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Original Article

# Assessing Cell Viability: Comparative Analysis of Calcium Hydroxide, Triple Antibiotic Paste, and Their Synergistic Impact on human Dental Pulp Stem Cells

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## Abstract

**Objective** The American Association of Endodontists recommends the use of calcium hydroxide ($Ca(OH)_2$) or triple antibiotic paste (TAP) as preferred medicaments in regenerative endodontic treatment. Although both medicaments showed an excellent antibacterial property, their impact on the viability of human dental pulp stem cells (hDPSCs) when used in combination remains uncertain. Previous studies have indicated that at certain concentrations, both $Ca(OH)_2$ and TAP can be harmful to cells. Therefore, it is aimed to assess the effects of $Ca(OH)_2$, TAP, and their combined application on the viability of hDPSCs in this study.

**Materials and Methods** Primary cultured hDPSCs, reaching 80% confluency and at passages 3rd to 4th, were subjected to 24-hour starvation. Subsequently, they were cultured in media supplemented with $Ca(OH)_2$, TAP at 0.1 and 1 mg/mL concentrations, and a combination of $Ca(OH)_2$ and TAP at equivalent concentrations, with Dulbecco's modified eagle medium serving as the control group. The viability and morphology of hDPSCs were assessed using both the quantitative 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and qualitative 4',6-diamidino-2-phenylindole staining.

**Statistical Analysis** First, the data were analyzed by one-way analysis of variance, followed by Bonferroni post hoc to compare between groups. All the tests were conducted at a significance level of 95% ($p < 0.05$).

**Results** In this study, a notable variation in hDPSCs viability was observed among all groups, with the lowest viability recorded in the combination of $Ca(OH)_2 + TAP$ at 1 mg/mL ($p < 0.05$).

**Conclusion** $Ca(OH)_2$, TAP, and their combination are not toxic to hDPSCs and the use of their combination was superior in hDPSCs viability on $Ca(OH)_2 + TAP$ in the 0.1 mg/mL group.

**Keywords**
- calcium hydroxide
- dental pulp stem cells
- endodontic
- regenerative
- triple antibiotic

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## Introduction

Regenerative endodontic treatment (RET) aims to regenerate the dentin-pulp complex that has been damaged, allowing it to function physiologically like normal pulp. One strategy to support dentin-pulp complex regeneration and maintain pulp vitality is the use of medicaments in RET.[1–4] Regeneration of the dentin-pulp complex essentially consists of two parts, regeneration related to pulp vitality preservation, such as pulp capping and pulpotomy, and regeneration related to revitalization or revascularization in nonvital teeth with infected root canals.[5]

The American Association of Endodontists (AAE) recommends calcium hydroxide ($Ca(OH)_2$) or triple antibiotic paste (TAP) as the medicament of choices in RET. Nonetheless, RET can fail due to secondary or persistent endodontic infections that may be detected after a 1-year evaluation. Related to this matter, Almutairi et al emphasized the potential for long-term failure with 1.5% sodium hypochlorite irrigation alone.[6,7] Therefore, the use of medicaments in RET as disinfection agents is essential. Medicament use in RET procedures can enhance the sterilization of root canals and the remaining dental pulp, when only minimally invasive instrumentation can be performed or in some specific cases (such as Cvek pulpotomy) in which no instrumentation can be done during the RET procedure.[7]

The AAE has designated $Ca(OH)_2$ as the primary medicament for root canal treatment. The antibacterial effect of $Ca(OH)_2$ is based on several mechanisms, including chemical and physical actions.[8,9] TAP as the other AAE recommended medicament, can also be used in RET. Previous studies have reported that either $Ca(OH)_2$ or TAP have superior antibacterial capabilities and facilitate the dentinal pulp regeneration process. TAP comprises metronidazole, ciprofloxacin, and minocycline antibiotics in a paste form and is suggested as a viable substitute for $Ca(OH)_2$. TAP demonstrates efficacy against microorganisms associated with endodontic infections.[3,10–12]

It is advisable to use TAP at its safest concentration (1 mg/mL) since higher doses may lead to adverse effects on stem cells in situ.[13] Prasanti et al found a significant decrease in human dental pulp stem cells (hDPSCs) viability at TAP concentration of 0.1 and 1 mg/mL. The same result occurred at $Ca(OH)_2$ concentration of 0.1 and 1 mg/mL. These results indicate that TAP and $Ca(OH)_2$ have similar effects on the cell viability.[7] Fouad et al compared the use of medicaments at varying concentrations in three procedures, for apexification using $Ca(OH)_2$, revitalization using TAP 0.1 mg/mL, and revascularization using TAP 1 mg/mL.

The results revealed that antimicrobial medicaments at high concentrations exhibit greater efficacy in qualitatively and quantitatively reducing endodontic microflora. Nevertheless, the impact on dental pulp cell viability is still unknown. In this study, a TAP concentration of 0.1 mg/mL was used because this concentration is consistent with the pharmacologically active antibiotic concentration in blood plasma and is used in the revitalization treatment group with pulpitis conditions (vital pulp) where bacterial penetration was assumed to be less minimal compared with necrotic pulp conditions. In revascularization procedure with necrotic pulp and open apex, a concentration of TAP 1 mg/mL is used for root canal disinfection, which is higher than the concentration for revitalization procedure.[2,14]

Some root canal bacteria are resistant to $Ca(OH)_2$, the use of which is quite effective in eliminating obligate anaerobic bacteria. However, facultative anaerobic bacteria can survive after exposure to this medicament. *Enterococcus faecalis*, a Gram-positive bacteria that is a facultative anaerobic, exemplifies this resilience.[15] Kusgoz et al found that the combination of $Ca(OH)_2$ and TAP provides better antibacterial activity against *E. faecalis*, even at low concentrations than using $Ca(OH)_2$ alone.[15] Hanin et al demonstrated similar results in their study. The combination of $Ca(OH)_2$ and TAP showed a better bactericidal effect compared with when each was used alone. This enhanced efficacy may be attributed to the presence of minocycline in TAP, which is an effective antibiotic against both Gram-negative and Gram-positive bacteria, thereby increasing the effectiveness of $Ca(OH)_2$.[16,17]

Other study by Jamshidi et al, the use of TAP reduces the viability of stem cells from the apical papilla (SCAP) in both concentration and in time-dependent manners; higher concentrations result in lower SCAP viability. In contrast, the use of $Ca(OH)_2$ did not show any significant difference in SCAP viability concerning concentration and time. Even though, at concentrations of 0.1 and 1 mg/mL, TAP still showed viability above 70%.[18] Therefore, this study aimed to assess the combination of $Ca(OH)_2$ and TAP as medicament in RET and determine the ideal concentration of TAP when combined with $Ca(OH)_2$ with respect to hDPSCs viability. These aims served the larger goal of assessing the result of antibacterial activity of this combined medicament from the previous studies and supporting the role of target drug strategy (TDS) in endodontic treatment. Innovative drug delivery systems can open up promising new prospects for delivering medicaments to the radicular space for regenerative endodontics.[19]

## Materials and Methods

Ethical approval was obtained from the Faculty of Dentistry Universitas Indonesia Dental Research Committee (ethical clearance number: 04 /Ethical Exempted/FKGUI/II/2023 No. Protocol: 050070123). The hDPSCs were stored raw biological materials from prior research (No.78/Ethical Approval/FKGUI/X/2022; No. Protocol: 011060922). This research was conducted at Prodia Stem Cell (Prostem) Laboratory, Jakarta, Indonesia. Two operators performed the experimental study.

### Medicament: Calcium Hydroxide and Triple Antibiotic Paste

In this study, $Ca(OH)_2$ was in form of 1 mg, a pure powder preparation diluted with Dulbecco's modified eagle medium (DMEM) until reaching concentration of 1 mg/mL. The TAP used in this study was a mixture consisting of three antibiotics (ciprofloxacin, metronidazole, and minocycline) at 1:1:1 ratio. Each medicament was diluted with DMEM until it reached the concentrations of 0.1 and 1 mg/mL.

The combination of $Ca(OH)_2$ and TAP was made from a 1-mg pure powder preparation of 1:1 ratio with the combined antibiotic mixtures (ciprofloxacin, metronidazole, and minocycline) using a measuring spoon. The combined medicament was diluted with DMEM until concentrations of 0.1 and 1 mg/mL were reached.

### Dental Pulp Stem Cell Culture

hDPSCs at passage 3rd to 4th were incubated in a humidified atmosphere of 5% $CO_2$ at 37°C until reaching 80% confluence. Subsequently, the cells were subjected to starvation by substituting the cell culture supplement with DMEM (ThermoFisher Scientific Inc., Waltham, Massachusetts, United States) supplemented with 5% fetal bovine serum (ThermoFisher Scientific Inc.) for a duration of 24 hours. After undergoing the serum starvation methods, the hDPSCs were cultured in six mediums; $Ca(OH)_2$, TAP 0.1 mg/mL, TAP 1 mg/mL, $Ca(OH)_2$ + TAP 0.1 mg/mL, $Ca(OH)_2$ + TAP 1 mg/mL, and control group for 24 hours before viability assay.

### MTT Assay and DAPI Test

The hDPSCs from six different medium were incubated in 96-well plates, which contain $5 \times 10^3$ cells in each well. These samples of control and experimental groups expression was measured after 24 hours' incubation. After 24 hours of incubation, the viability of hDPSCs was evaluated through a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 10 µL of MTT reagent (ThermoFisher Scientific) was added to each well and incubated at 37°C in 5% $CO_2$ for 4 hours. Subsequently, the medium was aspirated, and 100 µL of dimethyl sulfoxide solution (Sigma Aldrich, Saint Louis, Missouri, United states) was added to dissolve the formazan crystals, followed by a 30-minute incubation at 37°C. The absorbance of each well was measured at 570 nm using an enzyme-linked immunosorbent assay reader. Additionally, a 4',6-diamidino-2-phenylindole (DAPI) staining quality test was conducted after 24 hours of culture. The cells were fixed with 1 mL of methanol for 5 minutes, washed thrice with phosphate-buffered saline, and incubated with 500 µL of DAPI dye for 30 minutes. Images were captured using fluorescence microscopy (Axio Observer microscope, Zeiss, Germany) at a magnification of 50 µm.

In this study, the optical density (OD) value was measured with a microplate reader at a wavelength of 450 nm. After that, the cell viability value is calculated according to the following formula:

$$\text{Cell viability (\%)} = \frac{\text{Test group OD value} - \text{OD value } \textit{blank well}}{\text{Control group OD value} - \text{OD value } \textit{blank well}} \times 100\%$$

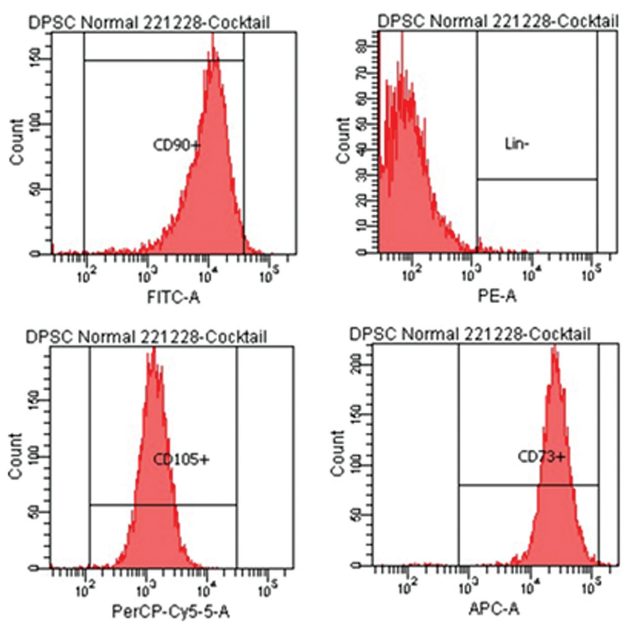
### Statistical Analysis

The gathered data were analyzed using a one-way analysis of variance, followed by Bonferroni post hoc to compare between the study groups. All the tests were conducted at a significance level of 95% ($p < 0.05$) and analyzed using IBM SPSS Statistics Software, version 26.0. (IBM Corp., Armonk, New York, United States).

## Results

Following isolation and culture, the hDPSCs were subjected to flow cytometry analysis to confirm their mesenchymal stem cell phenotype. The findings indicated a high expression of CD90+ (98%), CD105+ (99.7%), and CD73+ (94%), with a minimal expression of LinNeg (0.5%). These results suggest that the hDPSCs were positive for the evaluated mesenchymal markers and negative for the assessed hematopoietic markers (►**Fig. 1**).

Viability test of hDPSCs was conducted, assessed by the percentage of live cells, and calculated using the MTT assay




Tube: Cocktail

| Population | #Events | %Parent | %Total |
|---|---|---|---|
| All Events | 10,000 | #### | 100.0 |
| P1 | 6,538 | 65.4 | 65.4 |
| CD90+/Lin- | 6,443 | 98.5 | 64.4 |
| Q2 | 6 | 0.1 | 0.1 |
| Q3 | 89 | 1.4 | 0.9 |
| Q4 | 0 | 0.0 | 0.0 |
| Q1-1 | 1 | 0.0 | 0.0 |
| CD73+/CD105+ | 6,509 | 99.6 | 65.1 |
| Q3-1 | 17 | 0.3 | 0.2 |
| Q4-1 | 11 | 0.2 | 0.1 |
| Q1-2 | 1 | 0.0 | 0.0 |
| Q2-2 | 4,289 | 65.6 | 42.9 |
| Q3-2 | 27 | 0.4 | 0.3 |
| Q4-2 | 2,221 | 34.0 | 22.2 |
| CD90+ | 6,410 | 98.0 | 64.1 |
| CD105+ | 6,521 | 99.7 | 65.2 |
| CD73+ | 6,500 | 99.4 | 65.0 |
| Lin- | 33 | 0.5 | 0.3 |

**Fig. 1** Stem cell marker test results CD90 +, CD105 +, and CD73+ and LinNeg.

after being exposed to different concentrations for 24 hours. The collected data was subsequently transformed into percentages to represent cell viability. Cell viability was determined by calculating the absorbance values of each treatment group, and cytotoxicity was assessed by evaluating the percentage of DPSCs that perished following exposure to the medicaments over the 24-hour observation period. ►**Table 1** shows the results of cell mean viability values between groups at 24-hour observation. Mean viability value of all group is > 70%. In the experimental groups, the highest mean viability value was found in the TAP 0.1 mg/mL treatment group, and the lowest mean viability value was observed in the $Ca(OH)_2$ + TAP 1 mg/mL treatment groups.

Based on the post hoc test (►**Table 2**), there was a significant difference in the mean viability between treatment group of $Ca(OH)_2$ + TAP 0.1 mg/mL compared with the TAP 0.1 mg/mL and TAP 1 mg/mL groups, also between treatment group of $Ca(OH)_2$ + TAP 1 mg/mL compared with $Ca(OH)_2$, TAP 0.1 mg/mL, TAP 1 mg/mL, $Ca(OH)_2$ + TAP 0.1 mg/mL, and control groups, and between the TAP 0.1 mg/mL group compare with the $Ca(OH)_2$ and control groups. There was no significant difference between the treatment group $Ca(OH)_2$ with TAP 1 mg/mL, $Ca(OH)_2$ + TAP 0.1 mg/mL, and control groups, and also between the treatment group with combination of $Ca(OH)_2$ + TAP 0,1 mg/mL with $Ca(OH)_2$ and control groups.

►**Fig. 2** shows the results of microscopic image of cells after 24-hour incubation with a magnification of 50 µm with different morphology of cells for each group compared with qualitative DAPI staining tests result. The results showed that the hDPSCs stained blue due to the binding of DAPI dye to the cell's deoxyribonucleic acid (DNA). In ►**Fig. 2** of DAPI test results **(A.2)**, in the control group with DMEM, staining of cell nucleus covers more than 90% of the area, and the nucleus is uniform in size (white arrow). In ►**Fig. 2B.2** and **E.2**, in the Ca $(OH)_2$, TAP 0.1 mg/mL, and the combination of $Ca(OH)_2$ + TAP 0.1 mg/mL groups, there are changes in the morphology of the cell nucleus, both in size and shape (white arrow). In ►**Fig. 2F.2**, in the group with the combination of $Ca(OH)_2$ + TAP 1 mg/mL, there is a reduction in cell nucleus staining in amount and sizes to below 90% of the area (white arrow).

**Table 1** Comparison of hDPSCs viability between all groups at a 24-hour of observation

| Group | Mean (%) ± standard deviation | *p*-Value |
|---|---|---|
| DMEM | 100 ± 0.00 | 0.00[a] |
| $Ca(OH)_2$ | 100.18 ± 3.37 | |
| TAP 0.1 mg/mL | 107.14 ± 4.30 | |
| TAP 1 mg/mL | 101.02 ± 4.82 | |
| Ca $(OH)_2$ + TAP 0.1 mg/mL | 98.09 ± 2.42 | |
| Ca $(OH)_2$ + TAP 1 mg/mL | 95.12 ± 2.00 | |

Abbreviations: Ca $(OH)_2$, calcium hydroxide; DMEM, Dulbecco's modified eagle medium; hDPSCs, human dental pulp stem cells; TAP, triple antibiotic paste.
[a]One-way analysis of variance (ANOVA), $p < 0.05$.

## Discussion

RET aims to regenerate the dentin-pulp complex that has been damaged so that it can function physiologically like normal pulp. The use of medicaments is crucial in RET because the instrumentation involved is minimal or even without any instrumentation in the procedures. The success of RET is highly influenced by the disinfection of the root canals and the materials used for disinfection.[7]

$Ca(OH)_2$ is recommended as an intracanal medicament in RET procedures because of its high antibacterial ability. When $Ca(OH)_2$ particles reach the dentinal tubules, they encounter microorganisms that develop in the dentinal tubules and act as a source of dissociated calcium ions. Based on a study conducted by Komabayashi et al, the size, shape, and orientation of the calcium particles of $Ca(OH)_2$ can affect the depth of penetration of $Ca(OH)_2$ into the dentinal tubules. The larger the particle size and the more rectangular the shape, the deeper the $Ca(OH)_2$ penetration into the dentinal tubules. The $Ca(OH)_2$ particles will dissolve in water and release hydroxyl ions. This mechanism increases the pH in the dentinal tubules, thus allowing the elimination of bacteria.[17,20,21]

In addition to $Ca(OH)_2$, the AAE also recommends TAP as an intracanal medicament in RET procedures. TAP contains minocycline, a semisynthetic derivative of tetracycline, which is effective against Gram-negative and Gram-positive bacteria, including *E. faecalis*. TAP can diffuse into the dentinal tubules as far as 350 µm.[17] The combination of Ca $(OH)_2$ and TAP has a better antibacterial effect when used together compared with when used alone. This may be due to

**Table 2** Analysis of hDPSCs viability differences between all groups at a 24-hour of observation

| Group | DMEM | $Ca(OH)_2$ | TAP 0.1 mg/mL | TAP 1 mg/mL | $Ca(OH)_2$ + TAP 0.1 mg/mL |
|---|---|---|---|---|---|
| $Ca(OH)_2$ | 1 | | | | |
| TAP 0.1 mg/mL | 0.01[a] | 0.01[a] | | | |
| TAP 1 mg/mL | 1 | 1 | 0.04[a] | | |
| $Ca(OH)_2$ + TAP 0.1 mg/mL | 0.15 | 0.12 | 0.00[a] | 0.00[a] | |
| $Ca(OH)_2$ + TAP 1 mg/mL | 0.00[a] | 0.00[a] | 0.00[a] | 0.03[a] | 1 |

Abbreviations: Ca $(OH)_2$, calcium hydroxide; DMEM, Dulbecco's modified eagle medium; hDPSCs, human dental pulp stem cells; TAP, triple antibiotic paste.
[a]Post Hoc Bonferroni, $p < 0.05$.

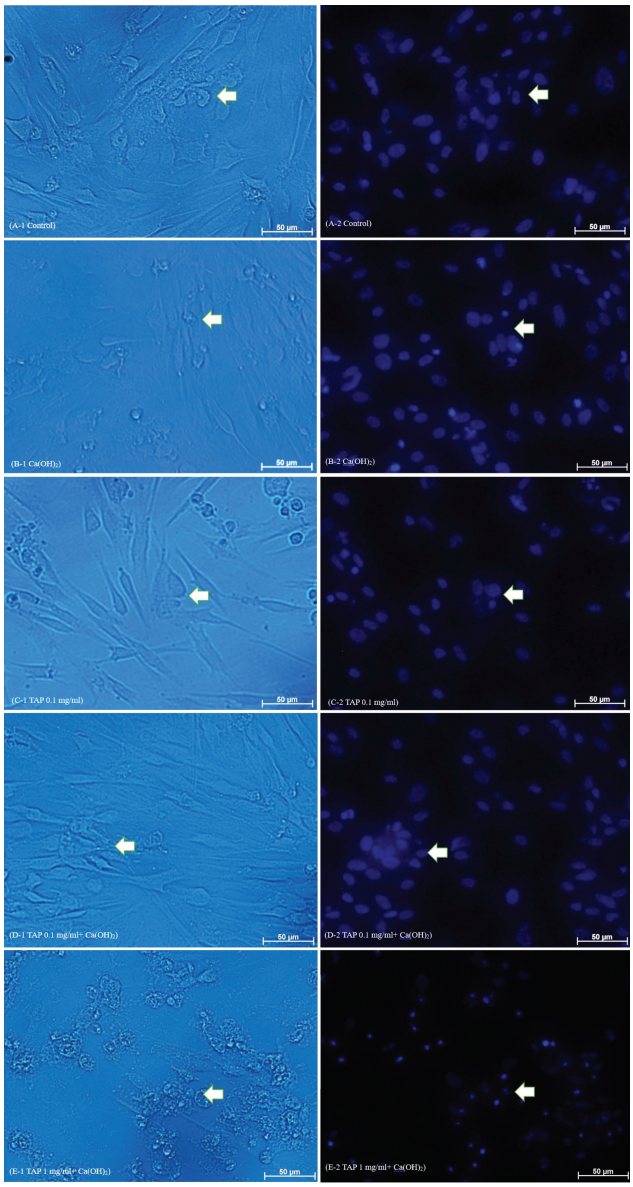


**Fig. 2** Microscopic image of cells after 24 hours' incubation with a magnification of 50 µm (1) and 4',6-diamidino-2-phenylindole (DAPI) test result (2). (A.1–2) Control group of Dulbecco's modified eagle medium (DMEM) medium, (B.1–2) calcium hydroxide ($Ca(OH)_2$) group, (C.1–2) triple antibiotic paste (TAP) 0.1 mg/mL group, (D.1–2) TAP 1 mg/mL group, (E.1–2) $Ca(OH)_2$ + TAP 0.1 mg/mL group, (F.1–2) $Ca(OH)_2$ + TAP 1 mg/mL group.

the combined effectiveness of the two medicament.[16,20] Research by Pedrinha et al states that the vehicle of an intracanal medicament also affects its antibacterial ability. Intracanal medicaments with a more liquid consistency, which use a water-soluble vehicle (aqueous or viscous), allow for a higher pH and calcium ion release. In addition, the ability of the medicament to diffuse into the dentinal tubules is also better.[20]

Research on RET medicaments and their modification based on the role of TDS in endodontic treatment is still limited, so this study aims to determine the differences in the cytotoxicity of $Ca(OH)_2$, TAP, and their combination on hDPSCs viability, thus can be an initial guide in determining the type of single or combination medication in RET.

hDPSCs are multipotent stem cells with good regenerative and differentiation capabilities. They could differentiate into odontoblasts. Cell viability is the cell's ability to survive without losing metabolic function and proliferation capacity, which can be determined by measuring the increase in the number of cells, protein levels, and DNA. Therefore, cell viability serves as a potential indicator of the cytotoxicity associated with a material.[22–24]

In this study, the MTT assay was employed to evaluate cell viability, which is widely recognized as one of the most common colorimetric assays used for assessing cytotoxicity and cell viability. The MTT assay serves as a reliable method to measure cellular metabolic activity, providing valuable insights into cell viability, proliferation, and cytotoxicity.[25,26] The results of the research showed that all groups had mean viability values of $>70\%$. According to the International Organization for Standardization (ISO) standards, viability $>70\%$ indicates that the material is not toxic to cells. In the $Ca(OH)_2$ and TAP 0.1 and 1 mg/mL treatment groups, the viability values were above 100%, indicating a higher viability compared with the control group. Therefore, it can be assumed that the material may also facilitate cell proliferation in the cell cycle.[13,25]

The highest viability value was observed in the TAP 0.1 mg/mL treatment group, while the lowest viability value was found in the $Ca(OH)_2$ + TAP 1 mg/mL treatment group, yet the reduction was still above $>70\%$ (the lowest 95.25%, see ►**Table 1**), so it can be concluded that all types of medicament including the combination of $Ca(OH)_2$ + TAP in 0.1 and 1 mg/mL concentration are still not toxic to hDPSCs (according to ISO). This is not in line with the result of previous studies that stated a significant decrease in hDPSCs viability in the TAP concentration of 0.1 and 1 mg/mL group, this study concluded that $Ca(OH)_2$ and TAP in 0.1 and 1 mg/mL were toxic to hDPSCs. The different results of both studies may be because of the different methods used in the study, therefore in this study the result of MTT assay in quantitative analysis was supported by the qualitative results of DAPI staining test.

The viability mean of the TAP 1 mg/mL group was higher than that in the $Ca(OH)_2$. Although, in the $Ca(OH)_2$ group, the mean viability value was above the control group. This is possibly due to the potential of $Ca(OH)_2$ to create a good microenvironment for hDPSCs that might induce proliferation that can continue with osteogenic differentiation and mineralization through the mitogen-activated protein kinase pathway. On the other hand, even though the $Ca(OH)_2$ group had slightly higher mean of hDPSCs viability compared with control, it was lower than in the TAP 0.1 and 1 mg/mL groups. The difference in cell viability between the $Ca(OH)_2$ and TAP groups may be due to the high pH (12.5–12.8) of $Ca(OH)_2$, which can lead to immediate cell apoptotic upon contact, so it can be concluded that TAP was less toxic to $Ca(OH)_2$.[7,27,28] This aligns with the research conducted by Jamshidi et al, where TAP concentrations of 0.1 and 1 mg/mL

provided good viability in SCAP and will be toxic with higher concentrations.[18]

The research results also indicate that the mean hDPSCs viability was lowest in the combination medicament groups $Ca(OH)_2$ + TAP 1 mg/mL, and significantly different compared with $Ca(OH)_2$ or TAP 0.1 and 1 mg/mL and control, but not significantly different compared with $Ca(OH)_2$ + TAP 0.1 mg/mL (►**Table 2**). This result may be attributed to the higher concentration of TAP medicament in combination with $Ca(OH)_2$, that can lead to the reduction of cell's viability. The low pH of TAP (4–4.6) in combination with high pH of $Ca(OH)_2$ (12.5–12.8) may cause the release of hydrogen ions from the hydrochloride groups in minocycline hydrochloride and ciprofloxacin hydrochloride, leading to an unfavorable environment for the cell. However, even though the mean viability results in the combination groups $Ca(OH)_2$ + TAP 0.1 mg/mL and $Ca(OH)_2$ + TAP 1 mg/mL were reduced compare with control, $Ca(OH)_2$ and TAP groups were still viable to cells (> 70%), which means that the combination medicaments at these concentrations are nontoxic and safe for use in RET yet better to use in combination of $Ca(OH)_2$ + TAP 0.1 mg/mL.[15,27,29] This result was in line with the previous study conduct by Kusgoz et al that concluded superior antibacterial activity of combination $Ca(OH)_2$ + TAP compared with single use of TAP or $Ca(OH)_2$. It can be summarized that $Ca(OH)_2$ + TAP 0.1 mg/mL is safer for cells yet superior in antibacterial action.[15,27,29]

Nevertheless, the DAPI staining test showed that the reduction in nucleus staining to below 90% of the area, and changing of size, shape, and number of the nucleus appears in an uniform (white arrow) was found in the $Ca(OH)_2$ + TAP 1 mg/mL group (►**Fig. 2–F.1–2**), the other groups were still similar with the control group. This result showed that it is safer to use the combination of $Ca(OH)_2$ + TAP 0.1 mg/mL for hDPSCs, the increase concentration of TAP can increase the risk of cell hypoxia so that the qualitative picture shows changes in cell shape and cell nuclei (►**Fig. 2F.2**). The DAPI staining depicting cell morphology indicates that the nuclei of cells in this group have undergone morphological changes that can confirm the results from the MTT assay, even though from MTT assay the result of viability of cell is still viable (►**Table 1**).[30] The limitation of this study is the absence of observations at different time intervals, which could span from 6 to 48 hours, to assess the potential increase and decrease in hDPSC viability over varying time periods.[27]

## Conclusion

The medicaments $Ca(OH)_2$, TAP, and their combination of $Ca(OH)_2$ + TAP 0.1 and 1 mg/mL were found to be nontoxic to hDPSCs. For safety reasons, the use of combination of $Ca(OH)_2$ + TAP medicament in endodontic regeneration, it is suggested that the TAP concentrations in 0.1 mg/mL be used.

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Conflict of Interest
None declared.

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## References

1 American Association of Endodontists. Glossary of endodontic terms. Gloss Endod Terms 2020;9:43
2 Fouad AF. Microbial factors and antimicrobial strategies in dental pulp regeneration. J Endod 2017;43(9S):S46–S50
3 Hargreaves KM, Berman LH. Cohen's Pathways of the Pulp. 11th ed. Elsevier; 2016
4 Kim SG, Malek M, Sigurdsson A, Lin LM, Kahler B. Regenerative endodontics: a comprehensive review. Int Endod J 2018;51(12): 1367–1388
5 Torabinejad M, Alexander A, Vahdati SA, Grandhi A, Baylink D, Shabahang S. Effect of residual dental pulp tissue on regeneration of dentin-pulp complex: an in vivo investigation. J Endod 2018;44 (12):1796–1801
6 Almutairi W, Yassen GH, Aminoshariae A, Williams KA, Mickel A. Regenerative endodontics: a systematic analysis of the failed cases. J Endod 2019;45(05):567–577
7 Prasanti ED, Margono A, Djauharie N. Effect of triple antibiotic paste, calcium hydroxide, ledermix® on viability of pulp mesenchymal stem cells. Int J Appl Pharm 2019;11(01):49–53
8 Kumar A. Intracanal medicaments – their use in modern endodontics: a narrative review. J Oral Res Rev 2019;11:89–94
9 Lakhani AA, Sekhar KS, Gupta P, et al. Efficacy of triple antibiotic paste, moxifloxacin, calcium hydroxide and 2% chlorhexidine gel in elimination of E. faecalis: an in vitro study. J Clin Diagn Res 2017;11(01):ZC06–ZC09
10 Suresh CB, Gopikrishna V. Grossman Endodontic Practice. 13th ed. Wolters Kluwer Health; 2014
11 AAE. Clinical Considerations for Regenerative Endodontics;. 2018
12 Hoshino E, Kurihara-Ando N, Sato I, et al. In-vitro antibacterial susceptibility of bacteria taken from infected root dentine to a mixture of ciprofloxacin, metronidazole and minocycline. Int Endod J 1996;29(02):125–130
13 Parhizkar A, Nojehdehian H, Asgary S. Triple antibiotic paste: momentous roles and applications in endodontics: a review. Restor Dent Endod 2018;43(03):e28
14 Fouad AF, Diogenes AR, Torabinejad M, Hargreaves KM. Microbiome changes during regenerative endodontic treatment using different methods of disinfection. J Endod 2022;48(10):1273–1284
15 Kusgoz A, Ozcan E, Arslan I, Inci M. Antibacterial activity of calcium hydroxide combined with triple antibiotic paste against enterococcus faecalis; an in vitro study. Cumhur Dent J 2013;16 (01):25–30
16 Azima Hanin SM, Anjaneyulu K, Muralidharan NP. Antimicrobial efficacy of calcium hydroxide and triple antibiotic paste combination on E. faecalis biofilm- an in vitro study. J Pharm Res Int 2020;32(18):120–126
17 Khanvilkar U, Pawar S, Bandekar S, et al. Scanning electron microscopy analysis of the intratubular radicular dentin penetration of calcium hydroxide, triple antibiotic paste, and nitrofurantoin. J Pers Med 2023;13(11):1554
18 Jamshidi D, Ansari M, Gheibi N. Cytotoxicity and genotoxicity of calcium hydroxide and two antibiotic pastes on human stem cells of the apical papilla. Eur Endod J 2021;6(03):303–308
19 Parhizkar A, Nojehdehian H, Tabatabaei F, Asgary S. An innovative drug delivery system loaded with a modified combination of

triple antibiotics for use in endodontic applications. Int J Dent 2020;2020:8859566
20 Pedrinha VF, Cuellar MRC, de Barros MC, et al. The vehicles of calcium hydroxide pastes interfere with antimicrobial effect, biofilm polysaccharidic matrix, and pastes' physicochemical properties. Biomedicines 2022;10(12):3123
21 Komabayashi T, D'souza RN, Dechow PC, Safavi KE, Spångberg LSW. Particle size and shape of calcium hydroxide. J Endod 2009; 35(02):284–287
22 Aslantürk ÖS In vitro cytotoxicity and cell viability assays: principles, advantages, and disadvantages. In: Genotoxicity - A Predict Risk to Our Actual World. Published online 2018:1–18. Doi: 10.5772/intechopen.71923
23 Karamzadeh R, Eslaminejad MB. Dental-related stem cells and their potential in regenerative medicine. Regen Med Tissue Eng Intechopen 2013:95–116
24 Eramo S, Natali A, Pinna R, Milia E. Dental pulp regeneration via cell homing. Int Endod J 2018;51(04):405–419
25 Dojindo. Measuring Cell Viability/Cytotoxicity: Cell Counting Kit-8. Available at: https://www.dojindo.eu.com/Protocol/Dojindo-Cell-Proliferation-Protocol.pdf
26 Riss TL, Moravec RA, Niles AL, et al. Cell Viability Assays. In: Markossian S, Grossman A, Arkin M, et al. Assay Guidance Manual. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2013
27 Bhandi S, Patil S, Boreak N, et al. Effect of different intracanal medicaments on the viability and survival of dental pulp stem cells. J Pers Med 2022;12(04):1–10
28 Diogenes A, Ruparel NB. Regenerative endodontic procedures: clinical outcomes. Dent Clin North Am 2017;61(01):111–125
29 Khoshkhounejad M, Sobhi Afshar M, Jabalameli F, Emaneini M, Sharifian M. Cytotoxicity evaluation of minimum antibacterial values of different medicaments used in endodontic regenerative procedures. Eur J Dent 2019;13(04):514–520
30 Lin S, Ke Z, Liu K, et al. Identification of DAPI-stained normal, inflammatory, and carcinoma hepatic cells based on hyperspectral microscopy. Biomed Opt Express 2022;13(04):2082–2090

 