Tea Catechin Epigallocatechin gallate inhibits *Streptococcus mutans* Biofilm Formation by Suppressing *gtf* Genes

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ABSTRACT

Objective: The anti-cariogenic properties of tea have been suggested for decades. Tea polyphenols, especially Epigallocatechin gallate (EGCG), have been shown to inhibit dental plaque accumulation, but the exact mechanisms are not clear at present. We hypothesize that EGCG suppresses *gtf* genes in *S. mutans* at the transcriptional level disrupting the initial attachment of *S. mutans* and thus the formation of mature biofilms. **Design:** In this study, the effect of EGCG on the sucrose-dependent initial attachment of *S. mutans* UA159 in a chemically defined medium was monitored over 4 h using a chamber slide model. The effects of EGCG on the aggregation and *gtf* B, C, D gene expression of *S. mutans* UA159 were also examined. **Results:** It was found that EGCG (7.8-31.25 µg/ml) exhibited dose-dependent inhibition of the initial attachment of *S. mutans* UA159. EGCG did not induce cellular aggregation of *S. mutans* UA159 at concentrations less than 78.125 µg/ml. Analysis of data obtained from real-time PCR showed that EGCG at sub-MIC level (15.6 µg/ml) significantly suppressed the *gtf* B, C, D genes of *S. mutans* UA159 compared with the non-treated control ($p < 0.05$). **Conclusions:** These findings suggest that EGCG may represent a novel, natural anti-plaque agent that inhibits the specific genes associated with bacterial biofilm formation without necessarily affecting the growth of oral bacteria.

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1. Introduction

Dental caries is one of the most prevalent and costly oral infectious diseases throughout the world (1). The etiology of dental caries is associated with bacterial metabolism of carbohydrates, leading to plaque acidification and demineralization of the dental hard tissues. Classic bacterial virulence factors contributing to the initiation and progression of dental caries consist of three components, i.e. stable biofilm formation, efficient acid production, and sophisticated environmental stress adaptation (2-5). *Streptococcus mutans,* one of the primary etiologic agents of dental caries (6, 7), produces glucosyltransferases (GTFs) which synthesize intracellular polysaccharides (IPS) and extracellular polysaccharides (EPS). The EPS, especially water-insoluble glucans, mediate the initial adherence of *S. mutans* and other oral bacteria on tooth surfaces and facilitate the formation of mature dental plaque biofilm (8-10). A recent study has shown that deletion of *gtf*B and *gtfC* genes in *S. mutans* resulted in diminished biofilm formation with minimal accumulation of bacteria and polysaccharides *in vitro* (10). This suggested that suppression of *gtf* genes may represent an alternative approach to disrupting biofilm formation.

Tea (infusion of dried leaves of *Camellia sinensis*) is the most popular and widely consumed beverage in the world today (11). Its polyphenolic component has been reported to possess antioxidant, antimicrobial, antimutagenic, antidiabetic, hypocholesterolemic, anti-inflammatory, and cancer-preventive properties (12-14). Its

anti-cariogenic activity has also been demonstrated in humans and experimental animals (15-21). Our previous study in adult humans found that rinsing with black tea extract resulted in a significantly reduced plaque pH fall and a lower plaque index compared with rinsing with water alone (11). Frequent short-term rinses with black tea also inhibited subsequent regrowth and glycolysis of human supragingival plaque bacteria (14). Our recent study has demonstrated that epigallocatechin gallate (EGCG), the antimicrobial monomeric component of tea catechins (the major polyphenolic component in tea) exhibited a wide range of physiological effects on *S. mutans*, particularly on virulence factors associated with its acidogenicity and acidurity (22).

Many researchers have reported that tea catechins, especially EGCG, reduced *S. mutans* cell adherence by suppressing the activity of GTF enzymes (16, 23, 24). The concentrations of EGCG reported for this inhibition were often in the mg/ml range which were sufficient to inhibit growth or viability of oral streptococci (15, 22). However, in the oral cavity, due to saliva dilution, sustaining such high inhibitory concentrations of polyphenols over a long period of time after tea consumption would be unlikely.

We have recently found that EGCG inhibited *in vitro* biofilm formation of *S. mutans* at an MBIC (minimum biofilm inhibition concentration) of 15.6 µg/ml, a concentration lower than the minimum growth inhibitory concentration (MIC) against *S. mutans* planktonic cells (22). This suggested the involvement of additional mechanism(s) by which EGCG may exhibit anti-plaque biofilm activity in the oral

cavity without necessarily inhibiting growth of oral bacteria. To our knowledge, the effect of tea polyphenols on *S. mutans gtf* gene expression at the transcriptional level has yet to be examined. Moreover, the mode of action of EGCG on the sucrose-dependent initial attachment of *S. mutans* towards biofilm formation has not been well documented. We hypothesize that EGCG suppresses *gtf* gene expression in *S. mutans,* thus inhibiting biofilm formation. In this study, we investigated: 1) the effect of sub-bacteriostatic levels of EGCG on the sucrose-dependent initial attachment of *S. mutans* to surfaces, and 2) the effect of EGCG on the transcriptional expression of *S. mutans gtf* B, C, D genes.

2. Materials and methods

2.1. Chemicals, test bacterium and growth conditions

Epigallocatechin gallate from green tea (EGCG, 95% HPLC) and all chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Corp (Saint Louis, MO, USA). *S. mutans* UA159 was grown in a chemically defined medium (CDM) (25) at 37°C in an anaerobic chamber (37 ºC, 10% H_2, 5% CO_2, and 85% N_2; Forma Scientific, Inc., Marietta, OH, USA). Artificial saliva used in attachment assay was prepared according to the formula as described previously (26).

2.2. Sucrose-dependent Initial Attachment Assay.

EGCG at sub-MIC concentration was used for the sucrose-dependent initial attachment of *S. mutans* cells. The minimum inhibitory concentration (MIC) and

minimum bactericidal concentration (MBC) of EGCG against *S. mutans* UA159 were pre-determined in CDM using a micro-dilution method as described previously (22).

The initial attachment of *S. mutans* UA159 on the glass surface was determined using a four-well chamber slide (culture area of 1.8 cm² per well; Nunc Lab-Tek, Rochester, NY, USA). *S. mutans* were collected in mid-log phase from the broth culture, washed two times with PBS, and re-suspended in CDM (1×10^6 CFU/ml) supplemented with 1% (w/v) sucrose and EGCG (7.8 - 31.25 µg/ml, 1/4 MIC - MIC). The cells suspension was placed onto artificial saliva pre-coated (37°C for 1 h) chamber slides and incubated under anaerobic condition. After 1h, 2h and 4 h incubation, cell free cultures supernatant were removed from respective chamber slide and the chambers were gently washed three times with deionized water to remove un-attached cells. The attached cells were stained with the fluorescent Live/Dead BackLight™ stain (Molecular Probes Inc., Eugene, Oregon, USA) and examined under a Leica DMRE microscope (Leica, Wetzlar, Germany). Images were captured at 20 × magnification using a digital camera (Meyer Instruments, Inc., Houston, TX, USA) and analyzed by Image-Pro Plus 5.1 (Media Cybernetics Inc., Bethesda, MD, USA). Total average coverage area of bacterial cells on the surface was obtained from at least 4 different images of the same sample. Control contained *S. mutans* UA159 cells grown in the absence of EGCG.

2.3. Bacterial Aggregation Assay.

Aggregation of *S. mutans* UA159 cells in the presence of EGCG was determined

according to the method modified from Matsumoto, *et al* (27). *S. mutans* UA159 cells were collected in mid-log phase by centrifugation, washed three times with PBS, re-suspended in PBS or CDM with 0.1% sucrose to a concentration, upon ten times dilution, yielding an OD_{660nm} of 0.3 in 96-well microtiter plate (200 µl). This highly concentrated cell suspension was then used to test the aggregation-inducing capability of EGCG. 100 µl of the cell suspension and an equal volume of twofold serial dilution of EGCG in PBS or CDM were mixed in the 96-well microtiter plate and incubated at 37°C for 2 h. 100 µl of the reaction mixture was then carefully transferred to a new 96-well microtiter plate without disturbing the precipitated cells at the bottom, and OD_{660nm} was recorded. The aggregation percentage of *S. mutans* cells were calculated according to the formula: Aggregation (%) = $100 \times [1 - (OD_{Exp} - OD_{Blk}) / (OD_{NTC} - OD_{Blk'})]$, where OD_{Exp} was OD_{660nm} of each experimental well (with serial concentrations of EGCG), OD_{NTC} was OD_{660nm} of the non-treated control (without EGCG), and OD_{Blk} and $OD_{Blk'}$ were OD_{660nm} readings of the blank for experimental wells and the non-treated control respectively. The minimum concentration that induced cellular aggregation was defined as the lowest concentration of EGCG promoting no less than 10% of cellular aggregation compared with the non-treated control.

2.4 RNA Isolation, Purification, Reverse Transcription and Quantitative Real-Time PCR.

S. mutans UA159 was grown in CDM supplemented with sub-MIC concentration of

EGCG (15.6 μg/ml). Cells were collected at late exponential phase by centrifugation and RNA was immediately stabilized using an RNAprotect Bacteria Reagent (QIAGEN, Valencia, CA, USA). Cells were then pelleted and re-suspended in 100 μl of lysis buffer (20 mM Tris-HCl, 3 mM EDTA, 20mg/mL lysozyme, 60 mAU/ml proteinase K, 1000 U/ml mutanolysin, [pH 8.0]) and incubated at 37ºC with gentle agitation for 45 min. The lysate was further sonicated by means of a cuphorn (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) on ice for 2 cycles of ultrasonication for 60 s and then purified using an RNeasy Mini Kit (QIAGEN). Reverse transcription was performed by use of a 1^{st} Strand cDNA Synthesis Kit with random hexamer primers (Invitrogen, Madison, WI, USA).

Real-time PCR was used to quantify *gtf* B, C, D mRNA expression with 16S rRNA as an internal control. All primers for real-time PCR were designed with Primer3 (28) and obtained commercially from Sigma-Aldrich Corp. (Table 1). Real-time PCR amplification was performed on the iCycler iQ detection system (Applied Biosystems, Foster City, CA). The reaction mixture (25 μl) contained 1 X SYBR green PCR Master Mix (Applied Biosystems), template cDNA, and forward and reverse primers (10 μM each). Thermal cycling conditions were the same as described previously (22). Threshold cycle values (C_T) were determined, and data were analyzed by StepOne™ Software v2.0 (Applied Biosystems) according to the $2^{-\Delta\Delta C_T}$ method.

2.5 Statistical Analysis.

All experiments were performed in triplicate and reproduced at least three separate

times. Differences between the experimental group and the untreated control group were analyzed by SPSS (version 15.0 for Windows). One-way analysis of variance (ANOVA) was performed, and a post hoc Tukey test was used for the comparison of multiple means. Significance was set at a p value of < 0.05.

3. Results

3.1. EGCG inhibits the sucrose-dependent initial attachment of *S. mutans* without inducing significant cellular aggregation *in vitro*.

EGCG inhibited the *in vitro* growth of *S. mutans* UA159 in CDM (MIC = 31.25 μg/ml) and was bactericidal at an MBC of 62.5 μg/ml. Given the importance of biofilm formation in the cariogenic virulence of *S. mutans* cells, inhibition of the formation of biofilm by increasing concentrations of EGCG was investigated in a chamber slide model. Fluorescent Live/Dead BackLight™ stain revealed sucrose-dependent initial attachment of *S. mutans* UA159 cells to glass surfaces toward biofilm formation (Figure 1A). The area of cell coverage of non-treated control was 500.4 ± 186.23 μm^2 at 1 h incubation, increasing to 7401.7 ± 1879.99 μm^2 at 2 h, and 34347.2 ± 5418.04 μm^2 at 4 h. In the presence of EGCG (7.8-31.25 μg/ml), a dosage dependent inhibition on the sucrose-dependent initial attachment of *S. mutans* UA159 cells to surfaces was observed. EGCG at 31.25 μg/ml inhibited the area of cell coverage of *S. mutans* UA159 cells by 79.57% at 1 h, 98.33% at 2 h, and 91.78% at 4h compared with the non-treated control (Figure 1B).

To determine whether the reduced bacterial attachment observed above was due

to possible cellular aggregation induced by EGCG, we investigated the effect of EGCG on aggregation of *S. mutans* UA159 cells in both PBS and CDM. Although a dosage-dependent aggregation inducing effect of EGCG on *S. mutans* UA159 cells was observed ranging from 78.125-1250 μg/ml, EGCG at the test concentrations employed in attachment assays (7.8-31.25 μg/ml) did not induce significant cellular aggregation (Figure 2). The minimum EGCG concentration that induced cellular aggregation in CDM was 78.125 μg/ml, which was more than two-folds higher than the concentrations used in the attachment assay (7.8-31.25 μg/ml).

3.2. EGCG inhibits *gtf* B, C, D genes expression of *S. mutans.*

In order to determine the effect of EGCG on the virulence factors associated with cells attachment and biofilm formation of *S. mutans*, real-time PCR was used to quantify *gtf* B, C, D mRNA expression with 16S rRNA as an internal control. Melt curves revealed the absence of non-specific products in all amplification reactions. EGCG at sub-MIC level (15.6 μg/ml) significantly inhibited the *gtf* B, C, D genes expression by 60.88%, 60.49% and 66.37%, respectively compared to the non-treated control (Figure 3, $p < 0.05$).

4. Discussion

Dental plaque is a complex bacterial biofilm community whose composition is governed by factors such as bacterial adherence, co-aggregation, and growth and survival in the environment (29). Biofilm organisms frequently express phenotypes

quite distinct from those of their free planktonic counterparts, *e.g.,* enhanced resistance to antibiotics or antimicrobial chemicals (30). Stable biofilm formation is considered one of the key factors of caries pathogenesis (5). *Streptococcus mutans*, a prominent member of the dental plaque community, synthesizes extracellular adherent glucans from dietary sucrose *via* GTFs, thus promoting the accumulation of oral bacteria on tooth surfaces (9). The early stage of *S. mutans* biofilm, characterized by the sucrose-dependent bacterial attachment to tooth surfaces, represents an important initial step towards the subsequent formation of the mature biofilm (31). Therefore, compounds capable of inhibiting this initial attachment of *S. mutans* would effectively prevent dental plaque formation and maturation.

In our previous study, we reported that EGCG disrupted *in vitro S. mutans* biolfilm formation at a minimum biofilm inhibition concentration ($MBIC_{90}$) of 15.6 μg/ml (22). In the present study, we have demonstrated that EGCG at sub-MIC levels was able to inhibit the sucrose-dependent initial attachment of *S. mutans* thus leading to inhibition of subsequent mature biofilm formatiom (Figure 1). It is known that bacterial aggregation may result in cellular clearance and reduced cell attachment onto surfaces (32, 33). Since tea polyphenols have been reported to induce cell aggregation (27), one may argue that the inhibition of initial attachment of *S. mutans* to surfaces by EGCG observed in this study could have been attributed to cellular aggregation. However, this is not the case because the minimum EGCG concentration (78.125 μg/ml) needed to induce cellular aggregation of *S. mutans* was more than twice the concentrations (7.8-31.25 μg/ml) that inhibited their attachment

to surfaces.

Tea polyphenols, especially EGCG, have been reported to inhibit activity of *S. mutans* GTFs through their interaction with enzyme proteins (23, 24, 34). The effective EGCG concentrations reported in these previous studies have mostly been above the milligram per milliliter level (16, 23, 24). The average concentration of tea catechins in a typical cup of tea (230 ml) is approximately 1 mg/ml (15). Immediately after tea consumption, EGCG may be concentrated enough in the oral cavity to inhibit growth and GTFs activity, thus reducing biofilm formation of *S. mutans*. However, a gradual decrease in EGCG concentration to the sub-MIC level may occur due to the dilution by saliva. At this point, EGCG, although at a lower concentration, may still be capable of suppressing *gtf* gene expression leading to disruption of *S. mutans* biofilm formation as demonstrated in this study.

5. Conclusion

Based on our current findings, we conclude that EGCG at sub-lethal levels is able to reduces *S. mutans* biofilm formation by suppressing *gtf* expression associated with cell adherence and biofilm formation. Given the difficulties of maintaining effective levels of various therapeutic agents to achieve antimicrobial efficacy in the oral cavity, EGCG represents a promising natural anticariogenic agent that prevents plaque biofilm formation without necessarily suppressing the growth of oral bacteria.

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Conflict of interest statement

The authors declare that there is no potential conflict of interest that would prejudice the impartiality of this scientific work.

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Figure Captions

Figure 1. Effect of EGCG on the sucrose-dependent initial *in vitro* attachment of *S. mutans*. A: Florescent stained images taken at 1 h (a-d), 2 h (e-h) and 4 h (i-l) incubation in the presence of EGCG (b, f, j: EGCG = 7.8 μg/ml; c, g, k: EGCG = 15.6 μg/ml; d, h, l: EGCG = 31.25 μg/ml; a, e, i: non-treated control). B: The coverage area (μm^2) of *S. mutans* UA159 on chamber slide surfaces. *EGCG significantly inhibited the coverage area of *S. mutans* UA159 compared with non-treated control.

Figure 2. Effect of EGCG on cellular aggregation of *S. mutans* UA159. A dosage-dependent aggregation of *S. mutans* induced by EGCG was observed with a minimum inducing concentration at 78.125 μg/ml.

Figure 3. Effect of sub-MIC levels of EGCG on *gtf*B, C, D genes expression of *S. mutans* UA159 grown in chemically define medium. *Significant inhibition was observed compared with non-treated control.

Table Caption

Table 1. Specific primers used for real-time PCR

Table(1)
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Primers	Sequences
16S rRNA	5'-AGCGTTGTCCGGATTTATTG-3'
	5'- CTACGCATTTCACCGCTACA-3'
gtf B	5'-CACTATCGGCGGTTACGAAT-3'
	5'-CAATTTGGAGCAAGTCAGCA-3'
gtf C	5'-GATGCTGCAAACTTCGAACA-3'
	5'-TATTGACGCTGCGTTTCTTG-3'
gtf D	5'-TTGACGGTGTTCGTGTTGAT-3'
	5'-AAAGCGATAGGCGCAGTTTA-3'

Figure(1)
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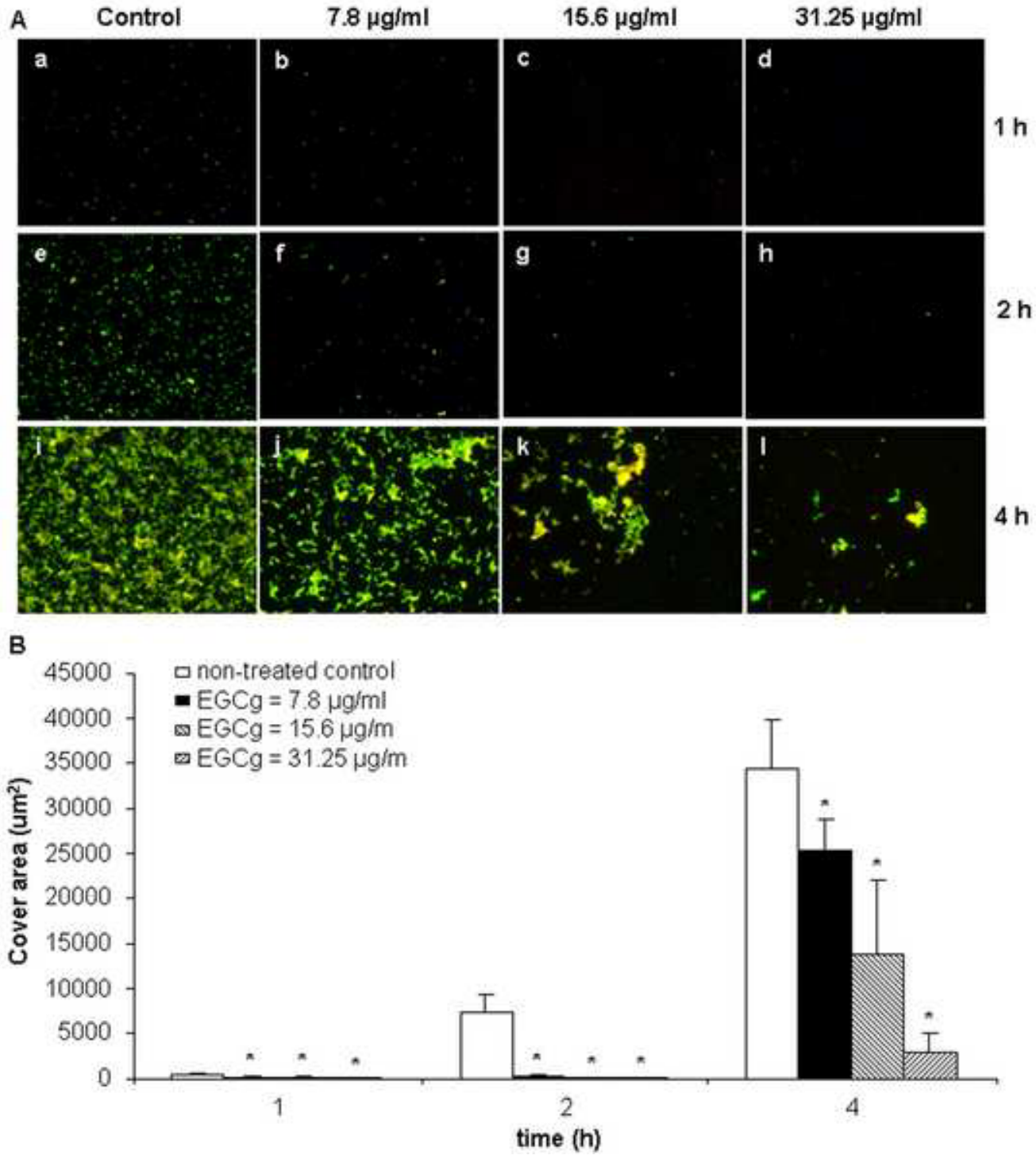

Figure2)
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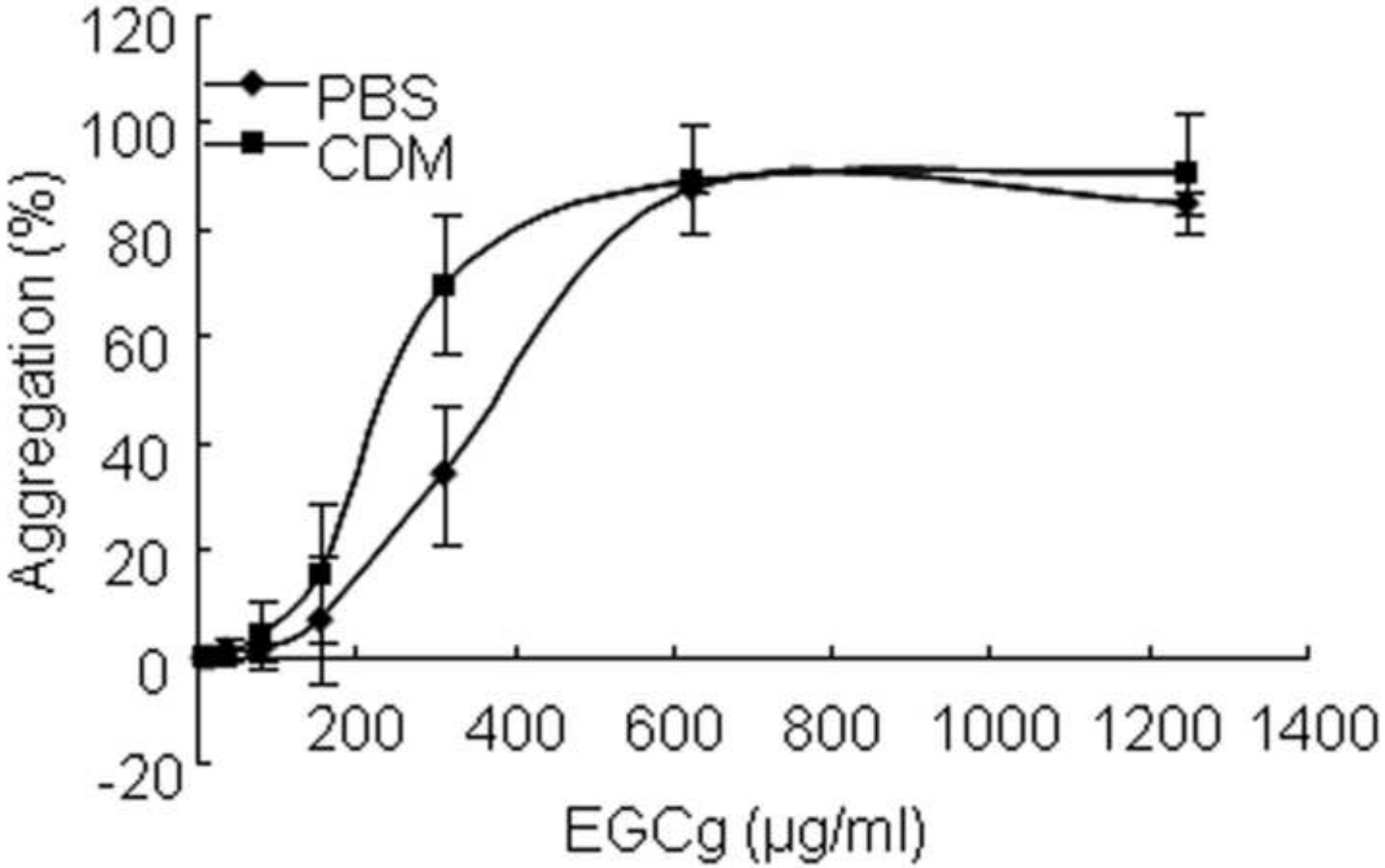

Figure(3)
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