In vitro oral epithelium cytotoxicity and in vivo inflammatory inducing effects of anesthetic rice gel

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\textbf{Summary} \textit{In vitro} cytotoxicity of lidocaine hydrochloride (LH) and prilocaine hydrochloride (PH) to oral epithelial cells, isolated from tissue specimens of healthy volunteers, were evaluated. Cell vitality after treating with 1-20% anesthetic solutions for 5 and 30 min was investigated using F-actin and 4',6-diamidino-2-phenylindole staining technique and observed by fluorescence microscopy. Vitality rate of more than 90% was found in all anesthetic groups at both durations whereas no survived cell was found in a positive control group (sodium dodecyl sulfate). Lactate dehydrogenase (LDH) assay was performed to confirm the safety of both anesthetic solutions. Cell culture medium after treating with LH or PH for 5 and 30 min were collected and analyzed using commercial kits. The results showed no significant difference between the test groups and negative control group (untreated culture) with low LDH levels. \textit{In vivo} inflammatory inducing effect of 5, 10, 20% LH or PH loaded rice gels was investigated in healthy volunteers. Tumor necrosis factor alpha (TNF-\(\alpha\)) in gingival cervicular fluid was determined by ELISA technique. It was found that the expression of TNF-\(\alpha\) was not different from the baseline. The expression of this inflammatory mediator caused by the commercial gel was higher than those of both anesthetic rice gels. It might be due to the effects of other excipients in the formulation of the commercial product. It is concluded that LH or PH possess no cytotoxicity to oral epithelium and the developed rice gel base and LH and PH rice gels do not induce inflammatory effect to oral tissues.

\textbf{Keywords:} Local anesthetic gel, lidocaine, prilocaine, epithelial cell, gingival tissue

\textbf{1. Introduction}

Local anesthetics are used in clinical setting for pain management during minor interventional treatments and postoperative care. Currently, they are extensively used in several procedures of dental treatment to eliminate pain (1,2). However, the drugs have possibility to produce various toxic effects in many tissues because of their chemical structures (3). Previous studies identified rates of incidence for systemic local anesthetic toxicity associated with various clinical forms of regional anesthesia (4). Local anesthetics can be classified into two groups based on the chemical moiety of amides [\(-\text{NH–CO–}\)] and esters [\(-\text{O–CO–}\)]. In general, local anesthetics exhibit their activity mainly by binding to specific receptor sites on the sodium (Na\(^+\)) channels innerves and blocking the movement of ions through these pores. Both chemical and pharmacologic properties of individual local anesthetics determine their clinical properties (5). Lidocaine and prilocaine belong to amino amide class of local anesthetics (6). Lidocaine was created in 1943 (7). Since it is non-addictive and well tolerated by most patients, lidocaine quickly became the standard by which we currently compare all anesthetic medications. By the mid-1960s, other local anesthetics including prilocaine, mepivacaine, and

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bupivacaine were available. Prilocaine has subsequently been widely reported in dental treatment. The formulation contained 4% prilocaine has been used as a primary buccal infiltration (8,9). It was reported that the effect of lidocaine and prilocaine as primary buccal infiltration was similar to each other when compared with articaine (8,10).

Local anesthetics including lidocaine applied topically have been reported to use for surgical anaesthesia with reduced cardiotoxicity and central nervous system toxicity (11,12). However, many studies have shown their cytotoxicity towards several cultured cells (13,14). Cytotoxicity of local anesthetic including lidocaine and prilocaine has been reported on mesenchymal stem cells and osteoblastic cells as well as human oral and tumor cells (15-18). Even though these local anesthetics have been long used in several complications, there is inadequate information about cytotoxic activity of these agents particularly for dental applications that the use of local anesthetics has been increased. Inflammation inducing effects of these anesthetics after clinical applications to gingival tissues have also not been well reported. Therefore, these need to be studied in detail.

2. Materials and Methods

2.1. Materials

Lidocaine hydrochloride (LH) and prilocaine hydrochloride (PH), BPC pharmaceutical grade, were obtained from Gufic Bioscince Ltd. (Mumbai, India). Sodium hydroxide and glacial acetic acid were from RCI Lab-scan Co., Ltd. (Bangkok, Thailand). ELISA kit (Biolegend®, Cat. No. 430203) was purchased from Biolegend Co., Ltd. (Tokyo, Japan). Alexa Fluor® 488-conjugated phalloidin was from Invitrogen (Carlsbad, CA). 4',6-Diamidino-2-phenylindole (DAPI) was from Biotium, Inc. (Hayward, CA). Commercial anesthetic gel (5% LH) was from Septodont Ltd. (Kent ME16 0JZ, UK). HEPES-buffered saline (fungizone), trypsin-EDTA, and Dulbecco’s modified Eagle medium (DMEM) were from Gibco-BRL, Life Technologies (Grand Island, NY). Fetal bovine serum was from Gemini (Calabasas, Calif). Protease enzyme (thermolysin) was from Sigma Chemical Co. (St. Louis, MO). Serum-free keratinocyte growth medium (KGM) was from Lonza (Walkersville, MD, USA). Trypsin-EDTA was of Invitrogen™ (Grand Island, NY, USA). The culture dishes were of Nunc™ (Roskilde, Denmark). All other chemicals and solvents were of AR grade or the highest grade available unless otherwise stated.

2.2. Preparation of anesthetic solutions and rice gels

Aqueous solutions of LH and PH at concentrations of 1, 5, 10, and 20% were prepared as followed. Exact amount of 1, 5, 10, and 20 g of LH or PH was dissolved in distilled water and the volume was adjusted to 100 mL. Rice gel base was prepared according to the method previously described (19). Exact amount of LH or PH was incorporated into certain amount of the prepared rice gel base using small amount of glycerin as wetting agent. Subsequently, the gel base was added until the total weight of the mixture was 100 g to obtain 5, 10, and 20% of drug in each riced gel formulation. The mixture was well triturated until the homogenous anesthetic transparent gel was obtained.

2.3. Tissue collection and epithelial cell culture

This experiment protocol (No. 22/2555) was approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University. Gingival tissue was obtained from 10 healthy human subjects (18-20 years old age), who were patients undergoing impacted teeth surgical removal at Faculty of Dentistry, Chiang Mai University. Informed consent was obtained. Tissue samples that appeared to be severely traumatized were excluded. Normal non-inflamed gingival tissues were collected and washed twice with cold HEPES-buffered saline containing 1% penicillin and 10 mg/mL streptomycin and 25 μg/mL amphotericin B, and subsequently cut into pieces, approximately 2 × 2 mm, and placed in the culture dishes, containing 0.5 mg/mL thermolysin and 1.125 mM Ca^2+ at 4°C for 90 min. After that, the epithelial sheet was separated from the connective tissue. The cells were isolated from the epithelial sheet by trypsinization with trypsin-EDTA, and cultured in serum-free KGM, while the connective tissues were placed on a 60 mm culture dish, which contained DMEM, supplemented with 10% fetal bovine serum and 1% penicillin/ streptomycin (Invitrogen™) until the cells spread from the tissue and their number was further expanded. After the cells reached 80% confluence, they were washed twice, trypsinized and then transferred to new culture flasks.

2.4. Cell vitality visualization

Epithelial cell vitality after treating with the anesthetic solutions (1-20% LH or PH) for 5 and 30 min was evaluated by visualization after staining. The cells were seeded in 96-well culture plates with sufficient density of cell for optimal well coverage and then incubated in a humidified atmosphere of 5% CO₂ at 37°C. After treating with the anesthetic solutions with different concentrations and durations, the cells were fixed with para-formaldehyde and stained with Alexa Fluor® 488-conjugated phalloidin for F-actin visualization and DAPI for nuclei staining (Invitrogen™). The cells were visualized and counted under fluorescence microscopy (Olympus BH2-RFC, Tokyo, Japan) with
from the standard curve of TNF-α standard (0-500 pg/mL), was performed as described in the instruction manual. Each sample was measured in duplicate and concentrations were derived from the standard curve. Percentage of relative amount of TNF-α expression causing by the test samples was calculated based on the control.

2.7. Statistical analysis

Statistical evaluation of LDH and TNF-α release experiment was performed by one-way ANOVA (Bonferroni test). Data were presented as mean ± SD. The value of \( p < 0.05 \) was considered to indicate significant differences.

3. Results

3.1. Epithelial cell culture

After epithelial cells from gingival tissue was isolated and cultured for 2 days in appropriated conditions, the morphology of polygonal or squamous shape was noticed. By using KGM, the cells could be subcultured. Cells cultured at the second to the fourth passages were used throughout this study.

3.2. Cell vitality after contacting with the anesthetic solutions

After treating with 1, 5, 10, and 20% anesthetic solutions for 5 and 30 min, high survival rates of epithelial cells were found as shown in Figure 2. It was noted that at longer contact time, the highest concentration of both anesthetic did not inhibit epithelial cell growth. Staining with DAPI and F-actin showed clearly that the cells could survive after 30 min exposure with solutions of either LH or PH whereas no cell was seen after contacting with SDS as presented in Figure 3.

3.3. Cytotoxicity by LDH assay

Detecting cytotoxicity induced by LH or PH solutions was assessed by LDH leakage into the culture medium. After exposure to the anesthetic solutions, the culture medium was centrifuged at 3,000 rpm for 5 min to separate the cells. Subsequently, the LDH in the cell free supernatant was determined using a commercially available kit from Sigma Diagnostics (LD50). The assay is based on the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD. The formation of NADH from the above reaction results in a change in absorbance at 340 nm. Aliquots of media and warm reagent were mixed in a 96-well plate (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and absorbance was recorded using a microplate spectrophotometer system (Spectramax 190 UV-Vis Microplate Reader, Molecular Devices, Sunnyvale, California, USA). Results were analyzed with the Soft max pro software (version 2.2.1) and are presented as percentage of control values. Cadmium chloride was shown not to interfere with the determination of LDH within a range from 0 to 2,000 μM.

3.4. TNF-α release

The amount of TNF-α in the gingival crevicular fluid of the volunteers after 2-min contact with LH and PH rice gels in comparison with the rice gel base and...
the commercial gel was shown in Figures 5 and 6, respectively. At the period of 0-3 h after removing gels, the rice gels containing either LH or PH and the commercial gel showed similar levels of TNF-α to the rice gel base which was about 1-2%. However, after 3 h, there was some difference occurred. The rice gel base and the PH gels for all test concentrations showed similar results with the steady TNF-α release but the commercial gel caused significantly slightly increase of TNF-α particularly at 24 h. Interestingly, LH gels showed gradually decrease of TNF-α along with the time and concentration. At 24 h, the gels with 10 and 20% LH showed significantly lower amount of TNF-α than at 0 h.

4. Discussion

Today, local anesthetics have been widely used in dental treatments for relieve of pain caused by the treatment procedure. Among several local anesthetics,
lidocaine and prilocaine were of the most commonly used drugs due to their anesthetic fast onset and suitable duration (24). In addition, they have been widely studied and reported on relative low side effects than the others in the same group based on chemical structure (25,26). The use of lidocaine and prilocaine gel for intrapocket anesthesia demonstrates the high potency for pain relief in scaling and root planing procedure (27). However, there was not well investigated on the toxicity and inflammatory inducing effects of lidocaine and prilocaine to gingival tissue which might be occurred during dental procedures. In the present study, these effects of both drugs were investigated using LH and PH as drug models in order to confirm the safety of lidocaine and prilocaine during being exposure to gingival epithelial cells. In the study, the epithelial cells were isolated from healthy human subjects with wide range of age. It was found that age of the subjects providing tissue samples did not appear to affect the success rate in the culture which was in good agreement with the previous report (28). After contacting with various concentrations of LH and PH solutions with different contact times, it is shown that the cells could be survived indicating that both drugs are fully safe. In fluorescent images of the epithelial cells staining with DAPI and F-actin, the nucleus and cytoplasms, respectively, of the cells were obviously seen confirming the cells could be survived after contact with lidocaine and prilocaine. For investigation of cytotoxicity using LDH assay, high LDH release indicates high amount of cell death (29). The results of LDH assay revealed that lidocaine and prilocaine at 5-20% had no toxic effect to the cells.

Inflammation in gingival tissue can lead to severe dental diseases. Oral inflammation can be occurred due to several factors including noxious stimuli, oral bacteria, and certain drugs (30). Mild inflammation has been reported when the cream containing both anesthetics was inserted subcutaneously to rats (31). For side effect comparison between the two anesthetics, prilocaine has been reported to increase higher level of methemoglobin than lidocaine (32). Many mediators including TNF-α are released from the host cells during inflammatory process. TNF-α is a cell signaling cytokine involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction. Therefore, determination of this cytokine can indicate the severity of inflammation. To investigate the inflammatory inducing effects of lidocaine and prilocaine, we applied the anesthetics in the form of gel formulation using modified rice as gelling agent in order to enhance the adhesion of the anesthetics to the gingival tissue along with the test period. The results indicated clearly that rice gel base as well as lidocaine and prilocaine rice gels did not induce inflammatory effects in volunteers. Therefore, we considered that lidocain and prilocain have no effect on inflammatory induction. The commercial lidocaine gel at the same drug concentration showed higher inflammatory inducing effect to the gingival tissues. It is considered that this effect might be due to the other excipients in the products.

In conclusion, the results of the present study demonstrate that both lidocaine and prilocaine are safe to use in oral cavity even in high concentration of 20% and prolong period of application. Rice gel base and rice gels containing these anesthetics also show no inflammatory inducing effect to the gingivitis.

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References


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