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Research Report

# The effects of ultrasonic scaling duration and replication on caspase-3 expression of *Sprague Dawley* rat's pulp cells

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***ABSTRACT***

***Background:*** *Ultrasonic scaling has been used commonly for stain and calculus removal in dental clinic for over 60years. Previous researches even had proved that ultrasonic scaling may give effects on the surface of tooth root. Ultrasonic wave exposure for 20 seconds or more can increase caspase-3 activity as an indicator of increased apoptotic cells associated with tissue damage.* ***Purpose:*** *This research was aimed to investigate the effects of ultrasonic scaling duration and replication on caspace-3 expression in dental pulp cells.* ***Methods:*** *The samples of this research were 54 male Sprague Dawley rats aged 2 months old divided into 2 groups, each of which consisted of 27 mice. The first group was induced with stain, while the second group was not. Each group was divided into 3 subgroups for ultrasonic scaling 1, 3, and 5 times. Each subgroup was divided into 3 sub-subgroups for duration procedure of 15, 30 and 60 seconds respectively. During scaling process, those rats were anesthetized using 0.1 ml of ketamine and 0.1 ml of xylol added to 2 ml of distilled water injected intramuscularly into their right thigh as much as 0.4 ml. Scaling was done on buccal surface of right first maxillary molar from cervical to occlusal. The teeth were decalcified and embedded in paraffin, then their sagittal plane was cut for thickness of 3μm and painted with immunohystochemistry for detecting caspace-3 expression of cell within dental pulp.* ***Results:*** *The results showed that the duration and replication of ultrasonic scaling procedures affected on the expression of caspace-3 cells as analyzed with Univariate Analisis of Variance test ($p<0.05$).* ***Conclusion:*** *It can be concluded that duration and replication of ultrasonic scaling procedure on teeth with and without stain enhauced the expression of caspace-3 in dental pulp cells.*

***Keywords:*** *Duration; replication; ultrasonic scaling; pulp cells; caspace-3*

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## INTRODUCTION

Ultrasound means sound that can not be heard by human ear at frequency above 20,000 Hz. In dentistry, ultrasonic waves are used for professional tooth cleaning, root canal cleaning, bone implant and surgical procedures.[1] Ultrasonic scaler can also be used for biofilm, stain and calculus removal of tooth surface.[2] Piezoelectric scaler moving back and forth 28000-36000 times even can be used to clean tooth surface.[3] If ultrasonic waves expose on liver cells of rats for 20 seconds or more, the activity of caspase-3 as an indicator of increased apoptosis associated with tissue damage.[4]

Ultrasonic waves can cause sonification. Sonification mechanism that occurs in ultrasonic scaling procedure even can cause acoustic microstreaming mechanism, turbulence flow, cavitation, and microjet formation resulting cavitation bubbles collapsed and making shock waves.[4] If this mechanism occurs on tooth surface with stain, the stain will be detached from the tooth surface causing partial removal of organic and inorganic materials from the tooth surface triggering to tooth surface damage. Microstreaming flow around the air bubbles generated by ultrasonic waves can get into bloodstream, and penetrate cell membrane through sonoporation molecular mechanisms.[5] Ultrasonic waves at a frequency of 20 kHz-2 MHz even can raise small air

bubbles resulting in cell injury.[6] It means that duration and cavitation of ultrasound exposure can affect on activity of cells exposure.[7] The increasing of caspase-3 expression after ultrasound wave exposure can become an indicator of the increasing of apoptosis cells.[8] This research was aimed to evaluate the effects of ultrasonic scaling duration and replication on caspase-3 expression in dental pulp cells of teeth with and without stain.

## MATERIALS AND METHOD

This research is a pra-experimental research since there were manipulation, repeatable measurements and control group, but without randomization approach.[9] This research used 54 male *Sprague Dawley* rats aged two months old, divided into two groups each of which consisted of 27 rats. The first group of mice were given a drink with a mixed solution of tea and coffee. The solution of tea was made of 2 grams of black tea powder in pouch packaging brewed into 100 ml of boiled water. Meanwhile, the solution of coffee was made of 2 grams of ground coffee without pulp brewed into 100 ml of boiled water. Both solutions were mixed and waited 10 minutes to be drunk by those rats for 21 days. On the other hand, the second group of mice were given a drink with water.

Each group of rats was divided into 3 subgroups with ultrasonic scaling 1, 3, and 5 times for each consisted of 9 rats. Each subgroup then was divided into 3 sub-subgroups each of which consisted of 3 rats with ultrasonic scaling duration of 15, 30 and 60 seconds respectively. During scaling process, rats were anesthetized using 0.1 ml of ketamine and 0.1 ml of xylol added to 2 ml of distilled water injected intramuscularly into their right thigh as much as 0.4 ml. Scaling was done on the buccal surface of their right first maxillary molar, starting from cervical line to occlusal surface, using supra-gingival stain tip. Tip was used on the tooth surface without pressure. The position of the tip was parallel to the axis teeth.

In addition, the volume of cooling water used was about 20 ml/ sec. The engine power used was moderate, and scaling process was done by one people. The procedure was then repeated started from the induction of stain into rats to scaling process 3 and 5 times with 21-day pause. After scaling, the rats were decapitated, and their teeth were extracted. The decalcified teeth then were embedded in paraffin, and their sagittal plane was cut for thickness of 3μm. Those were fixed on object glasses. Deparaffinization was conducted using xylol for 3 x 5 minutes, absolute alcohol, 96% alcohol, 70% alcohol respectively for 5 minutes, and dehydration was performed. The slides were washed using running water, and then washed by distilled water.

Samples were incubated with 3% $H_2O_2$ for 15 minutes and washed with running water, and later washed with distilled water. Cox2 retrieval then was conducted using both citrate buffer with pH 6 and decloac for 40 minutes at 95° C. After that, the samples were chilled for about 30 minutes. Those samples then were washed 2x times using PBS for 3-5 minutes, and incubated with blocking serum or normal serum for 15 minutes. The samples were drained and cleaned, then were incubated with anti-caspase-3 antibody for 1 hour, and washed 2x times using PBS for 3-5 minutes. The samples were incubated again with secondary antibody for 20 minutes, and then washed 2x times using PBS for 3-5 minutes. The samples were incubated again with trikkie avidin HRP for 10 minutes, and then washed 2x times using PBS for 3-5 minutes. Afterwards, the samples were dropped with chromogen DAB (1:50), then left for 2 minutes, and washed with water. Counterstain then was performed with hematoxylin mayer for 2 minutes, and washed with water. Those samples were dipped in alcohol with concentration from 70%, 96%, to 100%, and also xylol, followed with mounting, and then observed using a microscope at 400X magnification.[10]

## RESULTS

Data of the effects of ultrasonic scaling duration and replicatin on the expression of caspase-3 in the dental pulp cells with and without stain were presented in Table 1. The expression of caspase-3 in the dental pulp cells with and without stain for once scaling with a duration of 15 seconds showed relatively equal numbers. The expression of caspase-3 cells in the teeth with a stain was increased as the increasing of duration and number of replication of scaling. In contrast, the expression of caspase-3 cells in the teeth without stain was incresed domninantly due to the increasing of the duration of scaling. The expression of caspase-3 cells for once scaling with a duration of 60 seconds was higher than those for 3 and 5 times with the same scaling duration. Leverne test then was performed for homogeneity of the data. The results obtained was $p = 0.344$, which means that the data were homogeneous and qualified to be analyzed with Univariate Analisis of variance test.

Based on the results of univariate analisis of variance test (Table 2), duration and repetition of scaling either individually or communaly could affect significantly the expression of caspase-3 in the dental pulp cells with and without stain ($p < 0.05$). It means that there were significant effects of ultrasonic scaling duration and replication on the number of cells expressing caspase-3 in the dental pulp cells with and without stain.

Based on LSD test, it is also known that there were 9 types of relations without significant difference of the average number of caspase-3 expression ($p > 0.05$). LSD test showed that the expression of caspase-3 cells in the group induced with stain for once scaling with a duration of 15 seconds was not significantly different from that in the group without stain with the same scaling duration. Similarly, the expression of caspase-3 cells in the group induced with stain for three times of scaling with a duration

of 30 seconds had almost the same number to the group induced with stain for five times of scaling with a duration of 60 seconds.

The effects of ultrasonic scaling duration and replication on the expression of caspase-3 in the dental pulp cells with and without stain can be seen in Figures 1 and 2. Figure 1 shows that the increasing of the duration and replication of ultrasonic scaling could increase the expression of caspase-3 in dental pulp cells with stain. Figure 2 shows that the increasing of the duration and replication of ultrasonic scaling could increase the expression of caspase-3 in dental pulp cells without stain.

## DISCUSSION

Data in Tables 1 and 2 and in Figures 1 and 2 show the effects of ultrasonic scaling duration and repetition on the expression of caspase-3 in the dental pulp cells with and without stain. This is consistent with the explanation that the increase in temperature of ultrasonic scaling can damage the pulp and disrupt circulatory system resulting in dental pulpitis or necrosis.[11] The effects caused by ultrasonic wave exposure to tissue is thermal, mechanical (cavitation and microstreaming), and chemical (sonochemistry). Clinical effect is depending on the application technique used.[12]

The tip of ultrasonic scaler, actualy can get vibrations, and the movements are influenced by the design of the

**Table 1.** The average number of caspase-3 expression in dental pulp cells

| Replication and Duration (second) | Average±Standard Deviation of teeth with stain | Average±Standard Deviation of teeth without stain |
|---|---|---|
| 1x 15 | 14.60± 2.30 | 14.00 ± 2.88 |
| 1x 30 | 20.20± 3.11 | 23.00± 3.00 |
| 1x 60 | 33.00± 1.88 | 72.60± 4.77 |
| 3x 15 | 29.60± 4.72 | 44.20± 2.78 |
| 3x 30 | 51.60± 4.51 | 44.60± 2.07 |
| 3x 60 | 69.40± 5.18 | 52.60± 3.91 |
| 5x 15 | 43.20± 1.92 | 31.00± 1.87 |
| 5x 30 | 66.00± 4.36 | 40.00± 3.53 |
| 5x 60 | 80.40± 5.03 | 50.40± 3.65 |

**Table 2.** The results of univariate analysis of variance on the caspace-3 expression in dental pulp cells

| Source | F | Sig. |
|---|---|---|
| Intercept | 169346.844 | 0.000 |
| Tooth Stain (1) | 344.178 | 0.000 |
| Replication (2) | 8651.356 | 0.000 |
| Duration (3) | 13987.089 | 0.000 |
| (1) and (2) | 5086.422 | 0.000 |
| (1) and (3) | 463.089 | 0.000 |
| (1), (2) and (3) | 3719.511 | 0.000 |

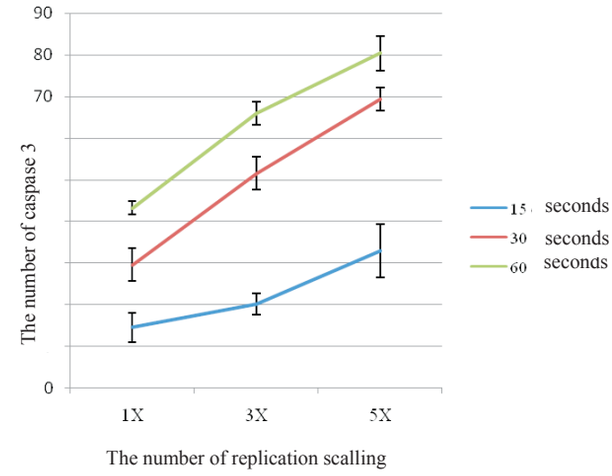


**Figure 1.** *Caspase-3* expression in dental pulp cells with stain.

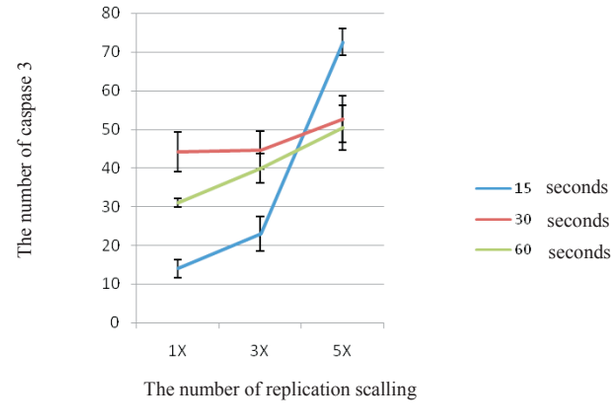


**Figure 2.** Caspase-3 expression in dental pulp cells without stain.

scaler. The movements of the tip then can trigger cavitation and microstreaming derived from the cooling water of the ultrasonic scaler.[13] Therefore, it can be said that the longer the exposure to ultrasonic waves is, the longer the cavitation and acoustic microstreaming mechanisms are. Similarly, some *in vitro* researches also show that the duration of the ultrasonic scaling can affect on tooth surface damage.[3] The damage of tooth surface then can lead to the increasing of cavitation and acoustic microstreaming mechanisms until air bubbles are collapsed and a shock wave is emerged.

However, ultrasonic equipment is still expected to be used for dental pulp treatment because it can cause more minimal damage than using bur with high speed.[14] Mechanism of ultrasonic scaler actually can clean tooth surface from materials due to chipping mechanism of scaler probe oscillating.[15] This mechanism usually occurs in areas with limited view and excessive scaling duration.[16] Thus, over-instrumentation of ultrasonic scaling must not occur since it can lead to damage, from tooth surface to dental pulp cells.

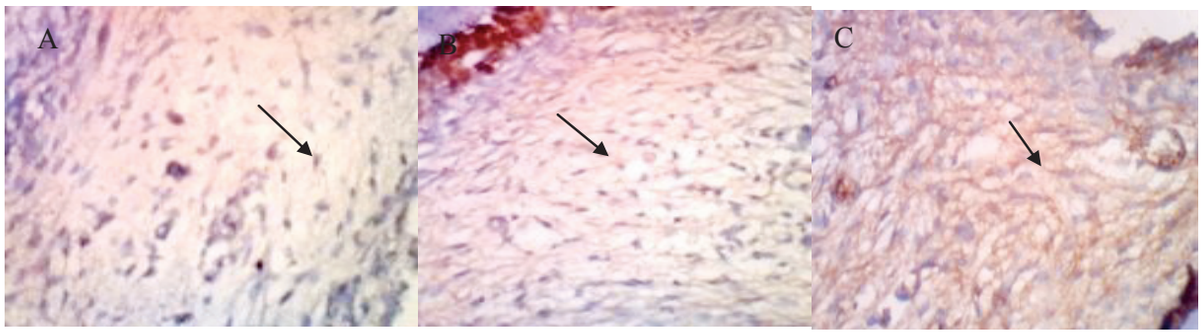


**Figure 3.** The expressions of caspase-3 in dental pulp cells with stain as follow: A) 1x of scaling for 15 seconds; B) 3x of scaling for 30 seconds; C) 5x of scaling for 60 seconds. The arrows show the expressions of caspase-3 with IHC staining, dark brown among light purple tissues.

In this research, the increasing of caspase-3 expression of dental pulp cell occurred as the increasing of duration and replication of scaling. Cell injury occur when the cooling water of ultrasonic scaler flows into the dental pulp chamber.[17] The changes of dental pulp cells, consequently, were detected in this research by the increasing expressing caspase-3of dental pulp cell.

The increasing of tooth temperature after ultrasonic scaling also cause damage in dentin and dental pulp tissue.[18] Over-instrumentation lead to tooth root damage, so the teeth will become more sensitive, thus, the longer the duration of ultrasonic scaling is, the higher the negative effects will be obtained.[19] In other words, certain effects of ulrasonic wave exposure to dental cells can be considered as normal, lysis, apoptosis or necrosis, depending on the duration of ulrasonic wave exposure.[11]

Ultrasonic scaling procedures can also cause cavitation and acoustic microstreaming mechanisms, shock waves and free radical bubbles.[21] These mechanisms then can lead to the changing of bubbles in the tissues both in size and number. Air bubbles will increasingly enlarge until they reaches maximum size and eventually burst, and the mechanisms occur repeatedly until the ultrasonic wave exposure is stopped.[22] Therefore, it is suspected as the cause change of the dental pulp cells resulting the increasing of caspase-3 expression.

The results of this research show that the number of caspase-3 expression in dental pulp cells after once scaling with a duration of 15 seconds on the tooth surface induced with stain was 14.60 ± 2.30, while that on the surface without stain was 14.00 ± 2.88. In general, it can be said that the number of caspase-3 expression in the group of teeth with stain was higher than in the group without stain. The number of caspase-3 expression in dental pulp cells after five times of scaling with a duration of 60 seconds in the group of teeth with stain was 80.40 ± 5.03, while that in the group without stain was 50.40 ± 3.65. It indicates that ultrasonic wave exposure to the surface of the teeth with and without stain cause similar mechanisms in the dental pulp cells since the enamel and dentin are porous, so the tissues in the pulp chamber can be affected by the complex mechanism.

One of indicators of apoptosis cells is caspase-3 expression.[23] Physiologically, apoptosis will be increased when deciduous teeth become permanent ones.[19] The results of univariate analysis of variance that duration and replication ultrasonic scaling individually or jointly significantly influenced on the number of caspase-3 expression in dental pulp cells with and without stain ($p<0.05$). It is suspected due to the complex mechanisms of the ultrasonic waves, including vibration, acoustic microstreaming, cavitation, and bubble rupture in the dental pulp chamber. These mechanisms can explain how the cooling water enters the pulp chamber and causes air bubbles in the pulp tissue and in the blood vessels of the pulp. In normal condition, air bubbles are small, but with ultrasonic scaling, the volume and number of air bubbles become excessive. After the size of the bubbles is maximum, the continuing ultrasonic wave exposure will cause the bubbles brust. As a results, the bubbles around the cells will damage, leading to tooth pain of patients during ultrasonic scaling. A mechanism that arises later is that the body create a balance with the increased number of apoptosis cells as one of indicators of the increased number of caspase-3 expression in dental pulp cells.

During ultrasonic scaling procedure, enamel structure consisting of organic and inorganic elements will also get impact pressure from the tip of the scaler to the tip of the tooth surface. The vibration of tip scaler and spray cooling can remove stain from the tooth surface. During scaling, cavitation and acoustic microstreaming mechanisms also occurs, as a result, the cooling water can enter the dental pulp chamber due to the nature of the porous enamel and dentin. These mechanisms occur because of exposure to the cell resulting in changes of the cell membrane, followed by membrane permeability, morphology and activity changes of cell membrane.[23] It takes a duration of 1-1.5 minutes for cleaning stain on the tooth surface using ultrasonic scaler.[24] The longer the duration is, the higher the negative effects will be obtained [25] and the higher the permeability of cytoplasm will occur.[26] It can be said that the ultrasonic

waves can alter the number and nature of the cell nucleus in apoptosis process.[27]

The results of LSD test, indicate that the number of caspase-3 expression in dental pulp cells the group of teeth without stain was not significantly different from the other groups of teeth without stain, compared with the group of teeth with stain. It is because the tooth surface without stain will make the tip directly contact with the enamel surface, but because stain is porous, air bubbles can get into the pulp chamber during the ultrasonic scaling. Sonification can trigger acoustic microstreaming, flow turbulence, microjet and shock wave leading to rupture of air bubbles.[2] This mechanism is suspected to be cause of damage to the surface of the organic structures and the dental pulp cells.

Sonoporation mechanism is used as a drug delivery by penetrating cell membranes.[5] Nevertheless, this mechanism can give negative effects, such as pulpitis or dental pulp necrosis.[8] At the time of the evaluation of the number of caspase-3 expression, there was the increasing of the number of small-sized bubbles which were more numerous and larger size as the increasing of the number of scaling replication and duration. Most preparations even showed an increase in the number and size of air bubbles in the blood vessels leading to the rupture of the blood vessels. This condition can indicate the occurrence of pulpitis or necrosis mechanism after the scaling. It then can be understood that one of the effects of ultrasonic waves on a tissues are the increasing of local temperature due to mechanical pressure passing the tissue. Ultrasonic pressure resulting in small bubbles on a live tissue can lead to distortion of the cell membrane influenced by flux and activity of ions.[20] The changes of bubble size that occur periodically even can cause expansion, contraction and death for the tissue. These side effects can be temporary or permanent. The effects of ultrasonic waves can also be reversible and irreversible disrupting cell membranes through cavitation mechanism. It can be concluded that ultrasonic scaling duration and replication on teeth with and without stain can improve the number of caspase-3 expression in dental pulp cells.

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