EGYPTIAN DENTAL JOURNAL
www.eda-egypt.org
VOL. 71, 2725:2733, JULY, 2025
PRINT ISSN 0070-9484 • ONLINE ISSN 2090-2360
CONSERVATIVE DENTISTRY AND ENDODONTICS
Submit Date : 09-05-2025 • Accept Date : 03-06-2025 • Available online: 15-07-2025 • DOI : 10.21608/edj.2025.382689.3467

THE ANTIMICROBIAL EFFICACY OF MODIFIED SALT SOLUTIONS AGAINST *E. FAECALIS BIOFILMS*: AN EX-VIVO STUDY

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ABSTRACT

Objectives: The aim of this study was to investigate the disinfecting effect of a modified salt solution (MSS) on *Enterococcus faecalis* biofilms in ex-vivo model.

Methods: Eighteen human dentine sections were standardized, inoculated with *E. faecalis* and incubated for three weeks to ensure bacterial biofilm maturation in the root canal space. Then samples will be allocated according to irrigation into 3 groups (6 dentine sections each): GI (Control) was irrigated with distilled water, GII irrigated with modified salt solution (MSS) and GIII with sodium hypochlorite (NaOCl) irrigation. Afterwards dentine sections were stained with 1:1 mixture of Syto 9/propidium iodide for LIVE/DEAD staining of *E. faecalis*, and were analyzed under conofocal laser scanning microscopy (CLSM). Comparisons between groups were done by One Way ANOVA test followed by Tukey's post hoc test for multiple comparisons.

Results: The results showed that both irrigants NaOCL and MSS dramatically increased *E. faecalis* death compared to the control. There was no statistically significant difference between MSS and NaOCl in dead *E. faecalis* detected in CLSM images.

Conclusion: MSS is potent antimicrobial against *E. faecalis* biofilm in dentine, with efficacy comparable to NaOCl.

KEYWORDS: Sodium hypochlorite (NaOCl) irrigation, modified salt solution, conofocal laser scanning microscopy, *E. faecalis* biofilm.

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INTRODUCTION

Apical periodontitis is an inflammatory reaction to microbial infection of the root canal system. Therefore, disinfection of the root canal is the main goal of root canal therapy. [1] Biomechanical preparation of the root canal system is a major step in root canal disinfection to eliminate bacteria and its by-products, however, complete eradication of the microorganisms often fails due to the morophological complexities of the root canal, in addition to biofilms that are considered as resistant structure to be removed. [2,3]

The existence of resistant microbial species such as variety of anaerobes, aerobes, and fungi is one of the major cause of failures in root canal therapy. [4]

Sodium hypochlorite (NaOCl) is the most widely used root canal irrigant solution because it is effective tissue-dissolving material and has adequate antimicrobial activity [5]. However; there are some drawbacks of NaOCl, as it has been found to be irritant to periapical tissues when extruded [6]. moreover, it is demonstrated to decrease the fracture resistance of teeth [7] and negatively affect the bond strength of adhesive restoration to dentine [8]. The inability of NaOCl to completely eradicate biofilms from inaccessible areas and preventing their regrowth [9,10]. so alternative irrigants are required to enhances the optimal disinfection of root canals with no irritating effect to the related vital tissues.

Therefore, a new disinfection strategy has been developed with a modified salt solution (MSS) [11,12] This MSS is a hypertonic solution composed of potassium sorbate and sodium chloride which is anticipated to diffuse into biofilms and accessory canals, isthmuses and ramifications. The antimicrobial action of MSS is based on a multiple-hurdle strategy including high osmotic pressure, weak acidity (low pH), and absence of oxygen [9]. The microorganisms try to make adaptation to these hurdles as they will do in the lag-phase bacterial growth cycle. Micro-organism needs energy to adapt however, there are several hurdles to overcome, so the microorganisms could be exhausted [10].

The proper combination of safe components could provide a synergistic impact, this is the charm of a multiple hurdle disinfection strategy. MSS applied in direct contact with the biofilms can inactivates multi-species of bacteria biofilms [11-13]. In addition, as it is a mixture of highly concentrated salt solutions, it is expected to be able to diffuse into more distant areas past the main root canal lumen.

Assessment of the penetration depth of microorganism inside dentinal tubule of root canal can be done by several methods as paper point sampling, scanning electron microscopy (SEM), transmission electron microscopy (TEM), Stereomicroscopy, and confocal laser scanning microscopy (CLSM). In a comparison to SEM, CLSM provides more detailed information about the intratubular penetration depth [10]

So the aim of this study was to explore the ability of MSS against *E. faecalis* biofilm in the irregularities of the root canal system beyond the main root canal lumen.

MATERIALS AND METHODS

Sample size calculation

Sample size calculation was done according to dead E. faecalis percent (%) reported by Du et al. 2014,in dentine sections irrigated by NaOCl and distilled water, mean ± standard deviation (SD) of Dead *E. faecalis* in dentine sections irrigated by distilled water is 4% ± 0.02, while in dentine sections irrigated by NaOCl is 26% ± 0.1, with effect size 3.05 when the (power) is 0.95 and the Type I error probability associated with this test is 0.05. The minimum sample size needed in the current study is 5, increased to 6 dentine sections in each group. The sample size was calculated using T test which was performed by using G. Power 3.1.4.9.

Eighteen dentine sections were required assuming the reported SD of 0.18 mm and anticipating no specimen loss during the study (alpha risk of 0.05 and beta risk of 0.2).

Sample dentine sections preparation

Collection and use of teeth was done according to ethics committee approval number, Faculty of Dentistry, Umm Al-Qura University, Makkah, Saudi Arabia (AWHR130924).

Nine human incisors with straight root, caries free and mature apices were collected and stored at 4°C till use. Dentine sections were prepared as described by **Ruiz-Linares *et al*. 2017** [14], cervical third of roots were obtained, splitted longitudinally into two halves, resulting in eighteen dentine sections, 220 to 800 grit silicon carbide papers were used to remove cementum and polish root canals to create relatively flat surfaces. Dentine sections were adjusted using a caliper to dimensions of 4 x 4 x 0.7mm (width x length x height).

Finally smear layer was removed by immersion in sodium hypochlorite for 15 minutes (JK-Dental, Egypt), then dentine sections were treated with 17% ethylenediaminetetraacetic acid (EDTA) (Produits Dentaires SA, Switzerland) for 5 min, then rinsed with distilled water and finally teeth were submitted for autoclaving at 121 °C for 20 minutes.

***E. faecalis* inoculation and biofilm formation**

E. faecalis American Type Culture Collection (ATCC) 29212 was subcultured in brain-heart infusion (BHI) broth media (Oxoid Ltd, Basingstoke, UK). Then, dentine sections were immersed in 1 mL BHI broth containing 10^8 *E. faecalis* in Eppendorf tubes for 3 weeks at 37°C under anaerobic conditions to develop *E. faecalis* biofilm, during this period, BHI was replenished every 2 days (Li *et al*. 2015) [15].

Treatments

Dentine sections were washed by distilled water for 1 minute to remove residual broth, then dentine sections were randomly allocated into 3 groups (n = 6) according to irrigant: group I; Distilled water, group 2; 2.5% NaOCl, group 3; MSS. Irrigation was performed by immersing each dentine section in 100 μL of the irrigant for 3 minutes, thereafter NaOCl group was inactivated using 5% sodium thiosulfate for 5 minutes and finally dentine sections were washed by phosphate buffered saline (**Ruiz-Linares *et al*. 2017**) [14].

Confocal laser scanning microscopic analysis

The fluorescence LIVE/DEAD BacLight Microbial Survival dye was used to prepare and colour each specimen as follows: Ten microliters of Acridine Orange (AO) stain (100μg/ml) (Sigma-Aldrich, Missouri, USA) and 20 microliters of Propidium Iodine (PI) stain (100μg/ml) (Sigma-Aldrich, Missouri, USA) were added respectively using a micropipette. The solution was then mixed using Thermo Scientific™ LP Vortex Mixer for 30s. The specimen was then left at room temperature (25ºC) for 15 minutes and kept in darkness. After 15 min the specimen was gently washed with 1 mm of sterile saline. Finally, the specimen was placed on a microscope glass slide and scanned by the confocal laser scanning microscope (Leica DMi 8 fluorescence microscope, Leica Microsystems, Germany) at magnification X40 and resolution:1024 × 1024 pixels.]. The root specimens were scanned at ×40 magnification. The excitation/emission wavelengths were 480/500 nm for SYTO9 and 490/635 nm for PI. CLSM images were acquired and analyzed using Leica Application Suite (Leica Microsystems, Germany) at a resolution of 1024 × 1024 pixels. Images were processed for background noise reduction (Leica Application Suite software)

The Propidium Iodide stain is excited at a wavelength of 458 nm for excitation and emission,

and the wavelength of 514 nm for the excitation of the Acridine Orange stain. The interaction of Propidium Iodide stain with dead bacteria results in the reemission of red fluorescent light, at the same time, the interaction of Acridine Orange stain with the live bacteria results in the re-emission of green fluorescent light. The red fluorescence (dead bacterial cells) and green fluorescence (living cells) were displayed simultaneously using dual-channel imaging By monitoring the light's fluorescent intensity, it was possible to determine the fluorescence from both live and dead bacterial cells. CLSM images were acquired by the software image J.

Statistical analysis:

Statistical analysis was performed with Statistical Package for Social Science (SPSS 20®, IBM, USA), Graph Pad Prism (Graph Pad Technologies, USA) and Microsoft Excel 2016. Normality of data was tested by using Kolmogorov and Shapiro Wilk's test, while homogeneity of data was assessed using Levene's. All data revealed normal distribution. Accordingly, comparison between groups was performed by using One Way ANOVA test followed by Tukey's Pots hoc test for multiple comparisons. The significant level was set to be at $P \leq 0.05$.

RESULTS

E. faecalis% following irrigation

CLSM imaging of dentine sections were analyzed for Live/Dead *E. faecalis* across three experimental groups. Control group (GI) presented in figure (1), from the figure it is evident that high green color intensity indicates high Live *E. faecalis* levels and low values of Dead *E. faecalis%*. CLSM imaging of dentine sections irrigated with MSS (GII) are presented in figure 2, red color intensity indicates higher Dead *E. faecalis%* following irrigation by MSS, confirming antimicrobial efficacy of MSS. Concerning GIII, CLSM images of dentin sections irrigated with NaOCl are presented in figure 3, it is evident from the figure that NaOCl performed antimicrobial effects.

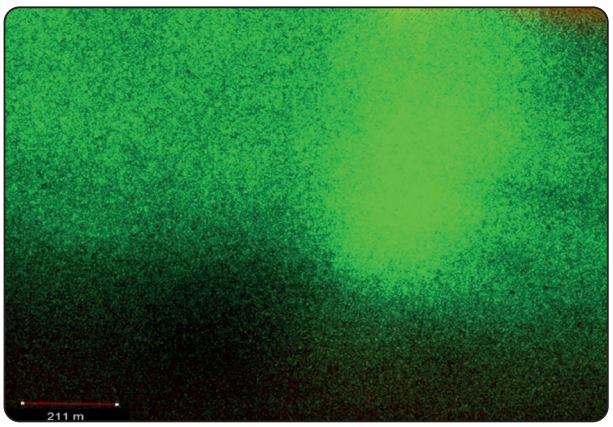

Fig. (1) CLSM image of dentin slice in GI. Green colour intensity indicates Live E. faecalis.

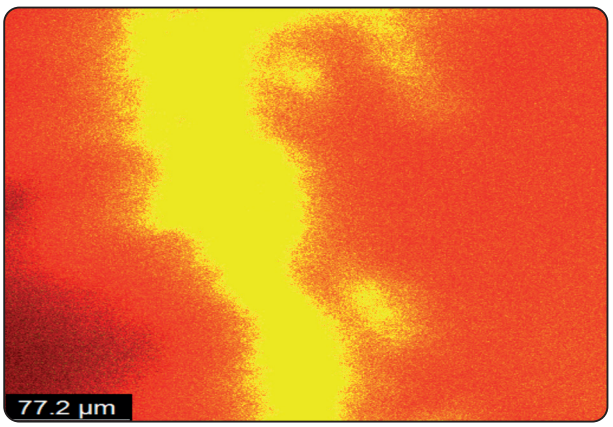

Fig. (2) CLSM image of dentin slice in GII. red color intensity indicates Dead E. faecalis evident by red staining. Accordingly, MSS increased the Dead E. faecalis percent.

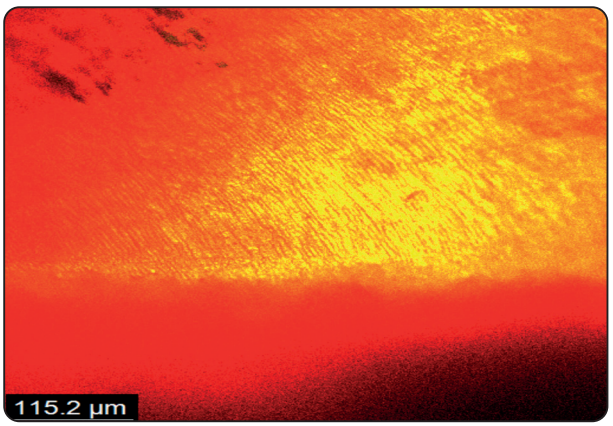

Fig. (3) CLSM image of dentin slice in GIII, red color intensity indicates antimicrobial action of NaOCl.

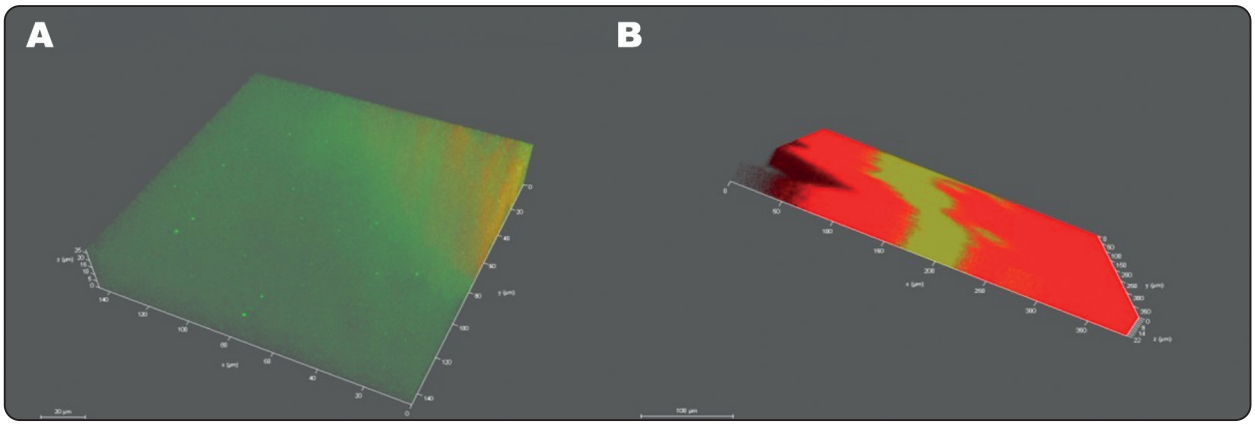

Fig. (4) CLSM 3 Dimensional image of dentine slices following irrigation. A: irrigated with distilled water. B: irrigated with MSS. From figure, higher intensity of red colour in MSS irrigated dentine slice is denoting the potent antimicrobial potentials o MSS increasing E. faecalis death.

The comparative analysis of Dead *E. faecalis%* across three experimental groups are presented as mean ± SD, minimum, median, maximam (Table 1). The One Way ANOVA test for comparison between groups showed statistically significant difference in Dead *E. faecalis%* between the groups (Table 2).

The results of a Tukey's Post Hoc test comparing Dead *E. faecalis%* between the three experimental groups in a pairwise manner showed a statistically significant more Dead *E. faecalis%* in GII compared to GI (Table 2). This indicates that MSS treatment dramatically increases *E. faecalis* death compared to the control. Similarly, Tukey's Post Hoc test showed statistically significant more Dead *E. faecalis%* in GIII compared to GI between GIII and GI (Table 2). This shows that NaOCl treatment also substantially increases *E. faecalis* death compared to the control. However, there is no statistically significant difference between GII versus GIII (Table 2). This suggests that while both treatments are highly effective compared to the control, neither treatment demonstrates a significant advantage over the other in terms of *E. faecalis* death.

TABLE (1) Descriptive results of Dead E. faecalis% across the three experimental groups

		N	Minimum	Median	Maximum	Mean	SD	SE
GI	**Control**	6	3.90	4.35	4.90	4.32	0.38	0.16
GII	**MSS**	6	84.06	90.69	99.24	91.04	5.49	2.24
GIII	**2.5% NaOCl**	6	87.61	92.67	96.44	92.70	3.04	1.24

TABLE (2) Comparison between E. faecalis Dead% in GI, GII, and GIII

Dead *E. faecalis%*	**GI**		**GII**		**GIII**		**P value**
	M	SD	M	SD	M	SD	
	4.317% [a]	0.3817	91.04% [b]	5.491	92.7% [b]	3.038	<0.0001*

Significant difference as P ≤ 0.05. Means with different superscript letters were significantly different as P ≤ 0.05.

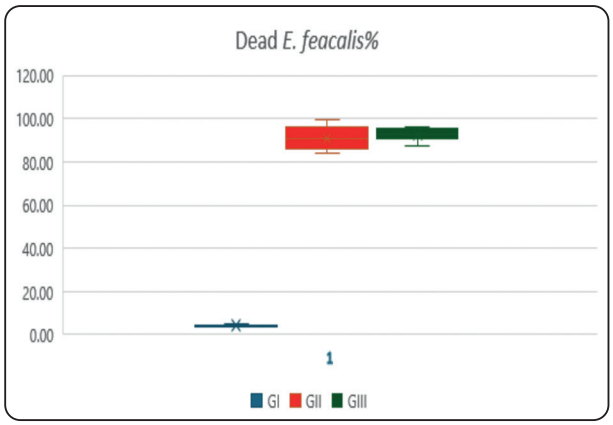

Fig. (5) Box plot representing Dead E. faecalis% in all groups.

DISCUSSION

Microorganisms are frequent cause of root canal treatment failure.[5,6] Accordingly, eradicating microorganisms from root canals is critical in endodontic therapy, current chemomechanical strategies are not capable to achieve this goal due complexity of the root canal system and dentine buffering capacity that impairing antimicrobial effect of the irrigants [16-18]. Microbial biofilms in root canals are main causative factor of apical periodontitis as inflammatory response [19]. The current study inoculated dentine sections with *E. faecalis* due to it's frequent correlation in persistent apical periodontitis (Dahlen *et al*. 2000) [16]. Although, monospecie infection is unlikely to occur, *E. faecalis* biofilm is adopted model to investigate efficacy of antimicrobials [20].

Bacterial invasion into the dentinal tubules has a significant role in endodontic infections and subsequent root canal treatment failures. *E. faecalis* and *Streptococcus mutans* are commonly implicated species due to their ability to survive in harsh environments and their highly resistant nature to the antimicrobial agents [21,22]. E. faecalis is commonly isolated from failed root canals persisting as biofilm, more resistant to antimicrobials [23]. Demonstrating the depth of bacterial invasion to the DT is crucial for developing more effective antimicrobial disinfection strategies. Confocal laser scanning microscopy (CLSM) has considered a valuable analyzing tool for assessment of bacterial penetration into dentinal tubules due to its ability to produce three-dimensional imaging with high-resolution, in addition to Live/Dead microorganism differentiation.[10, 21, 22]

The current study was conducted in dentin sections inoculated with 3 weeks old *E. faecalis* biofilms as advocated by Li *et al*. 2015 [15], this incubation period was done due to the fact that antimicrobial resistance of microorganisms embedded in biofilms are 1000-fold higher than planktonic microorganisms. More specifically, 3-weeks old *E. faecalis* biofilms are more resistant than young *E. faecalis* biofilms and harder to eliminate [21].

Biofilms are composed of microorganisms embedded in self produced matrix adherent to dentine surface [21], therefore biofilms are more difficult to eradicate than planktonic [23,24]. Unfortunately, biofilms persists even after mechanical root canal preparation [24,25], Currently, sodium hypochlorite (NaOCl) is the most common irrigating solution, because it is inexpensive, readily available, tissue-dissolving, a broad-spectrum antimicrobial, the antimicrobial action leads to breakdown of amino acids and proteins by the release of free chlorine. It has the potential to dissolve the necrotic pulp tissue and organic debris and lubrication properties [9,10, 26]. However, it is highly reactive and therefore it can be rapidly inactivated by dentine organic matter preventing its diffusion and does not remove tissue remnants in all root canal spaces [27]. NaOCl have several drawbacks including toxicity, corrosive potentials, damage to clothes and significant injuries to skin, eyes, oral mucosa upon contact [28]. NaOCl accident is dreadful experience and life threatening due to swelling in floor of the mouth compromising airway [28,29]. Aranda-Garcia et al. invistigated the effect of NaOCl on infected ex-vivo root canals. They resulted in immediately after disinfection, the root canals were free of bacteria using paper point sampling. But, after days regrowth of the canals

by the previously bacteria had occurred. Such a study indicate the inability of NaOCl to complete eradication of biofilms from inaccessible areas [9].

Live/Dead CLSM fluorescence imaging is a highly sensitive technique in microorganisms viability determination[10,11,14]. CLSM analysis is being advantageous over the conventional fluorescence microscopy in terms of better imaging resolution, eliminating scattered light and displaying microrganisms in dentine.

Development of safe root canal disinfectant seems challenging, MSS is hypertonic salt solutions combining potassium sorbate and sodium chloride that can synergistically kill *E. faecalis* biofilm in vitro [30]. Both components, potassium sorbate and sodium chloride are considered to be generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) of the United States of America and therefore used safely for human [31]. Its efficacy should be investigated in dentine models, because root canal antimicrobials are less effective in vivo than in vitro due to dentine inhibition of microbial killing [32, 33].

The effectiveness of MSS in bacterial eradication is proved by the results of this study. The significant increase in bacterial cell death observed in the MSS-treated group aligns with previous study demonstrating its highly antimicrobial effect [34].

Moreover, as observed in this study, the results highlight MSS as a good alternative to traditional root canal irrigants. MSS offers a potentially less cytotoxic alternative with comparable antimicrobial efficacy.

The statistical analysis of this study validated the effectiveness of both MSS and NaOCl, with both demonstrating significantly higher bacterial kill rates compared to the control. (Fig 1-5) (table 1,2).

Several investigations using in vitro dentine models demonstrated promising effects of MSS, however literature lacks study on antimicrobial effect of MSS in root canals in an ex-vivo situations that mimic clinical conditions using CLSM, as it is instrumental in providing a clear distinction between viable and non-viable bacteria, offering a robust method for evaluating disinfection efficacy [14].

The results current study demonstrated that there was no statistical significant difference in antimicrobial properties between NaOCl and MSS, this result is aligned with van der Waal *et al*. 2015 that showed that MSS have is a promising antimicrobial with efficiency equal to CHX and NaOCl against microbial biofilm when used as irrigation in dentin powder. Among the antimicrobial properties of MSS [11], Almeida *et al*. 2016 demonstrated that MSS can detach high proportions of *E. faecalis* biofilms verified by supernatant CFU, Propidium monoazide treatment in conjunction with quantitative polymerase chain reaction beside it's high multi hurdle antimicrobial strategies by hypertonic nature and inactivating biofilms via simultaneous action of hyperosmolarity, weak acid, and sorbic acid stresses [35].

Our results were in agreement with those results in a study done by Pereira *et al*. 2021 that concluded that both a modified salt solution (RISA) and NaOCl appeared to affect alteration on the remaining biofilm that hamper further biofilm removal [36].

The substantial increase in bacterial cell death observed in MSS and NaOCl treated groups suggests that these disinfectants effectively penetrate and eliminate *E. faecalis*, a bacterium notorious for its resistance in endodontic infections.

CONCLUSION

Within the limitations of this study, the statistical findings reinforce the promising antibacterial efficacy of MSS and being comparable to NaOCl treatments, highlighting the importance of alternative antimicrobial approaches in endodontic therapy. Further studies are required to evaluate its long-term effects on dentin structure, prevention of regrowth of bacteria and biocompatibility effect to the periapical tissues. Moreover further studies are

required to explore its potential in combination with other antimicrobial agents to enhance endodontic disinfection protocols.

Confocal laser scanning microscopy has revolutionized the study of bacterial penetration into dentinal tubules by providing detailed visualization and quantification of bacterial viability.

Financial support:

Nil.

Conflicts of interest

There are no conflicts of interest.

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