medium and weighed and dispersed in 3 volumes of citrate buffer containing 0.56 unit/ml papain (Sigma–Aldrich). The remaining solution was heated to 67°C overnight in a sealed tube to digest the proteoglycans. The absorbance of aliquots of the bead digest added to the DMB buffer was read at 595 nm using a UV/VIS spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan). GAG concentrations were estimated from a standard curve of chondroitin sulfate made up in 0.1% alginate in citrate buffer.

Statistical analysis

Unless otherwise stated, data are presented as the mean \pm the standard error of the mean (SEM) of at least five separate experiments ($n > 5$). Each experiment was carried out in triplicate. Significant differences in prior comparison were determined using a one-way ANOVA. Data were compared using repeated measures analysis of

variance followed by Scheffé's post hoc analysis. Data were entered into a database and analyzed by using SPSS statistical software, version 14.0.J (SPSS Inc, Chicago, IL). A probability of 5% was considered statistically significant.

Results

Cell viability profiles

The viability of chondrocytes in the bead was determined using confocal microscopy and fluorescent dyes that stained live cells green and dead cells red (Fig. 1). Cell viability decreased as the concentration of lidocaine increased. Figure 2 gives pooled data for five representative lidocaine concentrations from 7 separate experiments for cells cultured by chondrocytes. Cell viability decreased in a time- and dose-dependent manner in the concentration range of 0.125–1.0% lidocaine after 1 ($F = 139.73$, $P < 0.05$), 12 ($F = 152.53$, $P < 0.05$) and 24 h ($F = 140.23$, $P < 0.05$). After 24 h, chondrocyte viability was 90% or higher in the control group, while it was 84.0%, 58.9%, 24.5%, and 6.0% in 0.125%, 0.25%, 0.5%, and 1% lidocaine concentrations, respectively.

Transmission electron microscopy

Typical transmission electron micrographs of chondrocytes are shown in Fig. 3. In the control group, all cells appeared viable, with large nuclei, dotted with chromatin, and abundant rough endoplasmic reticulum (Fig. 3a, b); the cells appeared active throughout the bead. However, in the beads cultured with 1% lidocaine at high density, cells undergoing apoptosis were seen in the beads (Fig. 3c, d); the cells and nuclei were reduced in size, and chromatin condensation was visible in the nuclei. Cells with condensed and fragmented nuclei and condensed chromatin were visible (Fig. 3d). In the beads cultured up to 0.25% lidocaine, cells undergoing apoptosis were seen in the periphery; the cells and nuclei were reduced in size, and chromatin condensation was visible in the nuclei.

Lactate production rates and sulfate incorporation rates

Figure 4a shows the effect of lidocaine on lactate production by chondrocytes, a marker for total energy production. The rate of lactate production per live cell was significantly higher for cells cultured at high concentration than those at low concentration. The results of ANOVA showed the increase in lactate production rate as the concentration of lidocaine increased after 24 h ($F = 116.15$, $P < 0.05$). Similarly, the rate of sulfate incorporation per live cell was greater at high than at low concentration of lidocaine

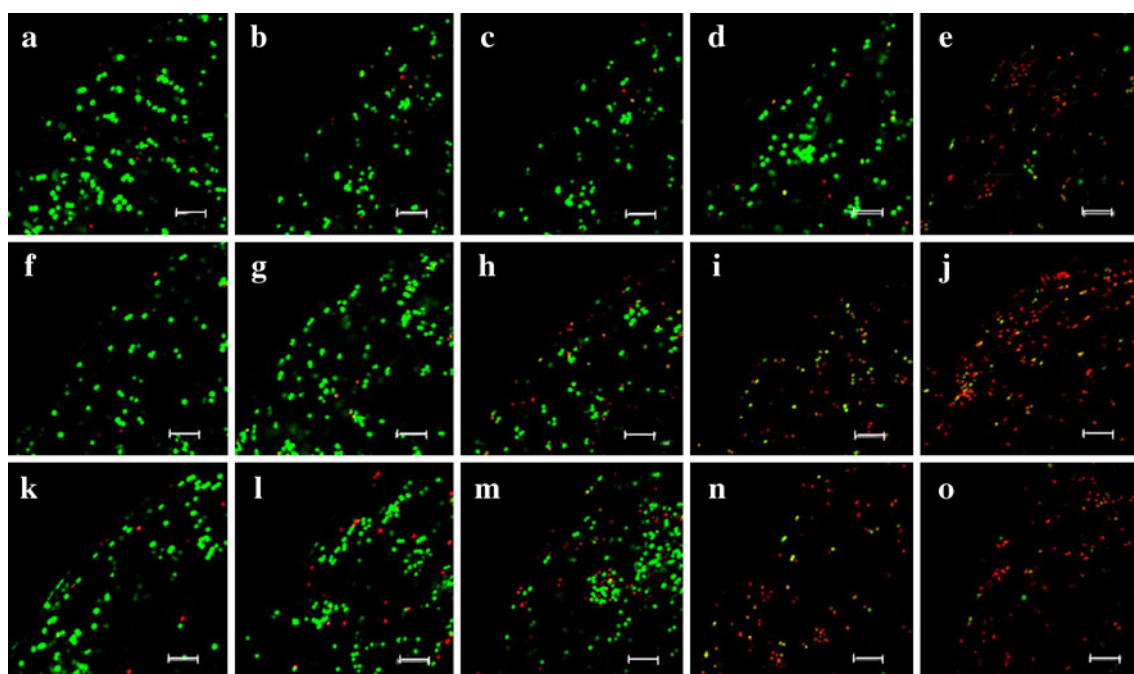


Fig. 1 Confocal microscopic micrographs of chondrocytes after 1 (a–e), 12 (f–j) and 24 h culture (k–o). a, f, k 0% lidocaine [control], b, g, l 0.125%-lidocaine, c, h, m 0.25%-lidocaine, d, i, n 0.5%-lidocaine, e, j, o 1%-lidocaine. Scale bar 100 μ m

($F = 55.14$, $P < 0.05$), though the difference was less marked than that seen in Fig. 4a. (Fig. 4b)

Glycosaminoglycan (GAG) content

Figure 5 gives pooled data for each lidocaine concentrations from 7 separate experiments for cells cultured by chondrocytes. After 24 h, a bimodal response was evident with the concentration of GAG accumulated falling as lidocaine concentration was increased from 0 to 1.0% (Fig. 5a). However, the GAG production per million cells increased as lidocaine concentration was increased and is greater at 1% lidocaine (Fig. 5b).

Discussion

The most important finding of the present study suggests that loss of healthy chondrocytes and proteoglycan production contributes to cartilage degeneration after intra-articular injection of lidocaine. Intra-articular injection of local anesthetics is a common practice. It may provide short-term pain relief in conjunction with steroids. A lot of studies on the effect of corticosteroids on articular chondrocytes had been carried out [4, 36], but many questions on the effect of lidocaine on articular chondrocytes remain unanswered. Lidocaine has been considered a benign and specific sodium channel blocker, and it is widely used as a local anesthetic. However, potency and ratio of effective

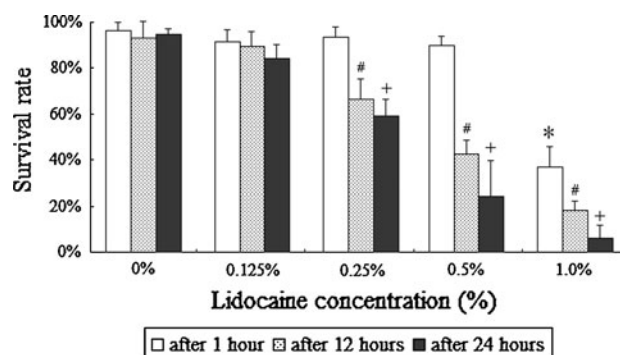


Fig. 2 Effect of lidocaine concentration on cell viability under the confocal microscope. * $P < 0.05$ by Scheffe's test for control group (0%-lidocaine) vs. 1% lidocaine group after 1 h culture. [#] $P < 0.05$ by Scheffe's test for control group vs. 0.25-, 0.5-, or 1% lidocaine group after 12 h culture. ⁺ $P < 0.05$ by Scheffe's test for control group vs. 0.25-, 0.5-, or 1% lidocaine group after 24 h culture

dose to overdose are important clinical characteristics of anesthetics. Recent evidence suggests that intra-articular injection of local anesthetics may have the adverse effect of damage to articular cartilage. The first such study was circumstantial, demonstrating increased inflammation in the articular cartilage of rabbit knee joints following the injection of 0.5% bupivacaine when compared with normal saline [5]. Then, in 2006, Chu et al. [3] demonstrated that 0.5% bupivacaine solution is toxic to bovine articular chondrocytes in vitro, even following only brief (15–30 min) exposures. Karpie et al. [15] showed that in vitro exposure of bovine articular chondrocyte cultures

Fig. 3 Electron micrographs of chondrocytes after 24 h culture. **a, b** Control group. **c, d** 1% lidocaine group. (Original magnification, **a, b** $\times 200$, **c, d** $\times 700$)

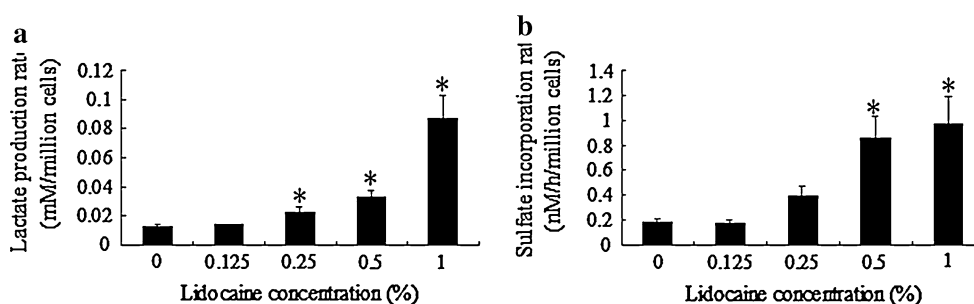
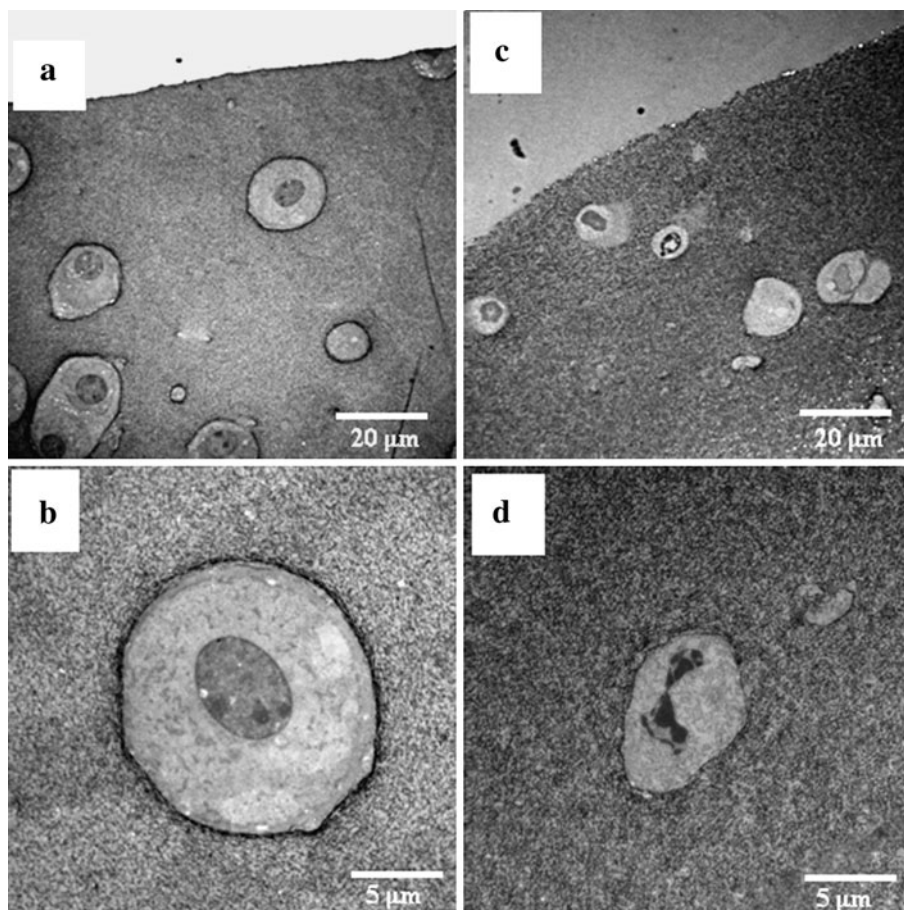


Fig. 4 Effect of lidocaine concentration on lactate production rate (**a**) and ^{35}S -sulfate incorporation rate (**b**). **a** The rate was significantly higher for cells cultured at 0.25, 0.5 and 1% lidocaine than control group ($*P < 0.05$ by Scheffe's test for control group vs. each group).

b Sulfate incorporation rates rise with increase in lidocaine concentration and with decrease in cell density ($*P < 0.05$ by Scheffe's test for control group vs. each group)

obtained from knee joints to lidocaine solutions results in significant cytotoxicity in a time- and dose-dependent manner, including concentrations as low as 1% and exposure times as short as 15 min. Similar, through less dramatic, in vitro toxicity to bovine articular chondrocytes was also observed with 1% lidocaine. These experimental findings raised concerns about intra-articular use of local anesthetics in human joints.

Synovial fluid exists about 0.5–4.0 ml in human healthy knee joint [23]. If we injected 3.0 ml of 1% lidocaine into

joint cavity, the concentration of lidocaine will theoretically be about 0.45–0.85% in the joint cavity. In this study, the chondrocyte cytotoxicity of lidocaine is also dose and time dependent. High-dose lidocaine (up to 0.25%) showed significant chondrocyte death (apoptosis). The mechanisms by which a local anesthetic might induce chondrotoxicity are not well understood. As noted previously, there is a relationship between anesthetic exposure and chondrocyte inflammation [2]. Lidocaine selectively up-regulates pro-inflammatory cytokines such as interleukin (IL)-1 β , IL-6,

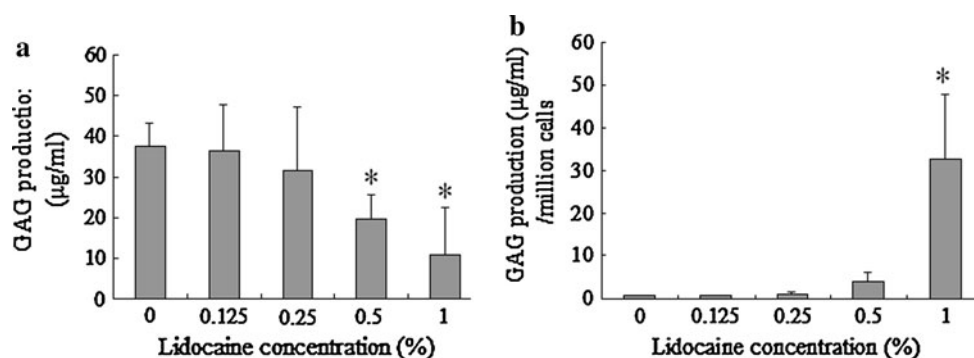


Fig. 5 Effect of lidocaine concentration on GAG concentration (**a**) and GAG accumulation per million cells (**b**) by chondrocytes after 24 h in culture. **a** The results of ANOVA showed the decrease in GAG accumulation/tissue volume as the concentration of lidocaine increased after 24 h ($F = 8.18$, $P < 0.05$). The rate was significantly lower for cells cultured at 0.5 and 1% lidocaine than control group

(* $P < 0.05$ by Scheffe's test for control group vs. each group). **b** On the other hand, GAG produced per million cells is greater at high concentration of lidocaine ($F = 6.89$, $P < 0.05$). The rate was significantly higher for cells cultured at 1% lidocaine than control group (* $P < 0.05$ by Scheffe's test for control group vs. each group)

IL-12, and tumor necrosis factor (TNF)- α , and down-regulates anti-inflammatory cytokines such as IL-4, IL-10, and IL-13, suggesting that it may impair resolution of ongoing inflammation. Apoptosis may also play a role in lidocaine-induced cytotoxicity. Manjo et al. [21] reported oncosis, a form of cell death accompanied by cellular swelling, organelle swelling, blebbing, and increase membrane permeability. They also showed that necrosis can occur after both forms (oncosis and apoptosis) of cell death. Therefore, its mechanism is based on failure of the ionic pumps of the plasma membrane induced by the changes in the extracellular osmotic environment. Lidocaine has been shown to induce apoptosis and/or necrosis in neuronal [9, 29] and muscle tissues [8]. Mitochondria are known as key players in the regulation of apoptotic pathways [26]. Grouselle et al. [12] demonstrated that local anesthetics, such as lidocaine and bupivacaine, could reach mitochondria in cell culture and collapse mitochondria transmembrane potential leading to programmed cell death. They also showed potential restoration of mitochondrial transmembrane potential after removal of the local anesthetic.

Articular cartilage has poor ability for tissue restoration. A decrease in proteoglycan, especially GAG, is noted from the early stage of cartilage degeneration and is a major factor precipitating cartilage abrasion and arthralgia that are seen in osteoarthritis [22, 38]. Cartilage tissues are avascular and aneural, and chondrocytes are embedded in the cartilage matrix. Chondrocytes essentially owes it to concentration gradient to receive nutrition and oxygen supply from the surrounding tissues and evacuate metabolic products [37]. Cartilage tissues have osmolarity of 350–500 mOsm [24], pH of 6.8–7.0 [40], and oxygen concentration of 1–5% [11]. So, the chondrocyte extracellular environment is relatively hypoxic, unlike the case of other tissues. In this severe extracellular environment,

chondrocytes account for only 1% or less of the total tissue, but still regulate the metabolism of the whole matrix [35]. The turnover of proteoglycans that make an important contribution to the mechanical strength of cartilaginous tissue occurs after about 20 years [30], while renewal of collagen takes more than 100 years [39], so the matrix is renewed slowly over an extremely long period. It is reported that such a harsh environment suppresses chondrocyte differentiation and maintains the natural characteristics of these cells. In this study, articular chondrocytes were observed in vitro, and GAG accumulation was found to decrease in relation to the concentration of lidocaine. It was decreased by about 71% at 1% lidocaine compared with the level in the control group (Fig. 5a). This result is in agreement with the results of confocal microscopic observation in which the cell viability rate decreased in dependence on the concentration of lidocaine (Figs. 1, 2), suggesting that the decrease in viable cells was the major reason for the decrease in GAG accumulation. However, GAG accumulation per cell (Fig. 5b) and lactate production per cell (Fig. 4a) increased in a dose-dependent manner with the concentration of lidocaine. This difference arose at least in part from a fall in the metabolic activity of the cells rather than because of the increased loss of GAG, as was shown by the higher rate of sulfated GAG production/viable cell (Fig. 4b) in cells cultured at low cell densities induced by lidocaine of higher concentration. Kobayashi et al. [17] examined how cell density influences the rate of GAG accumulation in a three-dimensional cell culture system.

They used alginate gels in the form of beads as a model system, so that cells could be recovered readily. This avascular constructs, unless experimentally perfused, rely on diffusion for supply of nutrients to the cells [25] simulating the condition seen in the cartilage tissue. In

avascular tissues and in constructs, there are steep gradients of oxygen and other nutrients between the surface and center of the tissue or constructs [16, 20]. The steepness of these gradients, and hence the nutrient concentrations in the construct, depends not only on the geometry and properties of the tissue or construct but also on the cell density and the cellular activity [31, 41]. Thus, in any particular construct or tissue, a decrease in cell density will lead to a corresponding rise in the concentration of nutrients such as oxygen and glucose and an increase in metabolic by-products such as lactic acid leading, once cell density has fallen sufficiently, to a rise in rates of cell metabolism and GAG synthesis [10, 41]. Diffusional nutrient transport is thus a limitation on the number of viable and active cells which can be maintained in any construct or tissue [42].

Joint pain is associated with a decrease in quality of life for people with osteoarthritis. Pain during activities of daily living is a common presenting complaint of individuals with osteoarthritis. Therefore, intra-articular injection of lidocaine is commonly used with hyaluronic acid and/or steroids to relieve joint pain. Lidocaine may modulate its ability to penetrate into intracellular spaces of cartilaginous tissue, allowing for reduced toxicity with even small dilutions from clinical levels. In this study, our physiological and morphological study showed that articular chondrocytes are unable to adjust to such non-physiological conditions lasting for a long time and this phenomenon plays a critical role in the development of cartilage degeneration and resultant osteoarthritis. Although study methods permitted reproducible quantitative analysis, limitations were that this was an *in vitro* study that used bovine cartilage. While these *in vitro* results cannot be directly extrapolated to the clinical setting, these data showing a dose-dependent manner between GAG production and lidocaine concentration provide both a potential mechanism for lidocaine-induced chondrotoxicity and a potential explanation for the importance of dilution in reducing the potential for chondrotoxicity in the clinical setting.

Conclusion

Cell viability and proteoglycan production (GAG accumulation/tissue volume) decreased as the concentration of lidocaine increased. These data suggest caution in prolonged exposure of diarthrodial joints to high concentration lidocaine and support using the lowest concentrations and volumes of lidocaine for the shortest period of time to achieve clinical treatment goals. Clinicians should be aware of this complication that repeated intra-articular

injection of lidocaine potentially worsens osteoarthritis by accelerating cartilage degradation.

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