In-Vitro Characterization of Eggshell-Based Cement as Novel Regenerative Endodontic Cement

Shaimaa NabilSulyiman[1*], Marwa M. S. Abbas[2], Aiah A. El-Rashidy[1]

1. Biomaterials Department, Faculty of Dentistry, Cairo university, Cairo, Egypt.
2. Oral Biology Department, Faculty of Dentistry, Cairo university, Cairo, Egypt.

Corresponding author: Shaimaa Nabil Sulyiman
E-mail:shaimaa.sulyiman@dentistry.cu.edu.eg

ABSTRACT

Background: Regenerative endodontic dentistry relies on the use of new bioactive materials which can enhance dentin-pulp complex regeneration. However, lack of bioactivity of calcium hydroxide is limiting its use.**Aim:** To characterize eggshell-based cement (ES), as a novel regenerative endodontic cement material, as compared to calcium hydroxide ($Ca(OH)_2$), in terms of average particle size, morphology, elemental composition, setting time and compressive strength.**Methodology:** ES and $Ca(OH)_2$were evaluated for particles size distribution, particle morphology and chemical composition, setting time and compressive strength. Results were statistically analyzed using ANOVA and Tukey's test.**Results:** ES particles size distribution ranged from 50 nm to 0.5 µm diameter and were composed mainly of calcium, phosphorus, magnesium, sodium fluoride and boron. Statistical analysis showed no significant difference in the setting time values recorded in the ES group, in comparison to $Ca(OH)_2$. Furthermore, ES group showed significantly higher compressive strength value recorded, in comparison to $Ca(OH)_2$.**Conclusions:** ES represents a promising novel natural biocompatible and bioactive dental cement with favorable setting time and compressive strength value.

Keywords: Eggshell; calcium hydroxide; bioactivity; regeneration Endodontic cement.

INTRODUCTION

Calcium hydroxide ($Ca(OH)_2$) has been used in dentistry for several decades and has become a mainstay therapeutic agent in the endodontic field owing to its numerous advantages. It has been used in several applications such as vital pulp therapy, pulp revascularization, apexogenesis, apexification, treatment of root resorption, intracanal medicament, and root canal sealers(N and Chandra S.M, 2014). Although this material exhibits several advantages, it also possesses many drawbacks. On the contrary, using calcium hydroxide in deciduous teeth is currently being discouraged since it may induce chronic pulpal inflammation and internal root resorption(N and Chandra S.M, 2014; Reddy *et al.*, 2020). During vital pulp capping, the formed osteodentine barrier is often incomplete and associated with tunnel defects formation due to vascular inclusions. Therefore, these defects may allow bacterial reinfection. In addition, long-term use of

$Ca(OH)_2$ may cause progressive calcification of the root canal space which affects the revascularization of the pulp tissue(N and Chandra S.M, 2014; Arandi, 2017). Moreover, in apexogenesis and apexification,$Ca(OH)_2$ affects the mechanical properties of the dentin when used for a longer period of time rendering the tooth susceptible to fracture. Also, $Ca(OH)_2$ has been shown to be less effective against Enterococcus faecalis and Candida albicans when used as an intracanal medicament, and root canal sealers(Mohammadi and Dummer, 2011).

Consequently, a new trend in regenerative endodontic dentistry is the development of new bioactive materials which are able to interact chemically with the body fluids leading to the formation of a biological bond between the host tissues and the materials(Cao and Hench, 1996; Gandolfi *et al.*, 2010), and enhance dentin-pulp complex regeneration(Gong *et al.*, 2016). Therefore, calcium carbonate-based cements ($CaCO_3$) are rapidly replacing $Ca(OH)_2$ in the regenerative endodontic field(Rahmanian, Azimzadeh and Eskandarizadeh, 2018).

A promising natural material for this application is the eggshell powder due to its numerous biological advantages. Eggshell is an agricultural waste, which is considered of no use and usually discarded as wastes(Abdulrahman *et al.*, 2014).The eggshell powder was described to be biodegradable, recyclable and biocompatible due to its unique bioceramic composition. It contains 94% $CaCo_3$ and 1% magnesium carbonate ($MgCo_3$) (Yosathai and Kavithaa, 2014).The regenerative action of eggshell on human maxillary cystic bone defects was detected by**Ashghar et al.,**to create nano-textured scaffolds from eggshells. The surfaces were characterized for the chemical composition and crystal phase structure. The attachment and proliferation of human fibroblasts on the nano-textured eggshell scaffolds were investigated and compared with their attachment and proliferation on counterpart flat substrates. The authors concluded that the nano-texturing added an extra advantage to the unique chemical composition of the eggshell and resulted in a more favorable fibroblast behavior in terms of attachment and proliferation, as compared to the flat constructs (Asghar *et al.*, 2012). Eggshell is also a valuable source for producing hydroxyapatite (HAp), the main inorganic mineral component of bone and teeth (Abdulrahman *et al.*, 2014). A split-mouth randomized controlled clinical trialevaluated the effect of eggshell-derived nano-HAp on bone healing ability following surgical removal of bilateral mandibular third molars at baseline, first, third, and sixth months. On the test side, the extraction sockets were grafted with eggshell-derived nano-HAp while on the control side, the sockets were allowed to heal with no intervention. At the end of the third month, the bone density of the test group reached the same density of the surrounding bone indicating full bone healing. In comparison to the control group, eggshell-derived nano-HAp caused improved and accelerated bone repair (Kattimani *et al.*, 2019).

Based on these data, eggshells could provide a cheap, natural, and bioactive alternative to $Ca(OH)_2$as an endodontic cement. Therefore, this study aimed tocharacterize the investigated groups in terms of average particle size, morphology, and elemental

composition. In addition to measuring the setting time and compressive strength of the prepared cements from both materials, in order toevaluate the ability of eggshell (ES) -based cementto be used as novel natural bioactive endodontic cement.

MATERIALS AND METHODS

Materials:

Calcium Hydroxide (*Dycal, Dentsply, US*).

Nano eggshell powder (*milled and sieved by Nano Gate Co., Egypt*).

Methods:

Preparation of the nano-sized eggshell powder:

The nano-sized eggshell powder was prepared according to the method described by Ahmed and Ahsan (Ahmed and Ahsan, 2008). A regular detergent was used to wash and remove any impurities from the eggshell, followed by soaking it in a 10% acetic acid solution for 10 min (Ito *et al.*, 2020; Rosnah *et al.*, 2021). Following its cleaning, and to allow for the detachment of the inner membrane layer, theeggshells were vacuum dried at 250 °C for 6 min. To obtain the nano-sized powder, the eggshells were first crushed with a morter and pestle, followed by ball-milling (*Planetary Ball Mill PM 100, Retsch, Germany*) at 200 rpm for 24 hours.

Preparation of the $Ca(OH)_2$ and ES disc-shaped specimens:

A total of 24 disc-shaped specimens were prepared. The specimens were prepared using polyvinyl chloride (PVC) molds with dimensions of 0.8 mm diameter and 1.6 mm thickness, according to *ISO specifications no* 9917-2:2017(ISO 9917-2, 2017).Mixing 0.5 gm NE powder with 0.5 ml deionized water (DIW) manually using a spatula and glass slab was found to give reasonable flow. $Ca(OH)_2$ cement, was mixed according to the manufacturer's instructions. For both types of materials, the obtained mix was placed into the PVC molds. The excess material was removed by stainless steel spatula and the set disc was removed from the mold after setting.

Characterization of the prepared ES and $Ca(OH)_2$ Cements:

Dynamic light scattering (DLS) (*NANOTRAC FLEX, MicrotracBEL, Japan*) was used to determine the particle size distribution profile of the prepared disc powder. Firstly, one disc from each group was prepared. The prepared discs were crushed into powder using mortal and pastel. Then, DIW was added to the powder of milled discs to form suspension prior to measurement. For DLS analysis, 1 mL of DIW was used to dilute 30 μL of each suspension, and for every analysis, three measurements were taken at room temperature.

Environmental scanning electron microscope (ESEM)(*Quanta 250 FEG (Field Emission Gun), FEI, Netherlands*)was used to determine the morphology of the prepared discs. Elemental analysis was performed using energy dispersive X-ray (EDX) analysis(*Model XL30 Philips, Germany*), with accelerating voltage 30 K.V., magnification (2000X, 8000X, 15000X)and resolution for Gun.1.

To measure the setting time, five discs from each group were prepared. The whole assembly was then stored in an incubator (37°C, >95% relative humidity) for at least 5 min. Then, the needle of a custom-made Vicat apparatus (300 g weight and 1mm diameter) was adjusted vertically onto the surface of the material. The setting time was

determined as the time when the indenter needle failed to create an indentation**(Figure 1)**. The time from the onset of mixing to the material setting was taken as the setting time. Five measurements were made for each disc.

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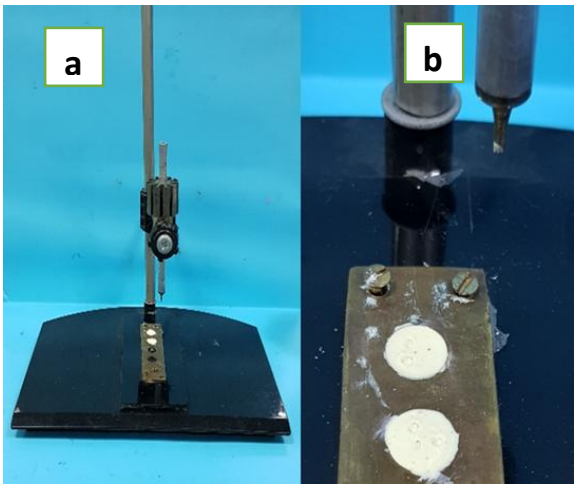

Figure 1. Setting time device and measurement.

To measure the compressive strength, five discs from each group were prepared. The whole assembly was then stored in an incubator (37°C, >95% relative humidity) for up to three times the setting time.After removing the specimens from the molds, their dimensions were confirmed using a digital caliper (Mitutoyo MTI Corporation, Tokyo, Japan). The samples were re-incubated at 37 °C and 95% relative humidity for 7 days. all samples were individually and vertically mounted on a computer-controlled materials testing machine (Model 3345; Instron Industrial Products, Norwood, MA, USA) with a loadcell of 5 kN and data were recorded using computer software (BluehillLite Software Instron® Instruments). Then the samples were statically loaded (in compression manner) using stainless-steel rod ended with flat plate (40mm x 60mm) attached to the upper movable compartment of the machine at a crosshead speed of 1 mm/min until failure**(Figure 2)**. The maximum failure load was recorded in N and converted into MPa. The compressive strength was calculated from the recorded peak load divided by sample surface according to the following equation;

Compressive strength (CS) = $4P/\pi d^2$, where P is the load (N) at the fracture point and d is the diameter (mm) of the cylindrical specimen.

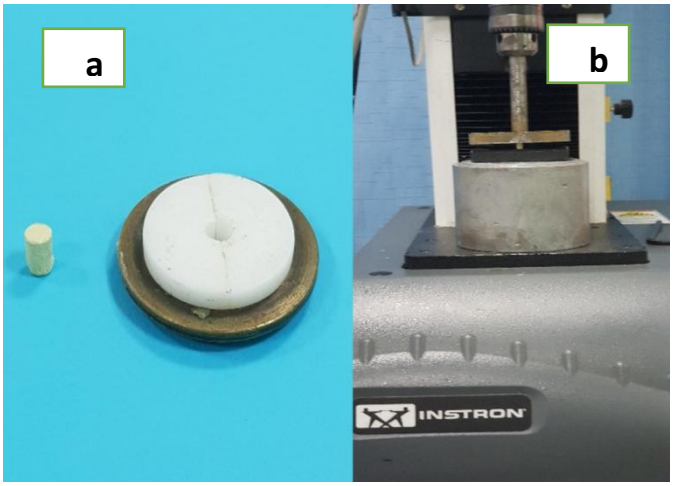

Figure 2. Compressive strength test,**a)** Samples prepared using Teflon molds. **b)**Specimens during compressive testing.

Statistical analysis

Data management and statistical analysis were performed using the Statistical Package for Social Sciences (SPSS) version 20. Numerical data were summarized using mean, standard deviation, median and range. Data were explored for normality by checking the data distribution and using Kolmogorov-Smirnov and Shapiro-Wilk tests. Since data were parametric numeric variables, the independent t test was used for comparison between groups.All p-values are two-sided. P-values ≤0.05 were considered significant.

RESULTS

DLS size distribution analysis:

The DLS pattern of the $Ca(OH)_2$ revealed the presence of sharp peaks at an average size from 0.3µm to 5µm in comparison to DLS pattern of the prepared ES revealed the presence of sharp peaks at an average size from 50 nmto 0.5µm **(Figure 3, 4)**.

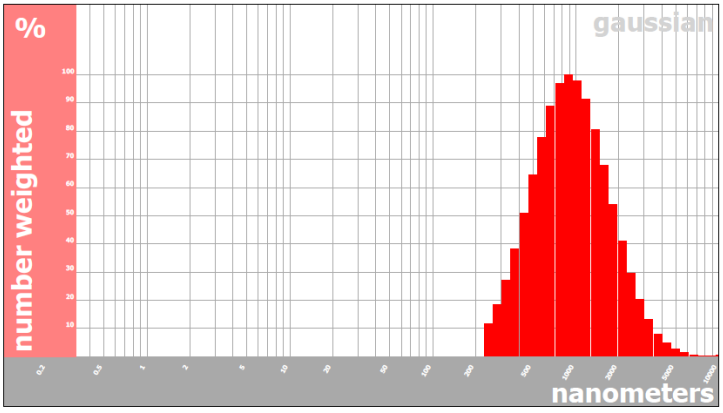

Figure 3. DLS pattern showing particle size disruption of $Ca(OH)_2$

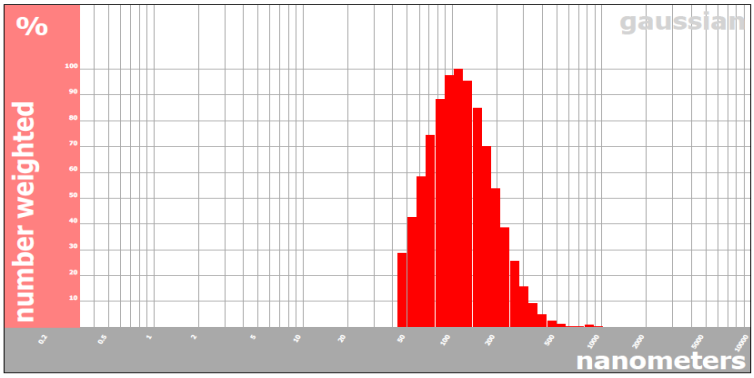

Figure 4.DLS pattern showing particle size disruption ofES

SEM-EDX morphologic and chemical analyses of $Ca(OH)_2$ and ES cement:

ESEM micrographs of average composition analysis $Ca(OH)_2$ cement showed the presence of a large irregular surface particles. **(Figures 5a, c and e).** EDX analysis **(Table 1, Figure 6a)** showed the presence of calcium (Ca), carbon (C), oxygen (O).

ESEM micrographs of average composition analysis ES showed the presence of smaller and regular surface particles in comparison to $Ca(OH)_2$. **(Figures 5b, d and f).** EDX analysis **(Figure 6b)** showed the presence of Ca, phosphorus (P), magnesium (Mg), C, sodium (Na), O, boron (B) and fluoride (F). **(Table 1)**.

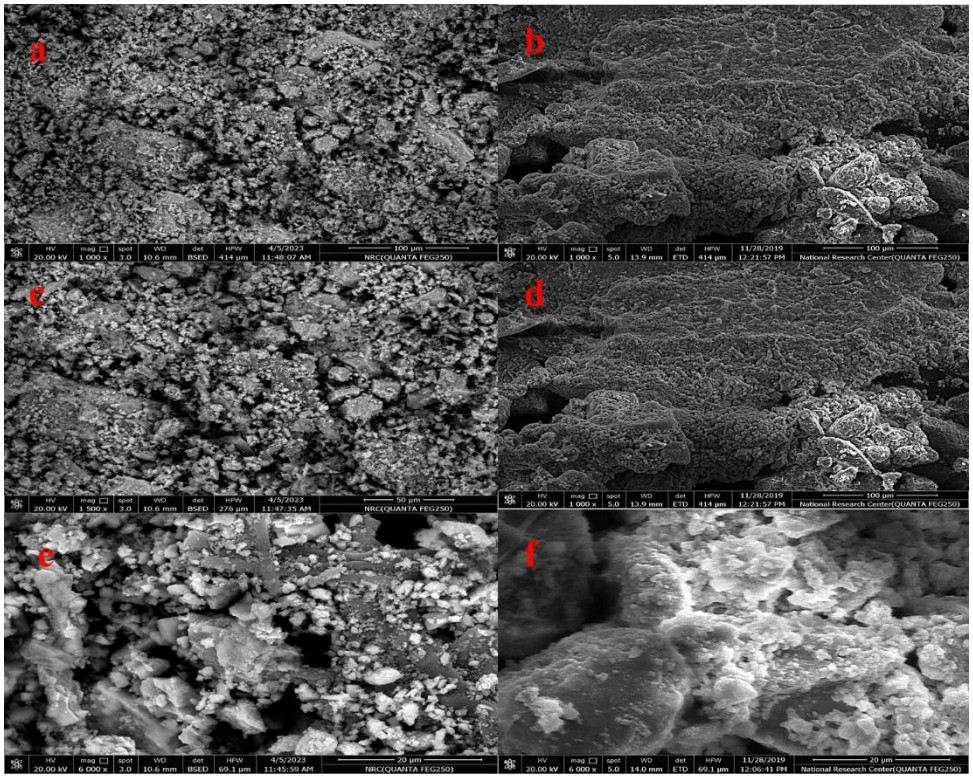

Figure 5. ESEM micrographs of average composition analysis $Ca(OH)_2$ and ES: **a)** 1,000 X of $Ca(OH)_2$ cement, **b)** 1,000 X of ES, **c)** 1,500 X of $Ca(OH)_2$ cement, **d)** 1,500 X of ES, **e)** 6,000 X of $Ca(OH)_2$ cement and **f)** 6,000 X of ES.

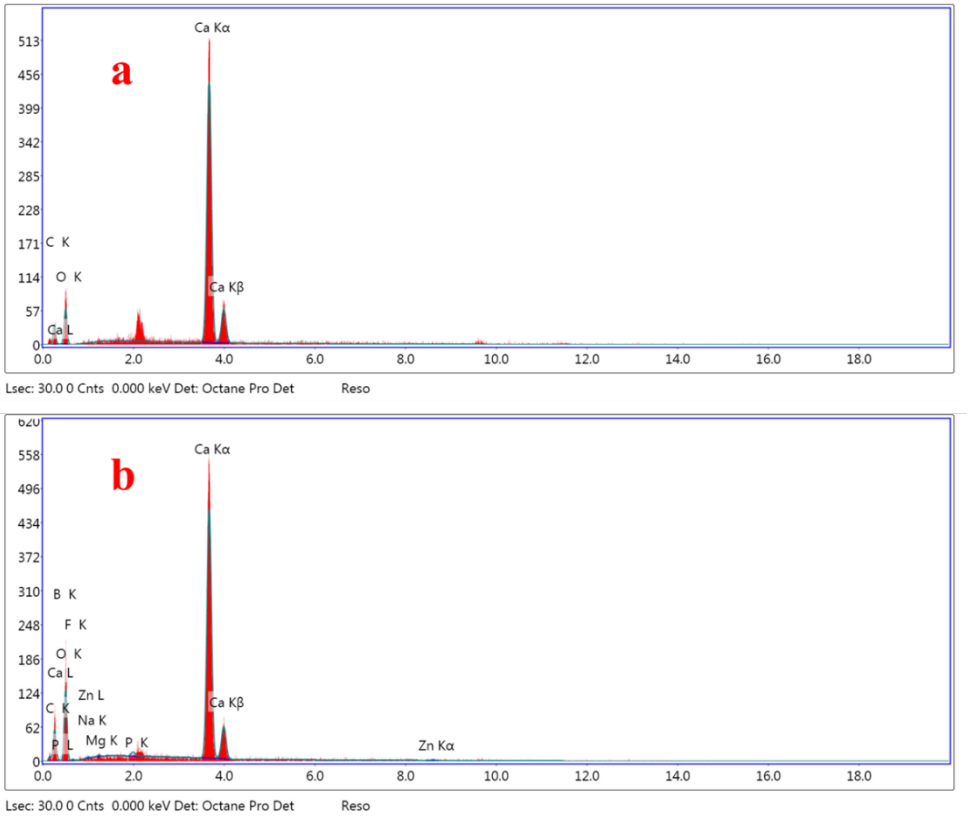

Figure 6. EDX spectra of average compositional analysis of: **a)**$Ca(OH)_2$ cement and **b)** ES discs.

Table 1. The average compositions (wt.%) of $Ca(OH)_2$ and ES discs as obtained from EDX elemental analysis.

Element	$Ca(OH)_2$	ES
C	9.4	11.84
O	37.12	44.96
P	-	0.5
B	-	1.04
Na	-	0.01
Zn	-	0.93
Mg	-	0.28
Ca	53.48	39.26
F	-	1.19

Setting timetest

Statistical results of the setting time test showed a higher setting time value recorded in the group ES, in comparison to $Ca(OH)_2$. However, the difference between both groups was not statistically significant **(Table 2, Figure7)**

Table 2. Comparison of setting time (min.sec)using independent t-test for intergroup comparison (vertical).

Setting	Groups	Mean±SD	Median	Min	Max	t	P

Time (min.sec)						value	value
	$Ca(OH)_2$	1.24±0.17	1.29	1.02	1.45	2.43	.063 ns
	ES	1.92±0.60	2.12	1.25	2.55		

Significance level p≤0.05, ns=non-significant

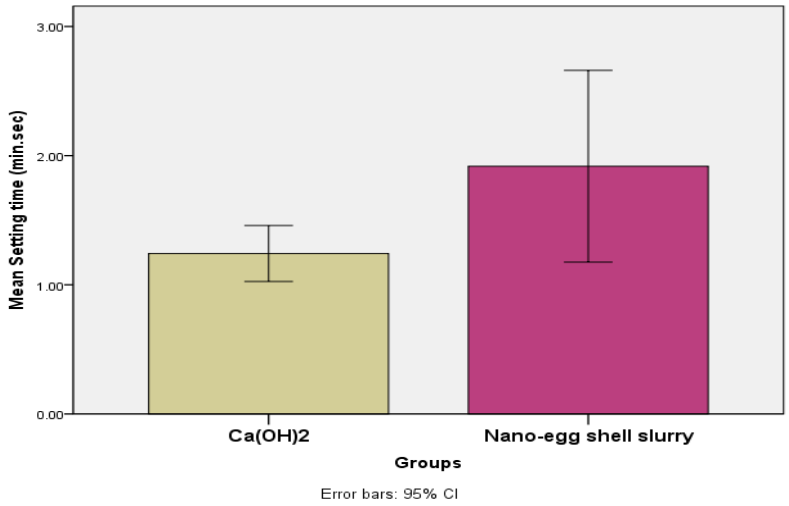

Figure 7. Bar chart showing mean value of setting time for both$Ca(OH)_2$ and ES group.

Compressive strength test

Statistical results of the compressive strength test showed a significantly higher compressive strength value recorded in the group ES, in comparison to $Ca(OH)_2$**(Table 3, Figure 8).**

Table 3. Comparison of compressive strength (MPa) using independent t-test for intergroup comparison (vertical).

Compressive strength MPa	Groups	Mean±SD	Median	Min	Max	t value	P value
	$Ca(OH)_2$	13.56±3.93	13.40	9.20	18.40	5.83	0.00*
	ES	24.96±1.92	25.10	22.40	27.20		

Significance level p≤0.05, *significant

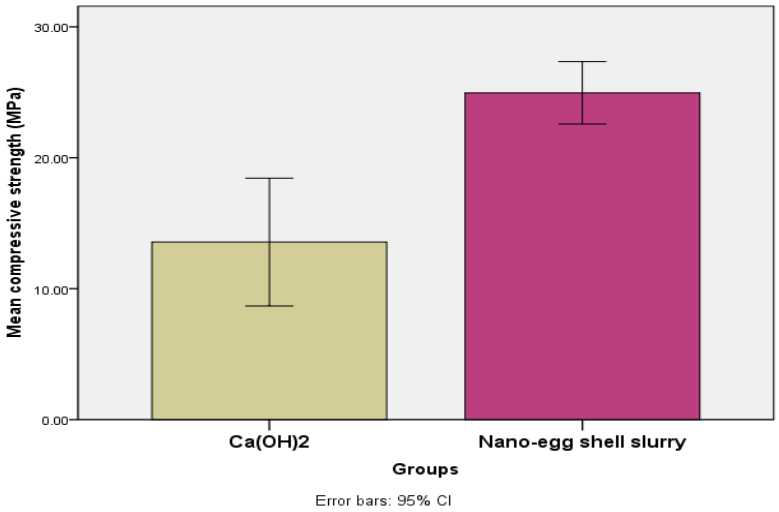

Figure 8. Bar chart showing mean value of compressive strength for both $Ca(OH)_2$ and ES group.

DISCUSSION

With the development of the modern concept of tissue engineering approach and the discovery of the potential of stem cells in dentistry, the regeneration of hard dental tissues has become a reality and a priority of modern dentistry. Regenerative endodontic procedures (REPs) utilize the concept of tissue engineering to restore the root canal system to a healthy state, allowing for the continued development and regeneration of the root and surrounding tissues. (Elnawam *et al.*, 2022). Indeed, the poor bioactivity and high alkaline effect of $Ca(OH)_2$ cement is considered unsatisfactory, as it affects the links between hydroxyapatite crystals and collagen, weakening the dentin and leaving the root more prone to fracture. To date, many efforts have been made to overcome the major drawbacks of $Ca(OH)_2$ cement (Elnawam *et al.*, 2019).A promising natural material for this application is the eggshell powder due to its numerous biological advantages. It was also reported to promote bone and dental mineralization and regeneration (Schaafsma and Pakan, 1999;J Rovenský, 2003).

The particle size distribution of the prepared $Ca(OH)_2$ and ES powder formed after discs milling was assessed by DLS analysis, as presented in **(Figures 3&4)**. DLS pattern revealed that the ES had smaller particles size distribution in comparison to the $Ca(OH)_2$, with presence of sharp peaks at an average size from 50 nm up to submicron size, as represented in **(Figure 3)**. It is already established that the nano-sized particles have greater surface area and energy, compared to their micro-sized counterparts, which accounts for their superior biological interactions (Jeevanandam *et al.*, 2018).For the discussion of the effect of particle size to be more meaningful, **Kobayashi et al.,** assessed the particle size the $Ca(OH)_2$ powder and reported that particles had a size that ranged between 0.5-2.5 µm and they had an irregular rectangular shape (Komabayashi *et al.*, 2009), which is in accordance with the particles size and particles morphology detected in the current study by DLS and ESEM, respectively. This relatively large particular size of the

$Ca(OH)_2$ cement may have been contributing factors in decreasing the surface area which affected the bioreactivity, solubility and regenerative power capacity of the material(Elham Soheilipour and Sanam Kheirieh, 2009).

Regarding ESEM micrographs of morphological analysis $Ca(OH)_2$ cement showed the presence of a large irregular surface particles. **(Figures 5a, c and e).** EDX elemental analysis **(Table 1, Figure 6a)** showed the presence of calcium (Ca), carbon (C), oxygen (O).The power of bioactivity and regenerative capacity of dental cement depends on the nature of the mineral particles and on the network structure of the cement which is responsible for ion release. The lack of minerals in $Ca(OH)_2$ cement except Ca, C and O could limit the cement's bioactivity. In addition, the presence of large irregular surface particles could be limiting the cement solubility which could be considered as another factor in decreasing the bioactivity of $Ca(OH)_2$. These findings are in agreement with the study conducted by **Gandolfi et al.,** who investigated the bio-interactivity, porosity, solubility and bioactivity of $Ca(OH)_2$. The author confirmed that the compositional elements and irregular surface particles could be limiting the cement solubility which decreases the bioactivity of $Ca(OH)_2$(Gandolfi *et al.*, 2015).

On the other hand, ESEM micrographs of morphologicalanalysis ES showed the presence of smaller and regular surface particles in comparison to $Ca(OH)_2$. **(Figures 5b, d and f).** EDX analysis **(Figure 6b)** showed the presence of Ca, P, Mg, C, Na, O, B and F,**(Table 1)**.The difference in the composition, particle shape and size of ES, as compared to $Ca(OH)_2$ cement **(Figure 5a, c and e),**could increase the rate of surface reaction and dissolution of ES, and thus leading to increasing rate of ion release from the ES in tissues as comparison to $Ca(OH)_2$ cement (Gandolfi *et al.*, 2015a; Tiskaya *et al.*, 2019). The release of ions plays an important role in the bioactivity because for mineralization to occur, bioavailable Ca, P, Mg, F, Na and B are essential (Mony *et al*., 2015; El-Rashidy *et al.*, 2018).

Regarding Ca and P, they are the main constituents of HAp, thus, the bioavailability of these two elements is a prerequisite for any dental hard tissue mineralization process. The calcium ion activity was measured to be 2-3 times higher in the predentin as compared to the dental pulp. (Lundgren and Linde, 1992). In addition, reverse transcription polymerase chain reaction (RT-PCR)revealed that human dental pulp cells (hDPCs) in cultures with elevated Ca ion concentrations express significantly high levels of osteopontin (OPN) and osteocalcin (OCN) mRNA expression (An et al., 2012).

P is an element that plays an important role in a variety of biological activities, it is the second most important component of bone and dental hard tissues after Ca. In addition, it is found in the phospholipids and biological membranes, P aids in the transmission of nerve sensations. It is a vital human intracellular anion because of its mobility, and it helps to maintain the acid-base balance in the body by forming buffer systems in the body fluids. It is stored in the form of phosphoproteins and HAp crystals in the teeth and bone. It was proven that the proper amount of inorganic P is required for the activity of odontoblasts, osteoblasts, and

osteocytes in the process of tooth and bone matrix mineralization (Ż et al., 2021).

Mg is the fourth most abundant cations in human bodies and is essential for ATP-dependent phosphorylation of DNA, RNA, and enzymes. Mg influences bone mass, bone turnover, bone-related hormones and cytokine levels. Its insufficiency has been indicated as a risk factor for periodontitis, dental caries and osteoporosis. Mg also plays an important role in dental hard tissue formation. **Salem et al.,** reported that the Mg ions increased the proliferation rate of human dental pulp cells (hDPCs). At optimal concentrations, the Mg exerts a dentinogenic effect where it promotes the differentiation of hDPCs into odontoblasts as evidenced by the increased activity of ALP. In this way, the Mg induces dentin matrix formation and mineralization and promotes the formation of osteodentin/ reparative dentin. That is why the incorporation of Mg in pulp capping materials was considered a new strategy for improving dental hard tissue regeneration (Jawed *et al.*, 2021; Kis *et al.*, 2021; Salem, Zhang and Chou, 2021).

F has an important role in the mineralization of hard tissues. the action of F on the teeth and bone appears to be mediated by several mechanisms. F can directly interact with the teeth and bone mineral matrix physicochemically. In vitro fluoridation of teeth and bone leads to the conversion of carbonated HAp to carbonated fluoroapatite. This is accompanied by changes in the apatite crystallinity and enhancement of the mechanical properties. F can influence the matrix metalloproteinases, thus affecting the composition of the remodelling matrix and its mineralization. In addition, in tooth remineralization, the F speeds up the mineralization process by bringing calcium and phosphate ions together (Everett, 2011). It is worth mentioning that previous researches confirmed that combining Sr with F increased the apatite crystallinity and was related to significantly decreased acid reactivity of the resultant carbonated HAp (Herbison and Handelman, 1975). Moreover, it was discovered that the interaction of Sr and F at sufficient levels resulted in a synergistic remineralizing effect on artificial enamel lesions *in vitro*(Thuy *et al.*, 2008).

While B is one of the trace elements that play an essential role in the formation of dentin and bone. B was reported to promote the *in vitro* odontogenic and osteogenic differentiation of human tooth germ stem cells (hTGSCs). The hTGSCs cultured with B compounds showed higher activity of alkaline phosphatase (ALP), which is a cell membrane-associated enzyme that appears early during odontoblastic and osteoblastic differentiation (Taşlı *et al.*, 2013).

Setting time varies with the specific application of dental cements. For luting and lining cements, a long working time and short setting time are required while for root canal sealers longer working and setting time is desirable. In the present study, the setting time test results showed a higher setting time value recorded in the group ES, in comparison to $Ca(OH)_2$. This could be due to setting time being influenced by powder reactivity, particle size, solvent, fluid concentration, and liquid-to-powder ratio(Twati *et al.*, 2009). However, the difference between both groups was not statistically significant. Whereas, the results of this study were in agreement with *ISO specifications no* 9917-2:2017 (setting time of dental cement avarages from 2-7

min).Hence, ES succeeds to satisfy the standards used for cements.

Compressive strength test is commonly performed to assess the mechanical properties of restorative materials. According to *ISO 9917*, the minimum compressive strength required for pulp capping agents is 50 MPa, however, ideally, these agents should have a compressive strength equal to that of dentin or the permanent restorative material applied over them. According to **Croll et al.,** compressive strength is the best quality control measure that can be considered to produce high-quality restorative material. Thus, compressive strength is commonly measured as a preliminary test to assess the clinical efficacy of dental materials(Croll and Nicholson, 2001; Twati *et al.*, 2009). The results of the present study revealed a significantly higher compressive strength value recorded in the group ES, in comparison to $Ca(OH)_2$. **El-Araby et al.,** showed that replacement of proton with Ca ions during polymerization of Dycal results in chelation of calcium phenolates, which bind to each other with secondary bonds only, causing low mechanical properties of Dycal. Thus, Dycal has high risk of fracture and cannot provide adequate support for the restorative material, if applied alone(El-Araby and Al-Jabab, 2005). However, ES is mainly composed of $CaCO_3$, therefore, it has higher compressive strength value than $Ca(OH)_2$. Similar to our findings, **Natale et al.,** showed that Dycal had lower compressive strength than other $CaCO_3$-containing liners such as MTA and Biodentine(Natale *et al.*, 2015).

This study had an in vitro design. Thus, the generalization of results to the clinical setting must be done with caution to evaluate the potential of ES cement in the regenerative endodontic field. This will enable better simulation of the clinical conditions. Also, it will help to identify areas requiring special attention to enable the implementation of successful dental care practices.

CONCLUSIONS

Within the limitation of the current study, it could be concluded that ES can be considered a promising regenerative endodontic material in terms of its unique chemical composition, setting time and compressive strength. Yet, further investigations should be performed regarding the usage of ES as a regenerative endodontic material in an animal models to properly evaluate its potential for clinical translation.

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AUTHOR CONTRIBUTIONS:

All authors have read and agreed to the published version of the manuscript.

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