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Comparative study on the effect of Grape seed extract and sodium fluoride on demineralized Cementum of Human premolar samples

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Abstract:

Aim: This study aimed to investigate: both effect of Grape seed extract and NaF on demineralized cementum of human premolar samples using Scanning electron microscope and Energy-Dispersive X-ray Analysis (EDX)-Polarized light microscope (PLM).

Materials and Methods: Thirty maxillary first premolars were assigned to three main groups (I,II,III) as the following: GI was a control group, GII was treated with grape seed extract and GIII was treated with sodium fluoride. Group I was equally divided into two subgroups, five premolars each: a negative control (received no treatment) and a positive control (after immersed in demineralizing solution). The specimens were subjected to pH cycling twice daily for two weeks, then analyzed by scanning electron with EDX and polarized light microscopy.

Results: SEM analysis showed regular narrow cracks in some areas with decreasing signs of resorption on the mineralized cementum in both groups II,III. Polarized light microscopic analysis showed mineral precipitation band on the surface of treated cementum lesions without area of demineralization. Wide birefringent zone known as remineralizing zone (RZ) was also observed in GII. While in GIII, thin birefringent zone was also observed.

Conclusion:Based on the data obtained in this *in vitro* study, it is suggested that grape seed extract promote remineralization of artificial cementum carious lesions probably through a different mechanism than that of sodium fluoride. GSE might be considered an effective natural agent in treatment of early carious lesions.

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Introduction:

Root caries is especially prevalent among the elderly population due to gingival recession and the exposure of susceptible root surface (*Tyagi et al., 2014*).

The management of incipient caries (white spot lesions) might include primary prevention of caries progression, non-invasive treatment or minimally invasive operative treatment of the incipient lesion (*Machale et al., 2013*). For this purpose, newer proposed concepts for treatment were introduced as the use of natural products to enhance remineralization that could have a protective effect against demineralization (*Mirkarimi et al., 2013; Heshmat et al., 2014 & Jawale et al., 2017*).

Grape seeds extract are rich in polyphenolic compounds having significant human health benefits. These polyphenolic compounds are called flavonoids (*Fine, 2000 & Joshi et al., 2001*). It verified that GSE, composed mainly of PA, has antimicrobial activity (*Aldini et al., 2003*). Proanthocyanidins are present in flowers, nuts, fruits, bark, and seeds of various plants, as a defense against biotic and abiotic stressors (*Prabhakar et al., 2012*). Moreover, it can positively affect the tooth structure, thus offering a new therapy for carious lesions (*Elumalai, et al., 2018*).

Some studies were performed to evaluate the effect of GSE on the remineralization of artificial root caries. The effect of grape seed extract was compared with that of fluoride in a study which reported that grape seed extract positively affected the remineralization processes of artificial root caries lesions, most likely through a different mechanism than that of fluoride. So GSE might serve to be a promising adjunct or alternative to fluoride in the treatment of root caries during minimally invasive therapy (*Xie et al., 2008 & Benjamin et al., 2012*).

Benjamin et al; (2012) evaluated the potential remineralizing effect of grape seed extract (GSE) on artificial root caries lesions. The sections of human teeth were divided into four treatment groups including: 6.5% GSE, sodium monofluorophosphate (220ppm) with 0.05% calcium glycerophosphate, 0.5% calcium glycerophosphate and control (no treatment).

GSE revealed less demineralization and more remineralization as compared to the other groups.

The effects of three flavonoids, including proanthocyanidin (PA), naringin (NR) and quercetin (QC) on remineralization of artificial root caries were compared. All three flavonoids showed positive effects on artificial root caries remineralization, which were significantly lower than that of 1000 ppm fluoride (*Dogan et al., 2004 & Epasinghe et al., 2016*).

Epasinghe et al; (2017) evaluated the effect of proanthocyanidin (PA) in combination with tri-calciumphosphoshate (TCP) and fluoride (F) on artificial caries lesion. Lesion depth and mineral loss was evaluated using microradiography and confocal laser scanning microscopy. The lowest lesion depth and mineral loss were observed in the TCP +F+PA group. The addition of (PA) to (TCP)+F significantly reduced collagen degradation depth, when compared to TCP only group. Lesion depth was the lowest in the PA and TCP+F+PA groups following collagenase degradation.

MATERIALS & METHODS

Samples:

30 maxillary first premolars were selected and stored after cleaning and removing debris in 0.1% thymol solution until used. The decayed and damaged teeth were excluded.

Grouping:

The teeth were divided into three groups (I,II,III) ten premolars each, GI was divided into two subgroup-ve GI, +ve GI five teeth in each subgroup so they were, GII, GIII. According to the procedure done: group I (control group), group II (treated with grape seed extract) and group III (treated with sodium fluoride).

Sample Preparation:-

In the middle third of the buccal surfaces of all teeth a window 4x2 mm² was created by covering the selected area with adhesive tape (Silkplast Adhesive Tape, Pharmaplast, Egypt) then the remaining of the buccal surfaces was covered by nail varnish (Maybellene, france). After the varnish was applied the adhesive tape

was removed. Testing done by using ESEM and EDXA to obtain the negative control.

creation of lesions:

by immersing Experimental specimens individually into the demineralizing solution (10ml/tooth for 96 hours at room temperature) (Tschoppea et al., 2011& Benjamin et al., 2012). Testing done by using ESEM and EDXA to obtain the positive control.

preparation of solutions:

A 6.5%(w/v) grape seed extract solution was prepared A 6.5% in phosphate buffer, 1000 ppm Sodium fluoride solution. The two previous solutions obtained from Elalamia for chemicals in Tebeen area. Artificial saliva as buffering solution was used to imitate the oral environment with the following: NaCl (0.67 g/l), C₆H₁₄O₆ (24 g/l), KCL (0.96 g/l), CaCl₂ (0.1168 g/l), MgCl₂ (0.0408 g/l), C₈H₈O₃ (1 g/l), KH₂PO₄ (0.274 g/l), H₂O (964.938 ml/l) PH 7 (Silva et al., 2015). The artificial saliva was prepared at the Laboratory of Pharmaceutical Sciences, Faculty of Pharmacy, Cairo University. The demineralizing solution was used to induce caries like lesion on cementum surfaces. The solution was freshly prepared in Faculty of Pharmacy, Ain Shams University. [50 mM acetate, 2.25 ml calcium chloride (CaCl₂), 2H₂O, 1.35 ml Potassium Dihydrogen phosphate (KH₂PO₄); 130 mm Potassium chloride (KCl) for pH=5.0].

pH cycling: (Table 1)

<i>pH cycling method</i>	<i>Time</i>
<i>1. First immersion in remineralizing agent (GSE or NaF)</i>	<i>10 Minutes</i>
<i>2. Artificial saliva</i>	<i>30 Minutes</i>
<i>3. Demineralizing solution</i>	<i>3 Hours</i>
<i>4. Artificial saliva</i>	<i>8:20 Hours</i>
<i>5. Second immersion in remineralizing agent (GSE or NaF)</i>	<i>10 Minutes</i>
<i>6. Artificial saliva</i>	<i>30 Minutes</i>
<i>7. Demineralizing solution</i>	<i>3 Hours</i>
<i>8. Artificial saliva</i>	<i>8:20 Hours</i>

Examination was done to the middle third of labial surface, which was cleaned from any remaining solution then examined by scanning electron microscope, Energy Dispersive X-ray Analysis (EDXA) and polarized light microscope.

Results

Scanning electron microscope: (SEM) examination:

- **Group I (Control group):**
- **Negative control no treated cementum (-ve I):**
SEM examination of the roots of premolar teeth of the control group demonstrated the cementum where examination of the cervical root surface showed large number of evenly distributed sharp's fibers bundles, in between them the intrinsic fibers, and regular narrow cracks with no signs of resorption on the mineralized cementum were detected.

The cervical thirds of the roots, which are covered by acellular cementum, exhibited a smooth surface (*Fig.1 A&B*).

- **Positive control (demineralized cementum) (+ve I):**

SEM examination of the roots of premolar teeth of the positive control group demonstrated An irregular widely cracked cementum surface of the cervical third which are covered by acellular cementum Some areas appeared with detachment of Sharpey's fibers, and area of resorption was detected (*Fig.1 C,D, E&F*).

GroupII (treated with Grape seed extract):

After pH cycling:

SEM examination of the cervical root surface showed regular narrow cracks with decrease signs of resorption on the mineralized cementum and the cervical thirds of the roots, which are covered by acellular cementum, exhibited a smooth surface, dispersed mineral precipitation and calcific deposits were observed (*Figs.1G&H*).

Group III C (treated with NaF):

After pH cycling:

SEM examination of the cervical root surface showed regular narrow cracks in some areas with decreasing signs of resorption on the mineralized cementum and the cervical thirds of the roots, which are covered by acellular cementum, exhibited a smooth surface and calcific deposits were observed (*Figs.1I&J*).

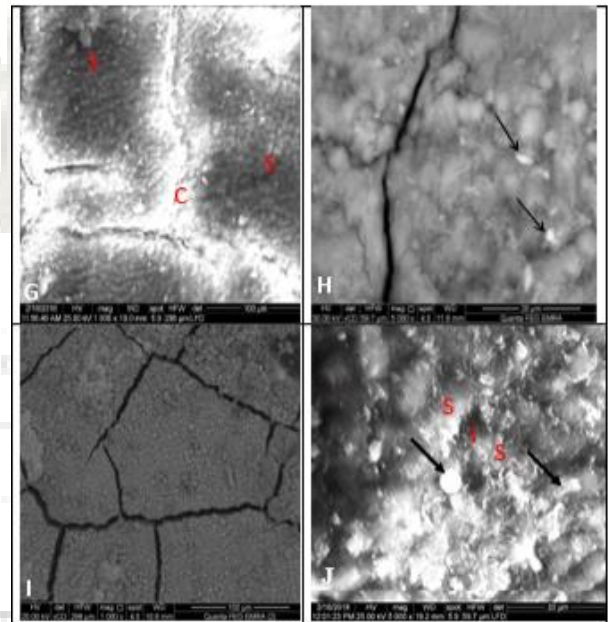
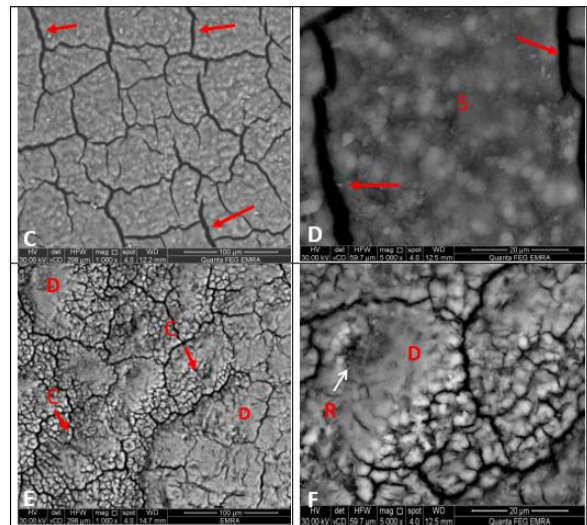
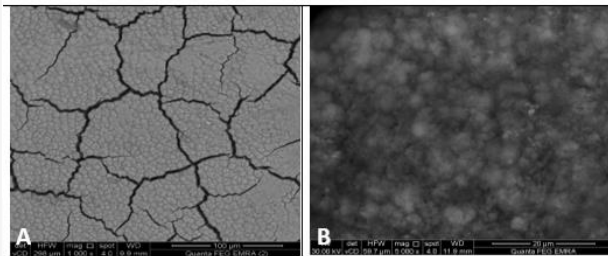


Fig. (1): Scanning electron micrograph of :

- A) Negative GI showing smooth surface with regular narrow cracks with no evidence of resorption. (Orig. Mag.x1000)
- B) Higher magnification of the previous Figure showing sharpy's fibers bundles and intrinsic fibers inbetween (Orig. Mag.x5000)
- C) Positive GI showing an irregular widely cracked rough cementum surface (redarrows) (Orig. Mag. X 1000)
- D) Higher magnification of the previous Fig. showing an irregular widely cracked cementum surface (red arrows) and Sharpey's fibers bundles (S) (Orig. Mag. X 5000)

- E) Positive GI showing irregular cementum surface with detachment of sharpy's fibers, and multiple cracks (C) (Orig. Mag.x1000).
- F) Higher magnification of the previous Fig.28 showing irregular cementum surface with detachment of sharpy's fibers (D), and area of resorption (R) (Orig. Mag. X 5000)
- G) GII showing regular narrow cracks in cervical part of cementum (C) and sharpy's fibers bundles (S) (Orig. Mag.x1000).
- H) Higher magnification of the previous Fig. 31 showing sharpy's fibers bundles without detachment. Calcific deposits were also observed (black arrows) (Orig. Mag.x5000).
- I) GIII showing regular narrow cracks in cervical part of cementum with no evidence of resorption (Orig. Mag.x1000).
- J) Higher magnification of the previous Fig.34 showing sharpy's fibers bundles (S), and in between the intrinsic fibers (I) Calcific deposits were also observed (black arrows) (Orig. Mag. X 5000)

- **Polarized light microscope:**

Group I (Control group):

- Negative control (Base line) no treated cementum (-ve I):

The microscopic analysis of the samples of negative group IC showed sound cementum surface with no signs of demineralization and no negative birefringence (**Fig. 2 A**).

- Positive control (demineralized cementum) white spot lesions (+ve I):

Microscopic analysis of the samples of positive group IE revealed a dark brown stain associated with demineralization effect caused by acid on the cementum surface (negative birefringence) (**Fig.2B**).

GroupII (treated with Grape seed extract):

Microscopic analysis of this group revealed that mineral precipitation band appeared on the surface of treated cementum lesions without area of demineralization. Wide birefringent zone known as remineralizing zone (RZ) was also observed (**Fig.2C**).

Group III (treated with NaF):

Microscopic analysis of this group revealed that mineral precipitation band appeared on the surface of treated enamel lesions without negative birefringent zone. Thin birefringent zone was also observed (**Fig. 2D**).

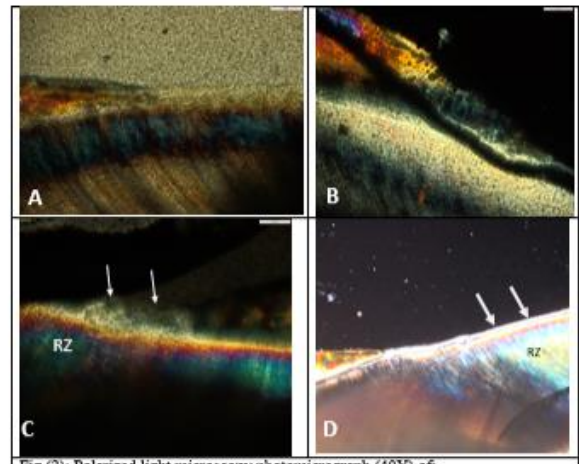


Fig (2): Polarized light microscopy photomicrograph (40X) of:

- Negative GI showed absence of negative birefringence with no area of demineralization in enamel surface
- Positive GI dark brown stain area.
- GII showed remineralizing zone (RZ) and mineral precipitation band (white arrows).
- GIII showed remineralizing zone (RZ) and mineral precipitation band (white arrows).

Statistical analysis:

Calcium:

The highest mean value was recorded in Group I (negative control) (38.76 ± 1.79), followed by Group II-GSE (38.1 ± 1.28), then group III-Naf (36.12 ± 1.36), with the least value recorded in Group I (+ve control-demineralized) (32.82 ± 1.84). ANOVA test revealed a statistically significant difference between groups ($p=0.00$). Tukey's post hoc test revealed no significant difference between group I (negative control) and Group II-GSE (Table 2, Fig. 3, 5)

Phosphorus:

The highest mean value was recorded in Group I (negative control) (22.26 ± 1.30), then Group II-GSE (20.26 ± 0.45), then group III-Naf (20.00 ± 0.74), with the least value recorded in Group I (+ve control-demineralized) (18.65 ± 0.43). ANOVA test revealed a statistically significant difference between groups ($p=0.00$). Tukey's post hoc test revealed no significant difference between Group II-GSE and group III-Naf (Table 2, Fig.4, 5)

Table (2) Descriptive statistics and significance of difference between groups regarding Ca and P (Wt%) in cementum (ANOVA test)

Significance level $p \leq 0.05$, *significant
Tukey's post hoc test: Within the same comparison, means sharing the same superscript letter are not significantly different

Cementum		Mean	Std. Dev	Std. Error	95% Confidence Interval for Mean		Min	Max	F	P
					Lower Bound	Upper Bound				
Ca	Group I (negative control)	38.76 ^a	1.79	.57	37.48	40.05	36.59	40.98	28.282	.000*
	Group I (+ve control-demineralized)	32.82 ^c	1.84	.58	31.50	34.13	30.36	34.78		
	Group II-GSE	38.10 ^a	1.28	.40	37.18	39.01	36.59	39.72		
	Group III-Naf	36.12 ^b	1.36	.43	35.15	37.09	34.78	37.98		
P	Group I (negative control)	22.26 ^a	1.30	.41	21.33	23.20	20.88	24.01	33.754	.000*
	Group I (+ve control-demineralized)	18.65 ^c	.43	.14	18.34	18.96	18.07	19.09		
	Group II-GSE	20.26 ^b	.45	.14	19.94	20.59	19.88	20.90		
	Group III-Naf	20.00 ^b	.74	.23	19.47	20.53	19.09	20.90		

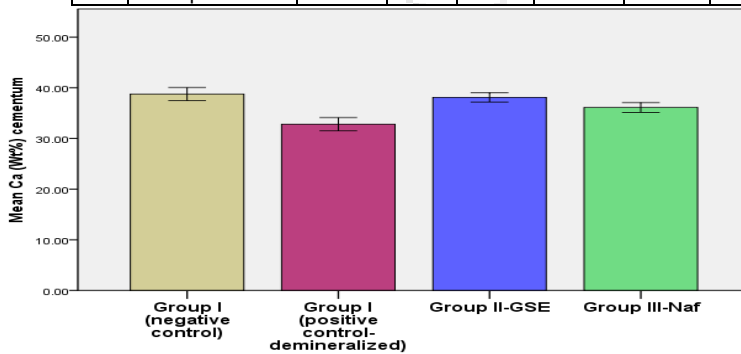


Fig. (3) Bar chart illustrating mean calcium (Wt%) in cementum in different groups

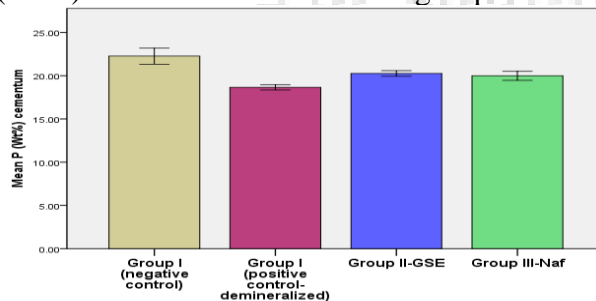


Fig. (4) Bar chart illustrating mean phosphorus (Wt%) in cementum in different groups

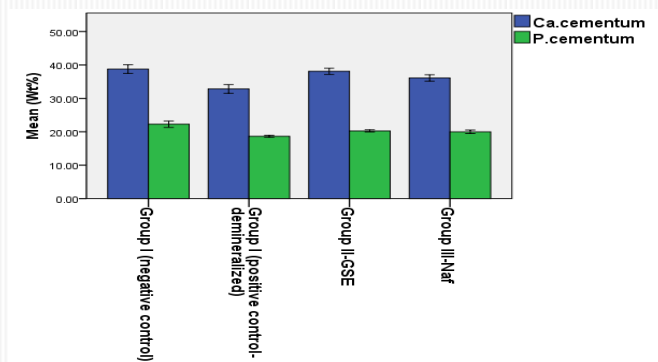


Fig. (5) Bar chart illustrating mean calcium and phosphorus (Wt%) in cementum in different groups

Discussion

Dental caries is one of the most commonly encountered dental diseases. New preventive and therapeutic approaches have rapidly evolved in the past few years to manage dental decay (*Hannig and Hannig, 2010 & Vaderhobli, 2011*).

Several reseaches focused on natural products to be used as new therapeutic agents. Grape seed extract (GSE) is a rich source of proanthocyanidin (PA), mainly composed of monomeric catechin and epicatechin, gallic acid and polymeric, and oligomeric procyanidins. PA, can positively affect the tooth structure, thus offering a new therapy for carious lesions (*Elumalai, et al., 2018*).

SEM photomicrograph demonstrated the generalized smooth surface architecture and regular narrow cracks of cementum (-ve GI). Surface irregularities, widely cracked and rough cementum surface of the cervical third which are covered by acellular cementum of demineralized cementum (+ve GI) were noted after the demineralization process. Then the surface architecture of the cementum exhibited smoother area after treated with both GSE (GII) and NaF (GIII). These results were in agreement with those of (*Juntavee et al., 2018*). They showed that among the groups tested by the X-ray diffraction, the remineralization of cementum upon using NaF is possibly associated with the exchange of minerals between the cementum surface and the surrounding environment, as is found in the enamel.

The process of root caries, this involves two steps: in addition to the dissolution of hydroxyapatite by acid challenge, as in the enamel, there is also the degradation of the organic matrix by proteases saliva, or bacteria. It is well known that the structure and the stability of the collagen matrix are essential for its correct mineralization. In addition, PA increase the synthesis of collagen and decreases the rate of enzymatic degradation of the collagen matrix (*Silva et al., 2015*).

In the present study, it was found that the recovery of mineral content was more in GIIC compared to GIIC group. This revealed with a significant increase in Ca/P ratio after remineralization with GSE. This result in contrast with *Xie et al., 2008* who revealed that The remineralization effect of GSE appears to be distinct from that of fluoride treatment based on a microhardness tester.

In the present study, GSE treated cementum (GIIC) demonstrated increased Ca/P value, wider precipitation band than NaF (GIIC). This effect was not contributed by fluoride since the fluoride concentration in 6.5% GSE solution was less than 0.01 ppm. According to *Xie et al., 2008 and Silva et al., 2015* probably after treatment with the grape seed extract, it is combined with the Ca²⁺ from the remineralizing solution and may enhance remineralization. Suggested mechanism of its action is that it contributes to mineral deposition on the superficial layer of the lesion. GSE is said to form visually insoluble complexes when mixed with remineralizing solution at pH 7.4. GSE may interact with the organic portion of root dentin through PA – collagen interaction, thereby stabilizing the exposed collagen matrix.

In current study, polarized light microscopy photomicrograph of GIIC (treated with GSE) showed remineralizing zone (RZ) and wide mineral precipitation band than in GIIC (treated with NaF) and supported by less lesion depth. These findings were in accordance with those of *Benjamin et al., 2012*. They reported that GSE inhibits demineralization and promotes remineralization of artificial root carious lesions better than fluoride and Calcium

glycerophosphate. Their results based on The relative optical density of GSE that was significantly higher when compared with the other group which implies a higher degree of remineralization.

Conclusion

it is suggested that grape seed extract promote remineralization of artificial cementum carious lesions probably through a different mechanism than that of sodium fluoride.

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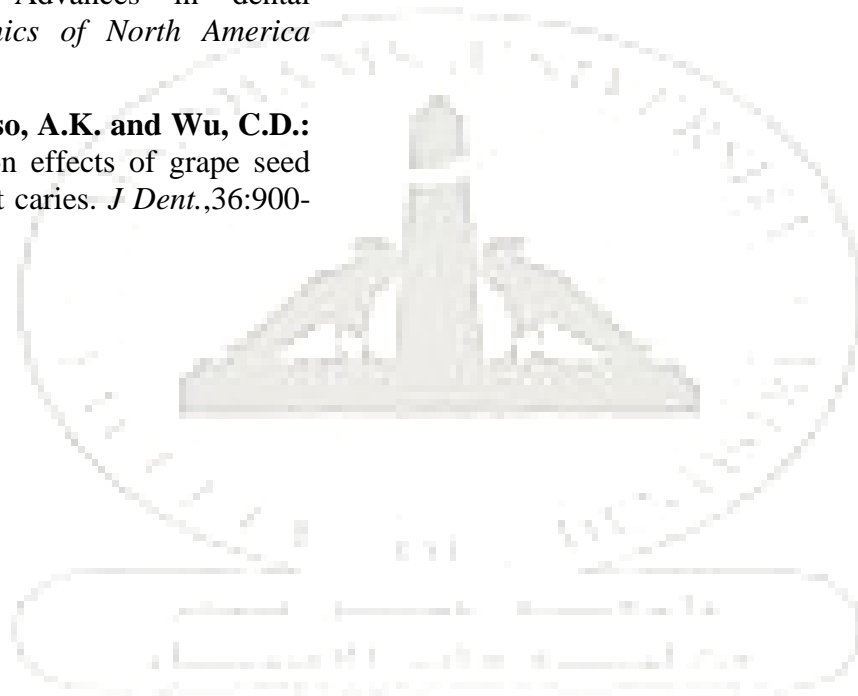
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