BMC Oral Health

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Oxidative stress impairs the calcification ability of human dental pulp cells

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Abstract

Background: The relationship between internal root resorption and oxidative stress has not yet been reported. This study aimed to add molecular insight into internal root resorption. The present study was conducted to investigate the effect of hydrogen peroxide (H_2O_2) as an inducer of oxidative stress on the calcification ability of human dental pulp cells (hDPCs) and the involvement of inositol 1, 4, 5-trisphosphate (IP3).

Material and methods: hDPCs (Lonza, Basel, Switzerland) were exposed to H_2O_2. Cell viability and reactive oxygen species (ROS) production were then evaluated. To investigate the effect of H_2O_2 on the calcification ability of hDPCs, real-time PCR for alkaline phosphatase (ALP) mRNA expression, ALP staining, and Alizarin red staining were performed. Data were compared with those of hDPCs pretreated with 2-aminoethyldiphenylborate (2-APB), which is an IP3 receptor inhibitor.

Results: H_2O_2 at concentrations above 250 μM significantly reduced cell viability ($P<0.01$). More ROS production occurred in 100 μM H_2O_2-treated hDPCs than in control cells ($P<0.01$). 2-APB significantly decreased the production ($P<0.05$). H_2O_2-treated hDPCs showed significant reductions in ALP mRNA expression ($P<0.01$), ALP activity ($P<0.01$), and mineralized nodule deposition compared with negative control cells ($P<0.01$). 2-APB significantly inhibited these reductions ($P<0.01$, $P<0.05$ and $P<0.01$, respectively). Data are representative of three independent experiments with three replicates for each treatment and values are expressed as means ± SD.

Conclusion: To the best of our knowledge, this is the first study documenting the involvement of IP_3 signaling in the calcification ability of human dental pulp cells impaired by H_2O_2.

Keywords: Oxidative stress, Human dental pulp cells, 2-aminoethyldiphenylborate

Introduction

Internal root resorption in permanent teeth is characterized by destruction of intraradicular dentin and dentinal tubules along the root canal wall [1]. It occurs in pathological conditions, including trauma, infections, or unknown causes [1, 2]. Concern and curiosity about resorption of dental structures are not recent. Numerous theories have been proposed as a possible cause of internal root resorption. One study reported that dental pulp cells have an innate ability to attenuate dentin resorption by inhibiting osteoclastogenesis [3]. Therefore, it is assumed that the damaged dental pulp cells are probably related to the onset of internal root resorption. However, the underlying pathology is not fully understood.

Dental pulp cells can differentiate into odontoblasts and generate a mineralizing matrix, particularly during reparative dentin formation associated with injury or disease [4, 5]. Odontoblasts, which are organized in a layer

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at the dentin-pulp interface, secrete type I collagen, osteocalcin (OCN), alkaline phosphatase (ALP), and other noncollagenous proteins [6]. The impairment of reparative dentin formation in permanent teeth may be a key factor implicated in internal root resorption.

Oxidative stress is a damaging response and refers to excessive intracellular levels of reactive oxygen species (ROS). Hydrogen peroxide (H_2O_2) is one of the major ROS. At low physiological levels in the nanomolar range, H_2O_2 is the major agent signaling through specific protein targets that engage in metabolic regulation and stress responses to support cellular adaptation to a changing environment and stress. Many previous studies reported the effects of oxidative stress on pulp cells [7–9]. One study showed that the oxidative stress of human dental pulp cells mediated by H_2O_2 promotes reduction of odontoblastic capability [8]. On the other hand, Matsu et al. [9] found an increase in osteopontin (OPN) and osteocalcin (OCN) in H_2O_2-treated-human dental pulp cells. Further investigations seem to be needed to clarify the mechanism. In addition, the relationship between internal root resorption and oxidative stress has not yet been reported. This study focused on H_2O_2 as an inducer of oxidative stress.

2-Aminoethyldiphenylborate (2-APB) was originally described as a membrane-permeant inhibitor of the inositol 1, 4, 5-trisphosphate (IP_3) receptor, and it was also used as one of the store-operated Ca^{2+} entry (SOCE) inhibitors [10]. Yamamura et al. [11] reported that oxidative stress reduced SOCE, and the decrease was recovered by 2-APB in brain capillary endothelial cells.

The present study was conducted to add molecular insight into root canal resorption. In the current study, it was demonstrated for the first time that IP_3 signaling is involved in the calcification ability of H_2O_2-treated human dental pulp cells. A possible mechanism to explain the influence of the oxidative stress caused by H_2O_2 on the calcification ability of human dental pulp cells is presented. The present study may provide a clue to help clarify a mechanism of onset of internal root resorption.

Materials and methods

Reagents

Hydrogen peroxide (H_2O_2) and 2-aminoethoxydiphenylborane (2-APB) were purchased from FUJIFILM Wako Pure Chemicals Corporation (Osaka, Japan) and Bio-Techne Corporation (Minneapolis, MN, USA), respectively.

Cell culture

Human dental pulp cells (hDPCs) were obtained from Lonza (Basel, Switzerland). Cells were cultured in growth medium (GM) (Eagle's Minimum Essential Medium, ALPHA modification (MEM-α)) supplemented with 10% heat-inactivated FBS (Fetal Bovine Serum; Sigma-Aldrich, MO, USA), 100 IU/mL penicillin (FUJIFILM Wako Pure Chemicals Corp.), and 100 μg/mL streptomycin (FUJIFILM Wako Pure Chemicals Corp.) (medium A). The cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cell cultures between the sixth and ninth passages were used in this study.

To differentiate into odontoblast-like cells, the cells were cultured in osteo-odontogenic medium (OM), MEM-α supplemented with 2.5% heat-inactivated FBS (Sigma-Aldrich), 100 IU/mL penicillin (FUJIFILM Wako Pure Chemicals Corp.), 100 μg/mL streptomycin (FUJIFILM Wako Pure Chemicals Corp.), 10 mM β-glycerophosphate (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), and 50 mg/mL ascorbic acid (FUJIFILM Wako Pure Chemicals Corp.).

WST assay

Cell viability was assessed by the WST assay using the Cell Counting Kit-8 according to the manufacturer's protocol (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Briefly, cells were plated at a density of 5.0×10^3 cells/well in 96-well plates and maintained in 0.1 mL of medium A. After 24-h incubation, culture medium was replaced to serum-free medium with H_2O_2 at concentrations of 0, 50, 100, 250, 500, and 1000 μM (final concentration) and incubated for 24 h in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Then, CCK-8 solution was added, and the cells were incubated for another hour. Absorbance at 450 nm was then measured with the reference wavelength at 620 nm using a microplate reader (Multiskan™ FC; Thermo Fisher Scientific, CA, USA). Data are representative of three independent experiments with three replicates for each treatment.

Intracellular reactive oxygen species measurements

Measurement of intracellular ROS was performed by dichlorodihydrofluorescein diacetate (DCFH-DA) (ROS Assay Kit-Highly Sensitive DCFH-DA-; Dojindo Laboratories). Cells were seeded at a density of 1.0×10^4 cells in 6-well plates and incubated overnight in medium A. The medium was replaced to serum-free medium with 10 μM 2-APB or PBS for control cells. One hour later, the cells were stimulated with 100 μM H_2O_2 for 2 h in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. DCFH-DA solution was added after 3-h incubation, and the cells were then incubated for another 30 min. Fluorescence signals were observed using an all-in-one fluorescence microscope (BZ-X700; Keyence, Tokyo, Japan). Data are representative of three independent experiments with three replicates for each treatment.

ALP staining

To evaluate alkaline phosphatase activity, the cells were stained by BCIP-NBT solution (SIGMA FAST™BCIP®/NBT; Sigma-Aldrich). hDPCs were seeded into 48-well plates at a density of 1.0×10^4 cells/well in medium A. After the cells reached 70–80% confluency, culture medium was replaced to fresh MEM-α supplemented with 2.5% FBS (Sigma-Aldrich), 100 IU/mL penicillin (FUJIFILM Wako Pure Chemicals Corp.), and 100 μg/mL streptomycin (FUJIFILM Wako Pure Chemicals Corp.) (medium B). The cells were treated with 10 μM 2-APB or PBS for control cells. One hour later, cells were stimulated with 100 μM H_2O_2 for 6 h. Then, medium was replaced to OM and cultured for 7 days. The medium was changed on the third day. After incubation, the cells were rinsed with PBS, fixed with 10% neutral buffered formalin, and stained with BCIP-NBT solution. The stained area was quantified by Image J software (National Institutes of Health, Bethesda, MD, USA). To briefly explain the quantification method, images were converted to RGB stack, and "blue slice" images were picked up (Fig. 2A(b)). The threshold of the blue slice images was adjusted, and a limited threshold area was highlighted in red, as shown in Fig. 2A(c). Thereafter, the % stained area was automatically measured by the software. Data are representative of three independent experiments with three replicates for each treatment.

Alizarin red staining

hDPCs were seeded into 6-well plates at a density of 1.0×10^5 cells/well and cultured in medium A. After the cells reached 70–80% confluency, the culture medium was replaced to medium B and treated with 10 μM 2-APB or PBS for control cells. One hour later, cells were stimulated with 100 μM H_2O_2 for 6 h. The medium was then replaced to OM and cultured for 28 days. The medium was changed every 3 days. To evaluate calcium deposition, Alizarin red S Solution (PG research, Tokyo, Japan) was used after fixation with 10% neutral buffered formalin. The images were captured by an all-in-one fluorescence microscope (BZ-X700; Keyence), and the stained area was quantified by Image J software (National Institutes of Health). Data are representative of three independent experiments with three replicates for each treatment.

Isolation of total RNA and reverse transcription

hDPCs were seeded into 6-well plates at a density of 1.0×10^5 cells/well and cultured in medium A until subconfluent. The cells were treated with 10 μM 2-APB or PBS for control cells. One hour later, the cells were stimulated with 100 μM H_2O_2 for 6 h. The cells were replaced to OM and cultured for 7 days. The medium was changed on the third day. Total RNA from each culture was extracted using RNA iso plus (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. cDNA was synthesized from 250 ng of total RNA in a final volume of 10 μL using the Rever Tra Ace qPCR RT Master Mix with gDNA remover kit (Toyobo Co., Ltd, Osaka, Japan) with a thermal cycler (Veriti™ 96-Well Thermal Cycler; Life Technologies, CA, USA).

Real-time PCR

Two-step qPCR was performed with the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, CA, USA) using THUNDERBIRD® Next SYBR® qPCR Mix (Toyobo Co., Ltd, Osaka, Japan). The cycling protocol was as follows: DNA polymerase activation at 95 °C for 30 s, followed by denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 10 s, for 40 cycles in fast mode. Gene expression was normalized to that of *Gapdh* mRNA in the same samples, using the $2^{-\Delta\Delta Ct}$ method. The sequences of relevant primers were as follows: *Gapdh*: forward 5′-AACGTGTCAGTGGTGGACCTG-3′; reverse 5′-AGTGGGTGTCGCTGTTGAAGT-3′; *ALP*: forward 5′-CGCCTACCAGCTCATGCATAAC-3′; reverse 5′-GTCAATTCTGCCTCCTTCCACC-3′. Data are representative of three independent experiments with three replicates for each treatment.

Statistical analysis

Each result is presented as a mean ± standard error of the mean. All experiments were performed in at least triplicate. Comparisons between two groups were evaluated by an unpaired two-tailed Student's *t*-test. For comparisons of more than two groups, one-way ANOVA was followed by Tukey's test for multiple comparisons. Significance was set at $p < 0.05$.

Results

hDPCs underwent severe cell death after high-dose H_2O_2 (250, 500 and 1000 μM) treatment for 24 h as determined by the WST assay (Fig. 1A). H_2O_2 at 50 and 100 μM did not decrease cell viability. ROS were increased in 100 μM H_2O_2-treated hDPCs (Fig. 1B). Therefore, in the present study, H_2O_2 at 100 μM, which is enough to promote ROS production, was used.

To determine whether H_2O_2 has an effect on the calcification ability of hDPCs in the present experimental system, the effects of H_2O_2 on hDPCs cultured in osteoinductive medium were tested via ALP activity. After osteogenic differentiation for 7 days, H_2O_2 at 100 μM significantly decreased ALP activity in hDPCs ($P < 0.01$) (Fig. 2A, B).

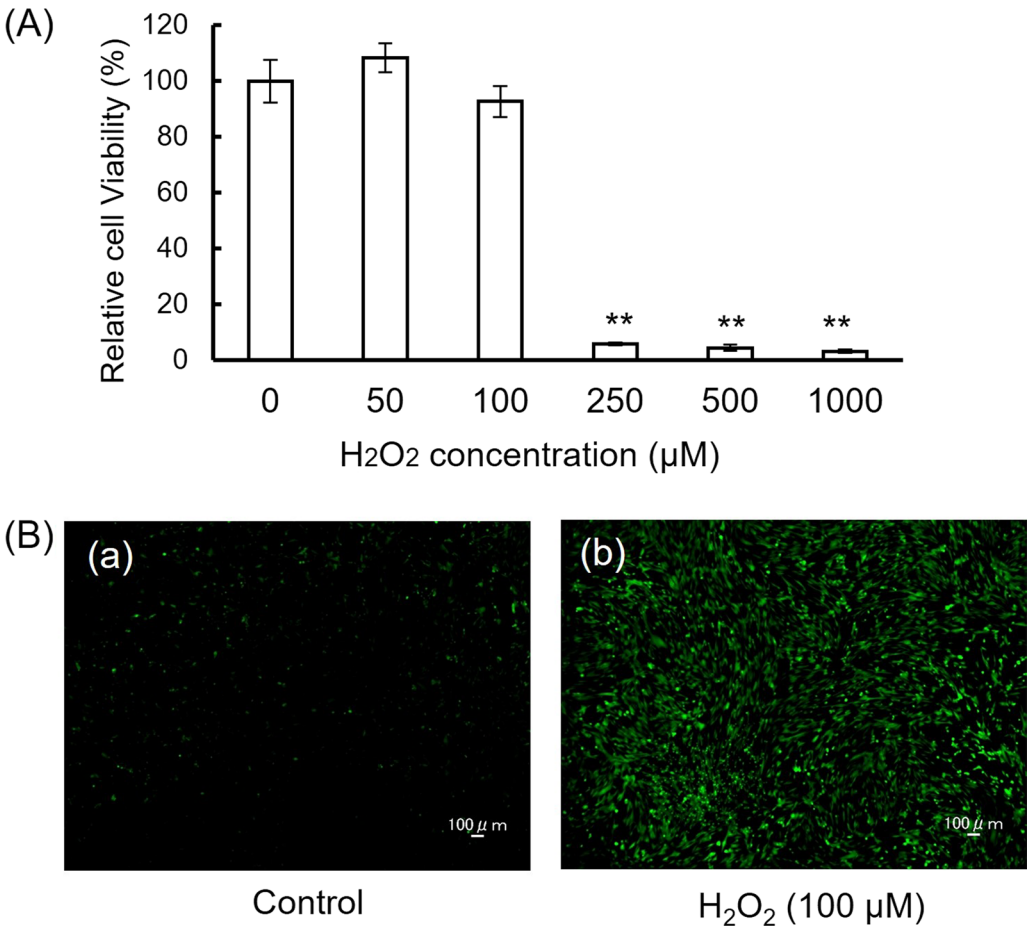

Fig. 1 Cell viability and ROS production of H_2O_2-treated human dental pulp cells. **A** Cell viability is assessed by the WST assay using Cell Counting Kit-8. hDPCs were treated with H_2O_2 at concentrations of 50, 100, 250, 500, and 1000 μM for 24 h. Data are presented as means ± SD of three independent experiments. $^{**}P < 0.01$, one-way ANOVA with Tukey's test. **B** ROS production in hDPCs. Fluorescence signals were detected with a confocal microscope. (a) Control, (b) H_2O_2 100 μM. Bars: 100 μm

Real-time PCR was used to determine the effect of H_2O_2 addition on the mRNA expression of ALP. H_2O_2 significantly decreased the ALP mRNA levels compared with control cells ($P < 0.01$). 2-APB reversed the decrease significantly ($P < 0.05$) (Fig. 3A).

The percentages of the BCIP-NBT stained area of the control group, positive control group (cultured in osteo-inductive medium), H_2O_2 group and H_2O_2/2-APB treated group were 24.4 ± 1.3%, 76.9 ± 5.9%, 45.2 ± 0.6%, and 62.6 ± 4.1%, respectively. H_2O_2 down-regulated ALP activity compared with positive control cells (cultured in osteo-inductive medium). 2-APB significantly rescued the decrease of ALP activity in H_2O_2-treated cells ($P < 0.01$) (Fig. 3B, C).

The percentages of the Alizarin red-stained area of the control group, positive control group (cultured in osteo-inductive medium), H_2O_2 group, and H_2O_2/2-APB treated group were 19.1 ± 3.6%, 67.1 ± 3.2%, 26.9 ± 4.8%, and 57.1 ± 1.5%, respectively. Alizarin red staining showed that 2-APB significantly increased H_2O_2-decreased hDPC calcification ($P < 0.01$) (Fig. 4A, B).

ROS were increased in 100 μM H_2O_2-treated hDPCs (Fig. 5A(a, b). 10 μM 2-APB inhibited the ROS production induced by 100 μM H_2O_2 (Fig. 5A(c)). 10 μM 2-APB did not affect the ROS production(Fig. 5A(d)). FITC fluorescence intensity of the H_2O_2/2-APB treated group was significantly decreased compared to that of the H_2O_2-treated group ($P < 0.05$) (Fig. 5B).

Discussion

H_2O_2 promotes ROS production in various cells, such as human periodontal ligament cells, human retinal epithelial cells, and vascular endothelial cells [12–14]. H_2O_2 can induce oxidative destruction of organs and tissues,

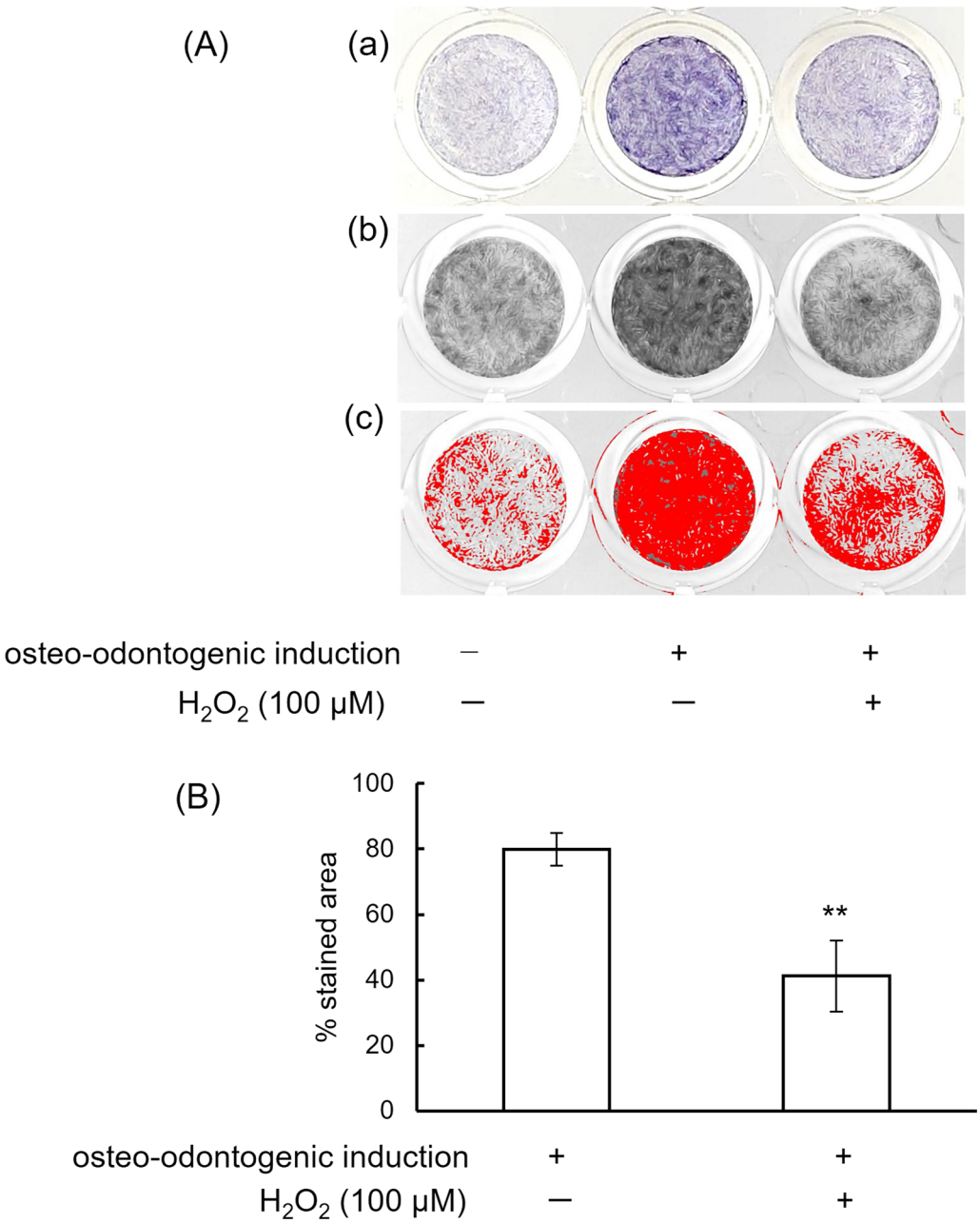

Fig. 2 H_2O_2 attenuates ALP activity of hDPCs. **A**(a) A macroscopic view of wells showing hDPCs cultured in growth medium, hDPCs cultured in osteo-odontogenic medium, and hDPCs treated with 100 μM H_2O_2 in osteo-odontogenic medium. (b) **A**(a) panels were converted to RGB stack, and "blue slices" were picked up. (c) The thresholds of the images of **A**(b) were adjusted, and a limited threshold area is highlighted in red. **B** The stained area was quantified using Image J software on digitized photomicrographs (**A**(c)) captured by a Windows-based computer. Values are expressed as means ± SD of three independent experiments. $^{**}P < 0.01$, Student's *t*-test

associated with other inflammatory responses when overexpressed in cells [15]. The effect of H_2O_2 in a living system is dependent on the type of cell, its concentration, its physiological state, and duration of exposure [16, 17]. Deng et al. [18] reported that, like other ROS molecules, a high concentration of H_2O_2 is cytotoxic to cells. In the present study, high-dose H_2O_2 (250, 500 and 1000 μM) significantly reduced the viability of hDPCs. Loss of cell viability is one characteristic of apoptotic cells. Therefore, in the present study, to avoid the influence of apoptosis, H_2O_2 at 100 μM, which is enough to promote ROS production without inducing apoptosis, was used.

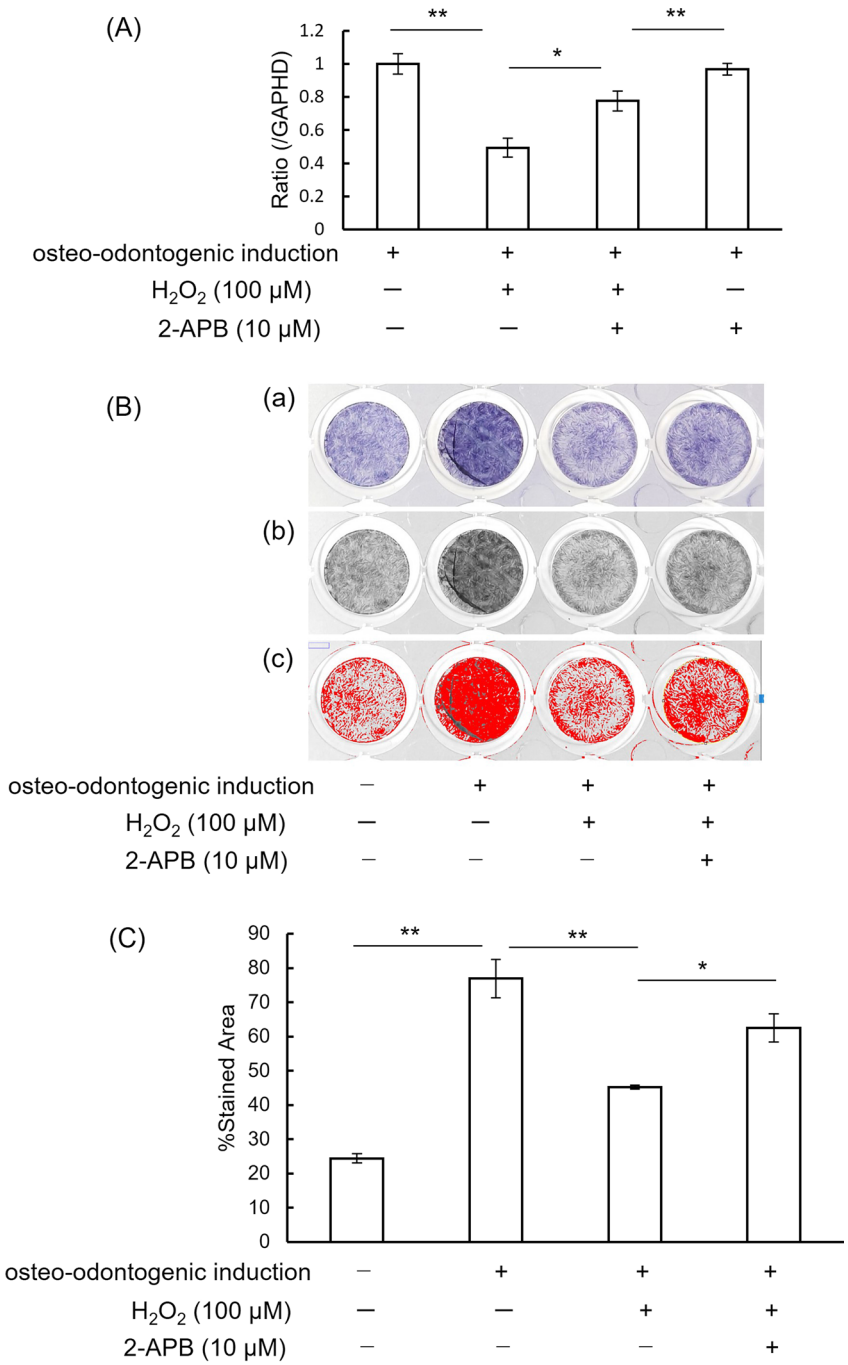

Fig. 3. 2-APB reverses H_2O_2-decreased ALP mRNA expression and ALP activity of hDPCs. **A** ALP mRNA was determined by real-time PCR. Graphs show the ratio of ALP mRNA to GAPDH mRNA. Values represent means ± SD of three cultures. *$p < 0.05$, **$p < 0.01$: differs significantly from the control. **B**(a) A macroscopic view of wells showing hDPCs cultured in growth medium, hDPCs cultured in osteo-odontogenic medium, hDPCs treated with 100 μM H_2O_2 in osteo-odontogenic medium, and hDPCs treated with 10 μM 2-APB before the addition of 100 μM H_2O_2 in osteo-odontogenic medium. (b) **B**(a) panels have been converted to RGB stack, and "blue slice" images were picked up. (c) The thresholds of **B**(b) images were adjusted, and a limited threshold area is highlighted in red. **C** The stained area was quantified using the software NIH Image J® on digitized photomicrographs (**B**(c)) captured by a Windows-based computer. Data are shown as means ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$, one-way ANOVA with Tukey's test

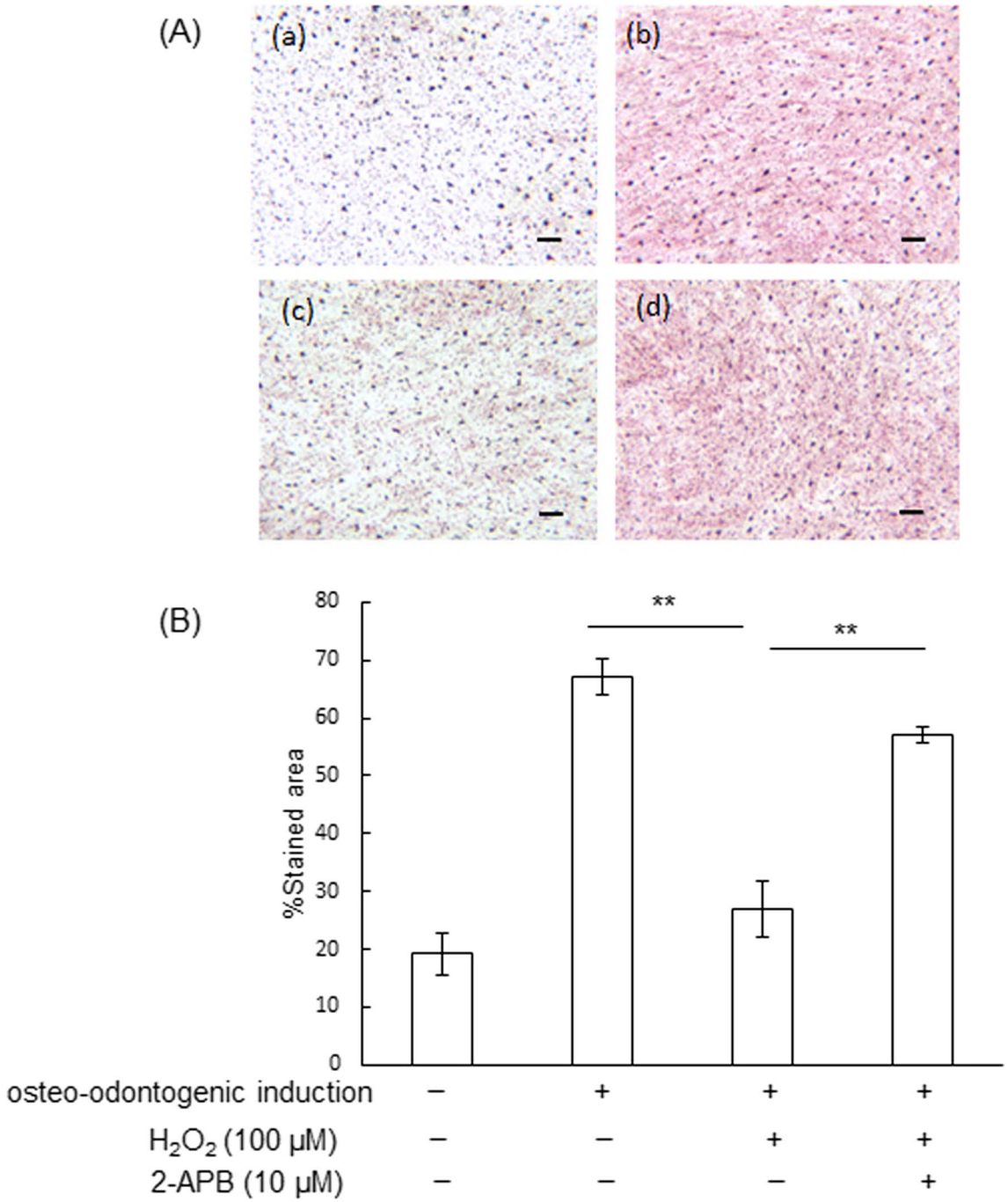

Fig. 4 Assessment of hDPC calcification with Alizarin red staining. **A** Panels show hDPCs cultured in growth medium (a), hDPCs cultured in osteo-odontogenic medium (b), hDPCs treated with 100 μM H_2O_2 in osteo-odontogenic medium (c) and hDPCs treated with 10 μM 2-APB before the addition of 100 μM H_2O_2 in osteo-odontogenic medium (d). Bars: 100 μm. **B** The amount of calcium was quantified using Image J software on digitized photomicrographs captured by a Windows-based computer. Data are presented as means ± SD of three independent experiments. $^{**}P < 0.01$, one-way ANOVA with Tukey's test

Alkaline phosphatase (ALP), involved with the initial phase of dentin matrix biomineralization, promotes dephosphorylation of extracellular matrix proteins, providing inorganic phosphate [19]. In the present study, H_2O_2 decreased ALP activity in hDPCs cultured in osteo-inductive medium. These data are in

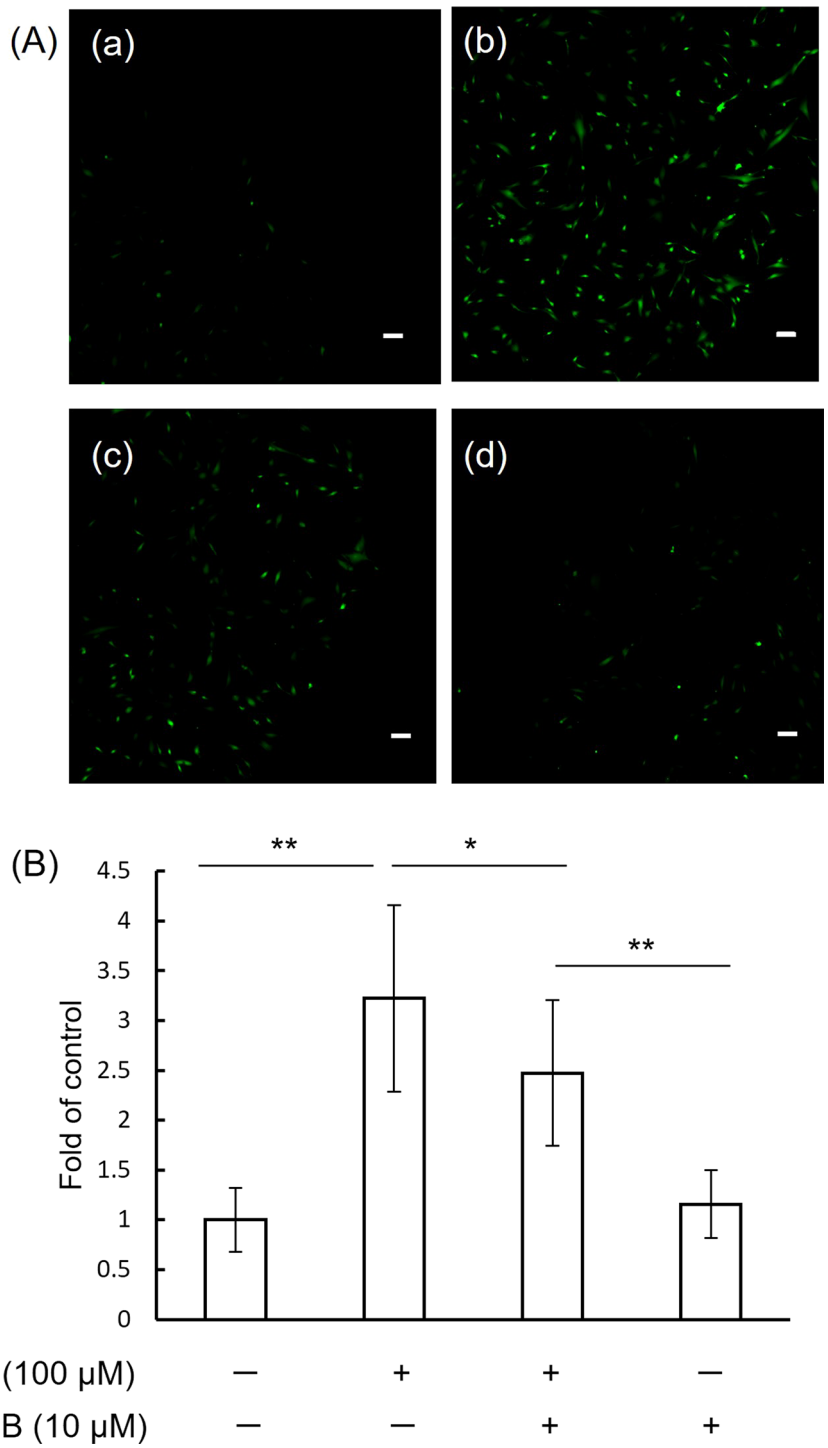

Fig. 5. 2-APB inhibits the ROS production induced by H_2O_2 in hDPCs. **A** ROS production in hDPCs. Fluorescence signals were detected with a confocal microscope. (a) control, (b) H_2O_2 100 μM, (c) H_2O_2 100 μM + 2-APB 10 μM, (d) 2-APB 10 μM. Bars: 100 μm. **B** The graph shows quantitative results of ROS production from three independent experiments. Per well, three pictures were taken at random. FITC fluorescence intensity from an area of 2 mm square from each picture was analyzed using Image J software on digitized photomicrographs captured by a Windows-based computer. Data represent means ± SD. *$P < 0.05$, **$P < 0.01$, one-way ANOVA with Tukey's test

agreement with those from previous studies [8, 20]. Correlated with ALP activity, the present study also showed decreased ALP mRNA expression by H_2O_2 in hDPCs.

2-APB has been described as a membrane-permeant inhibitor of the IP_3 receptor, and the inhibition of SOCE by 2-APB was taken as evidence for IP_3 receptor activation of calcium release-activated Ca^{2+} channels [21]. It has been reported that the increase in intercellular Ca^{2+} concentration regulates cell functions, such as proliferation, differentiation, and migration [22]. A previous study reported that oxidative stress reduced SOCE, and the decrease was reversed by 2-APB in brain capillary endothelial cells [11]. In the present study, the H_2O_2-decreased human dental pulp cell calcification ability was reversed by 2-APB. In addition, an important criterion for the characterization of odontoblastic cells is their ability to mineralize the collagenous matrix they secrete. In the present study, 2-APB rescued the H_2O_2-decreased amounts of mineralization of the extracellular matrix, judged by Alizarin red staining. Contrary to the present findings, it has been reported that vascular smooth muscle cell calcification was reduced by 2-APB [23, 24]. 2-APB is also used as an inhibitor of transient receptor potential channels (TRPCs), in which Ca^{2+} entry was inhibited directly, rather than as a result of the inhibition of IP_3 [25]. Further experiments are needed to clarify the detailed mechanism underlying the relationships between IP_3, SOCE, TRPC and the calcification ability of human dental pulp cells.

It has been reported that 2-APB reduced ROS production in neutrophils and prevented ROS-induced cardiomyocyte death [26, 27]. The present study showed for the first time that 2-APB reduces ROS production in hDPCs. Therefore, administration of 2-APB may represent a promising therapeutic strategy for the treatment of ROS-related endodontic disease. Further experiments beyond the scope of this paper are required to determine the relationship between ROS and endodontic disease.

In summary, H_2O_2 decreased the calcification ability of human dental pulp cells, and the reduction was reversed by 2-APB. To the best of our knowledge, this is the first study documenting the involvement of IP_3 signaling in the calcification ability of human dental pulp cells. The results of the present study add molecular insight into internal root resorption and may provide a clue to the development of a new therapeutic agent for such resorption in endodontic therapy.

Abbreviations

hDPCs: Human dental pulp cells; H_2O_2: Hydrogen peroxide; ROS: Reactive oxygen species; 2-APB: 2-Aminoethyldiphenylborate.

Acknowledgements

This work was in part supported by a Grant-in-Aid for Scientific Research (C) (JP25463218) of the Japan Society for the Promotion of Science.The authors would like to thank the Analysis Center of Life Science of Hiroshima University for the use of facilities.

Author contributions

SS mainly carried out the analyses of this study, performed the statistical analysis, and helped draft the manuscript. KT participated in study design and data interpretation and drafted the manuscript. TN and YT were involved in cell culture and data analysis. JN and SS helped draft the manuscript. HS participated in design and coordination and helped draft the manuscript. All authors critically revised the report, commented on drafts of the manuscript, and approved the final report.

Funding

Grant-in-Aid for Scientific Research (C) (JP25463218) of the Japan Society for the Promotion of Science financially support publishing.

Availability of data and materials

The datasets generated during or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

We, Katsuhiro Takeda (corresponding author), Satomi Shirawachi, Tomoya Naruse, Yohei Takahasi, Jun Nakanishi, Satoru Shindo, and Hideki Shiba hereby declare that we participated in the study and the development of this manuscript. We have read the final version and give our consent for the article to be published in BMC Oral Health.

Consent for publication

Not applicable.

Competing interests

The authors of the paper declare that there are no conflicts of interest.

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Received: 22 July 2022 Accepted: 20 September 2022
Published online: 03 October 2022

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