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Compressive Force Upregulates Notch Target Genes and *NOTCH2* mRNA in Human Dental Pulp Cells

Hataichanok Charoenpong*, Khitparat Kamoltham, Suchada Limsiriwong, Rutapakon Insawak, Apichart Veerawattanatigul, and Sirawish Lertchatripong

College of Dental Medicine, Rangsit University, Pathum Thani, 12000, Thailand

*Corresponding author; E-mail: hataichanok.c@rsu.ac.th

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Abstract

Dental pulp cells encounter compressive force in various situations. While mechanical force can produce various effects on dental pulp cells, the mechanisms underlying their response remain unclear. In this study, we examined the mRNA expression of Notch target genes and Notch receptors in human dental pulp cells (HDPCs) under mechanical compressive force. We utilized two in vitro compressive force application models, direct compression and hydrostatic compression. The results showed that there was an upregulation of Notch target gene, *HES1*, in HDPCs subjected to the compressive force generated by both models for 2 hours. Hydrostatic compression also upregulated *HES1* and *HEY1* mRNA expression following 6 hours of force application. *NOTCH2* was the only Notch receptor found to be upregulated in HDPCs following compressive force application, in which the upregulation was observed at 6 hours after hydrostatic compression. In conclusion, both hydrostatic and direct compressive forces can upregulate the mRNA expression of Notch target gene, *HES1*, in HDPCs. However, the hydrostatic compression model produced more prolonged activation of *HES1* and it also stimulated the upregulation of *HEY1* as well as *NOTCH2*.

Keywords**:** *dental pulp cells; mechanical force; compressive force; Notch signaling*

1. Introduction

Dental pulp is a soft tissue surrounded by hard tissue of the teeth. When teeth are displaced, such as during trauma, mastication, or orthodontic treatment, direct compressive force can be generated on dental pulp cells due to the compression of the dentin wall on pulp tissue. Dental pulp cells can also encounter hydrostatic compressive force, from tissue fluid during pulpal inflammation which can be induced by various stimuli for example tooth preparation, orthodontic forces, dental caries, trauma, and parafunctional habits. An increase in extravascular fluid from vasodilation and higher vascular permeability during inflammation causes an increase in intrapulpal pressure due to the physical constraint of the dental pulp. It was reported that intrapulpal pressure is approximately three times higher in inflamed dental pulp compared to normal pulp (Heyeraas, & Berggreen, 1999).

Mechanical forces can affect dental pulp cell behaviors including cell proliferation, adhesion, migration, osteogenic, and odontogenic differentiation (Marrelli et al., 2018; Rad et al., 2021). In addition, some studies have demonstrated the possible relationship between mechanical stress-induced dental pulp cells and subsequent stimulation of osteoclasts (Wang et al., 2017), which might be the mechanisms underlying root resorption. The response of dental pulp cells to mechanical force was reported to involve many signaling pathways including Erk1/2 and p38 mitogen activated protein

kinases, NFκB pathway, and Wnt/β-catenin pathway (Marrelli et al., 2018).

Notch signaling pathway is an evolutionarily conserved pathway that is involved in various biological processes during prenatal and postnatal development as well as in adult tissues. Notch receptors and Notch ligands are membrane-bound proteins, therefore, Notch signaling is activated in a juxtacrine manner upon binding of Notch ligand to its receptor on a neighboring cell. In mammals, four Notch receptors, NOTCH1-4, have been identified. Binding of Notch receptor to its ligand induces two consecutive cleavages of Notch receptor resulting in the release of Notch intracellular domain (NICD), which further translocates into the nucleus and eventually activates Notch target genes (Kopan, 2012). The well-known Notch target genes are Hairy/enhancer-of-split (HES) and Hes related with YRPW motif protein (HEY) family genes.

Notch receptors, ligands, and target genes were found to express in dental pulp and were upregulated following pulpal injury (Lovschall et al., 2005; Mitsiadis et al., 1999). Association between Notch signaling components and mechanical force was reported in various cell types. Shear stress was found to induce expression of Notch receptors, ligands and target genes in endothelial cells and limbal epithelial stem cells (Jahnsen et al., 2015; Kang et al., 2014; Mack et al., 2017; Masumura et al., 2009; Obi et al., 2009). Mechanical strain was reported to affect notch signaling components in vascular smooth muscle cells and umbilical vein endothelial cells (Loerakker et al., 2018; Morrow et al., 2007; Morrow et al., 2005). Compressive force was found to upregulate *NOTCH1* mRNA expression in human deciduous dental pulp cells (Peetiakarawach et al., 2015). However, in HDPCs that can be exposed to mechanical compressive force in various situations, the study on the relationship between notch signaling and mechanical force is still lacking. Therefore, this study aimed to investigate expression of Notch signaling components in HDPCs in response to mechanical compressive force.

To study the effect of compressive force in cell culture, many compressive force application models have been introduced (Manokawinchoke et al., 2021). These include direct compression and hydrostatic compression models which were used to deliver compressive force to many cell types including HDPCs, human periodontal ligament cells (HPDL), and human exfoliated deciduous teeth (SHEDs) (Govitvattana et al., 2013; Manokawinchoke et al., 2015; Satrawaha et al., 2011). In this study, we utilized both direct compression and hydrostatic compression models which may represent the types of compressive force encountered by dental pulp cells. We hypothesized that compressive force increases mRNA expression of Notch target genes and Notch receptors in HDPCs.

2. Objectives

This study aimed to investigate the effect of compressive force on mRNA expression of Notch target genes and Notch receptors in HDPCs.

3. Materials and methods

3.1 Cells Isolation and Culture

HDPCs were obtained by cell explant technique from non-carious third molars of healthy donors. Dulbecco's modified Eagle's medium (DMEM, GIBCO/Thermo Fisher Scientific, Rochester, NY) supplemented with 10% (v/v) fetal bovine serum (GIBCO), Glutamax (2 mM, GIBCO), penicillin (100 U/mL), streptomycin (100 mg/ml, GIBCO), and amphotericin B (0.25 µg/ml, GIBCO) was used for culture of HDPCs. Cells were maintained at 37°C in humidified atmosphere with 5% CO_2. After expansion until the 3rd to 6th passage, cells were used for compressive force application. HDPCs derived by this method were characterized in a previous study (Charoenpong et al., 2019).

3.2 Mechanical Force Application

HDPCs were plated at the density of 350,000 cells/well in 6-well plates. Cells were then starved in serum-free media for 3 hours prior to compressive force application. At the time of force application, the media were changed again. Compressive force was delivered to the cells by two different models of force application. Hydrostatic compression model used the computer-controlled apparatus introduced in a previous study (Manokawinchoke et al., 2015). Briefly, 6-well plate was attached to the cylinders and placed on the motor-driven platform. The computer-controlled motor drove the 6-well plate up towards the pestles that fit in the cylinders to generate hydrostatic pressure from the culture media to the cells at the bottom of the plate (Figure 1A). The platform under culture plate had the balance that sent the signal

back to the controller in order to constantly monitor the force level.

Another model of force application was the direct compression model. This model utilized metal weight containing-plastic cylinders that were fit into the wells of 6-well plate to deliver compressive force directly onto the cells (Figure 1B). This model was reported in many previous studies (Charoenpong et al., 2019; Govitvattana et al., 2013; Satrawaha et al., 2011). In this study, HDPCs were subjected to either model of compressive force application in which 1 or 2 g/cm^2 of compressive force was applied for 2, 6, or 24 hours as indicated. This range of force magnitude was applied to HDPCs in previous studies and was reported not to affect cell viability (Charoenpong et al., 2019; Satrawaha et al., 2011). Control groups were the cells cultured in the same condition and duration without force application. At least 3 lines of HDPCs were used and at least two independent experiments were performed.

3.3 Real-time Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted from control HDPCs and HDPCs subjected to mechanical force using TRIzol™ reagent (Invitrogen, Thermo Fisher Scientific). The RNA amount was measured using a spectrophotometer (NanoDrop2000, Thermo Scientific) and 1 µg of RNA was then converted to complementary DNA by reverse transcriptase (iScript™ Reverse Transcription Supermix, Bio-Rad, Hercules, CA). qPCR was then performed in a LightCycler® 480 II (Roche, Basel, Switzerland) with SYBR detection system (iTaq™ Universal SYBR® Green Supermix, Bio-Rad). The expression of target genes was normalized to the expression of GAPDH. Ct method was used to determine relative mRNA. Table 1 shows the primer sequences used in this study.

3.4 Data Analysis

Level of mRNA expression of each gene was calculated as fold change compared with the mRNA expression of that gene in control cells from the same donor. Statistical analyses were performed using IBM SPSS Statistics 21. The normality test was performed with Shapiro-Wilk test. Levene's test was performed to assess the homogeneity of variance. ANOVA was performed to compare the mean fold change of mRNA among 3 groups of data followed by the least significance difference (LSD) post hoc test when there was a significant change indicated by ANOVA in the data with normal distribution and homogenous variance. Independent t-test was used to compare the mean fold change of mRNA between two groups of data with normal distribution and Welch's t-test was applied if the equal variance was not assumed. Mann-Whitney U test was used to compare the mean fold change of mRNA between two groups of data with non-normal distribution.

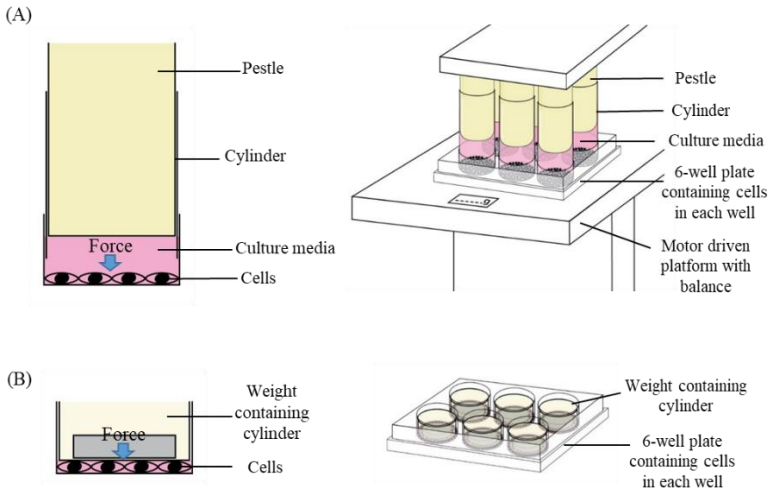

Figure 1 Schematic illustration of compressive force application models delivering compressive force to the cells. (A) Hydrostatic compression model. The pestles that fit with the cylinders generate the pressure to the cells through culture media when the motor-driven platform moves the 6-well plate up towards the pestles. (B) Direct compression model. This model delivers compressive force by direct compression of the weight containing cylinder onto the cells.

Table 1 Primer sequences used for qPCR

Gene	Sequence ID	Primer Sequences (5' to 3')
GAPDH	NM_002046.6	Forward- CACTGCCAACGTGTCAGTGGTG
		Reverse- GTAGCCCAGGATGCCCTTGAG
NOTCH1	NM_017617.5	Forward -GCCGCCTTTGTGCTTCTGTTC
		Reverse-CCGGTGGTCTGTCTGGTCGTC
NOTCH2	NM_024408.4	Forward -CCAGAATGGAGGTTCCTGTA
		Reverse-GTACCCAGGCCATCAACACA
NOTCH3	NM_000435.3	Forward -TCTTGCTGCTGGTCATTCTC
		Reverse-TGCCTCATCCTCTTCAGTTG
NOTCH4	NM_004557.4	Forward -AGCCGATAAAGATGCCCA
		Reverse-ACCACAGTCAAGTTGAGG
HES1	NM_005524.4	Forward -AGGCGGACATTCTGGAAATG
		Reverse-CGGTACTTCCCCAGCACACTT
HEY1	NM_012258.4	Forward -CTGCAGATGACCGTGGATCA
		Reverse-CCAAACTCCGATAGTCCATAGCA

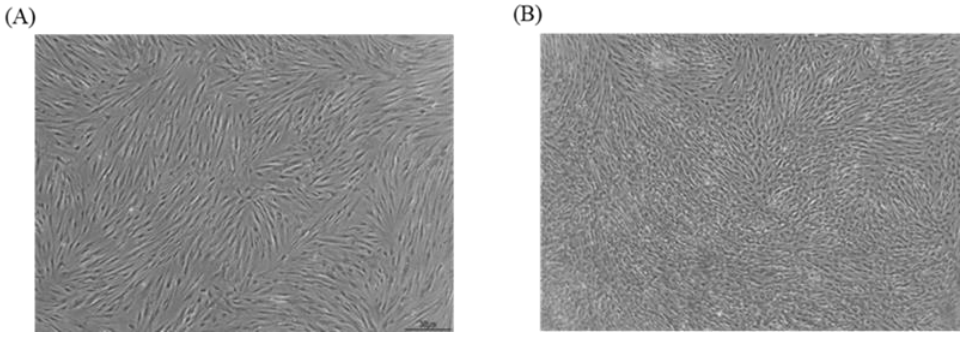

Figure 2 Morphology HDPCs. (A) HDPCs at lower confluence. Cells were spindle shaped with large nucleus and small cytoplasm. (B). HDPCs at high confluence. Cells became more polygonal in shape.

3.5 Ethical Consideration

This study was approved by Ethical Committee of Research Institute of Rangsit University (RSUERB2021-048).

4. Results

HDPCs derived by cell explant method showed the mesenchymal cell morphology, spindle shaped with large nucleus and little cytoplasm (Figure 2A). Cells became more polygonal in shape at higher confluency (Figure 2B). HDPCs derived by this method were previously characterized by flow cytometry and were found to be highly positive for the fibroblast markers, CD44, CD90, and CD105 while being negative for immune progenitors (CD34) and leukocytes markers (CD45) (Charoenpong et al., 2019).

To determine the effect of compressive force magnitude on Notch signaling activation, we applied a compressive force of 1 g/cm^2 and 2 g/cm^2 to HDPCs for 2 hours and investigated the mRNA expression of Notch target genes, *HES1* and *HEY1*. For data analysis, Shapiro-Wilk test indicated normal distribution of the data in all groups ($P \geq 0.05$). Levene's test indicated the homogeneity of variance among tested groups ($P \geq 0.05$). ANOVA was performed to compare the mean fold change of *HES1* and *HEY1* subjected to different magnitudes of force. The result showed that there was a significant difference in *HES1* expression among control cells, cell subjected to 1 g/cm^2 and cells subjected to 2 g/cm^2 of force in both hydrostatic ($P < 0.01$) and direct compression model ($P < 0.05$). LSD post hoc test further revealed the significant upregulation of *HES1* in HDPCs subjected to both 1 g/cm^2 and 2 g/cm^2 of force, from both hydrostatic and direct compression models, compared to control cells not receiving force ($P < 0.05$ and $P < 0.01$ as indicated) (Figure 3A and B). For *HEY1* mRNA expression, data analysis using ANOVA did not show any significant change ($P \geq 0.05$) following application of either hydrostatic or direct compressive force of both magnitudes at 2 hours of force application (Figure 3A and B).

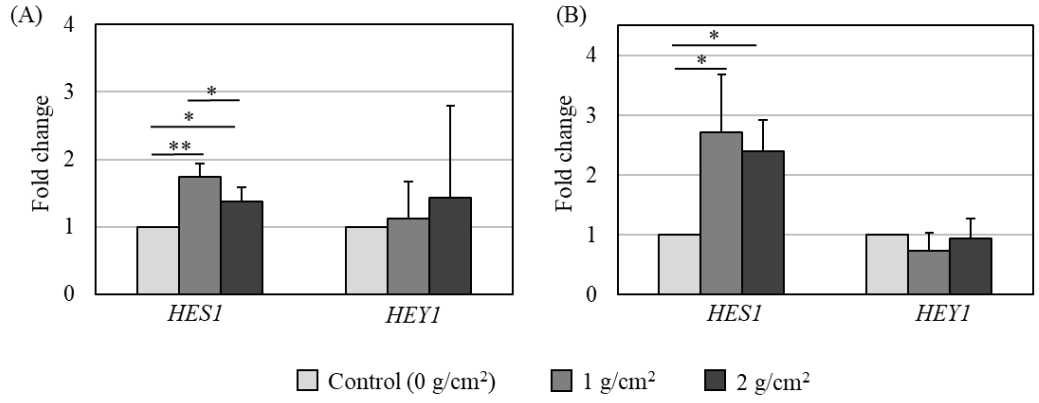

Figure 3 The fold changes of Notch target genes, *HES1* and *HEY1* mRNA expression following compressive force application in HDPCs for 2 hours by (A) hydrostatic compression model and (B) direct compressive model. Data are presented in mean±SD (n=3), **P* < 0.05, ***P* < 0.01.

Next, we investigated the effect of force duration on Notch signaling activation by applying compressive force of 1 g/cm^2 to HDPCs for 2 and 6 hours and determined the mRNA expression of Notch target genes. For data analysis, Shapiro-Wilk test indicated normal distribution of the data in all groups ($P \geq 0.05$) except for fold change of *HES1* in HDPCs subjected to hydrostatic compressive force for 6 hours ($P < 0.01$). Based on Shapiro-Wilk test, Mann-Whitney U test was further used to compare this groups to control. According to Levene's test, the equal variance was not assumed for *HES1* and *HEY1* expression in cells subjected to mechanical force by hydrostatic compression at 2 hours and for *HEY1* expression in cells subjected to mechanical force by direct compression at 2 and 6 hours ($P < 0.05$). For these data sets, Welch's t-test was performed to assess any significant difference comparing to control. For all other groups in which the data distribution was normal and equal variance was assumed ($P \geq 0.05$), independent t-test was used to compare the mean fold change of mRNA expression between controls cells and cells subjected to mechanical force. The results indicated that *HES1* mRNA expression was significantly increased by the compressive force generated from hydrostatic models at 2 and 6 hours after force application ($P < 0.05$) (Figure 4A). *HEY1* mRNA expression was also significantly upregulated by hydrostatic compression at 6 hours of force application ($P < 0.01$) (Figure 4A). Compressive force from direct compression models induced a significant increase in *HES1* mRNA expression in HDPCs only at 2 hours of force application ($P < 0.05$) (Figure 4B). At 6 hours, no upregulation of Notch target genes was observed for direct compression (Figure 4B).

Having observed the upregulation of Notch target genes, *HES1* and *HEY1*, following 2 and 6 hours of compressive force application in the hydrostatic compression model, we proceeded to investigate the expression of Notch receptors at these time points. Shapiro-Wilk test indicated normal distribution of the of all Notch mRNA expression in all groups ($P \geq 0.05$) except for the expression of *NOTCH1* in cells subjected to the force for 6 hours ($P < 0.05$). According to Shapiro-Wilk test, Mann-Whitney U test was selected for comparison between this group to the control. Levene's test indicated the homogeneity of variance of *NOTCH2* expression at 2 hours in control cells and cells receiving force ($P \geq 0.05$) in which independent t-test was further used for comparison between these groups. Welch's t-test was performed to for other *NOTCH* mRNA expression in which the equality of variance was not assumed as assessed by Levene's test ($P < 0.05$). The results showed that the only significant upregulation of Notch receptors was observed for *NOTCH2* after application of compressive force by the hydrostatic compression model for 6 hours ($P < 0.05$) (Figure 5). Expression of *NOTCH1*, *NOTCH3*, and *NOTCH4* did not change following application of compressive force in hydrostatic compression model for both 2 and 6 hours of force application (Figure 5).

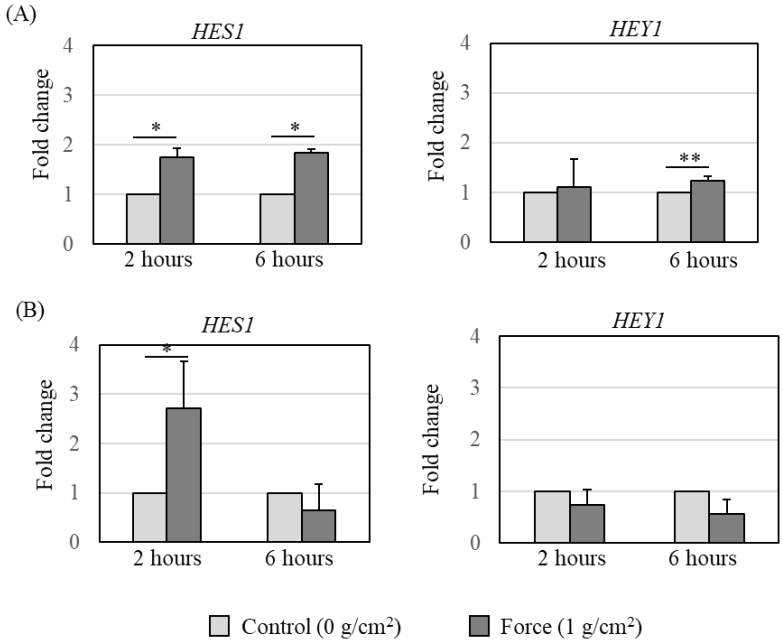

Figure 4 The fold changes of *HES1* and *HEY1* mRNA expression following compressive force application to HDPCs for 2 and 6 hours by (A) hydrostatic compression model and (B) direct compressive model. Data are presented in mean±SD (n=3), **P* < 0.05, ***P* < 0.01

For direct compression, we investigated the mRNA expression of Notch receptors at 2 hours of force application, which corresponded to the time point that we observed the upregulation of *HES1*. Shapiro-Wilk test indicated normal distribution of the data in all groups ($P \geq 0.05$). Levene's test indicated the homogeneity of variance of *NOTCH2* expression between controls cells and cells receiving force ($P \geq 0.05$) in which independent t-test was performed to compare the fold changes between these groups. For other *NOTCH* mRNA expression, the equality of variance was not assumed between control cells and cells subjected to mechanical force according to Levene's test ($P < 0.05$). Welch's t-test was then performed for comparisons between these groups and their controls. It was found that, although *HES1* was upregulated at this time point, there was no significant change in mRNA expression of all Notch receptors in HDPCs receiving direct compressive force compared to controls (Figure 6).

5. Discussion

Direct compression model using weight loading is the simplest model to deliver compressive force to the cells. However, the hydrostatic compression model produces a more harmonious load throughout the force application period with less deformation of cells shape and less physical damage to the cells compared to the direct compression model (Manokawinchoke et al., 2021; Natenstedt et al., 2015; Salinas et al., 2018). Although both models were used to deliver compressive force to various cell types in previous studies, none of the studies has compared the use of these two models. In the present study, we observed that both models seemed to produce the same response of HDPCs at early hours of force application, in that, 1 and 2 g/cm^2 of compressive force produced by both models can upregulated notch target gene, *HES1*, at 2 hours of force application while no upregulation of Notch receptor was observed. However, at longer duration, some difference can be observed. At 6 hours of force application, the upregulation of *HES1*, *HEY1*, and *NOTCH2* was observed only in the hydrostatic compression model. This suggested that the hydrostatic compression model produced more sustainable upregulation of Notch in HDPCs. Further studies on different aspects of cellular response and studies in other cell types would help to further clarify how cells response to compressive force generated by different models.

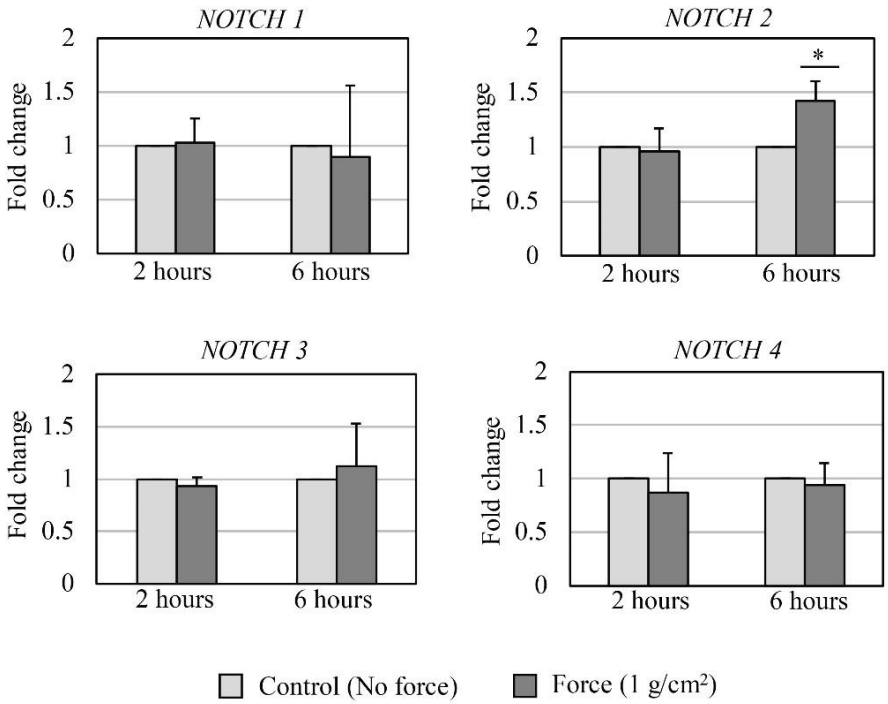

Figure 5 The fold changes of mRNA expression of Notch receptors following compressive force application by hydrostatic compression model for 2 and 6 hours. Data are presented in mean±SD (n=3), $*P < 0.05$

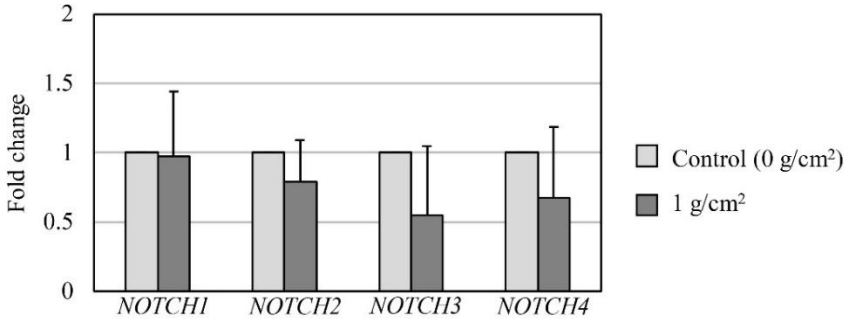

Figure 6 The fold changes of mRNA expression of Notch receptors following compressive force application by direct compression model for 2 hours. Data are presented in mean±SD (n=3)

In hydrostatic compression, mRNA of Notch target gene *HES1* was upregulated as early as 2 hours of force application while *HEY1* upregulation was not observed at this timepoint but can be observed after 6 hours of force application (Figure 4A). This might be due to the delayed response of *HEY1* gene after Notch signaling activation compared to *HES1*. Previous study using cells that have been engineered to express constitutively active form of Notch under the controlled condition found that *Hey1* upregulation required prolonged activation of Notch signaling while *Hes1* can be rapidly upregulated following only a short period of Notch activation (Nandagopal et al., 2018). Ziouti et al. (2019) also observed the delayed upregulation of *Hey1* following mechanical loading in osteocyte-enriched bone while *Hes1* upregulation was observed earlier after loading.

Activation of Notch signaling is a mechanosensitive process. Mechanical force generated from ligand endocytosis after binding of Notch ligand to its receptor, exposes S2 cleavage site on the negative regulatory region (NRR) of Notch (DuFort et al., 2011; Langridge, & Struhl,

2017; Meloty-Kapella et al., 2012). This allows Notch cleavage and releasing of NICD to activate Notch target genes. Previous studies reported that external mechanical force applied to ligand-bound Notch receptor or Notch receptor alone, can increase the cleavage at this S2 site (Gordon et al., 2015; Stephenson, & Avis, 2012). Therefore, it is possible that the mechanical force given to HDPCs in this study enhanced the mechanical force required to trigger Notch cleavage so that notch target gene, *HES1*, can be upregulated early at 2 hours (Figure 3) without upregulation of Notch receptors at this time point (Figure 5 and 6). However, to confirm whether compressive force affects Notch receptor cleavage and the release of NICD in HDPCs, further investigation is required.

Notch signaling is direct without signal amplification and Notch receptor cannot be "reused" since it is targeted to degradation thereafter (Bi, & Kuang, 2015; Henrique, & Schweisguth, 2019). This could be the reason that, in direct compression, in which no upregulation of Notch receptors was found (Figure 6), mechanical compressive force could not upregulate Notch target genes for longer than 2 hours, due to the lack of Notch receptors to replace those used. In contrast to hydrostatic compression in which an upregulation of *NOTCH2* was observed at 6 hours (Figure 5), more prolonged activation of mRNA of Notch target genes was observed (Figure 4). In addition, the stronger activation of *HES1* at 2 hours in direct compression (2.7 folds) compared to hydrostatic compression (1.7 folds) (Figure 4) may be responsible for the shorter duration of Notch target genes in the direct compression model, due to the larger number of Notch signaling components have been used at the early timepoint, causing less availability of them to generate signaling thereafter.

Mechanical compressive force may also increase the density of the cells and affect cell-cell contact. Since Notch signaling is activated in a juxtacrine manner, it was found that the amount of Notch signaling correlated with the contact area between cells (Sestan et al., 1999; Shaya et al., 2017). Extrinsic mechanical forces can affect cell shape and orientation, which affect cell-cell contact area (Matamoro-Vidal, & Levayer, 2019; Sumi et al., 2018). Therefore, it is also possible that the compressive force given to HDPCs increased cellular contact so that Notch signaling was enhanced. On the contrary, mechanical strain, which stretches the cells, was found to decrease Notch target genes in vascular smooth muscle cells (Loerakker et al., 2018; Morrow et al., 2005).

Notch signaling has been reported to be involved in various biological systems from development in embryo to maintaining homeostasis in adult. Dental pulp is surrounded by dentin which can be destroyed and regenerated throughout lifetime. Odontoblasts are dentin forming cells that can be differentiated from mesenchymal cells of dental pulp (Arana-Chavez, & Massa, 2004). Notch signaling has been reported to affect odontogenic differentiation process. However, the effect can be varied with activation by different Notch receptor-ligand complexes (Mitsiadis et al., 2017). In response to the mechanical force of Notch receptors, an increase in the expression of Notch1, Notch3, and Notch4 was observed in association with mechanical shear force in endothelial cells (Jahnsen et al., 2015; Mack et al., 2017; Masumura et al., 2009; Obi et al., 2009). Mechanical shear force also induced *Notch1* expression in limbal epithelial stem cells (Kang et al., 2014). In deciduous dental pulp cells, *NOTCH*1 was also found to upregulate in response to mechanical compressive force (Peetiakarawach et al., 2015). However, we observed that in adult human dental pulp cells, *NOTCH2* was the only Notch receptor upregulated following application of compressive force (Figure 5). The upregulation of *NOTCH2*, among all Notch receptors, observed in our study corresponds with the previous report in rat and human teeth that found Notch2 were the most prominent Notch receptor upregulated following pulpal injury (Mitsiadis et al., 1999; Mitsiadis et al., 2003). Upregulation of Notch2 following pulpal injury was observed mainly in undifferentiated cells that were likely to differentiate into odontoblasts (Cai et al., 2011; Mitsiadis et al., 1999; Mitsiadis et al., 2003). In vitro studies also reported that Notch2 was involved in osteogenic differentiation of dental pulp and PDL cells (Manokawinchoke et al., 2017; Manokawinchoke et al., 2020). In addition, it was also reported that compressive force enhanced the odontogenic differentiation of dental pulp cells (Marrelli et al., 2018; Miyashita et al., 2017; Yu et al., 2009). Therefore, it is possible that the upregulation of *NOTCH2* and Notch target genes in HDPCs following compressive force application may be related to odontogenic differentiation of these cells. However, it should be noted that, change in *NOTCH2* expression observed in this study was only 1.4-fold of control. Further study is

required to confirm the biological effect of an increase in *NOTCH2* expression in HDPCs subjected to mechanical force.

Further investigation of Notch ligands as well as investigation of protein expression of Notch signaling components should give more information about Notch signaling in dental pulp cells subjected to mechanical force. It should also be noted that, although statistically significant, fold changes of some gene expression following force application showed only slight change from control. Further study should be performed to investigate the biological effect of these changes. In addition, since dental pulp cells can be subjected to other types of force such as shear force, further study using other types of mechanical force should lead us toward better understanding of the dental pulp cells response to mechanical force. The results from this study could serve as the basic information for further studies that will lead to better understanding of molecular mechanisms occurring when dental pulp cells encounter mechanical force. This knowledge could be further applied to enhance the repair process of dental pulp and reducing the destructive processes including dental root resorption following the clinical situations such as tooth trauma, parafunctional habit and Orthodontic tooth movement.

6. Conclusion

Expression of Notch target gene, *HES1*, can be activated in HDPCs following compressive force application by both direct and hydrostatic compression models at an early time point. However, the hydrostatic compression model produced more prolonged activation of *HES1*. Hydrostatic compression also produced an upregulation of *HEY1* and *NOTCH2* at a later time point which was not observed in direct compression.

7. Acknowledgements

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