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Lidocaine inhibits osteogenic differentiation of human dental pulp stem cells *in vitro*

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Abstract

Objective: Lidocaine is an amide local anaesthetic commonly used for pain control, however, few studies have investigated the effect of lidocaine on the osteogenic differentiation of human dental pulp stem cells (HDPSCs). The present study aimed to determine the effect of lidocaine on HDPSC viability and osteogenic differentiation.

Methods: HDPSCs were incubated with 0, 0.05, 0.2, 0.5, and 1 mM lidocaine for 24, 48 and 72 h, after which, MTT assays were performed. HDPSCs cultured with the above lidocaine concentrations and osteogenic differentiation medium for 7 and 14 days were stained for alkaline phosphatase (ALP). Protein and mRNA levels of relevant osteogenic factors (bone morphogenetic protein-2 [BMP-2] and runt-related transcription factor 2 [RUNX2]) were examined using western blotting and real-time reverse-transcription polymerase chain reaction, respectively.

Results: Lidocaine did not affect the viability of HDPSCs, however, lidocaine reduced ALP activity in HDPSCs. Levels of ALP, BMP-2, and RUNX2 mRNA were reduced with lidocaine, and levels of BMP-2 and RUNX2 proteins were decreased, versus controls.

Conclusions: Lidocaine inhibits osteogenic differentiation markers in HDPSCs *in vitro*, even at low concentrations, without cytotoxicity. This study suggests that lidocaine may inhibit osteogenic differentiation in HDPSC-mediated regenerative medicine, including pulp regeneration and repair.

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Introduction

Stem cells have unique self-regeneration and multipotent differentiation abilities, and years of research in regenerative medicine have been devoted to studying the ability of stem cells to repair damaged tissues and partially restore organ functions.[1] Mesenchymal stem cells (MSCs) can be obtained from various tissues, such as bone marrow, adipose, and dental tissues, and are able to differentiate into many cell types, including osteoblasts, chondrocytes, neurons, and myoblasts. Compared with embryonic stem cells, they are advantageous based on a lack of ethical issues and immunological problems.[2–6] Bone marrow-derived mesenchymal stem cells (BMMSCs), obtained through bone marrow puncture, have conventionally been used in regenerative medicine, however, this method of collecting BMMSCs is associated with the drawback of potential complications, such as pain and infection.[7]

Human dental pulp stem cells (HDPSCs) are important essential MSCs that can be isolated from dental pulp tissues of extracted permanent human teeth. The phenotypes and functional properties of HDPSCs highly resemble those of BMMSCs. Furthermore, the source of these cells is abundant, unlike BMMSCs that are associated with the difficulty of bone marrow collection.[8,9] In addition, HDPSCs may promote specific cell differentiation through various endogenous and non-endogenous molecules.[10–12] Thus, HDPSCs are anticipated to become a promising resource in regenerative medicine, particularly in tissue reconstruction.[8,9]

Lidocaine is one of the most commonly used local anaesthetics for pain control. Widely used amide local anaesthetics, including lidocaine, prevent ion influx via inhibition of nerve conduction by binding to sodium channels.[13,14] A notable side-effect of local anaesthetics is cytotoxicity, resulting in the induction of cellular apoptosis or necrosis, although the complete underlying mechanism remains unknown. The cytotoxicity of local anaesthetics has been demonstrated in various cell types,[15–18] however, there are no studies on the effect of lidocaine on HDPSCs.

After isolation from different anatomical areas, and subsequent *in vitro* culture and frequent manipulations, stem cells are transplanted back into the human body to regenerate diseased or damaged tissues or organs.[19–22] Stem cell isolation procedures generally require the injection of local anesthetics,[20,23] thus, it is crucial to understand the potential effects of these agents on not only short-term cell survival, but also the polyphyletic differentiation of stem cells. Research into the effect of lidocaine injection on the collection or transplantation of HDPSCs, which have potential as an excellent cell source in regenerative medicine, is required. In particular, the ability of HDPSCs to differentiate into osteoblasts may be useful for bone regeneration.[8,9] Therefore, the present study was conducted to investigate the effect of lidocaine on HDPSC viability and osteogenic differentiation.

Materials and methods

Cell culture

HDPSCs were purchased from Lonza (Portsmouth, NH, USA, PT-5025) and maintained in Eagle's minimum essential medium (Corning, Manassas VA, USA), supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA), 1% penicillin–streptomycin (Gibco BRL), and 5 μg/ml Plasmocin (InvivoGen, San Diego, CA, USA), at 37°C in a humidified incubator with 5% CO_2.

Cell viability assay

To assess cell viability, HDPSCs were seeded into 24-well plates at a density of 2×10^4 cells/well and incubated with varying concentrations of lidocaine (0, 0.05, 0.2, 0.5, and 1 mM [Jeil Pharmaceutical, Daegu, Korea] diluted in normal saline) for 24, 48, or 72 h at 37°C with 5% CO_2. At the end of the culture period, cells were placed in fresh medium containing 0.5 mg/ml 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT; Duchefa Biochemie BV, Haarlem, The Netherlands) solution and incubated for 4 h at 37°C before adding 200 μl of dimethyl sulfoxide. The resultant dissolved blue formazan product was measured using a microplate reader at a wavelength of 570 nm.

Osteogenic differentiation of HDPSCs

To induce cell differentiation, HDPSCs were cultured in osteogenic differentiation medium (ODM) (α-minimum essential medium; Wellgene, Gyeongsan, South Korea), containing 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and 0.1 μM dexamethasone, for 7 and 14 days, as described below, refreshing the medium every 2–3 days.

Alkaline phosphatase (ALP) staining

To assess the effects of lidocaine on ALP activity, HDPSCs were seeded into 48-well culture plates at a density of 5×10^4 cells/well and incubated with ODM to induce differentiation, as described above, plus lidocaine at 0, 0.05, 0.2, 0.5, and 1 mM, for 7 and 14 days (at 37°C with 5% CO_2). For ALP staining, HDPSCs were rinsed with phosphate-buffered saline (PBS) containing 0.05% Tween-20 after aspirating the medium, then fixed (for 2 min at room temperature) and stained with the StemTAG™ Alkaline Phosphatase Staining Kit (CBA-300; Cell Biolabs, San Diego, CA, USA) following the manufacturer's instructions. Stained cells were observed and photographed with a phase-contrast microscope. To quantify ALP staining, the spectrophotometric absorbance of the samples was measured using a microplate reader (TECAN Infinite M200 PRO, Tecan Trading AG, Mannedorf, Switzerland).

Real-time reverse transcription–polymerase chain reaction (RT–PCR)

To assess the effects of lidocaine on levels of bone morphogenetic protein-2 (BMP-2), runt-related transcription factor 2 (RUNX2) and ALP mRNA, HDPSCs were seeded into 6-well plates at 2×10^5 cells/well, and incubated with maintenance media (control), ODM plus normal saline (vehicle) or ODM plus 0.5 mM lidocaine for 7 and 14 days. Total RNA was then isolated using 500 μl of TRIzol reagent (Qiagen, Hilden, Germany) and 1 μg of RNA was reverse-transcribed with M-MLV reverse transcriptase (Promega, Madison, WI, USA) using Oligo(dT)15 primers (Macrogen, Seoul, Korea), according to the manufacturer's instructions. The cDNA was amplified by real-time PCR in the following reaction: QuantiTect SYBR Green PCR master mix (Qiagen), 1 μl

(10 pmol/μl) each of forward and reverse primer, and 1 μl (1 μg/μl) cDNA template in a reaction volume of 25 μl, using two-step cycling that comprised 40 cycles of 15 s denaturation at 95°C and 30 s of amplification at 60°C in a Rotor-Gene Q thermal cycler (Qiagen). The primer sequences used in each PCR are listed in Table 1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalise the real-time PCR results. All samples were measured in triplicate, and the PCR results were quantified using the ΔΔCt method with Rotor-Gene Q series software (Qiagen).

Western immunoblot analyses

To assess the effects of lidocaine on levels of osteogenic differentiation marker proteins, HDPSCs were seeded into 6-well plates at 2×10^5 cells/well and incubated with maintenance media (control), ODM plus normal saline (vehicle) or ODM plus 0.5 mM lidocaine for 7 and 14 days. Total protein was extracted by washing cells twice with cold PBS then lysing in PRO-PREP protein extraction solution (Intron Biotechnology, Sungnam, Korea), according to the manufacturer's instructions. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Membranes were then blocked with 4% skim milk in Tris-buffered saline containing 0.1% Tween 20 (1 × TBS-T) for 1 h at room temperature, followed by incubation with primary antibody (anti-BMP-2 antibody [ab14933; Abcam, Cambridge, UK], 1:1000 dilution; anti-RUNX2 antibody [D130-3; MBL, Woburn, MA, USA], 1:1000 dilution; and anti-GAPDH antibody [NB300-221; Novus Biologicals, Littleton, CO, USA], 1:1000 dilution) overnight at 4°C. Membranes were then washed 3–5 times with 1 × TBS-T followed by incubation with goat anti-mouse IgG F(ab′)2, polyclonal (ADI-SAB-300-J) and goat anti-rabbit IgG, polyclonal horseradish peroxidase-conjugated secondary antibodies (both 1:5000 dilution; Enzo Life Sciences, Minneapolis, MN, USA) for 1 h at room temperature. Finally, membranes were washed 3–5 times with 1 × TBS-T and immunoreactions were visualised using an enhanced chemiluminescence detection system (ECL™ Select Western Blotting Detection Reagent; GE Healthcare Life Sciences, Pittsburgh, PA, USA) following the manufacturer's instructions. Signals were detected with a Fusion Solo X imaging system (Vilber; Paris, France).

Table 1. Primers used in real-time polymerase chain reaction to analyse the effect of lidocaine on gene expression in cultured human dental pulp stem cells.

Gene	Primer sequence
BMP-2	Forward: 5′-TTCCACCATGAAGAATCTTTGG-3′
	Reverse: 5′-AAACCTGAAGCTCTGCTGAG-3′
RUNX2	Forward: 5′-TCCCAGTATGAGAGTAGGTGTCC-3′
	Reverse: 5′-GGCTCAGGTAGGAGGGGTAAGAC-3′
ALP	Forward: 5′-GGACGCTGGGAAATCTGTG-3′
	Reverse: 5′-CCATGATCACGTCAATGTCC-3′
GAPDH	Forward: 5′-GGCGAGATCCCTCCAAAATC-3′
	Reverse: 5′-CAAATGAGCCCCAGCCTTC-3′

BMP-2, bone morphogenetic protein-2; RUNX2, runt-related transcription factor 2; ALP, alkaline phosphatase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Statistical analyses

Data are presented as mean ± SD obtained from at least three independent experiments. Between-group differences were analysed by Student's *t*-test, performed using SPSS software, version 26.0 (IBM, Armonk, NY, USA). A P value <0.05 was considered to indicate statistical significance.

Results

Effect of lidocaine on cytotoxicity in HDPSCs

Compared with HDPSC viability in the control group (no lidocaine), no cytotoxicity was detected upon the addition of lidocaine across any of the tested concentrations (0.05, 0.2, 0.5, and 1 mM) or incubation times (24, 48, and 72 h) (Figure 1).

Effect of lidocaine on ALP activity

ALP is a marker of osteogenic differentiation, with notably increased activity during active bone growth. In the present study, ALP activity in HDPSCs, shown by the level of ALP staining, increased with culture time in cells incubated in ODM (Figure 2a and b). Compared with the control group, fewer ALP-positive cells were observed upon treatment with higher concentrations of lidocaine, with absorbance levels being significantly lower versus controls in HDPSCs treated with 0.5 and 1 mM lidocaine at day 7, and cells treated with 0.2, 0.5 and 1 mM lidocaine at day 14 ($P < 0.05$; Figure 2a and b). Levels of ALP mRNA in the control group (ODM plus vehicle) were also higher on day 14 of culture than on day 7, and ALP levels were significantly reduced in cells treated with 0.5 mM lidocaine compared with controls at both time-points (Figure 2c). Lidocaine was thus shown to reduce ALP activity in HDPSCs, indicating the inhibition of osteogenic differentiation.

Effect of lidocaine on the expression of genes related to osteogenic differentiation of HDPSCs

To investigate the expression of osteogenic differentiation marker genes, HDPSCs were cultured with 0 or 0.5 mM lidocaine in ODM for 7 and 14 days, after which mRNA levels were determined using real-time RT–PCR. On both days, the mRNA levels were significantly reduced in HDPSCs treated with 0.5 mM lidocaine

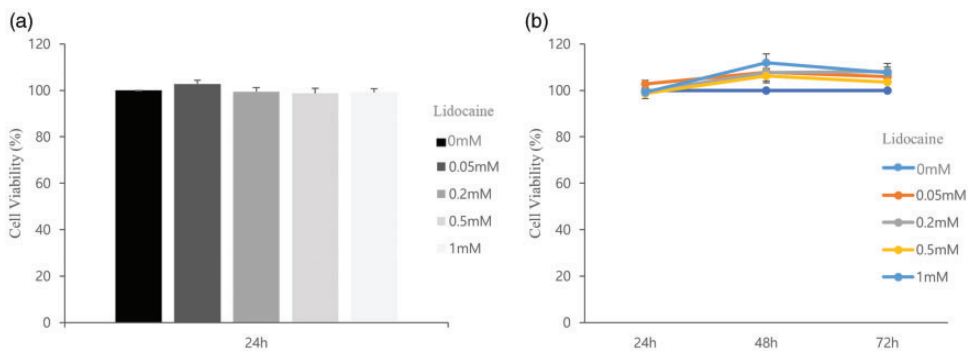

Figure 1. Effect of lidocaine on human dental pulp stem cell (HDPSC) viability. HDPSCs were incubated in medium containing lidocaine (0–1 mM) for up to 72 h, and cell viability was evaluated by MTT assay: (a) over 24 h, no toxicity was observed at any lidocaine concentration versus control group; and (b) for up to 72 h, lidocaine did not affect the viability of HDPSCs at any concentration. Data presented as mean ± SD.

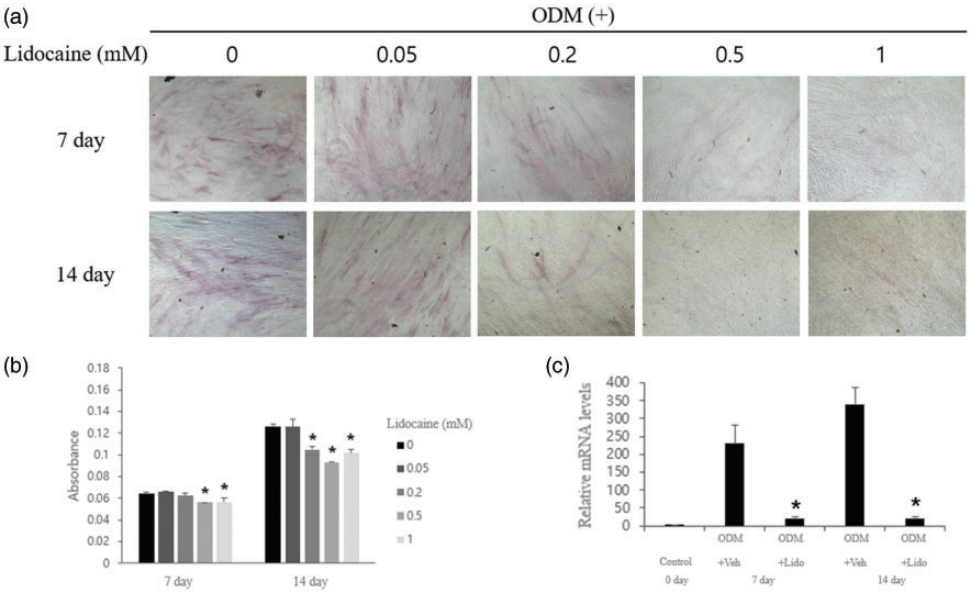

Figure 2. Effect of lidocaine on alkaline phosphatase (ALP) staining (to indicate osteoblast differentiation) in human dental pulp stem cells after 7 and 14 days of culture in control or osteogenic differentiation medium (ODM): (a) representative photomicrographs showing ALP staining performed after 7 and 14 days of osteogenic induction in the presence of 0–1 mM lidocaine (original magnification, × 100); (b) semiquantitative analysis, by absorbance readings, of ALP staining (shown in photomicrographs) revealed that lidocaine reduced the level of ALP (*$P < 0.05$ versus 0 mM lidocaine); and (c) levels of ALP mRNA, quantified by real-time reverse transcription–polymerase chain reaction, were reduced with lidocaine treatment (0.5 mM) for 7 and 14 days versus vehicle control. Data presented as mean ± SD (*$P < 0.05$ versus ODM + Veh). Lido, lidocaine; Veh, vehicle control.

versus ODM plus vehicle controls ($P < 0.05$; Figure 3). These results showed that lidocaine reduced the expression of BMP-2 and RUNX2, suggesting a decrease in osteogenic differentiation of HDPSCs.

Effect of lidocaine on levels of proteins related to osteogenic differentiation of HDPSCs

To assess the levels of osteogenic differentiation marker proteins, HDPSCs were cultured with 0 or 0.5 mM lidocaine in ODM for 7 and 14 days, after which protein levels were determined by western blotting (Figure 4). Protein levels of BMP-2 and RUNX2, well-known markers of osteogenic differentiation, were significantly reduced in HDPSCs on both days 7 and 14 of culture with lidocaine compared with the respective vehicle control groups, in agreement with the real-time RT–PCR mRNA levels. The results suggest that lidocaine may inhibit osteogenic differentiation by reducing the expression of *BMP-2* and *RUNX2* in HDPSCs.

Discussion

Tissue engineering using stem cells requires a source from which stem cells can be readily harvested, to allow for adequate numbers of cells while minimally affecting the donor site. HDPSCs are a rich source of MSCs that are associated with a less invasive harvesting method than BMMSCs, and

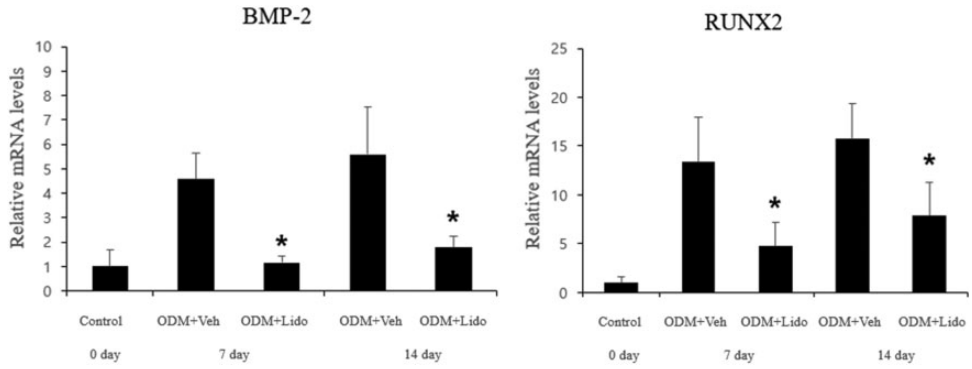

Figure 3. Effect of lidocaine on bone morphogenetic protein-2 (BMP-2) and runt-related transcription factor 2 (RUNX2) mRNA levels during osteogenic differentiation of human dental pulp stem cells (HDPSCs). Real-time reverse transcription–polymerase chain reaction results, normalised to glyceraldehyde 3-phosphate dehydrogenase levels, showed that BMP-2 and RUNX2 mRNA levels were significantly reduced at day 7 and day 14, in HDPSCs cultured with osteogenic differentiation medium (ODM) plus 0.5 mM lidocaine. Data presented as mean ± SD (*$P < 0.05$ versus ODM + Veh). Lido, lidocaine; Veh, vehicle control.

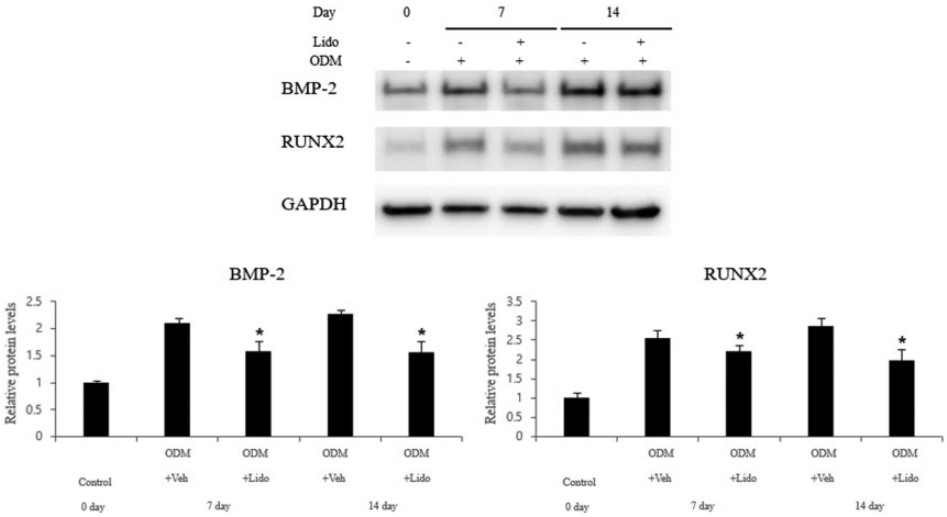

Figure 4. Effect of lidocaine on bone morphogenetic protein-2 (BMP-2) and runt-related transcription factor 2 (RUNX2) protein levels during osteogenic differentiation of human dental pulp stem cells (HDPSCs). Western blot results, normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels, showed that levels of BMP-2 and RUNX2 protein were significantly reduced at day 7 and day 14, in HDPSCs cultured with osteogenic differentiation medium (ODM) plus 0.5 mM lidocaine. Data presented as mean ± SD (*$P < 0.05$ versus ODM + Veh). Lido, lidocaine; Veh, vehicle control.

thus, are anticipated to prove useful in regenerative medicine.[7–9]

The viability and differentiation of mesenchymal stem cells are influenced by factors such as inflammation and co-treatment substances.[10–12,24] Teeth are commonly extracted after local anaesthesia with lidocaine, which is, thus, potentially present

during the isolation of HDPSCs. Therefore, identifying the effect of lidocaine on HDPSC differentiation is important to ensure successful treatment results. Nevertheless, the effect of lidocaine on the osteogenic differentiation of HDPSCs remains unclear. The present study aimed to determine the effect of lidocaine on HDPSC viability and osteogenic differentiation through a comparative analysis, and showed that low concentrations of lidocaine did not elicit cytotoxicity, but did have an inhibitory effect on the osteogenic differentiation of HDPSCs cultured *in vitro*. These data suggest that lidocaine injected for local anaesthesia may impair the survival and organ regeneration ability of HDPSCs.

Lidocaine has been reported to substantially reduce cell viability in a concentration-dependent manner, though low concentrations ($\leq$1 mM) have not been found to cause significant cytotoxicity.[16–18] In the present study, the MTT assay revealed no distinct cytotoxicity at lidocaine concentrations $\leq$1 mM. This result was considered in subsequent real-time RT–PCR and western blotting experiments, wherein 0.5 mM lidocaine was investigated, in order to avoid adverse effects on HDPSC survival. Although this low lidocaine concentration avoided cytotoxic effects, the osteogenic differentiation of HDPSCs showed considerable changes. Thus, it may be concluded that the lidocaine concentration used for cell culture was suitable to test the present hypothesis.

Stem cell differentiation into osteoblasts generally involves the expression of *ALP*, an enzyme found in the cell membrane at high levels during the early stage of osteogenesis.[25,26] In the present study, ALP staining was used to mark the early stage of osteogenesis. The vehicle control group showed darker staining than the lidocaine group, and an increase in lidocaine concentration led to diminished staining. Thus, lidocaine was shown to decrease the ALP levels in HDPSCs, suggesting that it reduced osteogenic differentiation in these cells.

An interesting pattern of osteogenic marker gene expression, at the mRNA and protein level, was observed during the induction of HDPSC osteogenic differentiation after treatment with lidocaine. After 7 and 14 days of HDPSC culture in ODM, mRNA levels of the osteogenic markers ALP, BMP-2, and RUNX2 were significantly decreased in the lidocaine group compared with vehicle controls, as were RUNX2 and BMP-2 protein levels, supporting the present hypothesis that lidocaine may affect osteogenic differentiation.

Of note, BMP-2 has been shown to induce *in vitro* osteogenic differentiation and *in vivo* osteogenesis. Osteogenic differentiation marker expression is increased as BMP-2 activates the Smad signalling pathway, which in turn activates RUNX2.[25,27] In addition, when BMP activity is inhibited by an antagonistic antibody for BMP-2, BMP-4, and BMP-7, osteogenic differentiation markers, such as ALP, are downregulated. This indicates a critical role of BMP-mediated signalling in RUNX2-mediated osteogenic differentiation.[28]

The master transcription factor, RUNX2, induces the expression of osteogenic differentiation markers such as *ALP*. During osteogenic differentiation, *RUNX2* is expressed at a very low level in uncommitted MSCs, but the level increases upon the proliferation of pre-osteoblasts.[25,29] As RUNX2-knockout mice show a defect in bone formation, RUNX2 clearly has an important role in osteogenic differentiation.[30] ALP is distributed across all tissues and, in bone tissue, a notable increase in ALP activity is found upon bone growth. Several studies have shown that ALP is a marker that can detect osteogenic differentiation during the early stage; a gradual increase in *ALP* expression is observed after 5 and 10 days of HDPSC culture.[25,26,31] Thus, lidocaine is presumed to

have an effect on BMP-2, RUNX2, and ALP, whereby the osteogenic differentiation of HDPSCs is inhibited with a consequent decrease in osteogenesis.

Mesenchymal stem-cell cytotoxicity has been reported based on the type of local anaesthetic drug. For example, while lidocaine has been shown to significantly reduce the viability of human BMMSCs, ropivacaine and bupivacaine displayed a lesser impact. Considering cartilage cytotoxicity and the additional cartilage dissolution associated with bupivacaine injection, ropivacaine has been reported to have low cytotoxicity against human BMMSCs and to be a safe choice as an anaesthetic for joint pain.[32] Another study also reported that lidocaine and bupivacaine had greater cytotoxicity against human adipose-derived MSCs than ropivacaine.[33] However, in a study investigating the cytotoxic effects of local anaesthetics against human BMMSCs during chondrogenic differentiation, no difference in MSC cytotoxicity was shown between bupivacaine, ropivacaine, and mepivacaine.[34] In a further study, lidocaine and mepivacaine were reported to exhibit less effect on the reduction in gene expression associated with chondrogenic differentiation in rabbit adipose-derived MSCs at the early phase of chondrogenesis, compared with bupivacaine and ropivacaine. In addition, lidocaine and mepivacaine were reported to have relatively low cytotoxic effects.[35]

The different cytotoxic effects of local anaesthetics against MSCs seen in the above studies are thought to be due to the different concentrations of local anaesthetics used, as well as various analytical methods, MSC types, and conditions used for differentiation. Similar to the results from other studies, the present study found that the local anaesthetic, lidocaine, had a negative impact on gene expression in MSCs. This finding suggests that local anaesthetics potentially impact all processes of MSC therapy and may affect cell therapy outcome. Therefore, the use of local anaesthetics should be minimized during MSC differentiation into various cells, and local anaesthetics other than lidocaine, as used in the present study, should be considered.

The results of the present study may be limited by several factors. First, the inhibitory effect of lidocaine on osteogenic differentiation was verified only *in vitro*, implying the need for future *in vivo* and clinical studies. Secondly, there is a lack of understanding regarding the mechanism by which lidocaine inhibits osteogenic differentiation. Ion channels are cell membrane proteins that mediate the membrane transport of certain ions. Many previous studies have reported that ion channels have an effect on cell growth and differentiation.[36–38] It is possible, therefore, that the inhibition of HDPSC osteogenic differentiation is associated with lidocaine-mediated inhibition of ion channels. However, studies into the role of ion channels in HDPSCs are lacking. Thus, further studies should investigate the basic mechanism underlying the effect of lidocaine on HDPSCs. In addition, as HDPSCs isolated from human tissues may have been exposed to lidocaine during the isolation process, the potential effect of such prior exposure cannot be ruled out. Nevertheless, the magnitude and duration of cellular effect in response to actual exposure to lidocaine in the present study appears significant.

Conclusion

The findings in this study suggest that lidocaine has no effect on HDPSC viability at low concentrations, but may inhibit osteogenic differentiation. Therefore, these data suggest that lidocaine, commonly used for pain control, may influence osteogenic differentiation in regenerative medicine using HDPSCs.

Author contributions

Eun-Ji Choi conceived and designed the experiments, and supervised the project. Eun-Jung Kim, Ji-Uk Yoon, Joo-Young Kim, and Hyang-Sook Kim performed the experiments and analysed the data. Eun-Jung Kim, Ji-Uk Yoon, Cheul-Hong Kim, and Ji-Young Yoon supplied the reagents, materials, and analysis tools. Eun-Jung Kim and Ji-Uk Yoon wrote the manuscript, and the final manuscript was approved by all authors.

Data availability statement

The data in this study are available upon request from the corresponding author.

Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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