Section A-Research paper
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Cytotoxicity, Solubility and Setting Time of Endodontic Sealers with and without incorporation of Chitosan Nanoparticles and Chlorhexidine: In Vitro Study

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ABSTRACT:

Context:

Any enhanced or modified cement or endodontic sealers must be examined to make sure its qualities meet standards before recommending its usage in therapeutic settings.

Aims: To evaluate and compare the Cytotoxicity, solubility, and setting time of endodontic sealers with and without incorporation of chitosan nanoparticles(CsNPs) and chlorhexidine(CHX).

Settings and Design: Experimental non clinical trial, in-vitro study.

Methods and Material: Samples were divided into following groups with 24 samples in each group:

Group A: AH Plus Sealer, Group B: AH Plus Sealer + CsNPs +CHX, Group C : CeraSeal sealer, Group D: CeraSeal sealer + CsNPs +CHX Materials and methods. Cytotoxicity testing was done using MTT assay, setting time (ST) was measured by using Vicat apparatus and solubility testing was done by weighing the set samples before ageing, at day 1, day 7 and day 30 on a digital weighing balance.

Statistical analysis used: One-way Analysis of Variance (ANOVA), Tukey-Kramer Multiple Comparisons Test.

Results:

When comparing the results of the biocompatibility testing, the values of Group D were comparable with the values of the control group. One-way Analysis of Variance (ANOVA), Tukey-Kramer Multiple Comparisons Test shows that there is a statistically significant difference is the values of Group D when compared with other group and control. The mean setting times for Group A, B, C and D were 901.11, 924.56, 197.11 and 204.67 mins respectively. High solubility percentage was recorded in all the 04 Groups at day 30, whereas at day 1 and 7. The percentage solubility with all the groups was < 3%. Highest solubility was recorded with Group B and C, i.e. AH Plus Sealer + CsNPs + CHX and Ceraseal sealer respectively.

Conclusions:

Thus the results of the Cytotoxicity, solubility, and setting time revealed Group D with Ceraseal sealer + CsNPs +CHX, had least cytotoxicity, less setting time and least percentage of solubility as compared to all other 03 endodontic sealer groups.

Key-words: Cytotoxicity, Solubility, Setting Time, MTT assay, Endodontic Sealers, Chitosan Nanoparticles and Chlorhexidine

INTRODUCTION:

Complete root canal cleaning, shaping, and hermetic sealing are essential in endodontic. The primary goals of endodontic therapy are therefore the eradication of bacteria from the root canal system and the avoidance of subsequent reinfection.[1-6]

An endodontic sealer is a substance that is used to bridge the space between a tooth's root canal system and the obturating substances used to treat root canal infections.[1] Root canal sealers can be classified as zinc oxide eugenol, calcium hydroxide, glass ionomer, resin-based, or calcium silicate-based. Some epoxy resin-based sealers, including AH plus (Dentsply De Trey, Konstanz, Germany), have been held up as the gold standard due to their superior sealing capabilities, high radiopacity, and long-term dimensional stability.[7-9] Due to their cytotoxicity, these sealers, however, have a negative impact on periapical tissue.[10]

Recently, a lot of products with syringe-based sealers based on calcium silicate have been created. These products have the benefit of being simple to use, absorbing dentinal tubule moisture, and not requiring mixing because the calcium silicate-based sealer hardens on its own.[10,11] Tricalcium silicates, dicalcium silicates, calcium aluminates, zirconium oxides, and thickening agents are the main ingredients in CeraSeal.

In order to effectively reduce microbial growth before filling the root canal with a sealer it has become crucial that Endodontic materials include specific components such as Chitosan nanoparticles (CsNPs), Chlorhexidine (CHX) that release chemicals for antibacterial activity to help limit infection inside the root canal system.[12]

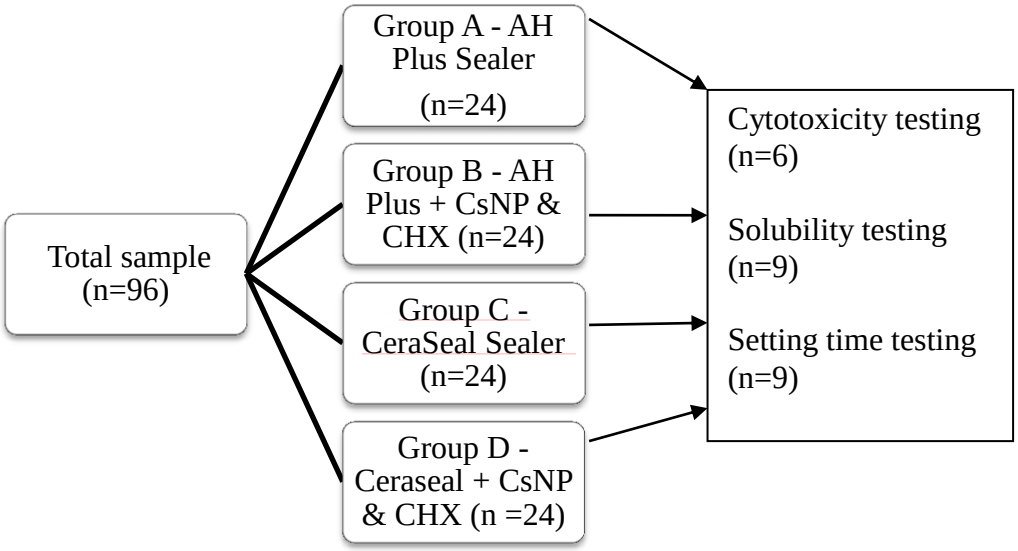
Chitosan is a natural biopolymer (poly[1,4-b-Dglucopyranosamine], a deacetylated derivative of chitin) which shows antibacterial potential when used as chitosan nanoparticles (CsNPs).[(13)] Because of its adaptability in a variety of forms, chitosan has drawn a lot of interest from the biomedical community.[14-16] 2% Chlorhexidine (CHX) as well has proven beneficial as an irrigating solution in endodontics. Therefore, the addition of antimicrobial compounds to endodontic cements could be predicted to enhance their antibacterial capabilities. Any enhanced or modified sealers / Cements must be examined to make sure its qualities meet standards before recommending its usage in therapeutic settings.[17]

Hence a study was planned to evaluate cytotoxicity, solubility and setting time of epoxy resin based sealers (AH Plus, DENTSPLY, GERMANY) and bioceramic sealers (CeraSeal, METABIOMED) with and without incorporation of chitosan nanoparticles (Nanoshel) and chlorhexidine(DENTOCHLOR,AMMDENT).

METHODS AND MATERIAL:

The present study was an in-vitro study and the scientific and ethical clearance for the study was obtained from the institutional review, scientific and ethical committee, wide letter no. DPU/484/21/2021 Dated 07/06/2021.

Study Commenced with Preparation and grouping of 04 different types of sealers and 24 samples were prepared of each endodontic sealer (total n=96). Samples were further divided and tested for **Cytotoxicity (n=6), Solubility (n=09), Setting time (n=09).**



Description of Procedure:

1. Preparation of sealers with chitosan Nanoparticles-

Two sealers AH Plus and Ceraseal sealers were used. In group A, the sealer was mixed on a mixing paper pad whereas, in group C, the sealer was poured into the moulds directly from the tube. In group B and D CsNPs and 2% CHX were added in the ratio of 10:100 by weight of the sealers and were mixed on a mixing paper pad.

2. Cytotoxicity testing (Figure 1)

Endodontic sealers of all the 4 groups were poured in the molds (diameter-5 mm, height-5 mm) and were incubated at 37^0 in an incubator with 95% relative humidity for 48 hours. After which the set samples were removed and were kept in ultraviolet chamber for 12 hours for sterilization and seeded with umbilical cord derived stem cells (UCDSCs) at the density of 10000 cells/. After the cells got adhered, they were incubated with different sealer groups for 48 h and the proliferation rate was determined by MTT assay. 10 ml of MTT (3-(4, 5- Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide, 5 mg/ ml) was added to each well and the absorbance was read at 570 nm using a micro plate reader and records were obtained using phase contrast microscope.

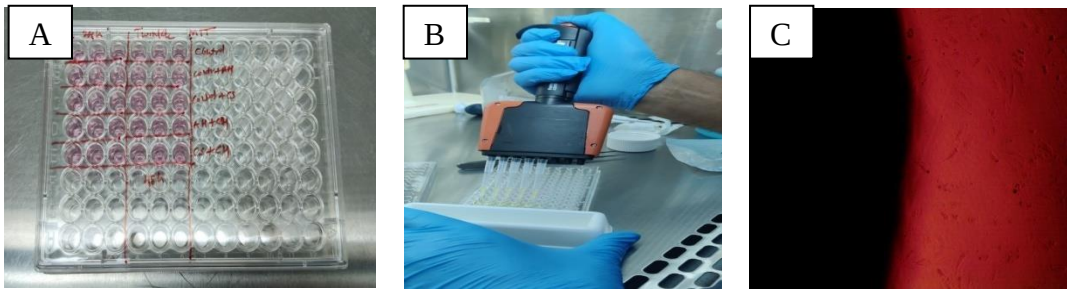


Figure 1 Cytotoxicity testing

3. Solubility testing (Figure 2)

Silicon molds (diameter-5 mm, height-5 mm) were first cleaned in an ultrasound bath for 15 min and the sealers were plugged into the molds avoiding air entrapment. All the specimens were stored in a dark container at 37ºC and 95% relative humidity for 72 hrs. The set sealer samples were removed from molds and weighed 3 times (accuracy 1mg) before aging. The immersion periods was of 1, 7, and 30 days. (n = 9 from each sealer for each time point) in 50 mL of distilled water. After which the specimens were removed, dried at 37ºC for 24 h., and weighed 3 three times, and the mean was recorded as the final weight. The difference of mass between the initial weight (before immersion) and the final weight was recorded as a percentage to determine the solubility percentage of each sealer.

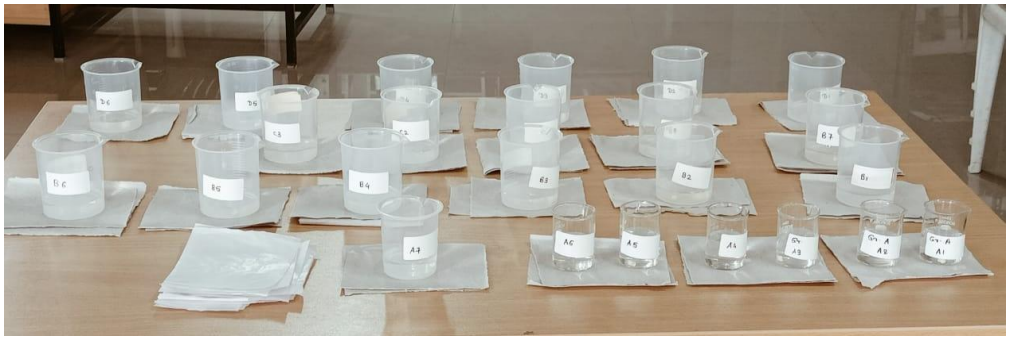


Figure 2 Solubility testing : Immersion periods (1, 7 & 30 days)

3. Setting time testing (Figure 3)

Acrylic molds (inner diameter 10 mm, height 4 mm) were prepared and each mold was labeled and placed on a glass plate, and then the respective sealer materials were mixed and packed into the molds. The whole assembly was then stored in an incubator (37ºC, >95% relative humidity) for at least 1 hour. The setting time was determined as the time when the indenter needle of a custom-made Vicat apparatus (300 g and 1 mm needle diameter) failed

to create an indentation. The measurement interval was adjusted from1 hour at the beginning to 5min in accordance with the setting process. The time from the onset of mixing to the sealer setting was taken as the setting time.

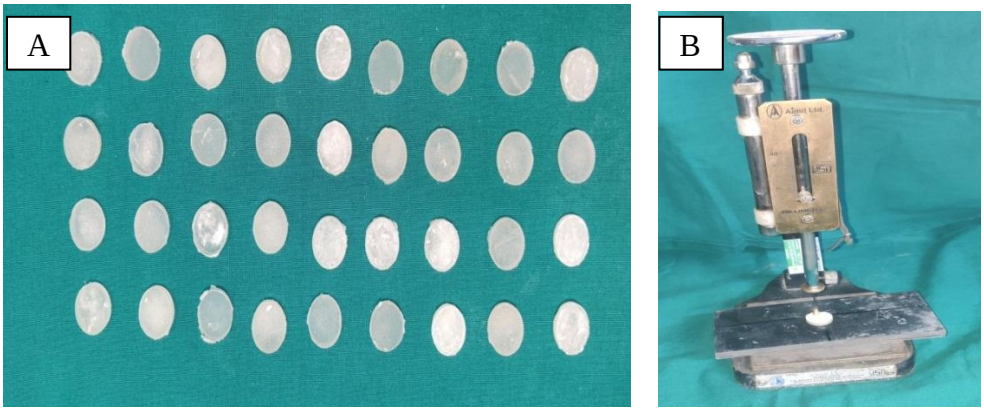


Figure 3 Setting time measurement: A. Moulds B. Vicat's apparatus

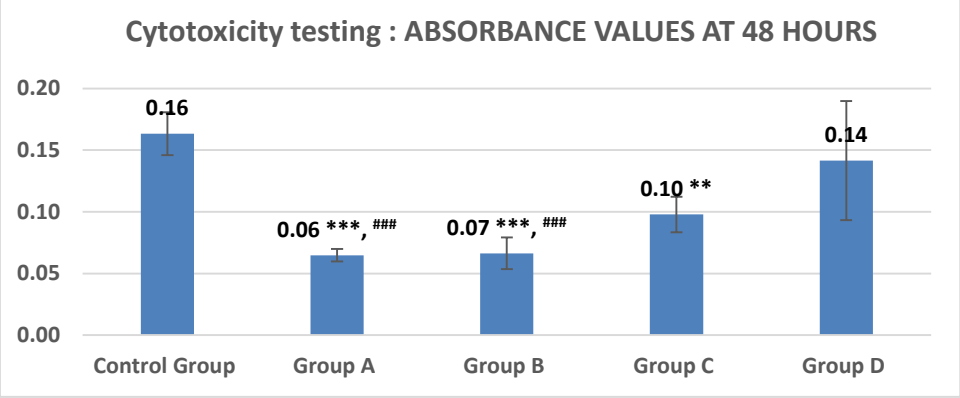
Statistical Analysis:

One-way Analysis of Variance (ANOVA), Tukey-Kramer Multiple Comparisons Test was used to draw the inferences.

RESULTS:

1. Cytotoxicity testing:

Cytotoxicity testing was done using ELISA plate reader. Cell viability was assessed by the absorbance values of all the 04 groups and compared with the control culture media at 48hours. Higher absorbance value indicates higher cell viability. The results are recorded as under:



Graph No. 1 : shows the absorbance measured in MTT assay using culture media and umbilical stem cell at 48 hrs. ** P<0.01, *** P<0.001 vs Control, ### P<0.001 vs Group D, One-way Analysis of Variance (ANOVA), Tukey-Kramer Multiple Comparisons Test shows

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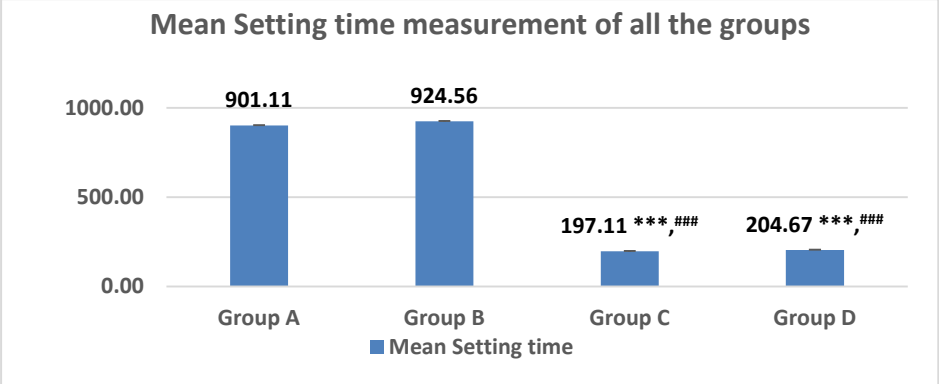
that there is a statistically significant difference is the values of Group D when compared with other group .

2. Setting time :

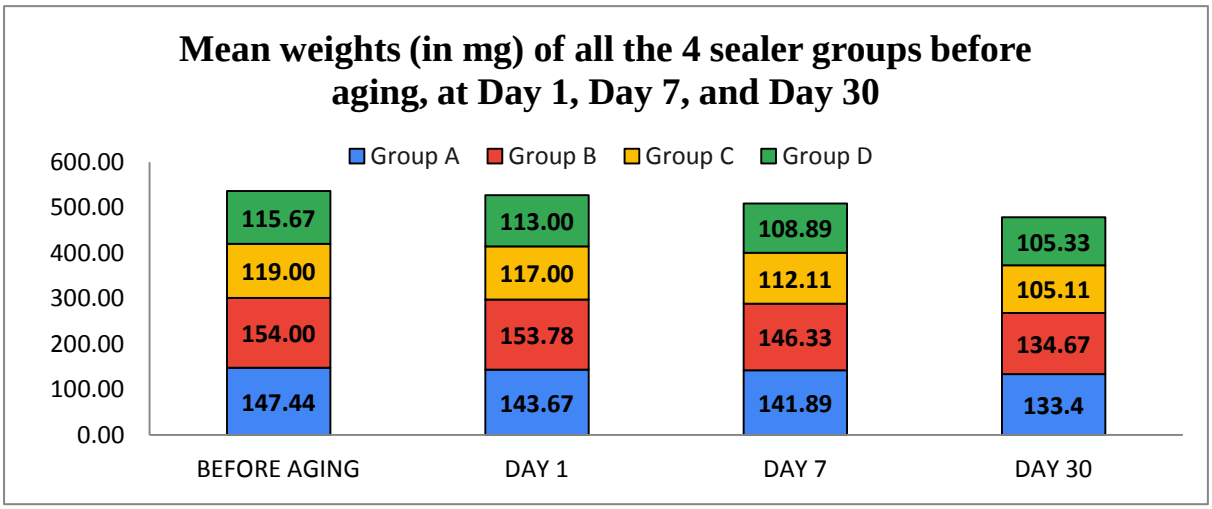
Setting time (ST) (minutes) of each sample of all the 04 Groups measured by using Vicat apparatus



Graph No. 2: shows the mean setting time of each group, *** P<0.001 vs Group A, ###P<0.001 vs Group B, One-way Analysis of Variance (ANOVA), Tukey-Kramer Multiple Comparisons Test which signifies statistically significant difference in setting time of Group C and D in comparison with Group A and B

3. Solubility testing:

Mean weights (in mg) of all the 4 sealer groups before aging, at Day 1, Day 7 and Day 30:



Graph No.3 One-way Analysis of Variance (ANOVA), Tukey-Kramer Multiple Comparisons Test revealed Mean weight declined in all the groups at day 1 when compared with the weights before aging but the difference was found high in the Group C then Group

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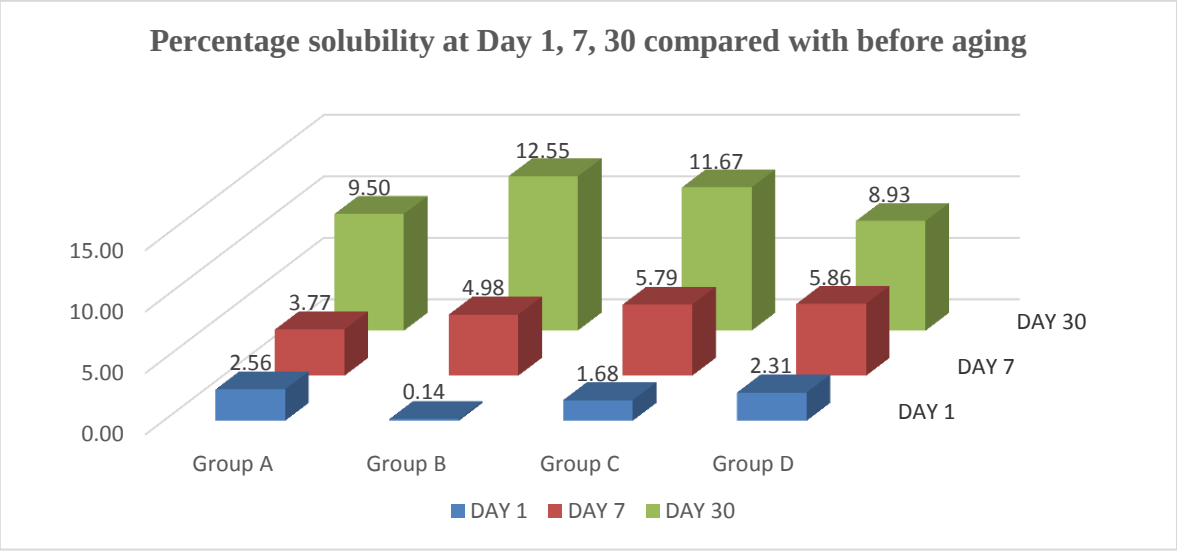
A, and B whereas Group D had no significant decline in mean weight at Day 1, reflecting least solubility .

At Day 7 the mean weight of Group B was highly reduced when compared with the mean weight before aging and Group C showed least reduction .

While Day 30 results revealed significant reduction in mean weight with the P value is 0.1595, One-way Analysis of Variance (ANOVA). Group B had least reduction whereas Group A showed higher decline as compared to other groups



Graph no.4: Percentage solubility at Day 1, 7, 30 compared with before aging

High solubility percentage was recorded in all the 04 Groups at day 30, whereas at day 1 and 7 the percentage solubility with all the groups was < 3%. Highest solubility was recorded with Group B and C, i.e. AH Plus Sealer + CsNP + CHX and CeraSeal sealer whereas amongst all the four groups , Group A and D had least solubility i.e. AH Plus Sealer and CeraSeal sealer + CsNP + CHX

DISCUSSION:

As the root canal sealer may come into direct touch with apical tissue through the apical foramen, biocompatibility is one of the most important factors for the sealer. The biocompatibility of sealers is one of the most important criteria for root canal obturation; hence this study was done to assess the biocompatibility.[18] The results show the absorbance measured in MTT assay using 48hours culture media and umbilical stem cell. One-way Analysis of Variance (ANOVA), Tukey-Kramer Multiple Comparisons Test shows that there is a statistically significant difference in the values of Group D which contains **CeraSeal sealer + CsNP+ CHX** when compared with other groups and control. (**Graph No. 1)**

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In Group D, CeraSeal Sealer and Chitosan are both present. Since CsNPs' antibacterial activity has already been established in most chitosan investigations.[1,14] it was confirmed in this study that CeraSeal Sealer's biocompatibility would be improved by the addition of CsNPs at a concentration of 1:10. The samples with CeraSeal sealer + CsNP + CHX had the best biocompatibility characteristics, followed by Group C, which contained only CeraSeal sealer ($p< 0.001$).

The calcium silicate-based sealer had much greater absorbance values than AH Plus as shown by the MTT experiment. A greater absorbance value suggests that there are more live cells because absorbance and cell count are connected.[19] The aforementioned findings are in line with earlier research that revealed AH Plus's initial toxicity was brought on by the resin and amine components of the epoxy resin-based sealer.[20-22] In all testing groups, calcium silicate-based sealers displayed higher biocompatibility. The biocompatibility of Ceraseal sealer may be related to the Ca2+ release and more alkaline environment.

In a therapeutic environment, a longer setting period may be viewed as a serious problem since it might increase the solubility of materials and create gaps that can lead to the growth of bacteria and reinfection of the root canal.[23,24] The setting time must not be more than 10% longer than what the manufacturer claims, according to ISO 6876/2012.[25]

The mean setting time was evaluated for all the groups using Vicat apparatus . One-way Analysis of Variance (ANOVA), Tukey-Kramer Multiple Comparisons Test signified statistically significant difference in setting time of Group C and D in comparison with Group A and B (**Graph No. 2)**.

According to the manufacturer, Ceraseal sealer needs 120 mins to set, whereas AH Plus needs at least 505 mins. This study's determination of a mean AH Plus sealer application time of 901 minutes was consistent with those of Duarte et al.[26] The addition of CsNPs and CHX into the sealer groups did not significantly alter the setting time of the sealers. All of the evaluated sealers in the current investigation demonstrated the longer setting times needed by the ISO standard. The setting time of calcium silicate-based sealants was quicker than that of AH Plus.

Depending on how the sealers are made, the setting time will vary.[7] Dry root canals may result in longer setting times for calcium silicate-based sealers because moisture in the dentinal tubule also triggers the reaction that causes the sealer to set.[27,28] To give enough time for the obturation of one or more root canals, the setting time of a root canal sealer is crucial.[29] Temperature, particle size, constituents, and humidity all affect setting time.[30]

The mass loss of a substance during a period of immersion in water is known as solubility.[31] The solubility of a root canal sealer should not exceed 3% by mass, as stated in ANSI/ADA Specification 57. A highly soluble root canal sealer will invariably allow gaps to grow inside the material and between the material and the root dentin, creating pathways for leaking from the oral cavity and periapical tissues.[32]

In the current study, the percentage of the difference of weights at day 1, 7 and 30 were calculated. The results showed that Mean weight declined in all the groups at day 1 when compared with the weights before aging but the difference was found high in the Group C then Group A, and B whereas Group D had no significant decline in mean weight at Day 1, reflecting least solubility**.** At Day 7 the mean weight of Group B was highly reduced when compared with the mean weight before aging and Group C showed least reduction. While Day 30 results revealed significant reduction in mean weight with the P value is 0.1595, One-way Analysis of Variance (ANOVA). Group B had least reduction whereas Group A showed higher decline as compared to other groups (Graph no.3). High solubility percentage was recorded in all the 04 Groups at day 30 ,whereas at day 1 and 7 the percentage solubility with all the groups was < 3%. Highest solubility was recorded with Group B and C, i.e. AH Plus Sealer + CsNP + CHX and CeraSeal sealer whereas amongst all the four groups , Group A and D had least solubility i.e. AH Plus Sealer and CeraSeal sealer + CsNP + CHX (Graph no.4).

The International Standard Organization 6876 and ANSI/ADA specification No. 57 both call for a solubility result that must show a weight loss of no more than 3%. Our results didn't meet these standards. There are contrasting accounts in the literature. Our study's findings primarily on day 30 did not maintain the international requirements of solubility.

Low solubility and excellent dimensional stability are crucial characteristics of a root canal filler material, in addition to strong biocompatibility. The sealer's high solubility and dissolution encourages bacterial infiltration which can lead to further reinfection. The ISO Standard 6876185 outlines the specifications for root canal sealing materials: the maximum dry mass loss after 24 hours of storage in deionised distilled water should be less than 3%.

According to the study's findings, compared to the gold standard, AH Plus, both the modified and unmodified calcium silicate-based sealers are more biocompatible and have equivalent qualities.

These findings suggest that a calcium silicate-based sealer modified with CsNPs and CHX would be preferable to an epoxy resin-based sealer for root canal therapy. A longer

investigation involving long-term clinical results is required because the modification is still relatively new and has limitations due to the paucity of research findings.

CONCLUSION

Within the limitation of this study, it can be concluded that:

A significant difference was present between both epoxy resin based sealer and calcium-silicate based sealer. Modified Ceraseal sealer was more biocompatible, least soluble and showed lower setting time when compared to other groups. Based on these findings, a calcium silicate-based sealer modified with CsNPs and Chx would be preferable to an epoxy resin-based sealer during root canal therapy.

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