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Effect of Various Disinfection Methods on Water Sorption and Solubility of Heat-Cure Acrylic Resin with and without Resilient Liner (*In-vitro* Study)

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ABSTRACT

Background: Resilient denture liners are beneficial to edentulous patients with severely resorbed ridges. Although disinfection of relined and non-relined acrylic resin dentures was highly recommended for advancing oral health status, these techniques may affect denture materials' physical criteria. **Objective:** To evaluate the impact of various disinfection methods on the water sorption and solubility of relined and non-relined heat-cure acrylic resin dentures. Material and Methods: 60 heat-cure samples were constructed, then randomly sectioned to two main groups; Group I (heat-cure acrylic resin) and Group II (relined heat-cure acrylic resin). Either group was sectioned to three equal subgroups (1 Control (Distilled water) and 2 disinfection groups; Sodium hypochlorite (NaOCl) and Chlorhexidine mouthwash). All specimen were daily disinfected for 30 days, washed with tap water then stored in distilled water at 37°C. Water sorption and solubility's degree were calibrated by the electronic balance and calculated with a specific formula. Data were collected, tabulated, and statistically analyzed. Results: Water sorption between Group I and different solutions showed significant difference; the lowest significantly in Control, while NaOCl and Chlorhexidine had insignificant difference. In Group II, water sorption significantly was the lowest in Control and the highest in Chlorhexidine, while NaOCl had insignificant differences with other solutions. Regarding water solubility relined and non-relined samples demonstrated insignificant differences between them as P=0.85, 0.68, and 0.13 regarding Control, NaOCl, and Chlorhexidine respectively. Conclusion: Plain acrylic resin samples demonstrated the lowest water sorption in Control, while displayed was an insignificant difference after immersion in NaOCl and Chlorhexidine. Concerning water solubility, an insignificant difference was apparent between the plain and relined samples in all immersions employed.

Keywords: Heat-cure acrylic resin, Resilient liner, Disinfection, Water sorption, Water solubility.

1. Introduction

The denture base is demarcated as the denture's fragment leaning on the underlying areas, holding teeth, transferring the dense occlusal forces to the supporting structures, recompensating the absent dentition and the accompanying alveolar tissues in both maxilla and mandible. Heat-cure acrylic resin is the utmost considerably employed form of non-metallic denture base materials for its insolubility, convenience, affordability, non-toxicity, ease of process, and repair together with its reasonable esthetics (Gupta and Mansi, 2012; Gujjari *et al.*, 2011).

Nevertheless, it also demonstrates few demerits such as the grand residual monomer content triggering tissue hypersensitivity and dimensional instability as a consequence of water sorption and solubility besides the polymerization shrinkage, thus manipulating its durability and inducing rough surface anomalies. Moreover, that blend of salivary glycoprotein and immunoglobulins labeled as

'pellicle' which coats the entire denture's surfaces acts as a substrate for attachment of debris and microorganisms, hence affording an appropriate habitat for cultivation of fungal, microbial, and bacterial pathogens which is remarkably advanced in its rough surfaces (Kutkut *et al.*, 2018, Zaki *et al.*, 2023).

Additionally, the habitually occurring modifications accompanying poly-methyl methacrylate (PMMA) dentures as advanced bone loss are a critical drawback. Accordingly, the patient experiences a lack of retention, support, and stability inducing pain, soreness, and inflammations, especially in cases where excessive denture pressure is applied on thin, sharp, excessively resorbed ridges, massive bony undercuts, and acquired or congenital palatal abnormalities ending-up with functionless denture (Zaki *et al.*, 2023; Malika *et al.*, 2021).

The resilient denture liner is advantageous and considered one of the modalities used for treating such symptoms via coating the prosthesis' mucosal surface, hence it is vastly mandatory. Its merits include; even distribution of the biting load, wide dispersion of the chewing forces, providing proper balanced occlusion, absorbing prosthesis' extra pressure, and relieving all the underlying tissues from the grand mechanical stress. Sequentially, the pain is alleviated, the patient's adaptation to the prosthesis is simplified, the denture's required modifications are diminished and the patient's quality of life is remarkably enhanced (Hamzah and Luma, 2023; Ewa *et al.*, 2021).

Apart from the soft liner's benefits, it bargains a few shortcomings clinically such as; color alterations, lack of resilience induced by the plasticizer leakage as well as, surface roughness and porosities as a consequence of water sorption and solubility together with deviations in the material's features. Consequently, distortion takes place stimulating stress at the denture liner bond leading to failure of their attachment, together with creating a good atmosphere for bacterial and fungal pathogens leading to inflammations and engorgement (Sudhapalli, 2016; Hussein, 2015).

Therefore, one of the approaches employed for counteracting these problems is chemical cleaning via utilizing commercially accessible solutions and household ones such as sodium hypochlorite. Furthermore, oxygen exposure, and denture cleansers which are applicable in versatile forms are extra chemical cleaning methods. Dentures and their liners not only encounter modifications from the oral atmosphere but are also influenced by the employed hygiene practices. This occurs by absorbing their remaining solvable constituents from the surrounding medium and internally diffusing the liquid available in their area, thus occupying the spaces and voids existing within their polymetric chain (Malika *et al.*, 2021; Hamzah and Luma, 2023; Mustafa and Zeynep 2014).

Water sorption and solubility have a remarkable impact on both the denture base material and the soft liner's clinical success. When placed in an aqueous atmosphere such as the versatile denture cleaning solutions, they absorb relatively few amounts of water which applies notable outcomes on the mechanical, chemical, and dimensional criteria of both the PMMA dentures and their liners, as well as leaching out some of their unreacted monomer which is nearly insoluble in the oral fluids (Porwal *et al.*, 2017; Mohamed *et al.*, 2016). Hence, this contemplate aimed to evaluate the impact of various disinfection methods on the water sorption and solubility of heat-cure acrylic resin denture base with and without soft lining material.

2. Materials and Methods

2.1. Materials

- 1. Conventional heat-cure acrylic resin (Acrostone; Acrostone dental factory industrial zone -Salam city A.R.E-WHW Plastic, England).
- 2. Acrylic resin-based auto-polymerized soft liner (Acrostone; Acrostone dental factory industrial zone -Salam city A.R.E-WHW Plastic, England).
- 3. Separating medium (Acrostone; Acrostone dental factory industrial zone -Salam city A.R.E-WHW Plastic, England).
- 4. Dental plaster (Type 3 model dental stone; Elite Rock Stone- Zhermack Clinical, Italy).
- 5. Denture adhesive (COREGA; Adhesive Ultra Corega Cream; (gsk) GlaxoSmithKline Brazil Ltda., BRAZIL; CA).
- 6. Silica gel, static dehumidification, SUD-SHEMIE®.
- 7. Distilled water (Aqua Chemicals Egypt, Dest Al-Ashraf Kom Hamada, Beheira Governorate, Egypt).

- 8. Chlorohexidine mouthwash (Liquid Orovex Mouthwash-Clove-Antiseptic chlorhexidine mouthwash; Macro Group Pharmaceutical S.A.E, Macro Capital, Egypt).
- 9. Sodium hypochlorite (Clorex; Household Cleaning Products Company of Egypt, 14 G Ahmed Kamel, Al Lasilki, Maadi, Cairo Governorate, Egypt).

2.2. Methods

2.2.1. Study Design

Such in-vitro research was applied in the Fixed and Removable Prosthodontics Department, Oral and Dental Research Institute, National Research Centre, Cairo, Egypt. This work initiated by exposing both plain and soft-relined heat-cure acrylic resin specimen to three dissimilar methods of acrylic resin denture disinfection; Distilled water, Chlorhexidine mouthwash, and 2% Sodium hypochlorite (NaOCl). Water sorption and solubility were assessed for all specimen via employing the Electronic balance and determined by aid of specific formula after their daily disinfection accompanied by their storage at room temperature for 30 days in distilled water.

2.2.2. Specimen Grouping

An overall number of 60 heat-cure acrylic samples were generally fabricated and similarly grouped owing to the samples' form into two prime Groups (30 samples/Group); Group I (Plain heat-cure acrylic resin) and Group II (Relined heat-cure acrylic resin). Each group was further segmented to three identical sub-groups (10 samples/Sub-Group) owing to the type of disinfection method employed into; Sub-Group A (Control) (Distilled Water), Sub-Group B (Sodium Hypochlorite), and Sub-Group C (Chlorhexidine Mouthwash).

2.2.3. Sample Size Calculation

The sample size was measured counting on preceding studies as references (Hamzah and Luma, 2023; Dehis *et al.*, 2018).

Hence, the passable sample size in each test was 10 specimen /sub-group with an effect size of 1.35, to be 80% certain that a two-sided 95% confidence interval's limits will eliminate a difference in means of more than 1.5.

2.2.4. Metal Pattern Preparation

The disk-shaped metal pattern with dimensions of (50 mm. diameter x 0.05 mm. thickness) was fabricated rendering to ADA specification NO.12 (Mahmoud *et al.*, 2023).

2.2.5. Mold Preparation

The conventional metal flask was employed to get molds for the heat-cure acrylic resin disks for both sub-groups (A) and (B). The flask's inferior section was occupied with dental plaster which was assorted rendering to the manufacturer's guidelines (50 ml \ 100 gm). The metal pattern was coated with the separating medium, followed with a layer of plaster mix. Consecutive to the plaster establishment (30 min.), a separating medium enclosed both the plaster and metal patterns then the flask's superior section was occupied with an auxiliary plaster film while vibration thru a mold vibrator. The plaster was left to strengthen for (60 min.), then finally the flask was unfastened, the metal pattern was detached, and the mold was gained.

2.2.6. Specimen Fabrication

The molds utilized for construction of the heat-cure acrylic resin specimen for both sub-groups (A) and (B). The heat-cure acrylic resin was assorted and compressed subsequent to the manufacturer's references in a dry glass jar utilizing the stainless-steel spatula. When the dough phase was achieved, the resin was assembled into its space within the plaster mold created. Then, the hydraulic press aided in compressing the flask gradually for 5 min. to ensure an even flow of the acrylic resin. The flask was placed into the water bath curing unit (Water bath curing unit; Type 5518, KaVo EWL, Biberach, Germany.) for 30 min. at 70°C and extended for an extra 30 min. at 100°C for heat curing (Machado *et al.*, 2009). Afterward, flask segregation from the water bath took place and was dropped out to chill at 37°C previous deflasking, then finishing and polishing for all the acrylic resin disks employed in all groups, as displayed in Fig. (1).



Fig. 1: The heat-cure acrylic resin specimen.

2.2.7. Soft Liner Application to Group (II) Prepared Specimen

The auto-polymerized acrylic soft-liner disks were fabricated following their heat-cure acrylic resin specimen's curing, finishing, and polishing in **Group (II)**. The soft-liner was mixed in a dry glass jar utilizing the stainless-steel spatula, then once the dough phase was achieved it was assembled in the flasks employing the same heat-cure resin specimen's mold to accomplish a controlled soft-liner thickness. Sequentially, flasks were placed under pressure for 15 min. then recovered and fabrication took place succeeding the manufacturer's instruction. The attained soft-liner disks were applied to the prepared samples and appropriately attached via denture adhesive, together with proper border finishing by cutting away any excess with a sharp blade (Qanber and Hamad, 2021) ending up with the soft-relined heat-cure acrylic resin specimen, as displayed in Fig. (2).



Fig. 2: Heat-cure specimen relined with resilient liner.

2.2.8. Inclusion and Exclusion Criteria

Sample selection from both the plain and relined heat-cure acrylic resin samples employed in the current research was limited by some inclusion criteria. Such standards embrace; proper fabrication owing to the manufacturer's strategies in each specific step, rid of any cavities, crashes, bubbles, or impurities together with being appropriately smoothed, finished, and polished. All samples should have been perfectly fabricated subsequent to the metal pattern's required dimensions stated by ADA specification no.12. If any further conditions were available other than these in all specimen, then they were promptly omitted from this investigation.

2.2.8. Disinfection Methods of the Samples

- 1. Sub-Group A (Control): The samples were drenched in distilled water for 10 min. /day for 90 days.
- 2. **Sub-Group B (Sodium Hypochlorite)**: The specimen were soaked for 10 min./ day in a 2% NaO Cl solution for 90 days (Hussein *et al.*,2009).
- 3. Sub-Group C (Chlorhexidine Mouthwash): The specimen were disinfected in a 2% Orovex solution for 10 min./ day for 90 days (Hussein *et al.*,2009).

2.3. Procedure

2.3.1. Assessment of Water Sorption

Water Sorption estimation was consequent to effectual specimen's grinding via an abrasive paper and then flooded with water to ensure even, smooth, and parallel surfaces. Subsequently, specimen were all dried in a desiccator comprising Silica gel (Silica gel; static dehumidification, SUD-SHEMIE®), at 37°C for 24 hours. All disks were sited in an identical desiccator at 37°C for 1 hour, after which evaluated to an accuracy of 0.0001 g. employing an Electronic balance (Electronic balance; Mettler Toledo PM 460, Switzerland). This cycle was periodic with all specimen till achieving a constant weight that is supposed to be as each specimen's initial weight (W1) and took place prior to immersion of all specimen in their disinfectants owing to their groups by utilizing the Electronic balance, as displayed in figure (3).



Fig. 3: Delicate Electronic Weighing Balance.

2.3.2. Specimen Immersion and Disinfection

Throughout the research all the samples were disinfected, then immediately cleaned by means of running tap water and sequentially deposited in distilled water at $37 \pm 2^{\circ}$ C to preserve standardization and avoid temperature discrepancies till assessment. In the 3 months immersion period simulation was accomplished for physical investigation, one hour was counted to signify 6 immersions of 10 min., thus every 24 h resembled to 144 immersions. Hence, to accomplish the interval (90 days), 15 hours were essential. The specimen were estimated formerly and afterwards this immersion protocol.

2.3.3. Specimen Weighing

Accordingly, tweezers aided in detaching all the disks from the water, then with a clean dry hand towel they all were rubbed properly till no further noticeable moisture is realized, fluttered for 15 sec.in air and then weighed post their exclusion from the water by 1min. Their weight was frequently checked till approaching a persistent mass and the weight loss of each disk's weight loss did not exceed 0.5mg in every 24-hour period demonstrating the saturated water which counts as the specimen's gained weight (W2). Every specimen's water sorption was evaluated in (mg/cm2) thru a specific formula: Solubility $\% = (W1 - W3)/W1 \times 100$).

2.3.4. Assessment of Water Solubility

The water Solubility test took place by using the same desiccation technique previously defined until reaching a constant terminal weight (W3). The water solubility of each specimen was then calibrated utilizing according to the ISO standards 1567:1999. Utilizing the next formula:

Solubility $\% = (W1 - W3)/W1 \times 100)$.

W1 = The initial weight

W2 = The weight after absorption

W3 = The final weight after desiccation.

(ug/mm3) sorption = W2-W3/V Sample

(ug/mm3) solubility = W1-W3/V Sample

V Sample = $20 \text{ mm} \times 20 \text{ mm} \times 2 \text{ mm} = 800 \text{ mm}$ 3

2.4. Statistical Analysis

Statistical analysis was performed with SPSS 16 $\mbox{\ensuremath{\mathbb{R}}}$ (Statistical Package for Scientific Studies), Graph pad prism and windows excel and offered in 2 tables and 2 graphs. Investigation of the granted data was achieved utilizing Shapiro-Wilk test and Kolmogorov-Smirnov test for normality which discovered that data originated from normal data distribution. Consequently, comparison between 2 different groups was accomplished by Independent T- test, while comparison between 4 different subgroups was executed by means of One-Way ANOVA test trailed by Tukey's Post Hoc test for numerous comparisons. The significance level was set at P ≤ 0.05 .

3. Results

3.1. Evaluation of Water Sorption

Comparison between heat-cure and soft-relined heat-cure samples demonstrated insignificant difference between them as P=0.44, 0.36, and 0.41 regarding distilled water, Sodium hypochlorite, and Chlorhexidine mouthwash respectively, as accessible in Table (1) and Figure (4).

Comparison among different immersion solutions showed that there was a significant difference through different solutions in both groups as P=0.004, and 0.03 regarding heat-cure and soft-relined heat-cure. In heat-cure group, immersion in distilled water (6.4 ± 4.4) Significantly demonstrated the lowest water sorption, while there was insignificant difference in water sorption after immersion in sodium hypochlorite (14.47 ± 7.05) and Chlorhexidine mouthwash (14.9 ± 5.77). In soft-relined heat-cure group, immersion in distilled water (7.75 ± 3.14) Significantly revealed the lowest water sorption, while immersion in Chlorhexidine mouth wash significantly revealed the highest water sorption, while immersion in sodium hypochlorite (11.96 ± 4.72) demonstrated insignificant difference with other immersion solutions.

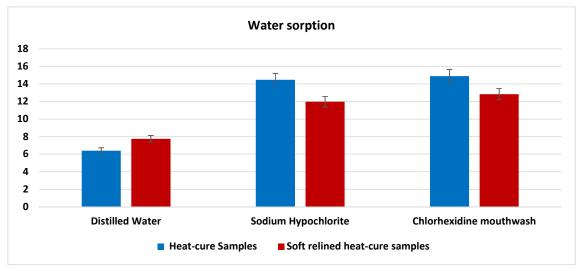


Fig. 4: Bar chart illustrating water sorption in both groups at different immersion solutions.

Evaluation of Water Solubility

Comparison between heat-cure and soft-relined heat-cure samples demonstrated an insignificant difference among them as P=0.85, 0.68, and 0.13 regarding distilled water, Sodium hypochlorite, and Chlorhexidine mouthwash respectively, as accessible in Table (2) and Figure (5).

Comparison among different immersion solutions showed that there was an insignificant difference through different solutions in both groups as P=0.62, and 0.53 regarding heat-cure and soft-relined heat-cure.

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Table 1: Mean and standard deviation of water sorption in heat-cure and soft-relined heat-cure samples after immersion in distilled water, Sodium hypochlorite, and Chlorhexidine mouthwash.

Sorption	Heat-cure samples		Soft relined heat-cure samples		Mean Difference	Std. Error	95% Confidence Interval of the Difference		P- value
	Mean	Standard Deviation	Mean	Standard Deviation		Difference	Lower	Upper	
Distilled Water	6.4 a	4.40	7.75 a	3.14	-1.35	1.71	-4.94	2.24	0.44
Sodium Hypochlorite	14.475 b	7.05	11.96 ab	4.72	2.52	2.68	-3.12	8.16	0.36
Chlorhexidine Mouthwash	14.9 b	5.77	12.825 b	5.21	2.08	2.46	-3.09	7.24	0.41
P-value	0.004*		0.03*						

*Significant difference as P<0.05.

Means with different superscript letters were significantly different as P<0.05.

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

Table 2: Mean and standard deviation of water solubility in heat-cure and soft-relined heat-cure samples after immersion in distilled water, Sodium hypochlorite, and Chlorhexidine mouthwash.

Solubility	Heat-cure samples		Soft relined heat-cure samples		Mean	Std. Error	95% Confidence Interval of the Difference		P- value
	Mean	Standard Deviation	Mean	Standard Deviation	Difference	Difference	Lower	Upper	_
Distilled Water	0.000120	0.000086	0.000107	0.00021	0.000013	0.00007	-0.00016	0.00013	0.85
Sodium Hypochlorite	0.000098	0.000036	0.000063	0.00027	0.000035	0.00086	-0.00024	0.00014	0.68
Chlorhexidine Mouthwash	0.000099	0.000034	0.000160	0.00012	0.000061	0.00039	-2.18	0.00014	0.13
P- value	0.62		0.53						

*Significant difference as P<0.05.

Means with different superscript letters were significantly different as P<0.05.

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

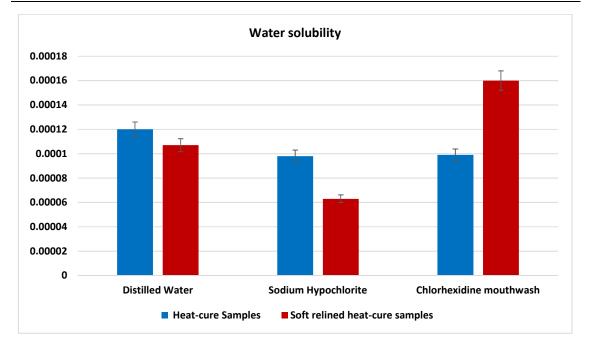


Fig. 5: Bar chart symbolizing water solubility in both groups at different immersion solutions.

4. Discussion

Acrylic resin bases are typically used in whole or partial dental prostheses to improve prosthesis support and maintain the artificial teeth in place and it provides both functional and aesthetic rehabilitation. Since acrylic resin is inexpensive, can be lined, looks attractive, and has a low density, it's considered as a good base material (Ahmed *et al.*, 2024).

Resilient liners improve denture adaptation, but their porosity increases the risk of microbial colonization and thereby tissue irritation. Resilient liners are easily subjected to breakdown for many reasons, such as staining, water absorption, loss of smooth texture, bacterial growth, and poor adhesion among the liner and the resin denture base (Dutta *et al.*,2023).

Acrylic-based resilient liners usually consist of polymers and monomers. The polymer is methacrylate polymers and copolymers, and monomer liquid comprising methacrylate monomer and plasticizers (ethyl alcohol and/or phthalate). These resilient liners absorb water through leaching out of plasticizers and other soluble substances into the water once immersed in it. Plasticizer's loss, not only accountable for material's softness, but also loses some of its initial softness (Chauhan *et al.*, 2021).

Owing to the resin molecules polar characteristics, acrylic resins captivate water gradually through time. Water absorption makes acrylic resin to be softer since the water can function as an acrylate plasticizer and weaken it. Resin polarity (which is defined by polar sites concentration) forms hydrogenwater bonds and controls the extent of water intake into polymer's webs. It also influences network structure (Narayanan, 2019).

The resilient liners degrade when they are submerged in cleaning or aqueous solution gradually (Palasuk *et al.*, 2019). Two processes are going on at once in these circumstances: the material may absorb water or cleaning solution, and plasticizers or other ingredients can leak out. Given their negative effects on the material's physical properties. In the oral environment, sorption and solubility must be calculated over time of usage (Kumar *et al.*, 2015).

As a result, huge water sorption may lead to swelling and stress at the liner/denture base boundary, enlarged distortion, diminished bonding, and augmented bacterial development through the liner and denture base. Sequentially, soft liners turn into more rigid progressively. Dibutyl phthalate plasticizer provides the flexibility of the resilient liner, but plasticizers may "leach out" of soft liners when they are embedded in an aqueous medium (Garg and Shenoy, 2016).

This study showed that a comparison between heat-cure acrylic resin with and without resilient liners demonstrated insignificant differences between them as P=0.44, 0.36, and 0.41 regarding distilled water, Sodium hypochlorite, and Chlorhexidine mouthwash respectively.

The water sorption and solubility were calculated rendering to ISO 1567: 1999. This specification states that water sorption of heat-cure acrylic resin cannot be greater than 32 μ g/mm3. The proportion at which the materials absorbed water or vanished their soluble compositions altered substantially with the material's nature, the plasticizer or filler's expanse, and their immersing solution. In the case of heat-cure materials, the mass loss per unit volume (soluble material) must not surpass 1.6 μ g/mm3, while for self-cured materials, it must not surpass 8.0 μ g/mm3 (Saini *et al.*, 2016, Council of Dental Materials and Devices, 1975).

The current contemplate disclosed the least sorption value (7.75 μ g/mm3) for relined heat-cure acrylic resin in distilled water, while the highest sorption value for heat-cure acrylic resin in Chlorhexidine mouthwash (14.9 μ g/mm3) and sodium hypochlorite (14.475 μ g/mm3) which were within clinically acceptable limits.

Rendering to earlier studies (Hussein, 2015, Saini *et al.*, 2016), the water sorption of dissimilar forms of acrylates is 10-25 μ g/mm3. In this research, the water sorption values were in harmony with those of others (Palasuk *et al.*, 2019). In comparison to distilled water, the value of denture cleanser sorption by heat-cure was much lower than relined heat-cure samples.

The plasticizer's inclusion, as in acrylic materials, affects the tested materials' compliance. Therefore, a perfect substance would have a minimal absorption rate and free from water or saliva soluble contents (Narayanan, 2019).

When distilled water was used instead of artificial saliva and disinfection solution, it showed the highest solubility of the denture base materials. This was because artificial saliva reduces water absorption and stops material from leaking from the matrix by lowering matrix swelling (Mostafa and Al-Sourori, 2023).

Acrylates frequently release the major residual monomer sum during the primary water loading days. This may be attributed to that heat-cure acrylic resins include varying amounts of leachable components (Mostafa and Al-Sourori, 2023). Relined heat-cure acrylic resin demonstrated the highest water sorption in a denture cleanser. This result may be attributed to Ethyl alcohol and a lower molecular weight plasticizers that dissolve more readily in ionic solutions than in water (Dutta *et al.*,2023).

5. Conclusion

Within the limitations of this study and based on the results obtained, it can be concluded that:

- **1.** Regarding Water sorption there was:
 - **a.**Significantly the least when disinfecting heat-cure samples with distilled water.
 - **b.**Almost no significant difference when disinfecting heat-cure samples with both NaOCL and Chlorhexidine mouthwash.
 - c. Significantly the least when disinfecting the relined heat-cure samples with distilled water.
 - **d.**Significantly the highest when disinfecting the relined heat-cure samples with Chlorhexidine mouthwash.
 - e. No significant difference when disinfected with NaOCL.
- 2. Regarding water solubility there was an insignificant difference between heat-cure and soft-relined heat-cure samples when disinfected with distilled water, Sodium hypochlorite, and Chlorhexidine mouthwash.

Authors' Contributions

Dehis WM was responsible for the laboratory work, writing the detailed methodology, review, and editing the whole manuscript. Hashem ABH was responsible for reviewing all the manuscript, reviewing the plagiarism, and submitting the manuscript to the journal. Alloush NT performed the interpretation of results and statistical analysis and writing the discussion.

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Conflicts of Interest: All authors reported that there are no conflicts of interest.

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