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ARTICLE

Antiviral Effect of Epigallocatechin Gallate on Enterovirus 71

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Oxidative stress is known to be a determinant of a host's susceptibility to pathogens. Natural compounds with antioxidant activity may provide a preventive measure against infection. Tea polyphenols were evaluated for their ability to inhibit enterovirus 71 (EV71) replication in Vero cell culture. Among the polyphenolic compounds tested, epigallocatechin gallate (EGCG) and gallocatechin gallate (GCG) potently inhibited replication of EV71. EGCG and GCG reduced the titer of infectious progeny virus by 95%. Quantitative RT-PCR analysis also revealed that EGCG suppressed replication of genomic RNA. It was accompanied by an increased cytoprotective effect. EGCG and GCG caused 5-fold increase in the viability of EV71-infected cells. The viral inhibitory effect correlated well with the antioxidant capacity of polyphenol. Mechanistically, EV71 infection led to increased oxidative stress, as shown by increased dichlorofluorescein and MitoSOX Red fluorescence. Upon EGCG treatment, reactive oxygen species (ROS) generation was significantly reduced. Consistent with this, EV71 replication was enhanced in glucose-6-phosphate dehydrogenase deficient cells, and such enhancement was largely reversed by EGCG. These findings suggest that EGCG may suppress viral replication via modulation of cellular redox milieu.

KEYWORDS: EGCG; enterovirus 71; G6PD deficiency

INTRODUCTION

Tea is an extract of the leaves from *Camellia sinensis* plant, which is rich in polyphenolic compounds. Green, black, and oolong teas are all derived from the same plant and differ mainly in the degree of fermentation (*1*). The nonfermented green tea has a polyphenolic composition similar to that of fresh green leaves (*2*). Green tea polyphenolic compounds include flavanols, flavandiols, flavanoids, and phenolic acids. Of these compounds, a group of flavanols known as catechins predominate and account for up to 30% of the dry weight of green tea leaves (*3*). Epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), epigallocatechin (EGC), and gallocatechin (GC) exemplify the members of this family. EGCG is the most abundant catechin, reaching 12−14% on a dry weight basis (*4*). The exact concentrations of EGCG and other catechins in tea vary, depending on the variety of tea bush, climate, horticultural practice, and the harvest season (*5*).

A number of biological and pharmacological activities, including bacteriostatic and bactericidal activity (*6*−*8*), anticarcinogenic activity (*9*), modulation of signaling (*10*), and antioxidant (*11*), have been described for tea polyphenols. As antioxidant, catechins are able to scavenge peroxyl radicals and inhibit chain reactions involved in lipid peroxidation (*12*). They have been shown to protect low-density lipoproteins from peroxidation and excessive α-tocopherol consumption (*13*). Among these compounds, EGCG is most effective in scavenging reactive oxygen species (*14*). It has been reported that EGCG can trap up to six superoxide anions or hydroxyl radicals in vitro while epicatechin only traps two radicals (*15*).

Enterovirus 71 (EV71) is a nonenveloped RNA virus within the family *Picornaviridae* (*16*). Since the initial identification of EV71 in California in 1969 (*17*), outbreaks of EV71 infection have occurred periodically throughout the world (*18*−*21*). The clinical manifestation of EV71 infection may include febrile illness, acute respiratory disease, hand-foot-and-mouth disease (HFMD), herpangina, myocarditis, aseptic meningitis, acute flaccid paralysis, brainstem and/or cerebellar encephalitis, Guillain−Barré syndrome, or combination of these clinical features (*21*−*23*). Children under 5 years of age are especially susceptible to these syndromes and may develop permanent neurologic sequelae or even succumb to such disorders (*24*). The largest epidemic of EV71 to date occurred in Taiwan in 1998: nearly 130 000 cases, of which 405 cases were severe, were reported over a period of 8 months (*18*, *19*). Since then, EV71 infection recurs every year in Taiwan and continues to circulate in the Asia−Pacific region (*21*−*25*).

It is known that oxidative stress affects the interactions between host and viral pathogens, and hence viral pathogenesis. Susceptibilities to infection with coxsackievirus, influenza virus, and coronavirus are modulated by redox environment (*26*−*28*).

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We have recently shown that deficiency in glucose-6-phosphate dehydrogenase (G6PD) enhances EV71 infection (*29*). G6PD-deficient cells are subject to increased oxidative stress and support EV71 replication more efficiently. Administration of *N*-acetylcysteine mitigates EV71 propagation and offers cytoprotective effect on infected cells. In the absence of an effective EV71 vaccine, elucidation of the relationship between the host's redox status and viral virulence is valuable to the development of therapeutic or preventive strategies against such clinically relevant viruses as EV71.

In the present study, we demonstrate that EGCG inhibits EV71 replication and formation of infectious progeny virions. The antiviral effect of EGCG correlates with its antioxidant capacity. Mechanistically, EGCG significantly reduced the EV71-induced oxidative stress. Moreover, EGCG inhibits the enhanced EV71 replication in G6PD-deficient cells. These findings suggest that EGCG exerts an antiviral effect through its antioxidant function.

MATERIALS AND METHODS

Chemicals. Unless stated otherwise, all chemicals were obtained from Sigma (St. Louis, MO). Cell culture media, fetal calf serum (FCS), antibiotics, and trypan blue were purchased from Invitrogen (Carlsbad, CA). Fluorogenic dyes, such as dichlorofluorescin diacetate (H_2DCFDA), MitoSOX Red, MitoTracker Green, and MitoTracker Red, were also obtained from Invitrogen.

Cell and Virus Culture. Vero cells (ATCC CCL-81) were cultured in modified Eagle's medium (MEM) supplemented with 10% FCS, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 μg/mL amphotericin in an atmosphere of 5% CO_2 at 37 °C. Primary human foreskin fibroblasts (HFF3) and its G6PD-deficient counterparts HFF1 were isolated as previously described (*30*, *31*). HFF1 was derived from the foreskin of a neonate who carries the Taiwan-Hakka ($G6PD^{1376T}$) variant of the G6PD gene. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 100 units/mL of penicillin, 0.1 mg/mL of streptomycin, and 0.25 μg/mL of amphotericin at 37 °C in a humidified atmosphere of 5% CO_2. HFF1 and HFF3 cells at a population doubling level of 15−20 were used.

EV71 (BrCr strain; ATCC, VR784) was propagated in Vero cells as previously described (*29*). Briefly stated, after the culture reached 80% of confluence, the cells were washed twice with phosphate buffered saline (PBS) and incubated with viral inoculum at 37 °C for 1 h. The flask was gently agitated at 20 min interval during the adsorption period. Afterward, the viral inoculum was removed, and fresh medium containing 1% of FCS and antibiotics was added. Cells were then incubated in a humidified atmosphere of 5% CO_2 at 37 °C. When over 90% of cells showed cytopathic effect, the viral supernatant was collected and clarified of cell debris by centrifugation. Any remaining viral particles were released from cell debris by three successive freeze−thaw cycles and subsequent centrifugation. Finally, all the supernatants were pooled and stored at −80 °C. To quantify the progeny virus production, we followed the same procedure, with the exception that 1×10^4 cells were seeded in each well of 24-well culture plate, and the progeny virions were harvested in 1 mL of DMEM/1% FCS. For catechin treatment, cells were pretreated with indicated concentrations of catechins for 1 h prior to viral infection. They were treated again with the same catechins after adsorption period.

Plaque Assay. Vero cells (3.8×10^5 cells/well) were seeded into a six-well culture plate, incubated overnight, and then infected with 10-fold serially diluted viral suspension. After 60 min of adsorption, the cells were washed once with PBS and overlaid with 0.3% agarose in MEM/2% FCS. Ninety-six hours later, the cells were fixed with 10% formaldehyde. The cells were stained with 1% crystal violet solution. The titer of virus was quantified as plaque forming unit (pfu) per milliliter of cell lysate.

Determination of Cell Viability. Cell viability was determined using 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) assay (*32*). In brief, cells were incubated with 0.5 mg/mL MTT at 37 °C for 2 h. After removal of culture medium, 100 μL of DMSO was added to solubilize the formazan formed. The absorbance (A) was measured using a microplate reader with a 570 nm test wavelength and a 690 nm reference wavelength. The $A_{570\ -690\text{nm}}$ of each well was corrected for the blank. The % viability was calculated as follows:

$$[(\text{corrected absorbance of each experimental well})/(\text{corrected absorbance of well containing uninfected, untreated cells})] \times 100\%$$

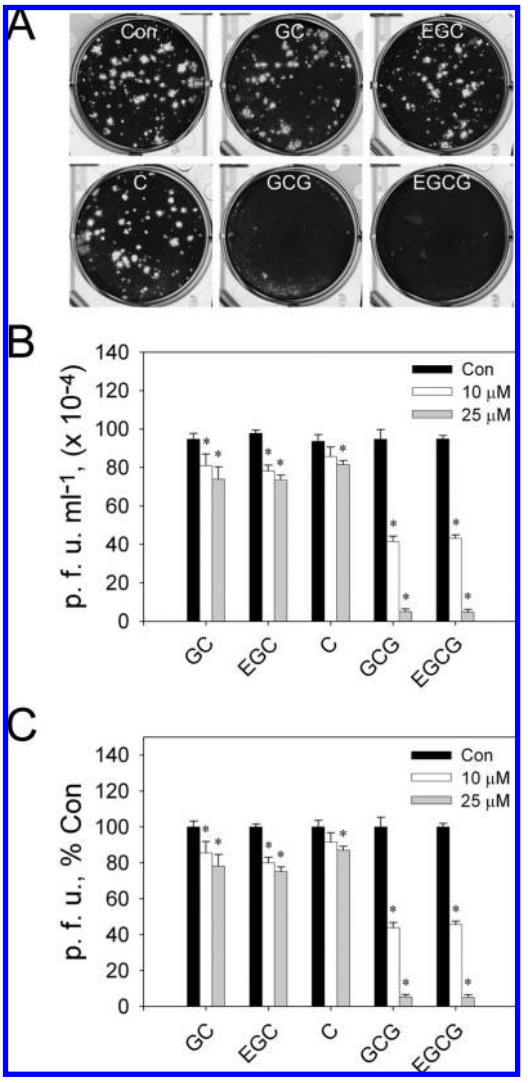

Figure 1. Inhibitory effect of tea polyphenols on production of infectious viral particles. (**A**) A typical plaque assay result shows that 25 μM GCG or EGCG inhibited production of infectious particles in Vero cells at 48 h after infection at moi of 1.25. (**B**) Vero cells were untreated (Con) or treated with 10 or 25 μM EGC, GC, C, GCG, or EGCG and infected with EV71 at an moi of 1.25. The progeny viral particles were quantified at 48 h pi. Data are expressed as pfu/mL lysate. (**C**) The number of viral particles produced in the treated cells is normalized to that of control, which is considered 100%. The results are presented as mean values ± SD of six experiments: (∗) $p < 0.05$, treated cells vs control.

Quantitative Reverse Transcription-Polymerase Chain Reaction (QRT-PCR) Analysis of EV71. Virus was allowed to adsorb on cells for 1 h. At the indicated time after infection, total RNA was extracted from infected cells using Viral RNA Extraction Miniprep kit (Viogene, Taiwan) according to the manufacturer's instruction. An amount of 1 μg of total RNA was reverse-transcribed into cDNA using SuperScript First-Strand Synthesis kit (Invitrogen, Carlsbad, California). Then 1 μL of a 1:100 dilution of the cDNA reaction was amplified with forward and reverse primers for EV71 genome and β-actin control, using LightCycler DNA Master SYBR Green I kit (Roche Diagnostics Corporation, Indianapolis, IN). The forward and reverse EV71 primers were 5′-ACTGACCAAGGACACTTCAC-3′ and

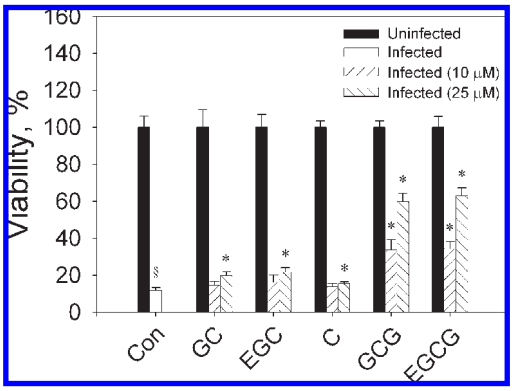

Figure 2. Cytoprotective effect of tea polyphenols. Vero cells were untreated (Con) or treated with the 10 or 25 μM EGC, GC, C, GCG, or EGCG and were uninfected and infected with EV71 at an moi of 1.25. Cell viability was quantified at 48 h pi. The solid bar within each treatment group shows the viability of the uninfected cells treated with 25 μM polyphenol under test. The results are presented as mean values ± SD of six experiments: (§) $p < 0.05$, infected cells vs uninfected cells within control group; (∗) $p < 0.05$, infected cells vs uninfected cells within each treatment group.

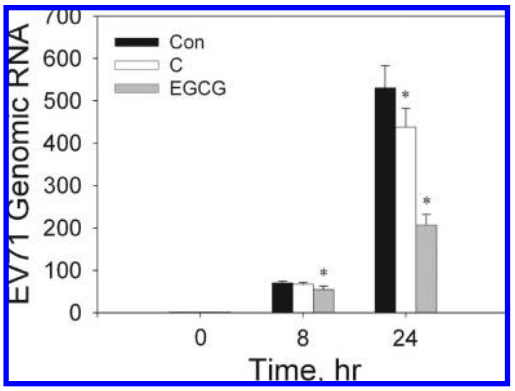

Figure 3. EGCG inhibits EV71 replication. Vero cells were untreated (Con) or treated with 25 μM C or EGCG and infected with EV71 at an moi of 1.25. At the indicated time, levels of EV71 genomic DNA were quantified by PCR. The results are presented as the mean ± SD of six experiments: (∗) $p < 0.05$, treated cells vs control.

5′-CCAGTGTGAGTTCCAAGTTT-3′, respectively. The forward and reverse β-actin primers were 5′-ATCGTGCGTGACATTAAGGAG-3′ and 5′-CCATCTCTTGCTCGAAGTCC-3′, respectively. The reactions were performed in LightCycler 1.2 real time PCR system (Roche Diagnostics Corporation, Indianapolis, IN) under the following thermal cycling conditions: 94 °C, 10 min; 35 cycles of 94 °C, 10 s; 57 °C, 10 s; and 72 °C, 30 s. The C_T value was normalized to that of β-actin.

Detection of Reactive Oxygen Species (ROS). To visualize the ROS formation, we microscopically examined the fluorescence of dichlorofluorescein (DCF) or oxidized MitoSOX Red derived from oxidation of their fluorogenic precursors. For dichlorofluorescin (H_2DCF)/MitoTracker Red double staining, cells were cultured on glass bottom culture dish (MatTek, MA). They were loaded with 100 nM MitoTracker Red and 5 μM H_2DCFDA for 20 min at 37 °C. They were counterstained with 5 μg/mL of Hoechst 33342 before examination under Zeiss LSM 510 Meta system (Carl Zeiss MicroImaging GmbH, Heidelberg, Germany). Confocal fluorescence images of labeled cells were obtained using Plan-Apochromat 100×1.40 NA oil immersion objective. During scanning of DCF, we used the 488 nm excitation line of an argon laser, beam splitter (HFT 405/488/561/633/KP720), and an emission window set at 505−550 nm. During scanning of MitoTracker Red, we employed the 561 nm excitation line of a DPSS laser and an emission window set at 575−615 nm. For scanning of Hoechst dye, we used the 405 nm excitation line of a diode laser and an emission window set at 420−480 nm. For MitoSOX Red/MitoTracker Green double staining, cells were loaded with 100 nM MitoTracker Green for 30 min and then with 2 μM MitoSOX Red for 20 min. The cells were counterstained with 5 μg/mL Hoechst 33342 before confocal microscopic examination. For scanning of oxidized MitoSOX dye, we used the 561 nm excitation line of DPSS laser and an emission window set at 575−615 nm. During scanning of MitoTracker Green, we employed the 488 nm excitation line of argon laser and an emission window set at 505−550 nm. All images were analyzed with Zeiss Zen software package (Carl Zeiss MicroImaging GmbH, Heidelberg, Germany).

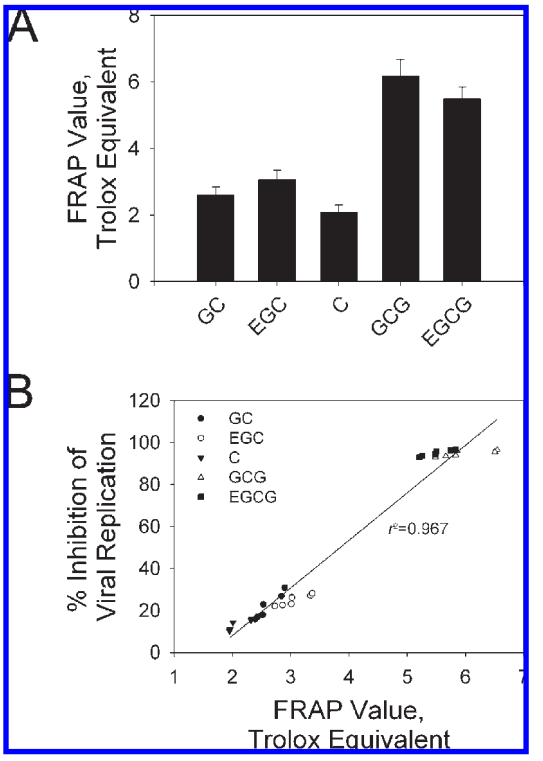

Figure 4. Correlation between antioxidant capacity of tea polyphenols and their antiviral capacity. (**A**) The antioxidant capacity of various tea catechins was determined by FRAP assay. The value on the ordinate (FRAP value, Trolox equivalent) is expressed as the millimolar (mM) concentration of Trolox with the same antioxidant capacity as a 1 mM solution of polyphenol under test. The results are presented as the mean ± SD of three experiments. (**B**) The correlation between viral inhibitory activity is plotted versus antioxidant capacity of tea polyphenols. The % inhibition of viral replication represents 100% minus the percentage of infectious viral particles produced after treatment with 25 μM solution of polyphenols (refer to **Figure 1C**). The squared Pearson's correlation coefficient r^2 is shown: $p < 0.001$.

ROS formation was also quantitatively analyzed by flow cytometric method. In brief, cells were loaded with H_2DCFDA/MitoTracker Red or MitoSOX Red/MitoTracker Green as described above. The loaded cells were washed twice with PBS and trypsinized for flow cytometric analysis as previously described (*29*). The mean fluorescence intensity (MFI) of channel for DCF or oxidized MitoSOX Red was quantified using CellQuest Pro software (Becton Dickinson, CA).

Ferric Reducing/Antioxidant Power (FRAP) Assay. The assay was performed as previously described with slight modifications (*33*). The FRAP reagent was freshly prepared by mixing 8 mM tripyridyltriazine (TPTZ), 20 mM $FeCl_3$, and 0.3 M sodium acetate (pH 3.6) in a ratio of 1:1:10. The absorbance of the sample was read at 590 nm. Antioxidant capacity of test compound was expressed as millimolar (mM) concentration of Trolox with the same antioxidant capacity as 1 mM solution of test compound.

Statistical Analysis. Results are presented as the mean ± SD. Data were analyzed by two-way analysis of variance (ANOVA). Tukey HSD test or t test was used to compare the mean values of groups. Pearson's

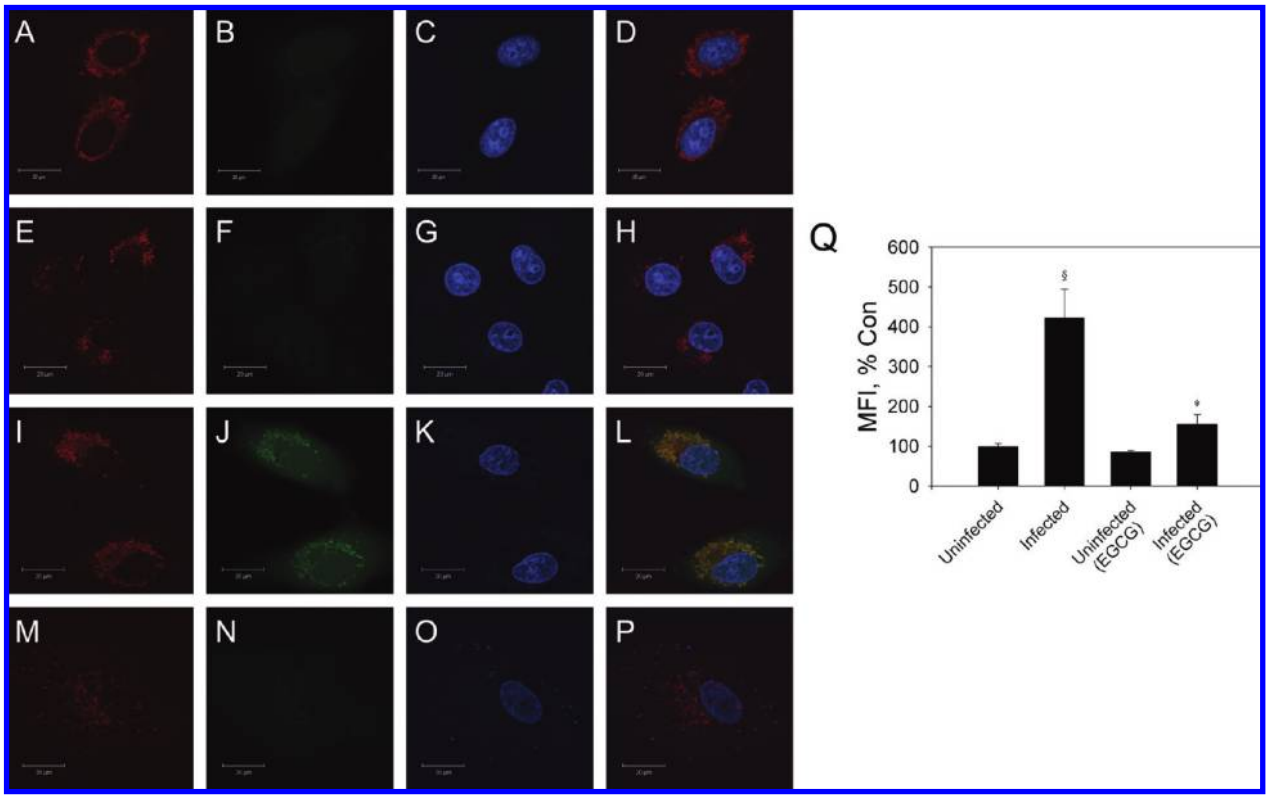

Figure 5. EGCG diminishes oxidative stress associated with EV71 infection. Vero cells were untreated (**A**−**D**, **I**−**L**) or treated (**E**−**H**, **M**−**P**) with 25 μM EGCG and uninfected (**A**−**H**) or infected (**I**−**P**) with EV71 at an moi of 1.25. At 48 h pi, the cells were loaded with H_2DCF and MitoTracker Red, counterstained with Hoechst 33342, and confocal images were acquired. The experiment was repeated thrice. Representative fields of MitoTracker Red (**A**, **E**, **I**, **M**), DCF (**B**, **F**, **J**, **N**), and Hoechst 33342 (**C**, **G**, **K**, **O**) labeled cells are shown. The corresponding images are overlaid (**D**, **H**, **L**, **P**): scale bar, 20 μm. (**Q**) Vero cells were untreated or treated with 25 μM EGCG and uninfected or infected with EV71 at an moi of 1.25. The cells were loaded with H_2DCF and analyzed by flow cytometry. The DCF MFI is plotted as shown. The results are presented as the mean ± SD of three experiments: (§) $p < 0.05$, infected cells vs uninfected cells; (∗) $p < 0.05$, EGCG-treated infected cells vs infected cells.

correlation coefficient was computed to characterize the correlation between antioxidant capacity of catechin and its inhibitory effect on viral replication. p values of less than 0.05 were considered significant. All the data analysis was performed with Statistical Package for Social Sciences, version 10.0 (SPSS Inc., Chicago, Illinois).

RESULTS

Inhibitory Effect of Catechins on EV71 Replication. To evaluate the antiviral effect of catechins on EV71, we tested their ability to inhibit the production of infectious EV71 virion in Vero cells. As shown in **Figure 1**, infection of Vero cells with EV71 at a multiplicity of infection (moi) of 1.25 yielded $(94.9 \pm 1.4) \times 10^4$ plaque forming units (pfu)/mL at 48 h postinfection (pi). EGCG and GCG significantly reduced the titer of EV71 progeny virus in a dose-dependent manner. Treatment with 10 μM GCG and EGCG resulted in 56% and 54% decrease in titer of progeny virus, respectively (**Figure 1B**). As their concentration reached 25 μM, the virus yields were further reduced to approximately 5% of control. GC and EGC showed lower inhibitory effect on EV71 replication. Treatment with 25 μM GC or EGC resulted in about 22% and 25% reduction in virus yield, respectively. Catechin (C) was relatively ineffective, reducing the virus yield by only 10% at a concentration of 25 μM.

Cytoprotective Effect of Catechins. The antiviral effect of EGCG and GCG was associated with their cytoprotective effect. As shown in **Figure 2**, the viability of Vero cells infected with EV71 at an moi of 1.25 was 12.0 ± 1.3%. Treatment with 25 μM GCG or EGCG increased the viability of infected cells by approximately 5-fold. Other catechins only had a modest cytoprotective effect. The viability of the infected cells increased to around 20% following treatment with 25 μM GC or EGC. Catechin (C) conferred minimal protection on the infected cells.

EGCG Suppresses Replication of Viral Genomic RNA. As EGCG and GCG have comparable inhibitory effect on EV71, and EGCG is more abundant than GCG, EGCG was used in most of subsequent experiments. To study whether EGCG affects replication of EV71 genomic RNA, we examined its effect on the copy number of viral genome using QRT-PCR. As shown in **Figure 3**, the copy numbers of viral genomic RNA in Vero cells at 8 and 24 h postinfection (pi) were 70.8- and 530.9-fold that at 0 h pi. EGCG treatment resulted in over 60% reduction in copy number of viral genome in cells at 24 pi. In contrast, C has a slight inhibitory effect (< 20%) on viral RNA synthesis.

Correlation between Viral Inhibitory Activity and Antioxidant Capacity. One of the important biochemical functions of catechins is their antioxidant activities. It is plausible that the antiviral activities of catechins may be attributed to their roles as antioxidants. To test such a possibility, we analyzed the antioxidant capacity of various catechins and examined its correlation with viral inhibitory activity. As shown in **Figure 4A**, GCG and EGCG had 3.1- and 2.8-fold higher antioxidant capacity than C. When FRAP values of catechins were plotted against their extent of inhibition of viral replication, there existed an excellent correlation between two parameters (**Figure 4B**). The viral inhibitory activity of catechins increased with their antioxidant capacity ($r^2 = 0.967$, $p < 0.0001$). These findings suggest that antiviral activity of EGCG is related to its antioxidant function.

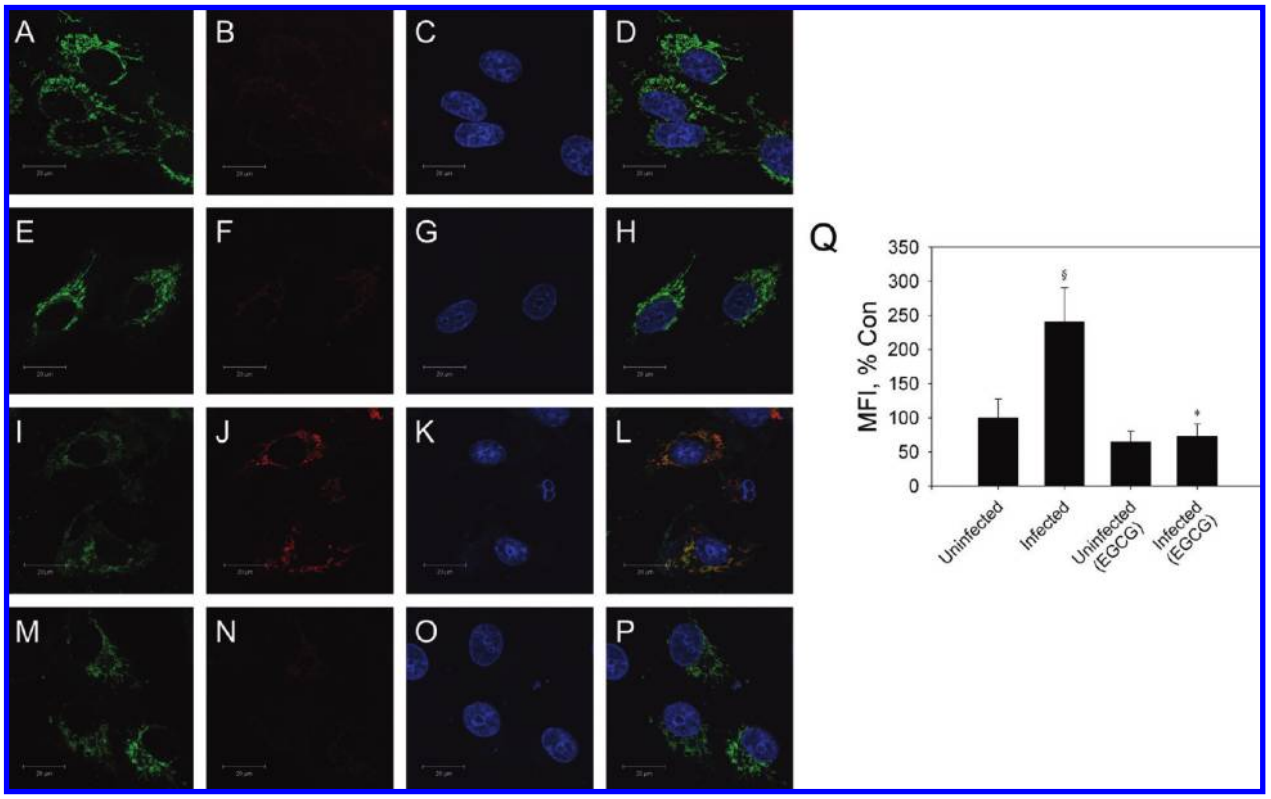

Figure 6. EGCG reduces EV71-induced superoxide anion generation. Vero cells were untreated (**A**−**D**, **I**−**L**) or treated (**E**−**H**, **M**−**P**) with 25 μM EGCG and uninfected (**A**−**H**) or infected (**I**−**P**) with EV71 at an moi of 1.25. At 48 h pi, the cells were loaded with MitoSOX Red and MitoTracker Green, counterstained with Hoechst 33342, and confocal images were acquired. The experiment was repeated thrice. Representative fields of MitoTracker Green (**A**, **E**, **I**, **M**), MitoSOX Red (**B**, **F**, **J**, **N**), and Hoechst 33342 (**C**, **G**, **K**, **O**) labeled cells are shown. The corresponding images are overlaid (**D**, **H**, **L**, **P**): scale bar, 20 μm. (**Q**) Vero cells were untreated or treated with 25 μM EGCG and uninfected or infected with EV71 at an moi of 1.25. The cells were loaded with MitoSOX Red dye and analyzed by flow cytometry. The MFI of oxidized MitoSOX Red is plotted as shown. The results are presented as the mean ± SD of three experiments: (§) $p < 0.05$, infected cells vs uninfected cells; (∗) $p < 0.05$, EGCG-treated infected cells vs infected cells.

EGCG Inhibited EV71-Induced Oxidative Stress. To further investigate the possibility that the antiviral effect of EGCG was associated with its antioxidant function, we monitored the change in ROS level in infected cells with or without EGCG treatment. The EV71-infected cells showed elevated DCF fluorescence (**Figure 5J**), consistent with our previous study (*29*). It appeared that ROS were mitochondrial in origin, as indicated by MitoTracker Red staining (**Figure 5I** and **Figure 5L**).Treatment with EGCG significantly reduced the intensity of DCF fluorescence in the infected cells (**Figure 5N**). In contrast, C did not efficiently quench virus-induced oxidative stress (Supporting Information Figure 1). Superoxide anion is a major free radical produced in mitochondria. We monitored the generation of superoxide anion in infected cells with the use of MitoSOX Red fluorogenic dye. EV71 infection led to increased generation of superoxide anion (**Figure 6I** and **Figure 6L**). The fluorescence diminished after treatment with EGCG (**Figure 6N**) but not after C treatment (Supporting Information Figure 1).

ROS generation was also quantitatively analyzed by the flow cytometric method. Mean fluorescence intensity (MFI) of DCF and oxidized MitoSOX Red in infected cells increased more than 4- and 2.4-fold, respectively (**Figure 5Q** and **Figure 6Q**). EGCG treatment caused over 60% reduction in MFI of these channels. Our findings indicate that EGCG can effectively suppress EV71-induced oxidative stress.

EGCG Diminished Virulence of EV71 in G6PD-Deficient Cells. Our previous studies have shown that G6PD-deficient HFF1 cells are subject to increased oxidative stress (*31*) and are more susceptible to EV71 infection than the wild-type HFF3 cells (*29*). To investigate whether catechins could mitigate the enhanced virulence of EV71 in HFF1 cells, we tested the effect of catechins on EV71-induced viability loss. As shown in **Figure 7A** and **Figure 7B**, the viabilities of infected HFF1 and HFF3 cells were 26.53 ± 3.54% and 46.19 ± 2.40%, respectively. Treatment with 50 μM EGCG effectively restored the viability of the former cells to 73.46 ± 6.72% and that of the latter cells to 83.23 ± 5.65%; GCG exerts its cytoprotective effect to a similar extent. Meanwhile, the other catechins had much less protective effect than GCG and EGCG.

To study whether catechins could inhibit the oxidative stress-enhanced EV71 replication, we evaluated the effect of catechins on formation of infectious progeny virions. Consistent with our previous study, the number of progeny virions derived from infection of HFF1 cells [$(21.60 \pm 2.34) \times 10^4$ pfu/mL] was significantly higher than HFF3 cells [$(11.00 \pm 1.75) \times 10^4$ pfu/mL]. After treatment with 50 μM EGCG, the abundance of progeny virus decreased by over 80% in both cell lines (**Figure 7C** and **Figure 7D**); GCG has a similar viral inhibitory effect. In contrast, GC, EGC, and C had much less viral inhibitory effect. Consistent with antiviral effect of EGCG, the copy number of EV71 genomic RNA in EGCG-treated infected cells was significantly lower than that of untreated cells (**Figure 7E**).

DISCUSSION

The present study establishes that EGCG and GCG, among tea polyphenols, exhibited the highest inhibitory effect on replication of EV71 and cytopathic changes (**Figures 1**−**3**). There was a positive correlation between antioxidant capacity of catechins

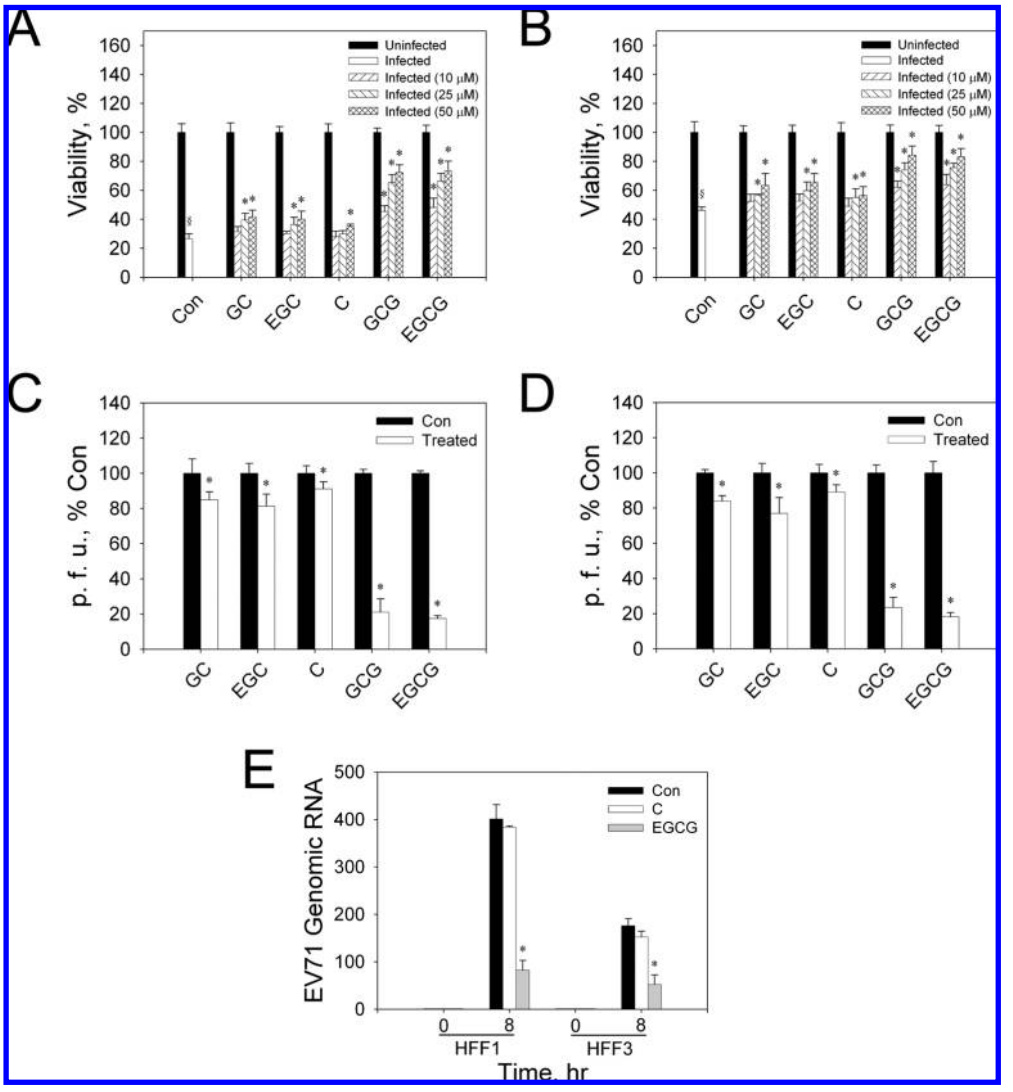

Figure 7. EGCG reduces the virulence of EV71 in G6PD-deficient cells. (**A**, **B**). HFF1 (**A**) and HFF3 cells (**B**) were untreated (Con) or treated with the indicated concentrations (10, 25, or 50 μM) of EGC, GC, C, GCG, or EGCG and uninfected and infected with EV71 at an moi of 1.25. Cell viability was quantified at 48 h pi. The solid bar within each treatment group shows the viability of the uninfected cells treated with 50 μM polyphenol under test. The results are presented as mean values ± SD of six experiments: (§) $p < 0.05$, infected cells vs uninfected cells within control group; (∗) $p < 0.05$, infected cells vs uninfected cells within each treatment group. (**C**, **D**). HFF1 (**C**) and HFF3 cells (**D**) were untreated (Con) or treated with 50 μM EGC, GC, C, GCG, or EGCG and infected with EV71 at an moi of 1.25. The progeny viral particles were quantified at 48 h pi. The number of viral particles produced in the treated cells is normalized to that of control, which is considered 100%. The results are presented as mean values ± SD of six experiments: (∗) $p < 0.05$, treated cells vs control. (**E**) HFF1 and HFF3 cells were untreated (Con) or treated with 50 μM C or EGCG and infected with EV71 at an moi of 1.25. At the indicated time, levels of EV71 genomic DNA were quantified by PCR. The results are presented as the mean ± SD of six experiments: (∗) $p < 0.05$, treated cells vs control.

and their antiviral activity (**Figure 4**). EV71 infection was associated with increased oxidative stress (**Figures 5** and **6**), which has been previously shown to enhance viral infectivity. EGCG treatment alleviated EV71-induced ROS generation (**Figures 5** and **6**), suggesting an antioxidant mechanism for its antiviral activity. Consistent with this, EGCG significantly suppressed the enhanced virulence of EV71 in G6PD-deficient cells (**Figure 7**). These findings suggest that the antiviral effect of EGCG may be attributed to its role as an antioxidant.

Tea is known to have antimicrobial activities, such as antibacterial, antifungal, and antiviral activities. Black tea extract was reported to inhibit growth of influenza virus in embryonated eggs and cell culture (*34*, *35*). Black tea extract and dark tea extract, to a lesser extent, were also found to have an inhibitory effect against rotavirus (*36*). Of tea constituents, polyphenols have been shown to inhibit viral infection (*37*−*44*). EGCG, or its derivative theaflavin digallate, suppresses the infectivities of influenza virus, rotavirus, poliovirus, coxsackievirus, and herpes simplex virus (*37*−*41*). Several mechanisms have been proposed for antiviral activities of catechins: the inhibitory effect on adsorption of influenza virus to cells (*37*); inhibition of neuraminindase activity of influenza virus (*39*); direct damage of herpes simplex virus (*40*); inhibition of reverse transcriptase of human immunodeficiency virus type I (*44*). Such mechanisms are unlikely to explain the antiviral effect of EGCG on EV71 for two reasons. First, the viral inoculum was not mixed with EGCG before infection of cell culture in our experiments. Second, EGCG had to be present in culture after the viral incubation phase in order for a complete cytoprotective effect to occur. This suggests that EGCG may inhibit at steps other than viral adsorption.

Oxidative stress is implicated as a host factor affecting the interactions between host and viral pathogens. Susceptibility to coxsachievirus and influenza virus infections is modulated by redox environment (*45*, *46*). We have recently shown that G6PD

deficiency enhances EV71 infection (*29*). Exogenous G6PD expression or *N*-acetylcysteine treatment suppresses EV71 propagation and confers a cytoprotective effect on the infected cells. Given the importance of oxidative stress in viral infection, the antiviral activity of EGCG is probably related to its role as antioxidant. Several lines of evidence advocate such a notion. First, EGCG and GCG, with potent antiviral activity against EV71, have a gallate group and a trihydroxy B ring. Such chemical groups play an important role in free radical scavenging abilities of catechins (*47*−*49*). It is consistent with our experimental findings. The viral inhibitory activity of catechins tested is in the order of EGCG ∼ GCG > EGC ∼ GC > C and is in excellent correlation with the FRAP values of catechins. Second, EGCG drastically reduces the EV71-induced oxidative stress, whereas C does not effectively do so. Moreover, EGCG and GCG suppress the enhanced EV71 replication in G6PD-deficient cells, as does exogenous G6PD expression or *N*-acetylcysteine treatment (*29*). These findings suggest that EGCG exerts its antiviral activity through its biochemical role as antioxidant.

The findings are consistent with our model for the relationship between the redox status of the host cell and EV71. After entry into a cell, EV71 viral RNA is translated into proteins and replication ensues. These processes may be sensitive to the intracellular redox milieu, being more active in an oxidized environment. Expression of viral protein(s) induces ROS generation from mitochondria. This sets up a vicious cycle of ROS production and viral replication. If antioxidant, such as EGCG, is present, the intracellular environment is shifted toward the reducing end. The cycle is impeded, resulting in diminution of viral replication and oxidative stress.

All in all, our present findings advocate the notion that the redox status of host cells modulates the infectivity of EV71. In addition, treatment with EGCG can reduce progeny virus production, suggesting its use as a therapeutic and/or prophylactic measure against enteroviral infections. This would be particularly helpful, as no effective EV71 vaccine is currently available. A concern about the pharmacological application of EGCG is its limited bioavailability. This can be solved or ameliorated by development of a ECGG prodrug (*50*) or coadministration with substances such as genistein and piperine (*51*, *52*). Prospectively, supplementation with natural antioxidants or their derivatives might be used as a preventive measure against clinically relevant viruses.

ABBREVIATIONS USED

C, catechin; DCF, dichlorofluorescein; EGC, epigallocatechin; EGCG, epigallocatechin gallate; EV71, enterovirus 71; GC, gallocatechin; GCG, gallocatechin gallate; G6PD, glucose-6-phosphate dehydrogenase; H_2DCF, dichlorofluorescin; H_2DCFDA, dichlorofluorescin diacetate; HFF1, G6PD-deficient fibroblasts; HFF3, normal fibroblasts; MFI, mean fluorescence intensity; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide; moi, multiplicity of infection; pfu, plaque forming unit; ROS, reactive oxygen species.

Supporting Information Available: Figure containing effect of catechin on EV71-induced oxidative stress. This material is available free of charge via the Internet at http://pubs.acs.org.

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