

EVALUATION OF THE ANTIBACTERIAL EFFECT OF NANO SALVADORA PERSICA ROOTS AS ROOT CANAL IRRIGANT USING CONFOCAL LASER MICROSCOPY (AN IN-VITRO STUDY)

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Abstract

Aim: The aim of this study is to evaluate the antibacterial effect of *Salvadora persica* nanoparticles and 2.6 % NaOCl as root canal irrigants against *E. Faecalis* bacterial biofilm using confocal laser microscopy. **Methods and Materials:** This study was performed on 48 recently extracted single canaled mandibular premolar teeth that were divided randomly into three groups. *E. Faecalis* bacterial incubation into root canals for three weeks to ensure biofilm maturation. The specimens were stained with the live/dead technique and evaluated using a confocal laser scanning microscope. Comparison between different groups was performed by using One Way ANOVA test which revealed significant difference between all groups regarding percentage of dead cells as $P < 0.05$, followed by Tukey's Post Hoc test for multiple comparisons. **Results:** Regarding percentage of dead cells *Salvadora persica* nanoparticles group ($52.17 \% \pm 2.31$) was significantly the highest, while control group ($1.47 \% \pm 0.24$) was significantly the lowest. **Conclusion:** *Salvadora persica* nanoparticles represented an efficient and natural root canal irrigant against *E. faecalis* biofilm.

Keywords: *Salvadora Persica*, Nanoparticles, Confocal Laser Microscopy, Bacterial Biofilm.

INTRODUCTION

A primary objective of root canal therapy is to eliminate as many harmful bacteria as possible from the root canal system while undergoing endodontic treatment. The development of pulpal inflammation and pulpal tissue necrosis, and therefore the formation of periapical lesions, are significantly influenced by microbial germs such the facultative bacteria *Enterococcus faecalis*¹. The removal of microorganisms is crucial. However, the anomalies and anatomical differences of the root canal system represent a challenge. Furthermore, mechanical instruments are unable to reach every dentinal canal wall, leaving untapped regions. In order to completely eradicate microorganisms from the root canal configuration without compromising the healing process, which is essential for the attainment of long-term endodontic root canal success, mechanical enlargement in addition to chemical disinfection is therefore very important^{2,3}.

Endodontic irrigants have been created using a variety of synthetic antibacterial agents for a number of years. Bacteria are growing increasingly resistant to conventional antimicrobial drugs, leading to the development of new, inexpensive, non-toxic alternatives. It has been discovered that natural plant extracts can successfully and effectively replace endodontic irrigants⁴.

Sodium hypochlorite, which has strong antibacterial qualities, has long been the preferred irrigant for root canal therapy. If it is forced past the root canal apex, though, negative consequences result. Natural materials derived from medical implants are increasingly being used in the dentistry industry to combat the chemosynthetic character of traditional irrigating solutions. *Salvadora persica*, often known as meswak, is a herbal substance that has been utilized in the dental sector and has been shown to have a potent antibacterial effect since it contains a variety of active components⁵.

The use of nanotechnology has improved the chemical and physical characteristics, as well as the antibacterial activity, of irrigating solutions. By using nanotechnology, this can be accomplished by reducing their solid state to the tiniest nanoparticles. *Salvadora persica* nanoparticles can maximize the solubility of its active components, enhancing their antibacterial effectiveness⁶.

MATERIALS AND METHODS

(A) Sample selection:

In this study, 48 mature single canaled human mandibular premolar teeth were obtained from the oral surgery department at October 6 University's Faculty of Dentistry. The presence of any fractures, cavities, craze lines, curvature, or external resorption in teeth was examined. Radiography was used to rule out any teeth samples that had internal resorption or calcification.

(B) Sample preparation and Sterilization:

An ultrasonic scaler (Suprasson P5 Booster, Satelec, France) was used to plane the chosen samples in order to remove any hard deposits, leftover bone calculus, or soft tissue remnants on the root. At the cemento-enamel junction, decoronation was carried out using a fine tapered diamond stone set on a high-speed hand piece (PANA AIR, NSK, Japan) with a generous supply of coolant flowing through it. The chosen samples' length was changed and fixed at 16 mm. Each tooth's root apices were sealed with a tiny piece of composite resin. This was done to ensure that the irrigating solution remained in the root canal during the contact period in order to get the maximum and consistent effect possible in order to simulate in vivo apical counter pressure, and to prevent seepage of the bacterial suspension during the autoclave incubation period. After which they were sterilized by autoclaving at 121°C for 30 minutes, twice, and then immersed in a 5.25 percent sodium hypochlorite solution for 14 minutes.

(C) Root canal preparation:

All selected samples were prepared as the follow:

Root canal patency and Pulp tissue extirpation from all root canals using # 15 -20 K-St St file (MANI, Inc, Japan). Root canal preparation by the crown down technique using ProTaper Next (PTN, Dentsply Maillefer, Switzerland) rotary system. ProTaper Next files were mounted on a contra-angle hand piece that was attached to a torque-limiting motor E-connect Pro endomotor rotary device (Changzhou Eighteenth Medical Technology Co., Ltd, China) at torque (0.8 N) and a speed of 300 RPM with a sequence X1, X2, X3 to a final size X4 (40/0.06). Irrigation was performed between each rotary file and the next with normal saline.

(D) Preparation of irrigating solutions:

2.6 % Sodium hypochlorite: 5.25% NaOCl solution was diluted to 2.6 % as half to half NaOCl and distilled water.

Salvadora perisca 60 % ethanolic extract preparation:

800 gram of *Salvadora Persica* chewing sticks (Botany garden, Mecca, Saudi Arabia), sun dried for 10 days. Then cut using a sharp knife, and then grounded into powder using commercially available food blender. 120 ml of 60% ethanol was added to 40 gram of powder in a sterile well capped flask, then left for 3 days at room temperature then filtered using number 1 filter paper. miswak extract was incubated at temperature 37°C until it became dry and then stored in sterile screw capped vials in the refrigerator. Serial dilutions of the extract was prepared using sterilized Ringer's lactate by taking 1 gram miswak alcoholic extract and dissolving it in 2.5 ml of Ringer's lactate in order to give 100% concentration.

Synthesis of polymeric PEG-PVP-linker biomaterial

PEG-PVP linker biomaterial was prepared by adding 2:1(w/w)-PEG-PVP dissolved in 25 ml of isopropanol dropwised with continues starring.

Synthesis of miswak encapsulation PEG-PVP biopolymer

A 1% of 25 ml of alkalized miswak was mixed dropwise with continuous stirring at room temperature to the polymer mixture namely as PEG-PVP linker biopolymer for 24 h, then filtration the mixture and 2.5 ml hydrochloric acid (HCL) was then added to neutralize the pH of the solution. The product was precipitated from the supernatant liquid by adding 2:1 (v/v) mixture of acetone and ethanol. The solution was then heated under reflux for 24 h. After the reaction, the solvents were evaporated under high vacuum then the miswak-PVP-PEGs were obtained encapsulated and sparingly soluble in 75 ml phosphate buffered (PBS) were prepared in 100 ml glass bottles. The mixture was stirred and vortexed several times for complete dissolution and encapsulation and stored at 4 °C.

Salvadora persica nanoparticles characterization:

Further evidence for their size, shape, and abundance was recognized by the transmission electron microscope (TEM) as shown in **(Figure1)**. The nanoparticles were shown in very small sizes ranges from 6.49 to 21.0 nm with an average size of 10.6 nm and spherical shape.

Dispersant name: water, dispersant RI: 1.330, viscosity (cP): 0.8872, material absorption: 0.100

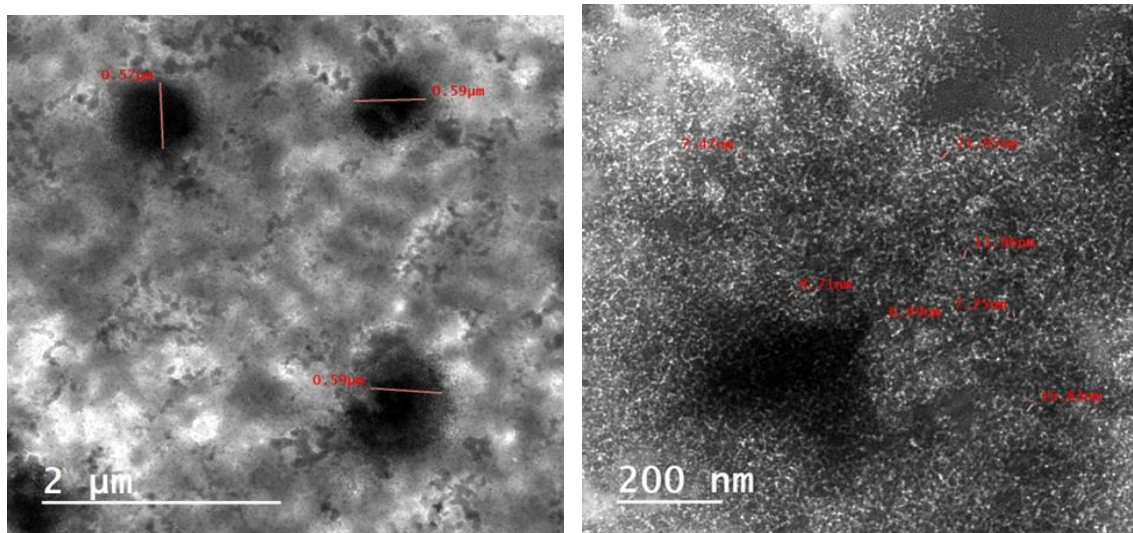


Figure (1): Salvadora persica nanoparticles characterization

Evaluation of antimicrobial effect:

(A) Sample sterilization:

Sterilization of samples after mechanical preparation by autoclaving at temperature 121 °C for 30 minutes for two sterilization cycles was done. Each tooth sample was stored in separate autoclavable eppendorf until time of inoculation.

(B) Microorganisms biofilm preparation:

Step one: Proper growth media preparation brain heart infusion (BHI) followed by pure bacterial culture of gram+ cocci (#29212, ATCC Manassas, VI), *Enterococcus faecalis* obtained from American type culture.

Step two: *Enterococcus faecalis* was introduced in 7 ml of brain heart infusion, incubated at temperature 37 °C for 24 hours. Then suspension prepared on the BHI plates under the same incubation conditions. Bacterial cells after that were resuspended in saline and adjusted to #1 mcfarland turbidity standard (3×10^8 cells/ ml).

Step three: 5 ml of sterilized brain heart infusion was mixed with five ml of bacterial inoculum. The samples were inoculated with *Enterococcus faecalis* by using micropettes in order to fill each root canal for two weeks to allow bacterial biofilm formation. Every 72

hrs interval this process was repeated, using pure cultured adjusted and prepared to #1 mcfarland standard.

Step four: Samples were kept under suitable atmospheric condition and humidity at temperature 37 °C for three weeks at the incubator to ensure complete biofilm formation.

Step five: Centrifuging the samples twice during incubation period 'time interval' was done.

(C) Biofilm confirmatory group:

16 Samples were used as control group to ensure complete bacterial biofilm formation before irrigating root canals that was scanned using confocal laser microscopy.

(D) Samples classification and irrigation:

32 Samples were classified according to final irrigation solution into two experimental groups.

Group (A): 16 sample were irrigated with 5 ml NaOCl 2.6% by plastic syringe with 23-gauge needle for 2 minutes.

Group (B): 16 samples were irrigated with 5 ml *Salvadora persica* nanoparticles 5 mg / ml by plastic syringe with 23-gauge needle for 2 minutes.

All root canals were dried with paper points after ten minutes and then irrigated with sodium thiosulfate to counteract effect of sodium hypochlorite. Samples were kept at low temperature -5 °C to prevent growth of microorganisms.

Method of evaluation:

1. Antibacterial effect using confocal laser scanning microscopy:

Sample cutting:

The extracted human teeth were sectioned to be available for imaging through using 0.3mm Isomet saw under constant cooling through using sterile distilled water. Two one millimeter sections were taken from each root, corresponding to the apical and middle thirds of each root. The dentin segments from the in situ experiment were not sectioned as they were flat before the infection so became unnecessary section for confocal microscopy analysis, with sample size 5*5*1 millimeter. All the needed samples were kept in ice box at temperature -4 °C for fixation of microorganism's growth.

Sample preparation for imaging by confocal laser microscopy:

- Dentin segments were washed carefully by 100 µl sterilized distilled water for one minute and then dried samples smoothly.
- Dentin segments were then placed at the bottom of the Eppender of tube.
- 10 µL of propidium iodide (PI) red fluorescence and 100 µL of each 0.01 % acidrine orange (AO) green fluorescence were added to the selected specimens to be stained in a dark room, then kept for 15 minutes after centrifuging for ten seconds.

- Specimens were removed from the tube and washed twice with 100 µl sterile distilled water to remove excess dye. After that specimens were transferred to glass covered slips, covered with immersion oil before performing any image acquisition.

Laser and stain specifications

Confocal illumination was performed by using argon laser microscope that was adjusted 460 nm emission for (PI) and to 500 nm for (AO). Fluorescence from the stained specimen cells was viewed through using cone focal laser scanning microscopy. Dye material has the ability to bind bacterial DNA emitting green fluorescence while bacterial RNA emitting red fluorescence, after staining, corresponding specimens were immediately analyzed by CLSM technique. PI enters only the dead cell membrane exhibiting red fluorescence, while AO stains the living cells exhibiting green fluorescence. For the acridine orange stain the excitation and emission for DNA was 500 and 526 nm respectively, while for RNA the excitation and emission was 460 and 650 nm. Sequential frame scan mode was used to prevent crosstalk.

Magnification of specimens:

Observing mounted specimens through using 40X magnification oil immersion objective with a numeric aperture of 1.4, confocal pinhole was set to diameter 164 Mm for channel two, and 88 Mm for channel one

Image analysis:

Images of confocal laser microscopy were analyzed using Zeiss laser scanning microscope software version ZEN 3.3 (2012). 5-10 µm in dentin structure as deep scans obtained from each specimen (twenty –forty sections of 2 µm step size in format of 1024 X1024 pixels).

Statistical analysis was performed with SPSS 20, Graph Pad Prism and Microsoft Excel 2016. All quantitative data were presented as minimum, maximum, mean and standard deviation. One Way ANOVA test was used to compare between different groups followed by Tukey`s Post Hoc test for multiple comparisons.

RESULTS

Regarding antibacterial effect Comparison between different groups was performed by using One Way ANOVA test which revealed significant difference between all groups regarding percentage of dead cells as $P < 0.05$, followed by Tukey`s Post Hoc test for multiple comparisons which revealed that % of dead cells of *Salvadora persica* nanoparticles group ($52.17 \% \pm 2.31$) was significantly the highest **figure (2)**, followed by 2.6% NaOCl group ($50.36 \% \pm 1.42$) while control group ($1.47 \% \pm 0.24$) was significantly the lowest **table(1)**, **figure (3)**.

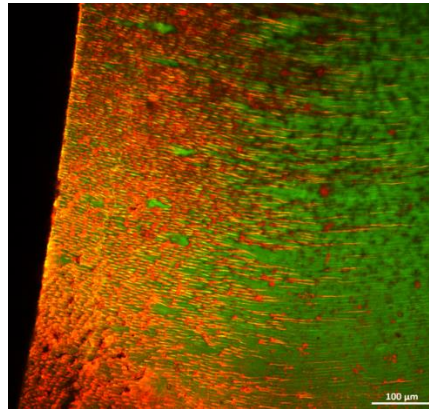


Figure (2): Live and dead cells shown by CLSM in *Salvadora persica* nanoparticles samples

Table (1): Mean and standard deviation of dead cells percentage in all groups and comparison between them using One Way ANOVA test followed by Tukey`s Post Hoc test:

Bacterial count reduction and Biofilm	% of dead cells	
	Mean	Std. Deviation
Salvadora persica Nanoparticles	52.17 ^a	2.31
Sodium hypochlorite solution (2.6%).	50.36 ^b	1.42
Control group	1.47 ^c	0.24
P value	<0.0001*	

*Significant difference as $P < 0.05$.

Means with the same superscript letters were insignificantly different a $P > 0.05$.

Means with different superscript letters were significantly different a $P < 0.05$.

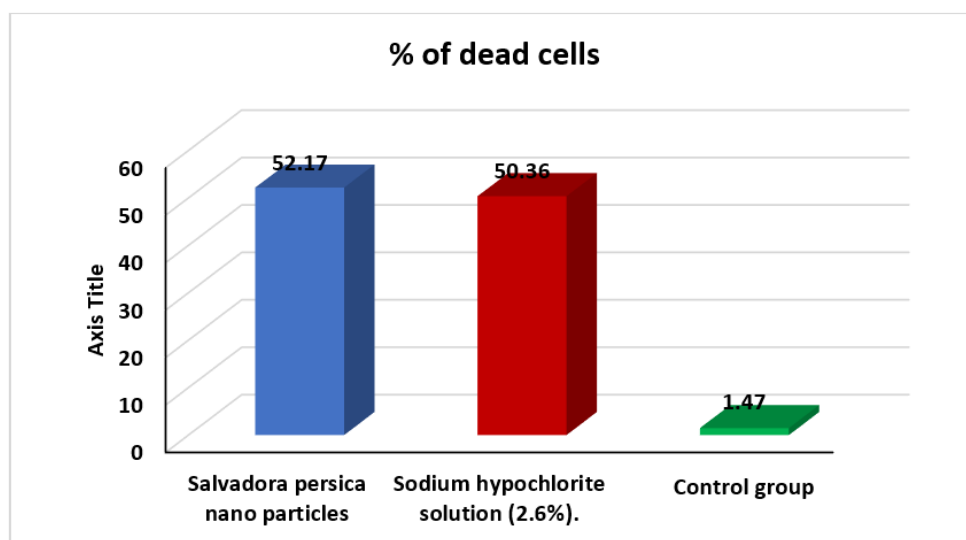


Figure (3): Bar chart showing percentage of dead cells in all groups

DISCUSSION

During root canal treatment, the conventional mechanical method of cleaning and shaping can't provide the desired removal of microorganisms penetrating dentinal tubules. Furthermore, after mechanical instrumentation 9.6 % - 47.6 % of root canal system is left untouched, this is covered with dentin chips, bacteria and pulp tissues that act as nidus for reinfection⁷.

The number of facultative anaerobes in the root canal system increases with the duration of the infection. One of the most prevalent pathogens that can be grown from retreatment-treated failing root canals is *Enterococcus faecalis*. By making the bacteria a thousand times more resistant to antibodies, phagocytosis, and antimicrobial chemicals, the biofilm that surrounds them helps the germs survive the destruction. This is because the extracellular matrix acts as a protective barrier. Additionally, through horizontal gene transfer, biofilms facilitate the transmission of antibiotic-resistant genes among several clinically relevant species, giving microorganisms the resistance to chemo mechanical preparation. For this reason, the best intracanal irrigant solutions are those with strong antibacterial properties to enhance the results of the instrumentation processes⁸.

Previous studies have reported that *S. persica* roots have antiseptic, antimicrobial, anticariogenic, and analgesic activities⁹. Trimethylamine, salvadorine, chloride, fluoride, and trace amounts of tannins, saponins, flavonoids, and sterol have all been shown to be present in *Salvadora persica*. It has been demonstrated that certain elements have strong antibacterial properties¹⁰.

Nanotechnology in the field of endodontics has become widely used in an effort to improve the antimicrobial efficiency of conventional antibacterial agents and to obtain promising results in endodontic therapy. Numerous benefits, such as their ultra-small size, high surface-area-to-volume ratio, and superior physical and chemical properties, can be obtained from antimicrobial nanoparticles. A recent development in the management of dental infections is the use of nanoparticles (NPs). Increased antibacterial impact is produced by NPs' ability to react with negatively charged bacterial cells due to their larger surface area and positive charge¹¹. Additionally, *Salvadora persica* root nanoparticle powder increases the bactericidal activity of the product by maximizing the solubility of its active components¹². Nevertheless, there aren't many studies comparing the antibacterial properties of *Salvadora persica* roots to sodium hypochlorite as an irrigating solution⁶.

The process of creating nanoparticles was done top-down. This method involves employing physical (lithographic) instruments or chemical processes to deconstruct the bulk material or solid until the desired tiny structures are formed, ultimately achieving the desired size and shape¹³.

Nanoparticles have the ability to infiltrate biofilms and engage in electrostatic interactions with bacterial cell walls, resulting in damage to cell membranes, heightened permeability, production of reactive oxygen species, disruption of cellular processes, degradation of proteins, damage to DNA, and ultimately, cell death.¹⁴

Human mandibular single-canaled teeth (premolars) were the study's sample of choice because they allowed us to assess the effectiveness of the tested irrigating solutions in the root canal system and replicate a clinical setting. In order to rule out anatomical variances and complexity concerns, the samples that were chosen were single-canaled teeth 15, 16. Every test sample was autoclaved to ensure secure handling. These samples were then kept in an aqueous solution with a disinfectant to prevent dehydration and the formation of more bacteria¹⁷. To standardize the specimens, teeth were decoronated to a length of 16 mm. The Protaper Next set of nickel titanium rotary tools¹⁸,¹⁹ was used for root canal preparation. Each tooth's root apex was capped with a little amount of composite resin to stop the bacterial suspension from seeping through while the tooth was in the autoclave for incubation. In order to replicate in vivo apical counter pressure, it will also help retain the irrigating fluid inside the root canal and guarantee its presence throughout the contact period²⁰.

The advancement of direct viable staining techniques and the confocal microscope (CLSM) have sparked interest in the study of bacterial viability in recent years, particularly in the field of biofilms. Some early research have reported using CLSM to analyze dentin infection and dental biofilms. The use of vital staining techniques to observe live bacteria in dentinal tubules and root canal walls, in situ hybridization to identify labeled bacteria, and three-dimensional visualization of structural organization in biofilms are some potential benefits of confocal microscopy for the study of dentin infection. When analyzing samples that are performed by sectioning decalcified dentin, optical sectioning, and the capacity to examine the subsurface of hydrated specimens may be helpful in avoiding impurities or artifacts located at the surface layer¹⁷.

As a non-destructive method of studying these ecosystems and the hydrated spatial arrangement at the cellular level, confocal laser scanning microscopy has shown to be an extremely useful tool in the recent years for studying the structure of biofilms. Targeting certain extracellular matrix elements and cell types is made possible by the use of fluorescent markers. It is possible to distinguish between live and dead bacteria using particular stains, as shown by red or green fluorescence. By employing a three-dimensional reconstruction of the biomass to examine the architecture of the biofilm, this technique enables us to have a comprehensive understanding of the efficacy of irrigating solutions and procedures in disturbing the biofilm structure²¹.

This study aimed to evaluate the antibacterial effect of *Salvadora persica* roots nanoparticles versus sodium hypochlorite. Using statistical results obtained by cone focal laser microscopy we calculated the mean value of percentage of dead cells. Both *Salvadora persica* nanoparticles and NaOCl showed antibacterial effect against *Enterococcus faecalis* biofilm. *Salvadora persica* nanoparticles showed the highest percentage of dead cells (52.17 %) that was significantly higher than NaOCl group (50.36%) as ($P < 0.05$), while control group showed the least percentage of dead cells (1.47 %). Actually, the wide range of chemical components in *S. persica* is responsible for its antimicrobial activity. Gram-negative bacteria are effectively inhibited by benzoyl isothiocyanate (BITC), a significant component found in *S. persica* roots. Because of its

lipophilic and electrophilic qualities, it has been suggested that BITC may penetrate bacterial membranes and inhibit their redox processes, which would disrupt the potential of the bacteria's membrane²².

Efficiency of NaOCl is attributed to its effectiveness in eliminating intracanal microorganisms. Hypochlorite acid which is a powerful oxidizing agent that produces an antibacterial effect by irreversible oxidation of hydrosulphuric groups of bacterial enzymes. As essential enzymes are inhibited, disturbance of the metabolic functions of the bacterial cell occurred which lead to death of bacterial cells. Highly toxic N-chloro composites that have antimicrobial effect can be formed by Chlorine adherence to bacterial cytoplasm components^{23, 24}.

Our study's results align with previous research demonstrating the antimicrobial efficacy of *Salvadora persica* extracts at varying concentrations against bacterial biofilms^{6, 25, 26, 27}. The antibacterial activity of miswak extract against *Streptococcus faecalis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* was also reported by **Darout et al.**²⁸. This activity may have been attributed to the extract's nitrate content. On the other hand, other studies have shown a significant antibacterial effect of sodium hypochlorite against common oral pathogens than *Salvadora persica* nanoparticles^{29, 30, 31}.

CONCLUSION

Salvadora persica nanoparticles represent an efficient, natural, promising root canal irrigant regarding reducing *E. faecalis* biofilm.

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