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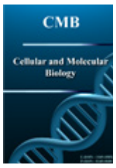

Original Article

EGCG inhibits the oxidative damage induced by TiO_2-NPs in human colon cell lines

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Abstract

To assess the protective effects of (-)-Epigallocatechin-3-gallate (EGCG), a natural antioxidant, against cellular oxidative damage induced by titanium dioxide nanoparticles (TiO_2-NPs), Human Colon cells NCM460 and Colon Cancer cells SW620 were selected for this study. The cells were divided into three groups: control group, TiO_2-NPs (80 μg/mL) exposure group, and EGCG (20 μmol/L)+TiO_2-NPs (80 μg/mL) co-exposure group. The study evaluated the precipitation rate of TiO_2-NPs influenced by EGCG in a cell-free system. It also measured the levels of ROS, MDA, and total antioxidant capacity in the cells of each group. The uptake of TiO_2-NPs by the cells was assessed using the SSC_e/SSC_0 ratio, and genome instability was evaluated. The results demonstrated that the addition of 20 μmol/L EGCG to the system resulted in greater sedimentation of TiO_2-NPs compared to TiO_2-NPs alone ($P<0.05$). The SSC_e/SSC_0 values in the co-exposure group were significantly lower than those in the TiO_2-NPs alone group ($P<0.001$). TiO_2-NPs induced a higher oxidative stress index in the cells ($P<0.001$), while the co-exposure group exhibited a lower REDOX index ($P<0.001$). The combination of EGCG and TiO_2-NPs did not significantly affect genome instability in either cell line. Importantly, EGCG showed a certain inhibitory effect on oxidative damage to colon cells induced by TiO_2-NPs, with no significant difference observed between normal and cancer cells in terms of this protective effect. Conducting a comprehensive investigation into the interaction mechanism between EGCG and TiO_2-NPs is crucial for establishing a scientific foundation that can guide the optimal utilization of the antioxidant properties of EGCG to mitigate the toxicity associated with TiO_2-NPs.

Keywords: EGCG; Genotoxicity; Oxidative stress; TiO_2-NPs

1. Introduction

In recent years, nanotechnology has experienced rapid advancements, with numerous nanoparticle applications permeating our daily lives, including medical devices, personal care products, electronics, and pharmaceuticals [1]. Among the most commonly utilized nanoparticles are titanium dioxide nanoparticles (TiO_2-NPs), which have led to a growing frequency of human exposure to these particles [2]. TiO_2-NPs exist in three distinct crystal forms: anatase, rutile, and the less common brookite [3-4]. Anatase, due to its superior photocatalytic activity, is considered the most toxic of these forms; paradoxically, it also boasts a broader range of applications compared to rutile and brookite [5].

Research on the adverse effects of TiO_2-NPs has been conducted across various *in vivo* and *in vitro* models, including bacteria, algae, zebrafish, mice, nematodes, plants, and different human cell lines [6-10]. Jaeger et al. reported that HaCaT cells exposed to TiO_2-NPs induced a mitochondrial "common deletion". Furthermore, these nanoparticles have shown ROS-mediated cytotoxic and genotoxic potential in human keratinocytes [11]. Shukla et al. explored the genotoxic effects on human epidermal cells (A431) exposed to TiO_2-NPs and concluded that the oxidative stress and ROS generated by the nanoparticles lead to oxidative DNA damage and the formation of micronuclei [12]. The consensus among these studies is that the toxic effects of nanoparticle exposure are primarily associated with the generation of ROS. Additionally, nanoparticles used in medical treatments and diagnostics can enter the bloodstream through various means and interact with endothelial cells, potentially damaging the endothelial layer [13-14]. This damage can trigger acute inflammation and lead to a variety of pathophysiological conditions, including thrombosis, neurotoxicity, and myocardial infarction [15-16]. Therefore, understanding the adverse effects of TiO_2-NPs on both the environment and human health, as well as identifying strategies to mitigate their impact, is of paramount importance.

Various natural compounds have been found to combat ROS by participating in electron exchange, thereby mitigating the damage caused by NPs exposure. A study demonstrated that co-exposure to nano-nickel oxide and

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curcumin effectively protected human airway epithelial HEp-2 cells and human MCF-7 cells from oxidative stress, cytotoxicity, and apoptosis induced by nano-nickel oxide [17]. Vitamin C demonstrated its efficacy in reducing oxidative stress, inflammation, and hepatic damage induced by ZnO-NPs in rats [18]. The effects of pre-and co-exposure to idebenone, carnosine, glycyrrhizic acid, and vitamin E with TiO_2-NPs were investigated to understand their potential in attenuating TiO_2-NPs-induced lipid peroxidation, inflammation, oxidative stress, elevated liver-function enzyme activity, as well as TNF-α and IL-6 levels in the livers of mice and rats[19].

(-)-Epigallocatechin-3-gallate (EGCG), the most prevalent polyphenolic compound in green tea, has garnered significant interest for its potent antioxidative, antitumor, and neuroprotective properties [20-21]. EGCG has a strong chelating and antioxidant activity due to its two gallocatechol rings which can directly remove free radicals with high efficacy. Despite these advantages, EGCG suffers from several pharmacochemical drawbacks, most notably its high instability, which leads to reduced bioavailability and diminished efficacy [22]. To address this, innovative formulations have been devised, encapsulating catechins within nanoparticles to enhance their bioavailability and stability [23-24]. In recent years, nanotechnology has emerged as a promising therapeutic approach, particularly in the realm of targeted drug delivery for diseases such as cancer, diabetes, and neurodegenerative conditions. This strategy leverages the unique properties of nanoparticles to improve the precision and effectiveness of treatments, offering a potential avenue for overcoming the limitations of traditional drug delivery methods [25-26].

This study took commonly used TiO_2-NPs as the model to investigate whether the antioxidant activity of EGCG can alleviate the cytotoxicity and genotoxicity induced by TiO_2-NPs. Additionally, the study aimed to examine the combined effects of EGCG and TiO_2-NPs on the anticancer activity of EGCG.

2. Materials and Methods

2.1. Chemicals

EGCG (purity ≥ 98%) was procured from Yuanye Bio-Technology (Shanghai, China). TiO_2-NPs (<25 nm, anatase) and cytochalasin-B were acquired from Sigma Aldrich (St.Louis, MO, USA). Cytochalasin-B was prepared at a concentration of 600 µg/mL in dimethyl sulfoxide (DMSO) and stored at -20 °C. Prior to use, it was diluted to the required concentration in the medium. The final DMSO concentration was kept below 0.25% (v/v), a level that was determined to have no cytotoxic or genotoxic impact [27].

2.2. Cell Culture

NCM460 and SW620 cell lines, both of which are adherent, were sourced from the Kunming Institute of Zoology, CAS (Yunnan, China). These cell lines were cultivated as monolayers in RPMI-1640 medium (Gibco, NY, USA), supplemented with 10% newborn calf serum (Gibco, NY, USA), 1% penicillin (5000 IU/mL)/streptomycin (5 mg/mL) solution (Gibco, NY, USA), and 1% L-glutamine (2mM) (Gibco, NY, USA). Cultivation took place in 25 cm^2 flasks (Corning, NY, USA), with the cells maintained at 37 °C in an atmosphere containing 5% CO_2.

2.3. MTT Assay

NCM460 cells were seeded into 96-well plates (Corning, NY, USA) at a density of 2.5×10^4 cells/mL and treated with varying concentrations of EGCG (0~40 µmol/L) and TiO_2-NPs (0~80 µg/mL). Following a 24-hour incubation period, 10 µL of 5 mM MTT was introduced into each well. Cells were then incubated with MTT at 37 °C for 4 hours. Subsequently, 100 µL of DMSO was added to dissolve the MTT. After a further incubation at 37 °C for 10 minutes, the absorbance at 570 nm for each well was measured using a microplate reader. This process was conducted in duplicate and repeated three times for each concentration.

2.4. Ultraviolet spectrophotometer detection

A UV spectrometer was utilized to assess the impact of EGCG on the sedimentation of TiO_2-NPs in a cell-free system, which was categorized into three groups based on the MTT assay results: a control group (RPMI-1640 cell culture medium containing 10% calf serum, without TiO_2-NPs and EGCG), TiO_2-NPs (80 µg/mL) group, and TiO_2-NPs (80 µg/mL)+EGCG (20 µmol/L) group. Each group was diluted to an equal volume with RPMI-1640 cell culture solution containing 10% calf serum, thoroughly shaken and mixed prior to the experiment. The initial absorbance value (A_0) was measured at 505 nm using the ultraviolet spectrometer. Subsequently, the absorbance value (A_e) was recorded hourly for a total of 12 hours as the solution was allowed to settle. The average values for each group were obtained from repeated measurements. The ratio of Ae to A0 was calculated to determine the sedimentation rate of TiO_2-NPs.

2.5. Flow cytometry

NCM460 cells were seeded into 6-well plates at a density of 1×10^5 cells/mL and cultured in the control group, TiO_2-NPs (80 µg/mL) exposure group, and TiO_2-NPs (80 µg/mL) and EGCG (20 µmol/L) co-exposure group for 24 h, 48 h, and 72 h. Following each treatment period, the culture medium was discarded, and the cells were washed twice with PBS (pH 7.2). Trypsin was subsequently added to digest the cells. A cell suspension was created by pipetting with RPMI-1640 medium, and the side scatter (SSC) value of each cell solution under red fluorescence (488 nm) was measured using flow cytometry. The ratio of SSC in the experimental group (SSC_e) to SSC in the control group (SSC_0) was calculated to assess the cell's capability of incorporating TiO_2-NPs.

2.6. REDOX detection

NCM460 or SW620 cells were seeded into 24-well plates and cultured with TiO_2-NPs (80 µg/mL) or TiO_2-NPs (80 µg/mL)+EGCG (20 µmol/L) for 24 h, 48 h, and 72 h. ROS detection kit (Beyotime, China, #S0033M), MDA detection kit (Beyotime, China, #S0131M), and total antioxidant capacity detection kit (Beyotime, China, #S0119) were used to determine the levels of REDOX indexes.

2.7. Cytokinesis-Block Micronucleus Cytome (CBMN-Cyt) Assay

NCM460 or SW620 cells were cultured for 72 hours in RPMI-1640 medium supplemented with either TiO_2-NPs (80 µg/mL) or TiO_2-NPs (80 µg/mL)+EGCG (20 µmol/L).

The medium was discarded after the treatment, and the cells were washed twice with PBS (pH 7.2). Fresh medium with 1.5 µg/mL cytochalasin B was added to each culture to block cytokinesis, followed by rinsing with PBS after a further 26 h. The cells were then processed according to the method described by Fenech [28]. This involved centrifuging the cells onto glass slides, fixing them in 100% cold methanol at -20 °C for 15 minutes, and staining with 10% Giemsa (San'ersi, Shanghai, China). The biomarkers of the Cytokinesis-Block Micronucleus Cytome (CBMN-Cyt) assay were evaluated at 1000× magnification under an optical microscope (Olympus, Tokyo, Japan) by a single observer, following the criteria established by Fenech (2006). A total of 1000 binucleated cells (BNCs) per group were analyzed to assess the frequency of micronuclei (MNs), nucleoplasmic bridges (NPBs), and nuclear buds (NBUDs), from which the frequency of genome instability (GIN) was determined.

2.8. Statistical Analysis

Statistical analyses were conducted using SPSS 22.0 for Windows (SPSS, Chicago, IL, USA). The normality of all datasets was assessed with the Kolmogorov–Smirnov test. To compare the values between the control and treated groups, an independent-sample t-test or one-way analysis of variance (ANOVA) was employed. Statistical significance was determined for differences with a *P*-value (two-tailed) less than 0.05. All graphical representations were created using GraphPad PRISM 5.0 (GraphPad, San Diego, CA, USA).

3. Results

3.1. EGCG promoted the deposition of TiO_2-NPs

In a cell-free system, the addition of 20 µmol/L EGCG to RPMI-1640 as the solvent significantly reduced the absorption ratio of TiO2-NPs (80 µg/mL) compared to TiO_2-NPs alone ($P<0.05$, Fig. 1). This suggests that EGCG enhances the sedimentation of TiO_2-NPs. This observation points to a potential interaction between EGCG and TiO_2-NPs that influences the physical behavior of the nanoparticles, with implications for their biological effects and applications.

3.2. EGCG inhibited the uptake of TiO_2-NPs by NCM460 cells

Following exposure to TiO_2-NPs for 24, 48, and 72 hours, there was a significant increase in the SSC_e/SSC_0 values ($P<0.001$, Fig 2), signifying that the cells had absorbed substantial quantities of TiO_2-NPs. Notably, after 24 and 48 hours of treatment, the SSC_e/SSC_0 ratios in the group co-exposed to EGCG and TiO_2-NPs were significantly lower than those in the group exposed solely to TiO_2-NPs ($P<0.05$, Fig 2), indicating that EGCG was able to partially inhibit the uptake of TiO_2-NPs by NCM460 *in vitro*.

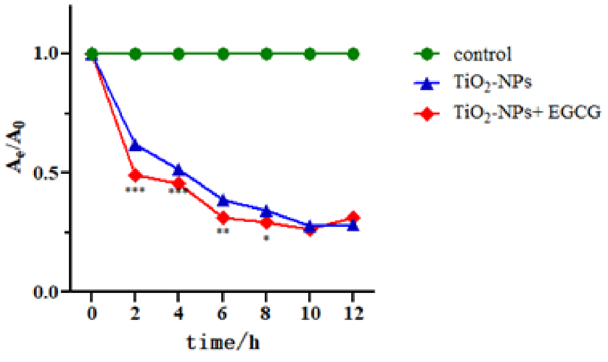

Fig. 1. Effect of EGCG on the TiO_2-NP sedimentation rate; * $P< 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. TiO_2-NPs.

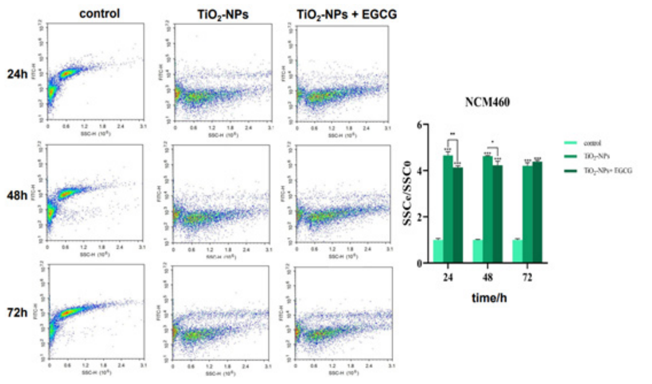

Fig. 2. Effect of EGCG on TiO_2-NPs uptake by NCM460 cells; *$P < 0.05$, **$P< 0.01$, ***$P< 0.001$.

3.3. Effects of EGCG on the ROS, MDA, and total antioxidant capacity of NCM460 and SW620 cells induced by TiO_2-NPs

Fig 3A shows that in NCM460 cells, the levels of ROS in the group exposed to TiO_2-NPs were significantly higher than those in the control group at 24, 48, and 72 hours ($P<0.001$). However, the ROS levels in the group simultaneously exposed to EGCG and TiO_2-NPs were significantly lower compared to the group exposed only to TiO_2-NPs ($P<0.001$). This indicates that EGCG potentially offers a protective effect, reducing the oxidative stress induced by TiO_2-NPs.

Fig 3B reveals that the MDA levels in the TiO_2-NPs group were significantly elevated when compared to the control group after 24 and 48 hours of exposure ($P<0.001$). Additionally, it was observed that the MDA levels in the groups co-exposed to EGCG and TiO_2-NPs were significantly greater than those in the group exposed solely to TiO_2-NPs ($P<0.001$). Following 72 hours of exposure, the MDA levels in both the TiO_2-NPs group and the EGCG+TiO_2-NPs group were notably lower than those observed in the control group ($P<0.001$). Moreover, the MDA levels in the EGCG+TiO_2-NPs group were significantly reduced compared to the TiO_2-NPs group alone ($P<0.001$).

Fig 3C presents the results for the total antioxidant capacity of the cells across each treatment group. After 24 hours of treatment, there was a significant reduction in the levels of intracellular total antioxidant capacity in both the TiO_2-NPs group and the EGCG+TiO_2-NPs group compared to the control ($P<0.001$), and there were no significant differences between the two treatment groups. After 48 h of treatment, the total antioxidant capacities of the TiO_2-NPs and EGCG+TiO_2-NPs groups were also significantly decreased ($P<0.001$). In comparison with the group exposed to TiO_2-NPs alone, the level of total antioxidant capacity in the co-exposure group was significantly reduced ($P<0.001$). After 72 hours of exposure, the level of total antioxidant capacity in the TiO_2-NPs group

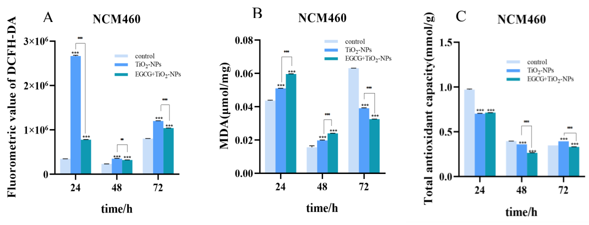

Fig. 3. Effects of EGCG on the REDOX indexes of NCM460 cells induced by TiO_2-NPs; $^{**}P< 0.01$, $^{***}P< 0.001$.

was significantly increased ($P<0.001$). The total antioxidant capacity in the co-exposure group was significantly lower than that in the group exposed to TiO_2-NPs alone ($P<0.001$). This indicates that for the 72-hour exposure, the cells in the TiO_2-NPs group may have initiated a compensatory response to enhance their antioxidant defenses, while the co-exposure to EGCG and TiO_2-NPs resulted in further depletion of these defenses.

To further investigate whether the effects of EGCG on TiO_2-NPs induced oxidative damage were different in normal and cancer cells, we also analyzed the REDOX markers in SW620 colon cancer cells and found that the response to each treatment group in the SW620 colon cancer cells was similar to that in the normal NCM 460 colon cells. TiO_2-NPs significantly induced oxidative stress in these cells ($P<0.001$, Fig 4), while the addition of EGCG was able to enhance the antioxidant capacity of the cells and significantly inhibit intracellular oxidative stress ($P<0.001$, Fig 4). Comparing Fig 3 and Fig 4, we found that SW620 cells exhibited a stronger antioxidant response than the NCM460 cells, suggesting variable sensitivities to oxidative stress and the protective effects of EGCG between different cell types.

3.4. Effects of EGCG on TiO_2-NP induced genotoxicity in NCM460 and SW620 cells

In this study, the CBMN-cyt assay was employed to evaluate the impact of TiO_2-NPs, both independently and in combination with EGCG, on the genomic instability (GIN) of NCM460 and SW620 cells. The results indicated that there were no substantial differences between the treatment groups and the control group (Fig 5). This suggests that the presence of TiO_2-NPs, with or without EGCG, did not significantly alter the genomic stability of the cells under the conditions of this study.

4. Discussions

Due to their wide application in various sectors including building engineering, environmental protection, and the food and cosmetic industries, the use of TiO_2-NPs has become increasingly prevalent [4]. Consequently, the potential risks associated with TiO_2-NPs exposure have also heightened. It is crucial to thoroughly investigate their effects on the human body and explore strategies to mitigate these risks. Further research is necessary to gain a comprehensive understanding of the potential health implications and develop effective measures for reducing the risk associated with TiO_2-NPs exposure.

Extensive studies in the field of nanotoxicology have revealed that the generation of free radicals is a primary mechanism for toxicity, which subsequently leads to DNA oxidative damage, cytotoxicity, and apoptosis, both *in vivo* and *in vitro* [6-12]. While the use of antioxidants has been recognized as an important strategy to counteract oxidative stress, the extent to which antioxidants can protect against nanoparticle-induced toxicity has not been thoroughly investigated. Therefore, this study aimed to assess the potential protective effects of EGCG against TiO_2-NPs in both normal NCM460 colon cells and SW620 colon cancer cells.

In our initial investigation, we examined the impact of EGCG on the sedimentation of TiO_2-NPs in a cell-free system. Interestingly, we observed a significant reduction in the absorption ratio when 20 μmol/L EGCG was introduced to the system alongside TiO_2-NPs (80μg/mL), compared to when TiO_2-NPs were added alone (Fig 1). This finding suggests that EGCG promotes the sedimentation of TiO_2-NPs. Subsequently, we utilized flow cytometry to measure the SSC_e/SSC_0 ratio, which assesses the uptake of particles by NCM460 cells. Notably, the SSC_e/SSC_0 values in the co-exposure group of TiO_2-NPs and EGCG were significantly lower than those in the group exposed to TiO_2-NPs alone (Fig 2), indicating that the presence of EGCG influenced the entry of TiO_2-NPs into the cells. Based on these findings, we hypothesize that one potential reason for EGCG's promotion of TiO_2-NPs sedimentation is the formation of larger aggregates through electrostatic interactions between TiO_2-NPs and EGCG. This aggregation may reduce the contact between TiO_2-NPs and cells. As a result, the number of particles entering the cells is reduced, thereby minimizing the impact of TiO_2-NPs on intracellular components. Additionally, the physicochemical properties of TiO_2-NPs bound to EGCG may be masked, potentially reducing their toxic effects after cellular entry.

Furthermore, our study demonstrated that exposure to TiO_2-NPs led to a substantial increase in ROS levels in both NCM460 and SW620 cells, indicating the induction of oxidative stress upon cellular entry. These findings

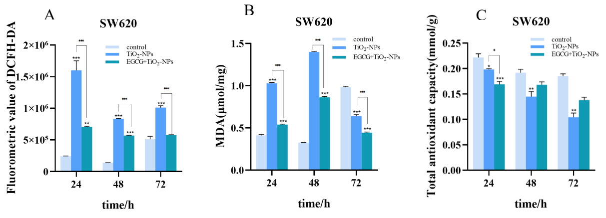

Fig. 4. Effects of EGCG on the REDOX indexes of SW620 cells induced by TiO_2-NPs; *P< 0.05, **P< 0.01, ***P< 0.001.

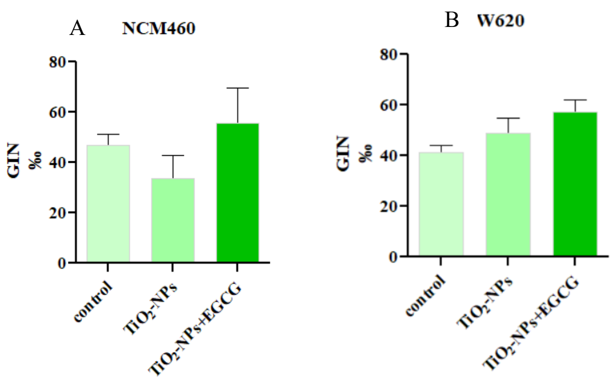

Fig. 5. Effects of EGCG on the GIN in NCM460 and SW620 cells induced by TiO_2-NPs.

align with previous studies by Jaeger et al. and Shukla et al. [11-12]. However, when EGCG was co-administered with TiO$_2$-NPs, we observed a significant reduction in ROS levels compared to cells exposed to TiO$_2$-NPs alone (Fig 3A). This suggests that EGCG effectively inhibits intracellular oxidative stress by limiting the entry of particles into the cells.

Malondialdehyde (MDA) is a byproduct of membrane lipid peroxidation and serves as an indicator of oxidative stress levels. Increased MDA levels can result in the oxidation of polyunsaturated fatty acids in biofilms, leading to cell damage through the decomposition products of lipid hydroperoxide. This process alters the configuration, structure, and permeability of the cell membrane, inducing oxidative stress. Our findings revealed that after 24 and 48 hours of exposure to TiO$_2$-NPs, both NCM460 and SW620 cells exhibited significantly elevated MDA levels, indicating intracellular lipid oxidation as a result of TiO$_2$-NPs exposure. Interestingly, even after co-treatment with EGCG and TiO$_2$-NPs, the MDA level in NCM460 cells remained significantly increased (Fig 3B), while it was significantly decreased in SW620 cells. This suggests that the unique self-oxidant and antioxidant properties of EGCG influenced cellular oxidative stress during its interaction with TiO$_2$-NPs. Previous studies have demonstrated that due to its specific structure, EGCG undergoes automatic oxidation in the medium and exhibits pro-oxidation activity for a certain period of time, leading to an oxidative stress response and oxidative damage [29]. Therefore, in the co-exposure group of TiO$_2$-NPs and EGCG, a certain amount of MDA might have been produced during the autoxidation process of EGCG, which was not metabolized in time, resulting in increased MDA accumulation in the cells. In SW620 cells, after TiO$_2$-NPs exposure, the total antioxidant capacity in the TiO$_2$-NPs groups was significantly decreased, and the addition of EGCG was able to enhance the antioxidant capacity after 48 and 72 h. Comparing the two cell lines, we observed that SW620 cells exhibited a stronger antioxidant response ability compared to NCM460 cells.

The GIN has been identified as a primary cause of many human genetic-environmental interaction diseases, and it is highly correlated with an increased risk of birth defects, immunodeficiency, and degenerative diseases, such as cardiovascular disease, Alzheimer’s disease, and cancers. Our previous study discovered that polyphenols such as resveratrol, tea polyphenols, and geranium can potentially induce high levels of GIN, leading to apoptosis in cancer cells. This may represent one of the mechanisms underlying their anticancer activity [30-31]. However, our current study did not find any significant differences between the treatment groups exposed to TiO$_2$-NPs alone, those co-exposed with EGCG, and the control group (Fig 5). This result contradicts our initial expectation. Previous research from our group has demonstrated that EGCG can reduce cellular GIN, and its pro-oxidation properties through autoxidation may underlie its antioxidant and anticancer effects [30]. In this study, we also observed the pro-oxidative effects of EGCG and its accompanying antioxidant activity. Interestingly, when cells were exposed to both EGCG and TiO$_2$-NPs, the short-term reduction in GIN rate mediated by EGCG was not evident. This may be due to the fact that, in the short term, EGCG primarily functions to counteract the TiO$_2$-NPs-induced intracellular hyperoxidation environment. It is possible that cells require more time to respond to oxidative stress, and therefore, in future studies, we will extend the treatment duration to explore this further.

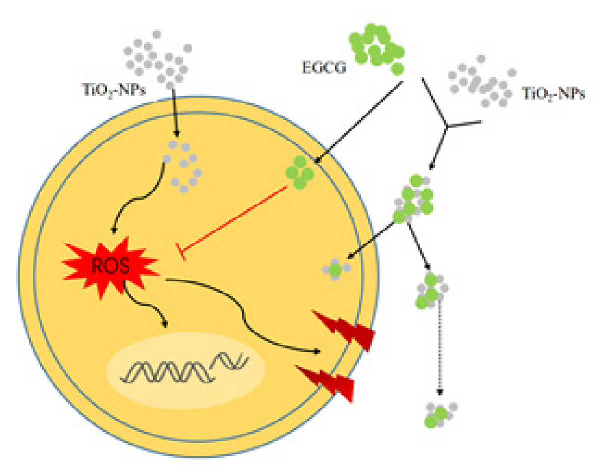

Fig. 6. Possible mechanism of EGCG’s inhibition of oxidative damage induced by TiO$_2$-NPs in colon cells.

5. Conclusions

In summary, the protective effect of EGCG on colon cells against TiO$_2$-NPs-induced damage can be attributed to two main aspects. Firstly, EGCG promotes the deposition of TiO$_2$-NPs, leading to a decrease in their uptake by cells. Secondly, the antioxidant activity of EGCG helps to reduce the oxidative stress caused by TiO$_2$-NPs (Fig 6). As a result, EGCG demonstrates a certain inhibitory effect on the oxidative damage inflicted by TiO$_2$-NPs on colon cells, with no significant difference observed between normal and cancer cells. Further investigation into the mechanism underlying the interaction between EGCG and TiO$_2$-NPs will contribute to a better understanding of how to utilize EGCG effectively in reducing the toxicity associated with TiO$_2$-NPs.

Conflict of interest

All authors declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Consent for publications

All authors have to write this sentence that they read and approved the final manuscript for publication.

Ethics approval and consent to participate

The authors have to declare that we do not use humans or animals in their research.

Availability of data and material

The authors have to declare that we embedded all data in the manuscript.

Author contributions statement

Han Wang, Xu Wang, and Juan Ni mainly responsible for the design of this study. Yundong Xu, Xiang Li, Yaping Tian, Qidan Li, and Yongzhen Zhang conducted the experiment. Juan Ni and Han Wang edited and refined the manuscript. All authors have read and approved the final manuscript.

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