Effects of Lidocaine on Migration, Proliferation, Collagen Expression and Cytotoxicity of Tendon Cells

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Original Article

Effects of Lidocaine on Migration, Proliferation , Collagen Expression and Cytotoxicity of Tendon Cells

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Background: Lidocaine is commonly used for the treatment of a variety of tendinopathies in combination with corticosteroids injection.

Purpose: The goal of this study was to evaluate the effect of lidocaine on migration, proliferation, protein expressions of type I and III collagen and cytotoxicity of tendon cells.

Methods: Rat Achilles tendon cells were divided into control and study groups (treated with 0.1%, 0.15%, and 0.2% lidocaine). Tendon cell migration and proliferation were assessed using transwell filter migration assay and MTT assay, respectively. Cytotoxicity of lidocaine was evaluated by the LDH-Cytotoxicity assay. The protein expression of type I and type III collagen were evaluated by immunocytochemical staining.

Results: Lidocaine significantly reduced tenodon cell migration (p < 0.001). Cell death was measured by LDH assay and cytotoxicity increased dose-dependently (p < 0.001). A dose-dependent lidocaine inhibition on tendon cell proliferation was demonstrated by MTT assay (p<0.001). Immunocytochemical staining revealed that the protein expression of type I and III collagen were not affected by lidocaine treatment

Conclusion: Lidocaine inhibits tendon cell migration and proliferation. Lidocaine induced cell death on tendon cells. However, the protein expression of types I and III collagen remained unchanged. (Tw J Phys Med Rehabil 2018; 46(2): 71 - 79)

Key Words: lidocaine, local anesthetics, tendons, tendon healing

INTRODUCTION

Most local anesthetics are classified as amides or esters.[1] Lidocaine is a local anesthetic in the amide class and is the most commonly used anesthetic for local infiltration. Lidocaine blocks both initiation and conduction of nerve impulses by decreasing ionic flux through the neuronal membrane by blocking sodium channels, which results in inhibition of depolarization with resultant blockade of conduction and causes local anesthesia.[2] Lidocaine is usually given as a 1 percent solution (10 mg/mL). Higher concentrations of lidocaine beyond 1 percent do not promote improved onset or duration of analgesia and may increase the risk of toxicity.[3] Complications during infiltration of local anesthetics are rare.

Avoidance of intravascular injection into major vessels and care not to exceed the maximum total dose of anesthetic helps to avoid complications in most patients. Systemic toxicity, such as central nervous system (CNS) and cardiovascular symptoms, is an unusual event, but can occur if the recommended dose is exceeded, if a major vessel is inadvertently injected with a large amount of anesthetic, or as an idiosyncratic response.

Tendons act as transducer of force from muscle to bone. Approximately 90% of collagen in normal tendons is type I and less than 10% is type III collagen.[4] Tendon cells (fibroblasts), the basic cellular component of tendon, are the source of collagen production, protein mediators of repair, and matrix proteoglycans.[5] In the initial inflammatory phase of the response to tendon injury, inflammatory cells enter the site of injury. In the regenerative phase that follows within days of injury, tendon cells in the epitenon migrate to the repair site, proliferate, and mediate tissue repair by synthesizing collagen. Different cells are postulated to contribute via production of different types of collagen at different time points.

Peri-tendinous injection of local anaesthetic, both alone and in combination with corticosteroids, is commonly performed in the treatment of tendinopathies, such as lateral and medial epicondylitis of the elbow, de Quervain's disease, patellar and pes anserine tendinopathies, Achilles tendinopathy, and biceps tendinopathy of shoulder.[6-11] Previous studies have shown that local anaesthetics and corticosteroids are chondrotoxic, but their effect on tenocytes remains unknown.[11-14]

The toxicity of local anesthetics on tendon fibroblasts has shown in vitro studies [15-18]. Lehner et al. reported that the treatment of rat tendon derived cells with 0.5% bupivacaine for 10min had detrimental effects on cell viability.[15] Piper et al. showed lidocaine was significantly toxic to the tenocytes in a dose-dependent manner after a 30-minute exposure to 1% and 2% lidocaine on bovine tendon fibroblasts by using the CellTiter-Glo viability assay and fluorescence-activated cell sorting (FACS) for live/dead cell counts.[16] Yang et al. concluded that lidocaine potentiated the deleterious effects of triamcinolone acetonide (TA) on cultured tenocytes derived from rat patellar tendons. Cells lost orientation and became attened after exposed to TA or 1% lidocaine and had more obvious morphologic change after the combination therapy with TA and 1% lidocaine. Tendon cell viability (Dojindo, Kumamoto, Japan) was lowered after exposed to TA or lidocaine and was significantly lower with TA and 1% lidocaine. When the tenocytes were incubated with the combination of TA and lidocaine, the expressions of tenocyte related genes (Collagen I, scleraxis (SCX), tenomodulin (TNMD) using RT-PCR) were significantly decreased when compared with TA or 1% lidocaine administration alone.[17] Sung et al. compared the cytotoxic effects of ropivacaine, bupivacaine, and lidocaine on human rotator cuff tendon fibroblasts and showed high cytotoxicity of lidocaine compared with the other agents, even in low concentration (1% lidocaine).[18]

Although lidocaine was shown to have deleterious effects on tendon cells by in vitro studies,[15-18] it is most frequently used in clinical practice as a diagnostic test and for the treatment of various musculoskeletal diseases and tendinopathies. The effects of lidocaine on tendon migration as well as the protein expression of type I and III collagen have not been studied extensively. The goal of this study was to evaluate the effect of lidocaine on the migration and proliferation of tendon cells and the protein expression of type I and III collagen; its cytotoxicity was evaluated as well.

METHODS

All procedures were approved by the Institutional Animal Care and Use Committee.

Primary Culture of Rat Achilles Tendon Cells

The Achilles tendons were obtained from Sprague-Dawley rats (weighting 200 to 250 g). The excised tendon was soaked in povidone-iodine for 3 min and washed twice in phosphate-buffered saline (PBS). Each tendon was then cut into small pieces of approximately 1.5–2.0 mm^3 (6 pieces in total from each tendon). The tendon fragments were placed individually in 6-well culture plates. After 5 min of air-drying to improve adherence, 0.5 mL of Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Grand Island, New York, USA), with 10% fetal bovine serum (FBS; Gibco, Life Technologies), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Life Technologies)

were added to each well. The explants were then incubated at 37°C in a humidified atmosphere of 5% CO2/95% air. After migrating from the explants, the cells grew rapidly and the confluence culture was subcultured using trypsin digestion at a 1:3 dilution ratio. Tendon cells between passages 2 and 4 showed proper growth rate and a normal fibroblast shape and were used in subsequent studies.

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide; Thiazolyl blue) Assay for Cell Viability

MTT assay was used to detect cell survival and proliferation in lidocaine-treated tendon cells. Cells were first seeded into 24-well flat-bottomed culture plate where lidocaine was added at the concentration of 0 (control group), 0.1%, 0.15%, and 0.2%. Twenty-four hours later, the cells were observed under a microscope and then MTT assay was performed. DMEM, containing 50 μg/mL MTT, was added to each well and the plate was incubated at 37 °C for one hour. Then, the MTT solution was discarded and the 1 ml dimethyl sulfoxide (DMSO) was added to each well. The colorless DMSO turned purple after the formazan crystals were dissolved by mixing using the micropipette. Aliquots were transferred from the wells into a 96-well plate and optical density (OD) was measured immediately at 570 nm with a spectrophotometer (NanoDrop 1000, Thermo Scientific, Wilmington, DE). Relative fold changes in OD_{570} value for lidocaine treated cells to the control cells were calculated. The experiments were performed in triplicate.

Transwell Filter Migration Assay

Transwell filters (Costar, Cambridge, MA, USA) with 8.0 μm pores were used for the migration assay. Tendon cells cultured for 24 h without and with lidocaine at various concentrations (controls versus cultures at 0.1%, 0.15%, and 0.2% lidocaine), were seeded at a density of 1.2×10^5 cells per filter. The inner chamber was filled with 250 μL of serum-free DMEM while the outer chamber was filled with 600 μL of DMEM with 10% FBS. Cells were allowed to migrate for 3 hours at 37 °C in an atmosphere of 95% air/5% CO_2. The cells were stained with Liu's stain and then washed twice in PBS.

Cells on the upper surface of the filter were removed using a cotton swab. Cells on the lower surface were counted under four random high-power microscopic fields (HPF) (100 × magnification) per filter and the mean number of migrating cells was calculated at each treatment concentration. The experiments were performed in triplicate.

Lactate dehydrogenase (LDH) assay for cytotoxicity

Cell death or cytotoxicity was classically evaluated by the quantification of plasma membrane damage. The LDH-cytotoxicity assay provides quantitated cytotoxicity based on the measurement of the activity of lactate dehydrogenase (LDH) released from damaged cells. Tendon cells were either left untreated or treated with 0.1%, 0.15%, 0.2% lidocaine for 24h, following which, 100 μL supernatant was transferred to an optically clear 96-well plate. The presence of LDH in the conditioned medium was detected using the LDH-Cytotoxicity Colorimetric Assay Kit (BioVision, Catalog#K311-400, USA) according to the manufacturer's instructions. Briefly, LDH reaction mixture was added to each well and incubated for 30 min at room temperature. Absorbance at 490 nm was measured with a multi-well spectrophotometer (NanoDrop 1000, Thermo Scientific, Wilmington, DE).

Immunocytochemistry

For direct microscopic examination of the immunocytochemical analysis, tendon cells were subcultured and then seeded directly on the collagen-coated glass coverslips, where cells behaved similarly as on the collagen-coated plastic dishes. Subconfluent tendon cells grown on glass coverslips were placed on the bottom of plastic dishes containing growth medium. The cells were treated without or with 0.1%, 0.15%, 0.2% lidocaine for 24h, then fixed in 4% paraformaldehyde in PBS (pH 7.5) for 15 min at room temperature. All immunostaining procedures were performed directly on the coverslips at room temperature. The coverslips were first immersed for 30 min in blocking solution that contained 1% bovine serum albumin (BSA) and 1% goat serum in PBS. After washings three times with PBS, the cells were incubated for 1 h with rabbit anti-rat monoclonal antibodies against type I or III collagen (Novotec, Saint Martin La Garenne,

France) diluted in blocking solution. The negative control was performed following the same procedures except the primary antibody was excluded from the incubation. The signal was detected with DAKO labeled streptavidian–biotin system and color was developed by incubation with diaminobenzidine substrate chromogen (DAKO, Via Real, Carpinteria, CA) for 5 to 10 min. After counterstaining the cell nuclei with hematoxylin, the coverslips were mounted on a glass slide with the cell layer down. The experiments were performed in triplicate.

Statistical Analysis

All data are expressed as the mean ± standard error of mean (SEM). Comparisons of transwell filter migration assay, MTT assay and LDH assay results between the control cells and cells treated with 0.1%, 0.15% and 0.2% lidocaine were performed using Kruskal-Wallis test. Mann-Whitney test was used to identify significant findings; a p-value less than 0.05 was considered statistically significant.
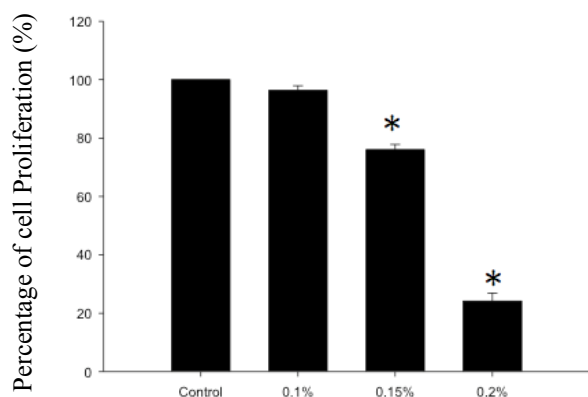

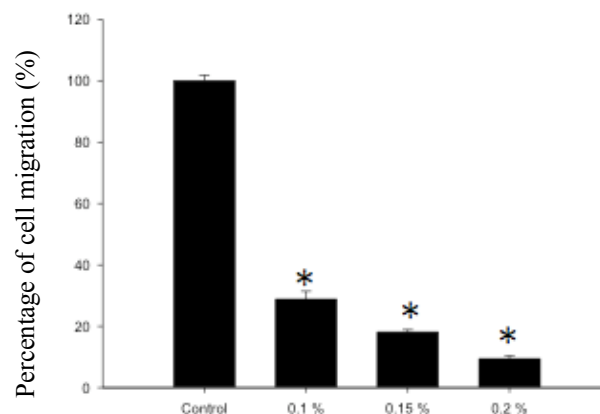Figure 1: MTT assay revealed decreased OD$_{570}$ value as a function of lidocaine dosage (*p<0.05)

Figure 2: Lidocaine dose-dependent inhibition of tendon cell migration in vitro using transwell filter migration assay (*p<0.05)
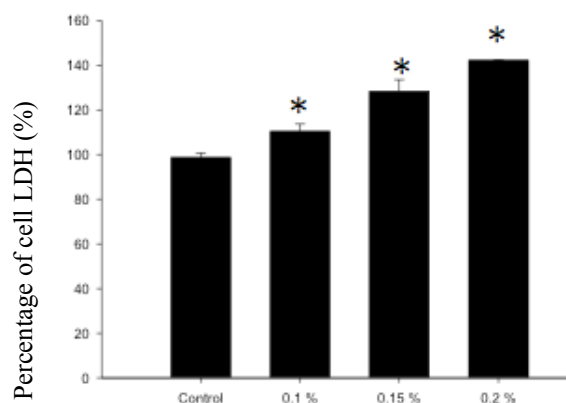

Figure 3: Cytotoxicity induced by lidocaine in vitro using LDH assay (*p<0.05)
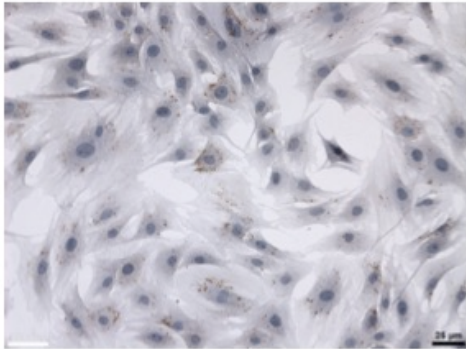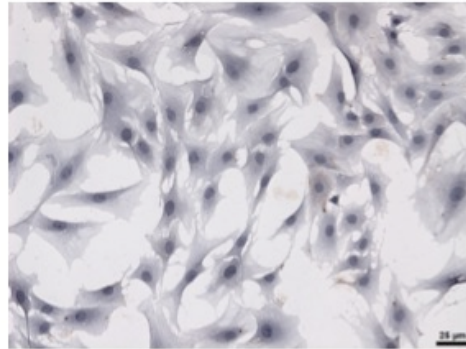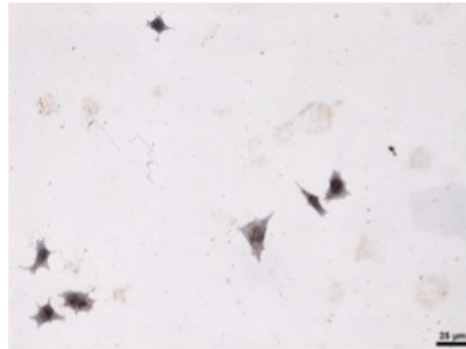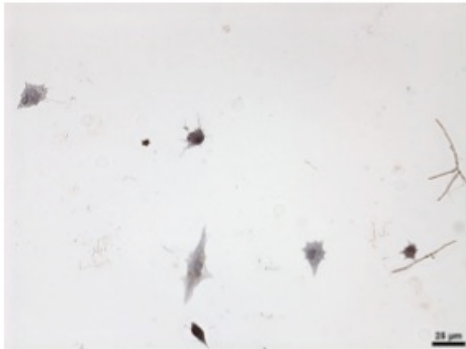
(A) Type I collagen staining (control)

(C) Type III collagen staining (control)

(B) Type I collagen staining (0.2% lidocaine)

(D) Type III collagen staining (0.2% lidocaine)

Figure 4: Types I and III collagen staining in tendon cells without lidocaine treatment (A) type I collagen, (C) type III collagen; and tendon cells with 0.2% lidocaine treatment (B) type I collagen, (D) type III collagen. Cytoplasm stained with brown color indicated positive type I or III collagen expression. The lidocaine-treated cells revealed the same staining (brown) for types I and III collagen in cytoplasm as control cells (200 x)

RESULTS

Effect of lidocaine on tendon cell viability

MTT assay showed lower OD_{570} values for tendon cells treated with 0.15% and 0.2% lidocaine (Figure 1). After 24 h, the respective OD_{570} values of 0.15% and 0.2% lidocaine were 76.0% ± 1.8% and 24.2% ± 2.7%, respectively, of those of control group. On the contrary, OD_{570} value of cells treated with 0.1% lidocaine, remained unchanged (96.4% ± 1.6% relative to controls). This indicates that the number of viable tendon cells decreased after at 0.15% and 0.2% lidocaine treatment.

Effect of lidocaine on tendon cell migration

The transwell migration assay was used to investigate the effect of lidocaine on tendon cell migration in vitro. A statistically significant ($p < 0.05$), dose-dependent inhibitory effect of lidocaine (28.8% ± 2.6%, 18.1% ± 0.8%, 9.4% ± 1.0% relative to the controls in cultures treated with 0.1%, 0.15%, and 0.2% lidocaine, respectively) was observed on tendon cell migration (Figure 2).

Effect of lidocaine on tendon cell death

The LDH assay showed a dose-dependent increase of tendon cell death (110.3% ± 0.07%, 128.3% ± 0.11%, 142.2% ± 0.006% relative to the controls in cultures treated with 0.1%, 0.15%, and 0.2% lidocaine, respectively) ($p < 0.05$) (Figure 3).

Effect of lidocaine on types I and III collagen expression

Expression of type I and III collagen was analyzed by immunocytochemical staining. In the control group, type I (Figure 4A) and type III (Figure 4C) collagen were expressed in the majority of tendon cells and localized exclusively in the cytoplasm (brown color). The stain reflects the amount of type I (Figure 4B) and type III (Figure 4D) collagen expression that remained constant after lidocaine treatment.

DISCUSSION

Three overlapping phases: inflammation, regeneration, and remodeling/maturation involve in healing of the injured tendon sequentially.[19] The initial phase involves an inflammatory response with an influx of inflammatory cells to the site of injury. During the regenerative phase, tendon cell from the epitendon and endotendon migrate to the repair site and proliferate.[20-21] Finally, production of the extracellular matrix (mainly collagen) begins once a sufficient number of tendon cells have colonized the repair site. Initially, type III collagen is rapidly deposited in a woven pattern for the first few weeks following injury.[20] The remainder of the repair process is characterized by a progressive transition to the deposition of type I collagen, which continues for an indeterminate period in the final maturation phase.[19] Therefore, tendon cell migration, proliferation, and synthesis of type I and III collagen are fundamental elements of the healing process in the injured tendon. To our knowledge, this study is the first to document the effects of lidocaine on the migration and collagen expression of tendon cells.

Recent studies have demonstrated the considerable toxicity of lidocaine in various human cell types, including mesenchymal stem cells,[22-23] chondrocytes and cartilage,[24] and rotator cuff tendons.[18,25] Studies in animal models have confirmed the toxic effect of lidocaine on bovine tendon fibroblasts[16] and rat patellar and rotator cuff tendons.[17,25] Recently, an in vitro study revealed that lidocaine significantly inhibited the proliferation and caused cell death in tenocytes from torn human rotator cuff.[25]

The use of corticosteroids (e.g., dexamethasone) as a treatment for tendinopathy has recently been questioned owing to the high risk of tendon rupture associated with their use. A systematic review supports the emerging clinical evidence that confirmed significant long-term damage to tendon tissue and cells associated with glucocorticoid injections. Local administration of glucocorticoids has significant negative effects on tendon cells in vitro, including reduced cell viability, cell proliferation, and collagen synthesis. Collagen disorganization and necrosis increase as shown by in vivo studies. The mechanical properties of tendons also significantly become weak[12] and the production of total collagen particularly collagen 1, and proteoglycans decreases.[13-14]

The results of this study revealed that lidocaine inhibited tendon cell proliferation in a dose-dependent manner, which was consistent with the results of previous studies. Accordingly, a dose-dependent increase in tendon cell death was observed after lidocaine treatment using LDH assay. The results of this study suggest that decreased tendon cell migration and proliferation might compromise the integrity of the cellular-repair process in the injured tendon during the regeneration phase. Therefore, physicians should be cautious while using lidocaine and steroids together to treat tendinopathy.

Animal studies revealed that lidocaine induced apoptosis and collagen necrosis and decreased biomechanical strength at the tear site of rat rotator cuff tendon.[25] The amount of type I (Figure 4B) and type III (Figure 4D) collagen expression remained constant after lidocaine treatment by immunocytochemical staining based on this study. However, quantitative analysis of type I and type III collagen expression was not performed. The effect of lidocaine on tendon healing is still not clearly understood. Therefore, further in vivo study is needed to clarify the exact role of lidocaine in tendon healing process.

In vitro data can not be always applied directly to the in vivo situation. However, when extrapolating to a clinical situation, too rapid to return heavy loading of the injured tendon structure after peri-tendinous injection with local anaesthetics and corticosteroids should be cautious. The underlying molecular mechanisms that are responsible for the adverse effects of lidocaine in this study are yet to be determined and need to be elucidated in further investigations. Moreover, we chose lidocaine

because it is widely used. Therefore, future studies should include other local anaesthetics.

CONCLUSION

In conclusion, lidocaine inhibits tendon cell migration and proliferation. Besides, lidocaine induced tendon cell death. However, the protein expression of type I and type III collagen remained unchanged.

COMPETIBG INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Wen-Chung Tsai and Jong-Hwei S Pang are responsible for ideation and experimental instruction. Hsiang-Ning Chang and Miao-Sui Lin are responsible for experimental procedures. Hsiang-Ning Chang and Wen-Chung Tsai are responsible for data analysis and manuscript writing.

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Lidocaine 對肌腱細胞移行，增生，膠原蛋白表現量之影響及毒性之研究

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局部麻醉劑 lidocaine 常和類固醇(如 dexamethasone)合併使用，作為注射藥物，用來治療多種肌腱相關疾病。

本研究目的為探討 lidocaine 對肌腱細胞移行、增生和第一型及第三型膠原蛋白表現量之作用，以及可能對肌腱細胞存在之毒性。

本研究方法為以不同濃度之 lidocaine (0.1%, 0.15%, 0.2%)處理從 Sprague-Dawley 大鼠培養出的肌腱細胞，與控制組比較進行後續實驗。利用 transwell filter migration assay 檢測肌腱細胞移行能力。以 MTT 試驗檢測肌腱細胞增生。使用 LDH-cytotoxicity assay 分析 lidocaine 對肌腱細胞潛在之毒性。以免疫組織化學染色法(immunocytochemical staining)觀測 lidocaine 對第一型及第三型膠原蛋白表現量之影響。

本研究結果顯示 lidocaine 顯著降低肌腱細胞移行能力($p < 0.001$)。Lidocaine 會加速肌腱細胞死亡且對肌腱細胞產生之毒性與 lidocaine 濃度呈現正相關($p < 0.001$)。MTT 試驗發現 lidocaine 會抑制肌腱細胞增生，lidocaine 濃度越高抑制肌腱細胞增生越顯著($p<0.001$)。免疫組織化學染色法呈現第一型及第三型膠原蛋白表現量與有無接觸 lidocaine 並無顯著差別。

本研究結論為 lidocaine 會抑制肌腱細胞移行及增生能力。Lidocaine 會加速肌腱細胞死亡，但對第一型及第三型膠原蛋白表現量無顯著影響。（台灣復健醫誌 2018；46(2)：71 - 79）

關鍵詞： 利多卡因(lidocaine)、局部麻醉劑(local anesthetics)、肌腱細胞(tendons)、肌腱修復(tendon healing)