Research Article

Tailoring of Sodium hypochlorite Lipid-free nanoemulsion: Ex-Vivo Comparative Study with Chlorhexidine HCl Nanoemulsion as an Antibacterial Root Canal Irrigant

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Abstract

Introduction and Aim: Sodium hypochlorite (NaOCl) has proven to be the chosen irrigator in modern practice due to its high tissue solution power, antibacterial, and lubricant properties. This study aimed to use a Lipid-Free Nanoemulsion (LF/NE) to improve the penetration efficiency, antibacterial effect, tissue dissolving, and cleansing ability of NaOCl. Moreover, masking NaOCl taste when used as a root canal irrigant.

Methods: NaOCl LF/NE were comprised of a mixture of surfactants (Tween20 and Tween 80) and cosolvents (Transcutol HP and propylene glycol). The desired size range for dispersed globules was achieved using an ultrasonicator. After that, it was left to equilibrate. To designate the best systems, pseudoternary phase diagrams were constructed. The drug content, dispersibility, droplet size, drug release, and antibacterial activity of the formulae were all evaluated. Ex-vivo study was performed for the selected formula. NaOCl LF/NE was compared with two different concentrations levels 0.75 and 1.6% versus 5.25% NaOCl normal particle size and prepared Chlorhexidine Hydrochloride (CHX.HCl) nanoemulsion (in previous work) for their penetration ability as a root canal irrigant, Antibacterial activity cleansing effect, and tissue dissolving were done.

Results: formula F3 with a composition of 33% Tween 80 and 33% Transcutol HP was selected. It had a small particle size (106 nm) and a maximum dissolution rate after 2 min. It was proven a thermodynamically physically stable system. The ex-vivo study results showed the deepest penetration with a mean depth of 75.71µm for 1.6% NaOCl LF/NE. The result was higher than the previous work of CHX.HCl as the mean for CHX.HCl nanoemulsion irrigant was 11.29µm.

Conclusion: NaOCl LF/NE had better ability in the cleansing of the root canals than both of NaOCl normal particle size and CHX.HCl with high efficacy as an antibacterial agent on Enterococcus faecalis.
Graphical abstract
Table 1: Compositions of Sodium hypochlorite LF/NE

<table>
<thead>
<tr>
<th>Formulae</th>
<th>Drug (1.5 %)</th>
<th>Surfactant (33%)</th>
<th>Cosolvent (33%)</th>
<th>Distilled water (32.5 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td></td>
<td>Tween 20</td>
<td>Transcuol HP</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>Sodium hypochlorite</td>
<td>Tween 20</td>
<td>Propylene glycol</td>
<td>Water</td>
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<tr>
<td>F3</td>
<td></td>
<td>Tween 80</td>
<td>Transcuol HP</td>
<td></td>
</tr>
<tr>
<td>F4</td>
<td></td>
<td>Tween 80</td>
<td>Propylene glycol</td>
<td></td>
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</table>

Introduction

Utilization of irrigation fluids that have an antibacterial action, mechanical tools, and irrigation help to prevent root canal bacterial infections [11]. The target of root canal treatment is to eliminate virulence factors from this system. [21]. This can be achieved by using mechanical tools combined with chemical methods [3]. For endodontics, sodium hypochlorite (NaOCl) is the most widely utilized irrigation solution [4]. One of NaOCl’s limitations is its inability to remove the smear layer [5]. Chlorhexidine hydrochloride is a broad-spectrum antibacterial agent with prolonged action and minimal toxicity. In contrast to NaOCl, the main advantages of CHX.HCl is its lacks foul odour and poor taste [6].

Lipid-free nanoemulsion (LF/NE) are composed of a mixture of surfactant and co-solvent forming micellar solution. The advantage of combining a surfactant and a co-solvent is that the surfactant has a significantly higher good solvent potential (as a micellar solution) than the co-solvent alone [7]. This study highlighted the significance of using nanotechnology to mask the distinct tastes and odours of sodium hypochlorite (NaOCl) and improve its activity as a root canal irrigant. Comparison of NaOCl LF/NE with two different surfactant levels 0.75 and 1.6% versus 5.25% NaOCl normal particle size and prepared in previous studies, the penetrating capability, antibacterial activity, cleaning effect, and tissue dissolving of chlorhexidine hydrochloride (CHX.HCl) nanoemulsion as a root canal irrigant were studied [6].

Material and Methods

Materials

Sodium hypochlorite (NaOCl), Propylene Glycol, Tween 80 (Polyoxyethylene (80), Tween 20 (Polyoxyethylene (20) Sorbitan Monolaurate), Sorbitan Monoleate) were provided by Gomhory Company, Cairo, Egypt. Transcutol HP and Labrasol (Caprylocaproyl Polyoxyl-8 Glycerides) were supplied as a gift by Gattefosse, Saint-Priest, France. Single-rooted noncarious human teeth were collected due to periodontal disorders. Oral and Maxillofacial Faculty, Faculty of Dentistry, Ain Shams University, Cairo, Egypt. E. Faecalis (strain ATCC29212) pure culture grown in brain heart infusion (BHI) broth (RC CLEANER, Ichung Dental Ltd., Seoul, Korea).

Methods

Pseudoternary phase diagram

Surfactants namely Tween 20, Tween 80, and Labrasol, co-surfactants like Transcutol and Propylene Glycol, and NaOCl are mixed. First, the NaOCl was combined with a surfactant and co-surfactant (S_max) mixture in the varying proportion of 1:1, 2:1, and 3:1. Then water was added dropwise during an aequous titration [8]. The results were plotted on the Golden Software Grapher program (Version 8.1.388) detect the emulsification regions

Preparation of LF/NE

Based on pseudoternary phase diagram results certain selected ratios of Tween20 and Tween 80, were mixed with Transutol HP and Propylene Glycol and sonicated (Ultrasonic Lc 60 H Sonicator, Elma, Germany) for LF/NE preparation. It is then left to equilibrate [9], Table (1).

Characterization of the Prepared LF/NE

Dispersibility Test

Disperibility tests were performed using the United States Pharmacopeia (USP) dissolution apparatus (Pharma Test, Hainburg, Germany). 1 mL of each formula was added to 500 mL of water at 37± 0.5°C. A standard stainless-steel dissolution paddle at 50 rpm was used to gently stir the mixture. Visual examination was done to determine the type of emulsion that developed. The formulation is classified as clear, translucent with a bluish tone, milky, or turbid. [10, 11].

Determination of Drug Content

3 mL of the formulation was diluted with 50 mL of water. 10 mL of 6 N acetic acid and 2 g of potassium iodide were added to the mixture. The liberated iodine was titrated with 0.1 N sodium thiosulfate VS, and 3 mL of starch TS was added near the endpoint. A blank analysis was carried out. Each1 mL of (0.1 N) sodium thiosulfate is equivalent to 3.722 mg of NaOCl [12].

Droplet Size Analysis

The average droplet size and polydispersity index for the prepared formulations were determined using the Malvern Zetasizer 2000 (Malvern Instruments Ltd., Malvern, UK). The measurements were carried out after dilution [13].

In-vitro Release Study

The United States Pharmacopeia (USP) apparatus type II (paddle) was used to perform the in-vitro dissolution test at 50 rpm (Pharma Test, Hainburg, Germany). Using 500 mL of distilled water at 37± 0.5°C as a dissolution media. 5 mL of the tested systems were added into the dissolution
medium at time 0. At various periods, 3 mL of dissolution media was withdrawn and replaced with fresh medium at the time intervals of 1, 2, 3, 4, 5, 6, 7 and 8 min. A UV spectrophotometer was used to measure the analysed aliquots at $\lambda_{\text{max}}$ 291.3 nm [14].

**Stability Studies of Prepared LF/NE**

**Drug Precipitation and Phase Separation**

Phase separation was checked after 48 hours of storage of a 2 mL sample of each formulation at room temperature. Additionally, each formula was diluted with distilled water to 10 mL and 100 mL and kept at 25°C for 24 hours before being tested for phase separation [15, 16].

**Refrigeration Thaw Cycle**

2 mL of each formula was kept at 2°C for 24 hours before being taken out and kept at 25°C and 40°C. The samples were then tested for drug precipitation and phase separation [15, 16].

**Centrifugation**

5 mL sample was centrifuged at 4,000 rpm for 5 minutes (Microcentrifuge Shanghai Surgical Instrument Factory, model 800, China). Phase separation and drug precipitation were then observed in the samples [15, 16].

**Ex-vivo Study of LF/NE Selected Formula.**

For root canal irrigation, NaOCl 0.75% (F3a) and NaOCl 1.6% (F3b) prepared in the best formula (F3) were utilized. All the tests were approved by the ethical committee at the Egyptian University Ain Shams.

**Evaluation of Irrigant Penetration Ability and Cleansing Effect:**

Thirty non-curious single-rooted human teeth with mature apices were used. Before using the extracted teeth, Patients signed consent letters of informed permission. The exterior root surfaces were debrided using a curette. The surfaces of the teeth were then disinfected with 0.5% NaOCl for 24 hours before being preserved in normal saline until they were needed. The crowns were extracted, and the tooth length was 16 mm from the root apex to the coronal edge [17]. Depending on the irrigation solution, the teeth were divided into three groups:

Group (A) [n=24] F3 which divided into two subgroups:

(I) [n=6] 5mL (F3a).

(II) [n=6] 5mL (F3b).

Group (B) [n=12] NaOCl normal particle size which divided into two subgroups:

Subgroup (I) [n=6] 5mL 2.5% NaOCl normal particle size.

Subgroup (II) [n=6] 5mL 5.25% NaOCl normal particle size.

Each subgroup was divided according to the method of activation of the irrigant

• Passive ultrasonic irrigation (PUI) [n=3] (Satelic P5, France, Irrigation tip size 25). According to the previous study on CHX, the optimum activation method was PUI [6] so, it was used in this study.

• No activation [n=3].

• Control groups [n=6]:

  • Positive control: [n=3] 5mL 5.25% NaOCl normal particle size (No activation).

  • Negative control: [n=3] 5 mL saline (No activation).

Mechanical preparation was performed to a master apical file size using a standardized methodology. All samples were longitudinally sectioned into two halves using a hammer and chisels [18, 19]. Then, all samples were analysed using a scanning electron microscope (SEM)

**Study of Antibacterial Effect:**

A total of 60 single-rooted, non-carious human teeth with developed apices were chosen. The upper and lower incisors and mandibular premolars were included in the teeth. The teeth were treated the same as the pervious test [17, 20]. Using a standardized technique, a master apical file size of 45 was mechanically prepared. The canals were washed for one minute with 2mL of 17% EDTA to eliminate the smear layer. Each sample's whole root surface, including the apical foramen, received two layers of nail paint. To facilitate handling and labelling, the teeth were next positioned vertically in dental stone blocks [20-22]. Samples were then autoclaved for 20 minutes at 121°C and 15lb pressure. Samples were transferred and processed using sterile instruments under aseptic conditions. Using a pure E. faecalis culture, root canals were infected. All teeth prepared canals were treated with an inoculum of pure E. faecalis suspension. Then, the blocks were placed inside sterile beakers and incubated for 24 hours at 37°C [22-24]. The teeth were divided into the following groups according to the irrigation solution:

Group (A) [n=24] F3.

Subgroup (I) [n=12] 5mL of the prepared (F3a).

Subgroup (II) [n=12] 5mL the prepared (F3b).

Group (B) [n=24] NaOCl irrigation solution of normal particle size.

Subgroup (I) [n=12] 5mL 2.5% NaOCl irrigation solution of normal particle size.

Subgroup (II) [n=12] the samples were with 5mL 5.25% irrigation solution of normal particle size.

Each subgroup was divided according to the method of activation of the irrigant:

Passive ultrasonic irrigation (PUI) [n=6] (Satelic P5, France, Irrigation tip size 25).

No activation [n=6].

Group (C) [12]:

Positive Control: [n=6] the contaminated root canals were irrigated with 5mL of normal saline.
Negative Control: \[n=6\] suspension injections were not given to the specimens, that is, the root canals were not contaminated and kept sterile as a negative control to ensure sterility and reliability of procedures. Each sample contained 5mL of test irrigator. 1mL of sterile normal saline was then used to irrigate each sample.

Evaluation of Bacterial Growth:

Using sterile paper with a point size of 35, root canal samples were obtained. After inserting the paper points into the canals until they reached the working length, they were kept in situ for 10 seconds. The colony-forming units (CFU) per plate were then counted after being transferred to agar plates. The plates were examined visually for bacterial growth after a 24-hour incubation period at 37°C. Clear plates are sterilized. Plates that were blurred were considered positive growth. For each plate, the average number of CFUs was counted, and the total number of CFUs was calculated. Pour plates and diluted ones were both utilized to count high CFUs and low CFUs, respectively [25].

Viable Count

The sample is applied to a melted agar medium before it solidifies. Colonies are evenly distributed across the medium after the desired sample dilution is plated. Serial dilutions will be needed if the sample contains more cells than the agar plate can hold, with a statistically significant range of 30 to 300 CFU. On the day of the experiment, the agar medium was autoclaved, and a tube containing 15 mL of melted agar material was prepared. E. faecalis ATCC29212 bacteria were used in the sample, which was made by mixing colony cells with sterile saline. After that, a 10 µL bacterial sample was diluted in saline to McFarland 0.5, which equals 108 CFU/mL. A 100-mL graduated cylinder was filled to the top with 1% sulfuric acid and 0.6 mL of a 1% solution of barium chloride dihydrate to create a turbidity standard. The sample was poured into the middle of the empty Petri dish once the lid was opened. Once the agar had fully solidified, the plate was inverted and left to incubate for 24 hours at 37 °C [26].

Statistical Methods

The statistical analysis was performed using IBM® SPSS® Statistical Version 17 for Windows [IBM Corporation, NY, USA, SPSS, Inc., an IBM Company].

Results

Preparation of LF/NE formulae

Trials were made to mix Labrasol with different ratios with Tween 80 and Tween 20 with co-surfactants, but all formulas produce a milky appearance during the water titration test, so a formula that contains Labrasol were excluded from further evaluation. The other systems were plotted on the Grapher program (Version 8.1.388) to determine the emulsification regions. Formulae in this study were selected from high emulsification region on pseudoternary phase diagram. This may be attributed to that NaOCl is miscible with the used tested vehicles (Fig.1).

Characterization of LF/NE formulae

Dispersibility Test

All the prepared formulae that appeared to be clear were included for further evaluation research.

Drug Content

The prepared formulae showed drug content ranged between 94.84-99.69% (Table 2).

Droplet Size Analysis

The droplet size of the prepared formulae F1, F2, F3, and F4 were 693±94.5, 719±97.6, 106±1.7, and 205±12.5 nm respectively, with polydispersity index 0.34, 0.81, 0.39, and 0.23. (Fig. 2)

<table>
<thead>
<tr>
<th>Formulae</th>
<th>Drug content (Mean±SD) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>98.38±0.31</td>
</tr>
<tr>
<td>F2</td>
<td>94.84±0.86</td>
</tr>
<tr>
<td>F3</td>
<td>99.69±0.21</td>
</tr>
<tr>
<td>F4</td>
<td>98.51±0.78</td>
</tr>
</tbody>
</table>

Table 2: Drug content of prepared Sodium hypochlorite LF/NE
In-vitro Release Study

The percentage of NaOCl released from the formulae showed in (Fig.2). The complete drug release for prepared NaOCl formulae ranged between 2 to 8 minutes. It was observed the formula F3 showed a higher dissolution rate than other formulas.

Stability Studies

All formulations were put through intensive thermodynamic testing using centrifugation, freezing, and thawing cycles. F3 and F4 passed the thermodynamic stability tests with no signs of phase separation. F1 and F2, which contain Tween 20 and the co-surfactants propylene glycol and Transcutol HP, respectively, showed gelling appearances (Fig.3)

Ex-vivo Study of LF/NE Selected Formula.

The concentration of the components under investigation was standardized at the beginning of the study to resemble that of a commercially available irrigant. The viscosity of the LF/NE (F3) was too high to use for irrigation. Serial dilutions were performed until the highest concentration that could be used for irrigation was reached. F3b was diluted to 1.6% w/v to give the higher concentration and F3a was diluted to 0.75% w/v giving the lower concentration. Reducing the concentration is better for biocompatibility and decreasing the cytotoxicity to the apical and periodontal tissues.

There was a statistically significant (P < 0.001) difference between the mean depths of irrigant penetration in DT at different irrigant concentrations. F3b was statistically highly significant (P < 0.001) recording the deepest penetration with a mean depth of 75.71µm and SD 0.007. NaOCl normal particle size 5.25% and F3a were statistically (P > 0.05) non-significant, which means that the higher concentration of F3b is more effective. The result was higher than the previous work of CHX as the mean for CHX nanoemulsion irrigant was 11.29µm. the statistical analysis was significantly (P > 0.05) different with a P-value of 0.23576. (Fig.4).

The effect of LF/NE irrigating solutions on E. faecalis was investigated by incubating the bacteria in tooth specimens and then calculating Colony Forming Units (CFU). It was observed that conventional irrigants into DT, they are unable to entirely remove E. faecalis. While, The tissue dissolving and debris removal of LF/NE irrigants were much more than the normal particle size irrigants (Table 3).

Discussion

NaOCl is miscible with all tested vehicles except Labrasol. All formulae that contain Labrasol in different percentages give a milky appearance this may be due to the
immiscibility of Labrasol with NaOCl. The ratio of surfactant and cosurfactant of more than 3:1 was not tested to avoid increasing the viscosity of preparation to ease its rheology during dental irrigation. Also, It was noted that formulae F3 and F4 have smaller particle sizes than other formulae. This could be due to the presence of Tween80, which has better emulsifying and solubilizing properties for sodium hypochlorite due to its high HLP value [26, 27]. LF/NE do not contain natural lipids and represent the most hydrophilic formulations. These types produce very fine dispersions and have greater drug payloads. This may be due to the increased drug solubility in the co-solvent and surfactants. As a result, the drug showed greater absorption and is released more quickly [28]. Formula F3 which has the smallest particle size (106 nm) showed a higher dissolution rate than other formulas, indicating that particle size and hence surface area of drug particles have a greater impact on dissolution rate. This is due to the fact that reducing the size below 1µm increases the solvation pressure, enhancing solubility and causing the solute-solute interaction to be disrupted, which greatly facilitates the solubilisation process [29, 30]. The prepared formulation must maintain its stability over various temperatures and maintain its ability to solubilize even when diluted [31]. Thermodynamic stability and phase separation studies were carried out to test formulae's ability to withstand stressful conditions. Formulae containing Tween 20 showed gelling appearance which may be a sign that they are unstable due to droplet aggregation which confirm the high capacity of Tween 80 as stabilizer to decrease surface free energy of droplets and prevents its aggregation in formulae F3 and F4 [32, 33].

F3 was chosen as the best formula since it has the smallest droplet size (106 nm), a rapid dissolution rate after 2 minutes, as well as being thermodynamically/physically stable system.

Ex-vivo Study was designed to compare the antibacterial performance of the new irrigation F3 to commercially available irrigation of normal-size NaOCl as an initial study in the evaluation of a new endodontic irrigant. The microorganism studied in this investigation was E. faecalis. E. faecalis was selected because it is one of the most frequently isolated species in persistent root canal infections. Additionally, E. faecalis may survive in treated root canals for a very long period with harsh conditions and a lack of nutrition. Extremely high and low pH values,..
as well as extremely high temperatures, are not barriers to E. faecalis survival [2, 34].

The effect of LF/NE irrigating solutions on E. faecalis was investigated by incubating the bacteria in tooth specimens and then calculating Colony Forming Units (CFU). Because of the low penetration depth of conventional irrigants into DT, they are unable to entirely remove E. faecalis [33].

NaOCl's taste and odour are two of its most unpleasant drawbacks. The use of the micellar solution of the reagent masked the disgusting taste and odour [36]. LF/NE of NaOCl is a tasteless and odourless clear irrigating solution. It was found that the higher concentration of nanosized delivery formulations (F3a and F3b) had a deeper penetration ability in DT due to the smaller particle size. The tissue dissolving and debris removal of LF/NE irrigants were much more than the normal particle size irrigants. F3b was statistically highly significant recording the deepest penetration and it was higher than the previous work of CHX. (Fig. 4).

When NaOCl was exposed to sunlight, the chlorine content decreased quickly. Heated NaOCl lost around 5% of its potency in just six hours [37]. The ultrasonically activated irrigant facilitates root canal cleaning and disinfection. The efficacy of ultrasonic instrumentation is due to the interaction of ultrasonic energy and the irrigating solution. Transient cavitation occurs when ultrasonic energy creates a bubble that expands to a certain size before the collapse. This collapse creates a vacuum pressure that kills microorganisms and cleans channel irregularities. When using conventional techniques like irrigation alone, such as on rough or difficult-to-reach surfaces, the cavitation implosion works successfully [38]. When F3b was activated by PUI, complete bacterial elimination was achieved and the deepest penetration and better cleansing effect was found when PUI was used.

**Conclusion**

The root canal cleaning performance of NaOCl LF/NE was superior to NaOCl normal particle size, and it was also more effective as an antibacterial agent. CHX nanoemulsion was an effective antibacterial agent but its cleansing ability was not as effective as the NaOCl LF/NE. We recommend that NaOCl LF/NE is a promising irrigant that should replace the currently used irrigants.

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**Conflict of Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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