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Studies on the Effect of Epigallocatechin Gallate-Carboxymethyl Chitosan Hydrogel on the Remineralization of Early Root Surface Caries

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Abstract: Objective: To observe in vitro the effect of epigallocatechin gallate-carboxymethyl chitosan hydrogel on the remineralization of early root surface caries. METHODS: Freshly extracted healthy bovine incisors were selected and prepared into dental bone blocks, and after establishing an artificial caries model, they were randomly divided into the epigallocatechin gallate-carboxymethyl chitosan (EGCG-CMCS) hydrogel group, the carboxymethyl chitosan (CMCS) hydrogel group, the epigallocatechin gallate solution (EGCG) group, the deionized water group, and the 2% NaF solution group, and each group was remineralized. The remineralization treatment was carried out; SEM was used to observe the surface morphology of the remineralized specimens, X-ray spectrometry was used to analyze the calcium-phosphorus ratio before and after remineralization of the specimen surfaces, a microhardness tester was used to detect the hardness value of the specimen surfaces, and a fluorescence microscope was used to detect the staining of the specimen slices. RESULTS: After remineralization treatment, scanning electron microscopy observation showed that spherical particles were deposited and flattened on the surface of the dentin in both the EGCG-CMCS hydrogel group and the 2% NaF group; the calcium-phosphorus mass ratio and atomic ratio of the dentin samples in the EGCG-CMCS hydrogel group and the dentin samples before demineralization were closest to each other, and there was no statistically significant difference between the two groups ($P > 0.05$); the microhardness results showed the hardness values of specimen sections stained via the 2% NaF and EGCG The microhardness results showed that those treated by 2% NaF and EGCG-CMCS hydrogel were better; the fluorescence intensity of the treated dental bone samples was the weakest in the EGCG-CMCS hydrogel group, followed by the 2% NaF group. Conclusion: Epigallocatechin gallate-carboxymethyl chitosan hydrogel has a good remineralization effect on early root surface caries and has the value of clinical application.

Keywords: Epigallocatechin Gallate; Carboxymethyl Chitosan; Remineralization; Root Surface Caries.

1. Introduction

Root surface caries is a common dental disease that is closely related to changes in the oral environment. Caries-causing bacteria produce acids during metabolism, which continue to erode the dentin and break the dynamic balance between mineralization and demineralization of the tooth, thus triggering the lesion[1] .

As a natural polyphenol derived from tea, EGCG has significant antioxidant and antibacterial properties [2][3]. Previous studies have shown that it can effectively inhibit the growth of caries-causing bacteria such as Streptococcus pyogenes in the oral cavity and reduce the production of acid products, thus minimizing the risk of tooth demineralization at source [4][5]. At the same time, EGCG can promote mineral deposition and enhance tooth remineralization [6], which provides a theoretical basis for its application in oral disease prevention and treatment. CMCS, combined with EGCG, is a biocompatible polymer. In the complex environment of the oral cavity, CMCS can form a stable membrane structure, which not only serves as a carrier for the continuous release of EGCG and prolongs its duration of action [7], but also increases the local concentration of EGCG at the lesion site and synergistically enhances the remineralization effect. In this experiment, we investigated the effect of EGCG-CMCS hydrogel on the remineralization of demineralized dental bone to provide an experimental basis for further analysis of the anti-caries mechanism of EGCG-CMCS hydrogel.

2. Materials and Methods

2.1. Main Materials and Instruments

EGCG (Shanghai Macklin Biochemical Technology Co., Ltd.);

CMCS (Beijing Solarbio Technology Co., Ltd.);

NaF (Shanghai Macklin Biochemical Technology Co., Ltd.);

Muscimol (Beijing Solarbio Technology Co., Ltd.);

Acid Resistant Nail Polish (Lumineau, China);

Artificial saliva (ISO/TR10271 standard): NaCl 0.4 g, KCl 0.4g, $CaCl_2 \cdot 2H_2O$ 0.795g, $NaHPO_4 \cdot 2H_2O$ 0.78g, $Na_2S \cdot 2H_2O$ 0.005 g, urea 1 g, and DDW 1000 ml, pH=6.8;

Artificial acid etching solution: $Ca(NO_3)_2$ 2.2 mmol/L, KH_2PO_4 2.2 mmol/L, CH_3COOH 50 mmol/L, pH=4.7;

Automatic Turret Digital Vickers Hardness Tester (KOZI HSV-50Z, China)

Scanning electron microscope (Thermo Fisher Apreo 2C, USA);

Ultrasonic shock machine (Jiemeng JP-080ST, China)

X-ray spectrometer (HORIABA EX-250, Japan)

Body microscope (Zeiss Stemi 508, Germany);

Constant temperature shaking bath (Dongxin SHA-CA, China)

Fluorescence microscope (OLYMPUS BX53, Japan)

2.2. Specimen Preparation and Root Surface Caries Modeling

Freshly extracted bovine incisors without caries, osteochondral hypoplasia, and cracks were selected. The periodontal soft tissues and contaminants on the root surface were removed, and the root was ultrasonically washed with deionized water for 10 min, and the crown and root of the treated isolated teeth were separated by a high-speed turbine at 2 mm below the enamel-cementum boundary, and the roots of the teeth were cut into 95 pieces of 4 mm*3 mm*2 mm osteoid blocks using a hard-tissue osteotome. After ultrasonic swishing for 1 min to remove the debris produced by sample preparation as well as the tarnish layer, the sample blocks were dried naturally, covered with a double layer of acid-resistant nail polish outside the experimental area of the osseous surface of the teeth and placed in a deionized aqueous solution containing 0.05% muscimol for storage at 4℃.

The demineralization of the odontoblast was placed in an artificial acid etching solution in a shaking thermostatic water bath at 37° C. After demineralization, it was ultrasonically swished and washed in deionized water for 5 min to form a model of early root surface caries. Success criteria for artificial caries: chalky color change on the surface of the dentin block, rough surface of the dentin block observed under the electron microscope, and honeycomb structure produced.

2.3. Preparation and Grouping of Drugs

2.3.1. Preparation and Grouping of Hydrogels

Preparation of EGCG-CMCS hydrogel: 0.2g of CMCS+ 20mg of EGCG was weighed in a 15.0ml centrifuge tube, placed on a sterile operating table, sterilized by UV irradiation for 1.0h, and then added with 10.0ml of sterilized deionized water, fully dissolved to form an EGCG-CMCS hydrogel, which was stored at 4.0° C and protected from light.

Preparation of CMCS hydrogel: 0.2g of CMCS was weighed in a 15.0ml centrifuge tube, placed on a sterile operating table, sterilized by UV irradiation for 1.0h, and then added with 10.0ml of sterilized deionized water to form a 0.2% CMCS hydrogel after full dissolution, and then stored in a protected place at 4.0° C. The CMCS hydrogel should be stored at a protected place at a protected place at a protected place at a protected time.

EGCG solution preparation: EGCG solution at a concentration of 2 mg/ml was prepared in distilled water after autoclaving.

Ninety-five odontoblasts successfully modeled for root surface caries were taken and randomly and equally divided into five groups totaling 19 odontoblasts each in A (EGCG-CMCS hydrogel), B (EGCG solution), C (CMCS hydrogel), D (2% NaF), and E (deionized water).

2.4. Remineralization

The samples of each group were rubbed with a small brush with the treatment solution of the group for 10min→acidic buffer treatment for 30min→artificial saliva immersion for 7h20min, and rinsed with deionized water for 1min before each replacement of the newly configured solution, 3 times/d, for a total of 30d, respectively, in a 37 ℃ shaking thermostatic water bath, and rinsed in deionized water for 5min to air-dry, respectively, at the end of the cycle.

2.5. Specimen Testing

Before demineralization, after demineralization and after remineralization of each group of samples, 5 samples were randomly selected for microhardness testing under the microhardness tester (in which the samples before demineralization and after demineralization were tested and returned to the original containers), 5 samples were randomly selected for observation of surface characteristics under the scanning electron microscope, 5 samples were randomly selected for detection of the compositional content of each element and changes in the compositional content of each element by X-ray spectrometry, and the calculation of the calcium-phosphorus mass ratio and the atomic ratio. Five samples were randomly selected and analyzed under a fluorescence microscope for the area of fluorescence, total fluorescence, and average fluorescence of the dental bone mass.

2.5.1. Scanning Electron Microscope Observation

The randomly selected samples of each group were wiped with acetone to remove the nail polish on the surface of the specimens, placed in the electron microscope fixative (2.5% glutaraldehyde) for 24 h and then washed by ultrasonic swirling with deionized water for 1 min, and then dehydrated with graded grades of ethanol (70%, 80%, 90%, 100%) in stages, each gradient was immersed for 20 min, dried naturally, and sprayed with gold. The ultrastructure of the dentin surface of each group was observed under a scanning electron microscope.

2.5.2. X-ray Energy Spectrum Testing

The calcium and phosphorus contents were detected by X-ray energy spectrum analyzer at three randomly selected points in the experimental area of each group of specimens in the above, and the calcium and phosphorus mass ratios and atomic ratios (Ca/P) were calculated respectively, and the average value of three times was taken as the final result of the experiment.

2.5.3. Microhardness Testing

Each group of osteoid blocks was placed in superhard gypsum to make osteoid specimens, and the specimens were fixed on the microhardness tester carrier table. The surface microhardness (SMH) of each specimen was measured by Vickers microhardness tester in the windowed area. Three sites were randomly selected for each specimen, and the Vickers microhardness tester was adjusted to a load of 100 g, loaded for 15 s, and the surface hardness value of each group of osteoid was measured in HV, and the average of the three measurements was taken as the value of its surface hardness.

2.5.4. Fluorescence Microscopic Observation

Dental bone blocks of each group were embedded in self-coagulating resin, and each block was sliced with a hard tissue cutter and then sanded to 300 μm slices with 320 mesh, 800 mesh, and 1,200 mesh sandpaper under bilateral running water, and finally polished with 4,000 mesh sandpaper, and then placed in a glass dish containing freshly configured 0.1 mmol/L rhodamine B staining solution in a thermostatic 37° C water bath for 1 h. After staining, the sections were placed in an ultrasonic swisher with deionized water for 10 min, dried, and then placed on slides. After the staining was completed, the dental bone sections were placed in an ultrasonic washing machine with deionized water for 10 min, dried, sealed on slides, and placed under a fluorescence microscope with the objective lens adjusted to 4x to observe

the fluorescence staining bands, and the fluorescence area of the dental bone blocks was analyzed by applying the software (A) in units of μm2; the total fluorescence (TF) was the total density (TF) of fluorescence within the range of the measurement; the total density (TF) of fluorescence (TF) was the total density of fluorescence (TF) within the range of the measurement. density (TF); average fluorescence density (AF): the average density of lesion fluorescence in the measured area, data collection and organization.

2.6. Statistical Analysis

SPSS 27.0 software was used for data entry and analysis. The various data of the experimental results were statistically analyzed, and the values were used ($\bar{x} + \sigma$), and the comparison of the data before and after the experiment in the same group was performed by the paired t-test; the comparison of the means between different experimental groups was performed by the one-way ANOVA, with a test level of P=0.05.

3. Results

3.1. Scanning Electron Microscope Observation Results

The results of scanning electron microscopy for each group are shown below: the surface morphology of the dentin blocks varied in each group. Before demineralization, the surface of dentin was flat, and after demineralization, the surface of dentin was porous and cracked. Group A (EGCG-CMCS hydrogel) showed irregularly arranged spherical particles on the surface, and there was no obvious pore structure, which indicated that it promoted the deposition of minerals, and the effect of remineralization was better. Group B (EGCG solution) had a flat surface, and there was still a small amount of smaller pore structure, and the effect of remineralization was limited. Group C (CMCS hydrogel) had an unsmooth surface, and there was no significant pore structure, which indicated that it promoted mineral deposition, and the remineralization effect was limited. group C (CMCS hydrogel) had a smooth surface. The surface of group C was not smooth, more heterogeneous, and still had more pore structures, indicating poor remineralization. Group D group (2% NaF) had an uneven surface, with a large number of spherical particles deposited on the surface, and no pore structures were seen, resulting in an obvious remineralization effect. Group E (deionized water) had a relatively large number of obvious cracks on the surface, with the dentin bone structure being severely destroyed, with little restorative effect. Group B (EGCG solution) had an uneven surface, with a small number of smaller pore structures, indicating that the remineralization effect was limited.

3.2. X-ray Energy Spectrometer Analysis Results

The calcium-phosphorus mass ratio to atomic ratio of each group is shown in Table 1: the EGCG-CMCS hydrogel group was the closest to the pre-demineralization calcium-phosphorus mass ratio to atomic ratio, with no statistically significant difference between the two groups (P>0.05), which indicated that its composition after remineralization was similar to that of natural dental bone, and it was effective in restoring the calcium-phosphorus ratio; the calcium-phosphorus mass ratio to atomic ratio was the highest in the 2% NaF group, and the lowest in the EGCG group. A two-by-two comparison between the groups showed that there was a statistical difference between the groups (P < 0.05), except for the EGCG-CMCS hydrogel group, which showed no statistical difference in the calcium-phosphorus mass ratio to atomic ratio from the pre-demineralization period (P > 0.05).

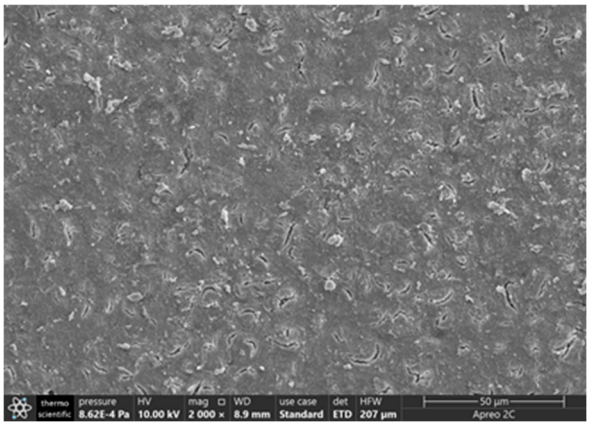
3.3. Microhardness Analysis Results

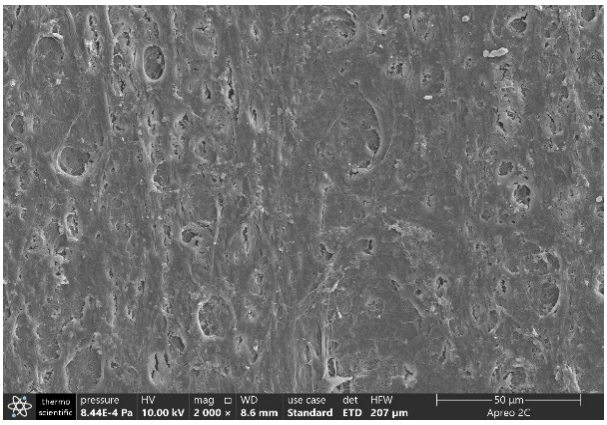
As shown in Table 2, there was no significant difference in the initial microhardness SMH0 between the samples of the dentin blocks in each group (P > 0.05). There was a significant decrease in microhardness SMH1 of the dentin samples after demineralization, and the difference in microhardness of the dentin surfaces of each group before and after demineralization treatment was significant, indicating that the root surface caries model was established effectively and that there was no significant difference in microhardness values SMH1 after demineralization between the groups (P > 0.05). Comparison of the values of microhardness SMH2 after remineralization and the changes of △SMH before and after remineralization after different drug treatments are shown in Table 2: the microhardness of the EGCG-CMCS hydrogel group, the EGCG group, the CMCS group, and the 2% NaF group after remineralization was significantly higher than that of all the groups before remineralization (P < 0.01), and the microhardness of the deionized water group after remineralization was significantly higher than that of all the groups before remineralization (P < 0.01). The microhardness of the deionized water group after remineralization was significantly higher than that before remineralization (P<0.05). The difference in microhardness in the 2% NaF group was the largest, followed by the EGCG-CMCS hydrogel group, indicating that the two were effective in enhancing the hardness, whereas there was no statistically significant difference in the difference in microhardness between the EGCG group and the CMCS hydrogel group (P>0.05), and the difference in microhardness in the deionized water group was the smallest.

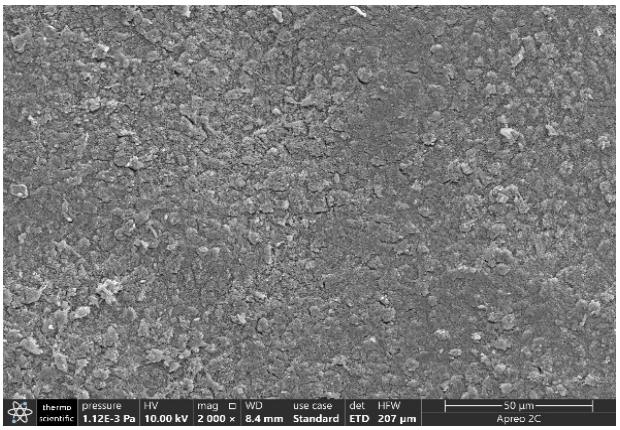
3.4. Fluorescence Microscopy Results

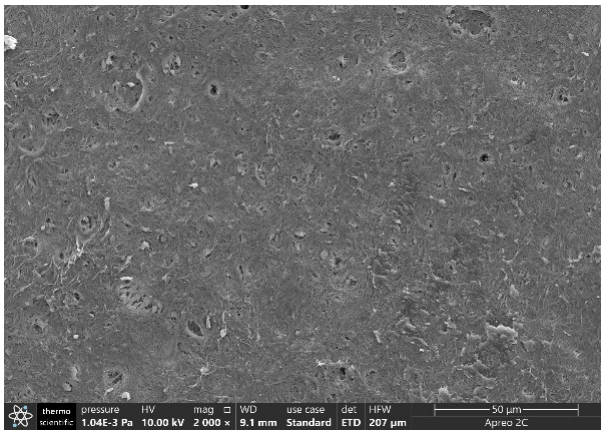
After demineralization, hydroxyapatite crystals were dissolved, and a large number of voids were created on the surface of the odontoblastic samples, which allowed the rhodamine B fluorescent dye to penetrate into them, and there was a significant fluorescence effect under the fluorescence microscope, and under the microscope, the fluorescent strips of odontoblastic samples before demineralization were stained more superficially, and the sparse structure of the collagen fibres of the odontoblastic matrix before demineralization and remineralization led to the bright fluorescent strips, the significant staining effect, and the darker thickness of the staining. . The staining of the samples after remineralization treatment with each group of drugs varied, as shown in Table 3: the fluorescence intensity of the dentin samples treated with the EGCG-CMCS hydrogel group was the weakest, indicating the best remineralization effect; the 2% NaF group was the second weakest, and its fluorescence intensity was lower than that of the EGCG group and the CMCS hydrogel group, indicating a better remineralization effect; the fluorescence intensities of the EGCG and the CMCS groups were not statistically different from each other, as the fluorescence intensities of the EGCG and CMCS groups were not statistically different from those of the CMCS group. area of EGCG and CMCS groups were not statistically different from each other (P>0.05), and there

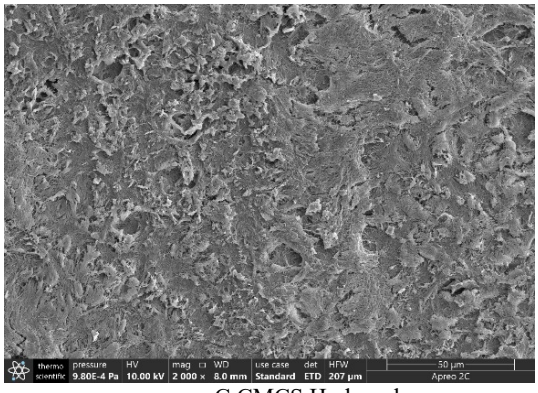
was no significant difference in the remineralization effect between these two groups. The fluorescence intensity of each treatment group was greater than that of the deionized water group (P<0.01), indicating that the remineralization effect was better than that of the deionized water group in all cases. There was no statistically significant difference in the mean fluorescence intensity between the groups (P > 0.05)

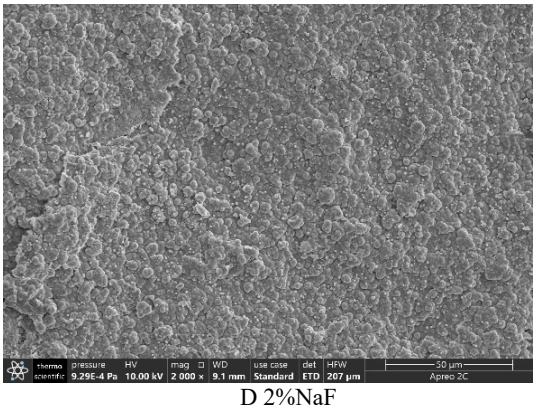

a pre-demineralization


b post-mineralization


A EGCG-CMCS Hydrogel


B EGCG solution


C CMCS Hydrogel


D 2%NaF

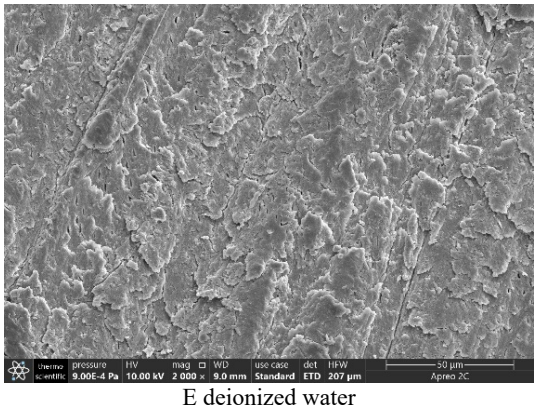

E deionized water

Fig 1. SEM of cementum surface (×2000)

Table 1. The measurement result of calcium and phosphorus ratio in each group (n=5, $\bar{x}\pm\sigma$)

Group	Quality ratio	Atomic ratio
EGCG-CMCS Hydrogel	2.61 ± 0.69^{z}	2.05 ± 0.25^{a}
EGCG solution	1.02 ± 0.25^{x}	0.77 ± 0.19^{b}
CMCS Hydrogel	1.51 ± 0.27^{y}	1.16 ± 0.22^{c}
2%NaF	8.56 ± 0.47^{m}	6.56 ± 0.35^{d}
DDW	2.11 ± 0.08^{n}	1.63 ± 0.06^{e}
pre-demineralization	2.82 ± 0.10^{z}	2.38 ± 0.25^{a}
F	497.81	393.30
P	<0.01	<0.01

Note: Comparisons between different symbols are statistically significant P<0.05, comparisons between the same letters are not statistically different P>0.05.

Table 2. Bone surface hardness of teeth in each group (HV. n=5,$\bar{x}\pm\sigma$)

Group	N	SMH_0	SMH_1	SMH_2	△SMH	T (*P*) (before and after remineralization)
EGCG-CMCS Hydrogel	5	51.50±4.62	23.92±3.50	41.18 ± 1.72^{a}	17.26 ± 4.62^{f}	11.81(*P*<0.01)
EGCG solution	5	45.63±4.63	25.50±2.96	38.22 ± 2.05^{b}	12.72 ± 2.89^{g}	43.56(*P*<0.01)
CMCS Hydrogel	5	46.76±4.38	23.53±3.00	36.28 ± 1.53^{c}	12.85 ± 2.51^{g}	20.28(*P*<0.01)
2%NaF	5	52.99±5.32	23.93±2.60	48.95 ± 5.42^{d}	25.03 ± 6.16^{h}	10.28(*P*<0.01)
DDW	5	52.99±6.17	25.73±2.99	32.39 ± 2.93^{e}	6.66 ± 3.46^{i}	4.42(*P*<0.05)
F	-	1.94	1.69	60.89	40.35	-
P	-	>0.05	>0.05	<0.01	<0.01	-

Note: Comparisons between different symbols are statistically significant *P*<0.05, comparisons between the same letters are not statistically different *P*>0.05.

Table 3. The measurement result of cementum remineralization fluorescence area in each group (n=5, $\bar{x}\pm\sigma$)

Group	Fluorescence total density TF ($\times10^4$. a.u.)	Fluorescent area A ($\times10^7.\mu m^2$)	Average fluorescence intensity AF (a.u.)
EGCG-CMCS Hydrogel	0.29 ± 0.24^{d}	0.13 ± 0.11^{q}	2.22 ± 0.01^{z}
EGCG solution	27.98 ± 20.14^{f}	12.61 ± 9.06^{w}	2.22 ± 0.01^{z}
CMCS Hydrogel	90.99 ± 62.87^{f}	41.00 ± 28.26^{w}	2.22 ± 0.01^{z}
2% NaF	2.01 ± 1.39^{c}	0.92 ± 0.63^{t}	2.20 ± 0.03^{z}
DDW	534.03 ± 304.85^{g}	243.61 ± 138.22^{h}	2.20 ± 0.03^{z}
F	8.54	8.60	1.88
P	*P*<0.01	*P*<0.01	*P*>0.05

Note: Comparisons between different symbols are statistically significant *P*<0.05, comparisons between the same letters are not statistically different *P*>0.05.

4. Discussion

In recent years, a variety of remineralization materials have been used in the prevention and treatment of early caries, each with its own mechanism of action. Conventional fluorides (e.g., NaF) enhance the acid-solubility resistance of dental tissues by promoting the formation of fluorapatite, but their action is mainly dependent on the concentration and persistence of local fluoride ions [8]. Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), on the other hand, stabilizes calcium and phosphorus ions through casein phosphopeptide and maintains a supersaturated state, which results in an increase in the concentration of calcium and phosphorus ions on the surface of enamel and their slow release, thus promoting remineralization of demineralized enamel[9]. Bioactive glass (BAG) forms a hydroxyapatite layer in acidic environments by releasing calcium and phosphorus ions, but the rate of mineralization is strongly influenced by the environmental pH [10]. Hydroxyapatite nanoparticles can provide a direct source of calcium and phosphorus, but their lack of antimicrobial activity makes it difficult to inhibit the demineralization process at source [11].

In contrast, the EGCG-CMCS hydrogel used in this

experiment combines the dual advantages of EGCG and CMCS.EGCG, as a natural polyphenol, not only reduces demineralization triggers by inhibiting the metabolic acid production of cariogenic bacteria (e.g., Streptococcus anomalies) [12], but also promotes orderly deposition of hydroxyapatite crystals by chelating calcium ions[13]. It has been shown that EGCG complexed with amorphous calcium phosphate (ACP) to form EGCG-ACP can release ACP to promote the orderly crystallization and growth of calcium and phosphorus, fill the dissolved enamel column sheaths, and have the ability to restore the microstructure and mechanical properties of enamel [14]. Some scholars have also used sodium fluoride (NAF) in combination with EGCG, which can produce synergistic bacteriostatic effects, retaining the bacteriostatic properties as well as the remineralization ability of NAF while reducing the dosage of NAF to improve safety [15]. As a biocompatible carrier, the carboxymethyl group of CMCS can enhance the adhesion to the dentin surface and prolong the slow release time of EGCG [16], while its negative charge property may attract calcium and phosphorus ions through electrostatic interaction [17],which synergistically promotes mineralization. This 'antimicrobial-mineralization synergistic' mechanism makes it more advantageous in restoring the calcium-phosphorus ratio and repairing the microstructure, especially its calcium-phosphorus ratio is closer to that of natural dentin, which avoids the problem of over-mineralization or compositional deviation that may be caused by fluoride.

The results of this experiment showed that the samples remineralized by EGCG-CMCS hydrogel had spherical particles deposited on the surface as seen under the scanning electron microscope and were smoother and denser than the demineralized dentin, which implies that the EGCG-CMCS hydrogel can promote the deposition of minerals on the surface of the dentin, and is able to repair the rough and porous structure caused by demineralization. The results of the X-ray spectrometry analyses showed that the calcium-phosphorus ratio of the EGCG -CMCS hydrogel group was closer to the calcium-phosphorus ratio of natural dentin, indicating that the hydrogel was closer to the composition of natural dentin in terms of the types and ratios of guided mineral deposition, which could help restore the natural structure and function of dentin. Under the fluorescence microscope, the EGCG-CMCS hydrogel group has the smallest fluorescence density and area, which can intuitively reflect its remarkable remineralization effect. In terms of microhardness, the difference in hardness of the EGCG-CMCS hydrogel group compared to the pre-demineralization samples was second only to that of the 2% NaF group, demonstrating its ability to effectively enhance the hardness of dental bone. Although the remineralization effect of EGCG-CMCS hydrogel was slightly lower than that of the 2% NaF group in terms of microhardness, the performance of EGCG-CMCS hydrogel was better than that of the 2% NaF group in fluorescence microscopy, and its calcium-phosphorus ratio was closer to that of natural dentin, which was superior to that of the EGCG solution, CMCS hydrogel and deionized water groups alone in all the indicators.

In this experiment, we chose bovine incisors as the experimental samples because their dentin structure is similar to human teeth and easy to obtain and handle. The PH cycle method used can more realistically simulate the oral environment by simulating the change of oral pH in the daily diet, reflecting the mineralization and demineralization dynamic processes of teeth in real life, so as to make the results of the experimental results more clinically referential. However, there are still some limitations in this study. The experiment was only conducted in an in vitro environment, which is different from the complex physiological environment in the oral cavity, such as the salivary flow rate and the diversity of microbial communities in the oral cavity, which may affect the remineralization effect of the hydrogel. Follow-up studies could further conduct intraoral experiments to verify the effectiveness and safety of EGCG-CMCS hydrogels in real oral environments. In conclusion, epigallocatechin gallate-carboxymethyl chitosan hydrogel has a good remineralization effect on early root surface caries and demonstrates potential clinical applications. Follow-up studies should be devoted to overcoming the existing limitations and promoting the early application of this material in clinical practice for the benefit of patients.

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