RESEARCH ARTICLE

Biomimetic remineralization of acid etched enamel using agarose hydrogel model [version 1; peer review: 5 approved]

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Abstract

Background: Minimally invasive dentistry aims to prevent progression of caries and treats non-cavitated lesions through non-invasive approaches to preserve the integrity of tooth structure. The aim of this research was to investigate the possible biomimetic effect of agarose hydrogel in remineralizing a human demineralized enamel model.

Methods: Mandibular third molars were distributed into three groups (G1, G2 and G3) according to the follow up time (2, 4 and 6 days respectively). Caries like lesion was prepared by applying 37% phosphoric acid gel for 1 minute and then remineralization was performed through applying agarose hydrogel on the demineralized surfaces. The specimens were placed in phosphate solution at 37°C for 2, 4 & 6 days. Scanning electron microscope (SEM), surface microhardness (SMH) and surface roughness analysis (SR) were performed to assess the regenerated tissue.

Results: SEM revealed mineral depositions on the demineralized enamel surface that increased in density by time resulting in a relatively smooth surface in G3. SR and SMH analysis revealed significant differences between the remineralized enamel surfaces of different groups ($p < 0.00001$) with the highest SR in G1 and the highest SMH in G3.

Conclusions: Agarose hydrogel application is a promising approach to treat early carious lesion. Further studies are needed to clarify the stability of agarose hydrogels in clinical application.

Keywords

Remineralization, agarose, enamel, microhardness, surface roughness.

Open Peer Review

Approval Status

	1	2	3	4	5
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17 Sep 2018	view	view	view	view	view

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4. **Mahmoud M. Al-Ankily** , British University in Egypt, Cairo, Egypt

5. **Mahmoud M. Bakr** , Griffith University, Nathan, Australia

Any reports and responses or comments on the article can be found at the end of the article.

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Author roles: **El Moshly S:** Conceptualization, Funding Acquisition, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Abbass MMS:** Project Administration, Supervision, Validation, Visualization, Writing – Review & Editing; **El-Motayam AM:** Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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Introduction

Biomimetic remineralization is a non-invasive therapeutic approach that has received great attention in the last decades. It aims to restore the dental tissues to its normal biological function and esthetics¹. Although several studies have proposed different methods to remineralize enamel lesions, their clinical applications are limited because they require difficult application conditions²⁻⁵. Agarose is a natural biocompatible polysaccharide that has been proposed as a matrix for crystal formation⁶⁻⁹. Therefore, the purpose of this study was to investigate the possible biomimetic effect of agarose hydrogel in remineralizing a human demineralized enamel model.

Methods

Specimens preparation

The experiment was done according to the recommendations and approval of the Ethics Committee of the Faculty of Dentistry, Cairo University for working on extracted human teeth (Approval no.18766). Mandibular third molars were collected after being surgically extracted due to impaction with patients' written consents. The roots of 47 tooth were removed using diamond disk (Komet, Rock Hill, USA, K6974) in low speed under water cooling. The crowns were divided mesio-distally and each half was embedded in self-cured acrylic resin (Acrostone Co. Cairo, Egypt, 01CCP50) exposing the uncovered enamel surface. Specimens were examined under stereomicroscope (Leica S8 APO, Leica Microsystems, Switzerland) and specimens with defects (erosions, cracks, visible stains, hypo-calcification) were excluded. Specimens were distributed into three groups (n = 31/ group), according to follow up time (Table 1). Specimens were demineralized using 37% phosphoric acid gel (Super Etch, SDI Limited, Australia, 8100040) for 1 min and rinsed with de-ionized water for 60 seconds.

Remineralization

Agarose (Vivantis, USA, PC0701) hydrogel and phosphate solution were prepared as previously mentioned by Cao *et al.*⁷. Agarose hydrogel was applied on the specimen using acrylic template of 2mm thickness to adjust the thickness of the applied hydrogel. After gelation of the applied hydrogels each specimen was placed into a container filled with 20 mL of phosphate solution and placed in an incubator at 37°C. The phosphate solution and the hydrogel were changed every 24 and 48 h respectively.

Scanning electron microscope (SEM) examination

Thirteen specimens from each group were mounted on the SEM plate with electro-conductor glue (Electron Microscopy Sciences, PA, USA, 12660) to examine their surfaces. The used SEM Model was Quanta FEG 250 (Field Emission Gun) with accelerating voltage 30 K.V.

Surface microhardness (SMH) analysis

SMH of 9 specimens from each group was measured using microhardness tester with Vickers diamond indenter in different areas of the specimens (Vickers diamond, 100 g, 5 s, HMV 2; Shimadzu Corporation, Tokyo, Japan). SMH was measured at baseline, after demineralization and after remineralization.

Surface roughness (SR) analysis

SR of 9 specimens from each group was measured using digital microscope equipped with a built-in camera (Digital Microscope U500X, Guangdong, China). The microscope is connected to IBM compatible computer. WSxM software (Version 5 develop 4.1, Nanotec, Electronica, SL) was used to analyze the photos and to create a 3D image of the specimen surface. The average SR was estimated using WSxM software and expressed in μm . SR was measured at baseline, after demineralization and after remineralization.

Statistical analysis

The mean SMH values and the mean SR values were statistically analyzed. One-way ANOVA followed by Tukey's post hoc test were performed to compare remineralizing potential at different time intervals (2,4,6 days). Furthermore, the same tests were used to compare enamel surfaces within the same group. The significant level was set at 0.05. Statistical analysis was performed with SPSS 18.0 for Windows (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA).

Results

SEM examination

Sound enamel has a smooth surface with some pits and scratches (Figure 1A, Figure 2A & Figure 3A). After acid etching different etching patterns were seen, most commonly type I and type II with scattered areas of type III (Figure 1B, Figure 2B & Figure 3B). After remineralization, G1 revealed partial occlusion of some rod cores with clearly thickened interprismatic substance (Figure 1C) while in G2 prismatic enamel

Table 1. Specimens grouping and intervention.

Group	Demineralization	Remineralization	Time of application	Follow up
G1 (n = 31)	37% phosphoric acid gel for 1 min.	Agarose hydrogel 2mm thickness	48 hours	2 days
G2 (n = 31)			96 hours, hydrogel changed every 48 hours.	4 days
G3 (n = 31)			144 hours, hydrogel changed every 48 hours.	6 days

G1 (2 days), G2 (4 days), G3 (6 days).

Figure 1. Scanning electron microscope (SEM) images for G1; sound enamel at baseline (A), demineralized enamel surface (B), remineralized enamel surface (C).

Figure 2. Scanning electron microscope (SEM) images for G2; sound enamel at baseline (A), demineralized enamel surface (B), remineralized enamel surface (C).

Figure 3. Scanning electron microscope (SEM) images for G3; sound enamel at baseline (A), demineralized enamel surface (B), remineralized enamel surface (C).

configurations became hidden by mineral depositions (Figure 2C). G3 revealed a relatively smooth surface with less clearly seen rod ends. Some rods' peripheries showed complete remineralization while others were still empty (Figure 3C).

SMH analysis

The mean SMH values of enamel at different intervals (2,4,6 days) are presented in Table 2. In G1, significant differences were revealed between baseline, demineralized and remineralized

enamel ($p < 0.05$) with the highest SMH at baseline. While in G2 and G3, there was a significant difference between the baseline and the demineralized enamel ($p < 0.05$), however there wasn't a significant difference between baseline and remineralized enamel. Furthermore, there were significant differences among the remineralized enamel surfaces of different groups ($p < 0.05$) with the highest SMH at G3.

SR analysis

The mean SR values of enamel at different intervals (2,4,6 days) are presented in Table 3. In G1, there were significant differences between baseline, demineralized and remineralized enamel ($p < 0.05$) with the highest SR at the demineralized enamel. While in G2 and G3, there was a significant difference between the baseline and the demineralized enamel ($p < 0.05$), however there wasn't a significant difference between baseline and the remineralized enamel. Furthermore, there were significant differences among the remineralized enamel surfaces of different groups ($p < 0.05$) with the highest SR in G1. The differences in SR at baseline, demineralized enamel and after remineralization in different groups were obvious when inspecting the 3D images in Figure 4.

Dataset 1. Raw surface microhardness (SMH) and surface roughness (SR)

<http://dx.doi.org/10.5256/f1000research.16050.d217398>

Dataset 2. Raw scanning electron microscope (SEM) images

<http://dx.doi.org/10.5256/f1000research.16050.d217399>

Discussion

Biomimetic synthesis of enamel like apatite structures under a physiological condition is an alternative restorative pathway¹⁰. Acid etching technique was used to mimic early enamel lesions because of the simplicity and reproducibility of this technique¹¹. SEM results of the present study are in agreement with previous studies⁶⁻⁹. Agarose hydrogel acted as enamel organic matrix to control the size and form of the formed hydroxyapatite crystals through the interaction between hydroxyl group of agarose and calcium. In addition, it acts as a mineral reservoir for continuing remineralization⁷. The SR analysis results confirmed the SEM results, as the SR values were gradually decreased between different groups which revealed a smoother enamel surface. SMH results are in accordance with previous studies^{7,9}. In the current work, the lower SMH than sound enamel could be attributed to incomplete compaction of formed crystals on enamel surface¹².

Conclusions

Agarose hydrogel model have a remineralizing potential to treat early carious lesion. Further studies are required to clarify the stability of agarose hydrogels in clinical application.

Table 2. Analysis of Surface microhardness (SMH) (Kg/mm²).

	SMH-B	SMH-D	SMH-R	P-value
G1 (2D)	254.377±24.73 ^a	171.138±15.23 ^c	196.864±9.74 ^{b,C}	< 0.00001*
G2 (4D)	251±35.88 ^a	171.84±32.42 ^b	218.485±14.76 ^{a,B}	0.000028*
G3 (6D)	256.842±24 ^a	175±8.98 ^b	242.433±14.36 ^{a,A}	<0.00001*
P-value	0.911069	0.918935	< 0.00001*	

Baseline (B), after demineralization (D), after remineralization (R).
Different upper and lower-case superscript letters indicate significant difference between tested groups at $P < 0.05$. Lower case superscript letters are used for comparison within the same row and upper case letters are used for comparison within each column.

Table 3. Analysis of Surface roughness (SR) (μm).

	SR-B	SR-D	SR-R	P-value
G1 (2D)	0.253±0.0009 ^c	0.274±0.0025 ^a	0.2663±0.002 ^{b,A}	< 0.00001*
G2 (4D)	0.256±0.0096 ^b	0.275±0.0026 ^a	0.258±0.003 ^{b,B}	< 0.00001*
G3 (6D)	0.254±0.00027 ^b	0.275±0.003 ^a	0.255±0.003 ^{b,C}	< 0.00001*
P-value	0.376997	0.508623	< 0.00001*	

Baseline (B), after demineralization (D), after remineralization (R).
Different upper and lower-case superscript letters indicate significant difference between tested groups at $P < 0.05$. Lower case superscript letters are used for comparison within the same row and upper case letters are used for comparison within each column.

Figure 4. Representative Surface roughness (SR) images of enamel specimens; baseline **A**, demineralized enamel **B**, remineralized enamel surfaces **C**, **D**, **E** (G1, G2, G3 respectively).

Data availability

Dataset 1: Raw surface microhardness (SMH) and surface roughness (SR) [10.5256/f1000research.16050.d217398](https://doi.org/10.5256/f1000research.16050.d217398)¹³

Dataset 2: Raw scanning electron microscope (SEM) images [10.5256/f1000research.16050.d217399](https://doi.org/10.5256/f1000research.16050.d217399)¹⁴

Grant information

The author(s) declared that no grants were involved in supporting this work.

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Reviewer Report 17 October 2018

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Mahmoud M. Bakr

School of Dentistry and Oral Health, Griffith University, Nathan, Qld, Australia

The study by El Moshy *et al.* is well designed and investigates an important topic with potential clinical applications.

However, there are a few issues that need to be addressed to improve the quality of the manuscript:

1. Introduction and discussion sections are extremely short and do not cite enough literature on the topic.
2. Discussion section besides being short is basically a repetition of some results without discussing technical aspects of the study and comparing it to previous similar studies.
3. The quality of some of the images could be improved.
4. The statistical analysis is not a true representation of the results and could be inaccurate. The effect of time is neglected in this case. Ideally a two way ANOVA should be used to illustrate the interaction between time and treatment. If no interaction was observed then the single main effects of time and/or treatment could be reported then. Using the post hoc analysis is not the best practice as it neglects the effect of time and will most likely lead to false-positive results.
5. Some images for illustration of the techniques used in the materials and methods section would be helpful.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 15 October 2018

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Mahmoud M. Al-Ankily

Oral Biology Department, Faculty of Dentistry, British University in Egypt, Cairo, Egypt

This report by El Moshy et al. examines biomimetic remineralization of acid etched enamel using agarose hydrogel model. The authors' inclusion of Agarose hydrogel model has a remineralizing potential to treat early carious lesion. The study, although it is small, adds knowledge to the existing literature.

Minor comments would help to improve the impact of this paper:

Methods

Specimens preparation: It is better to use premolars extracted for orthodontic treatment, the surface of enamel is usually intact and standard in enamel rods orientation, crystallization and size rather than mandibular third molars with the anatomical varieties, surgical instrumentation and surface irregulars.

Remineralization: (Agarose hydrogel and phosphate solution were prepared as previously mentioned) there is no previously mentioned information about hydrogels and Phosphate Solution Preparation.

Surface roughness (SR) analysis: The average SR was expressed in 1 µm only. how can you be sure

that you measure SR at baseline, after demineralization and after remineralization of the same 1 μ m every time. It is recommended to use another type of surface roughness analysis with wider surface area at least 25 μ m like AFM.

Results

SEM examination: Figures x3000 is not good enough to show baseline, after demineralization and after remineralization changes also x5000 is not so clear please do not minimize them to save the magnification benefits. It is recommended to use 20000 to 50000 to show the crystals, regenerated mineralized tissue and crystal orientation.

Discussion

Needs more details about mechanism of growth of the enamel crystals and the mechanical properties of the regeneration tissue.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 15 October 2018

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Mohamed Shamel

Oral Biology Department, Faculty of Dentistry, Modern Sciences and Arts University, Cairo, Egypt

The current study performed by El Moshy et al, is an interesting one that aims to investigate the possible biomimetic effect of agarose hydrogel in remineralizing a human demineralized enamel model.

Overall, the study is well constructed and presented with the results efficiently supporting the discussion and conclusions.

However some minor points might be helpful in adding to the study:

- SEM images used were cropped from the original images which caused some blurriness in the images.
- A more detailed statistical analysis needs to be performed on the relatively large amount of data obtained.
- The discussion section needs to be more detailed as it is too short in comparison to the results obtained.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 05 October 2018

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Nehad Samir Taha

Oral Biology Department, Faculty of Oral and Dental Medicine, Misr International University (MIU),
Cairo, Egypt

Excellent work:

1. The study design is appropriate to the work done.
2. Replication of this study could be applicable in the future based on the study results.
3. The statistical analysis is appropriate to the study.
4. In my opinion, the study results efficiently support the conclusions drawn.

But, I had some questions about:

1. The acrylic templates: how to use it, and whether it is standard in size.
2. No references for the etching pattern.
3. Some pictures are hazy and with no arrows.

Further comments:

1. In the Methodology: I would prefer if you could show us in one picture the differences between a defected specimen that you excluded and one that you choose.

2. In the Results:

- In Figure 1: no arrows to demonstrate the different types of acid etching.
- In Figure 1C: no arrows to show the closed rod cores.
- In Figure 2C: no arrows to show the areas of mineral deposition.
- No reference to the acid etching classification.
- Finally, I preferred to see a figure plate comparing the 3 states together to be easy to compare (i.e.: a plate of Figures 1A, 2A and 3A, a plate of Figures 1B, 2B and 3B, and a plate of Figures 1C, 2C and 3C).

3. The discussion is too short to clarify the findings of the study.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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Raneem Farouk Obeid

Department of Oral Biology, Future University in Egypt, New Cairo, Egypt

Radwa Taher el sharkawy

Faculty of Dentistry, Future University in Egypt, New Cairo, Egypt

Good work, but I have some comments to clarify my confusion:

1. In the Methodology: the acrylic template - why and how to use? And do you standardize the 2mm in this template?
2. In the Results:
 - In Figure 2 you mention acid etch type? Where is the reference of this classification and arrows to show the different type?
 - Picture C is hazy, please change it.
 - Figure 3: please put arrows to show us rod complete and empty one in Picture C.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Comments on this article

Version 1

Reviewer Response 26 Sep 2018

Radwa Elsharkawy, Future University in Egypt, New Cairo, Egypt

Interesting topic, here are some points to be considered:

1. In the methodology: specimens with defects (erosions, cracks, visible stains, hypo-calcification) were excluded and you mentioned that teeth are surgically extracted third molar, erosion and visible stains detected in erupted teeth not impacted ones.
2. Etching pattern in SEM results with no reference.
3. Discussion is too short.

Competing Interests: No competing interests were disclosed.

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