Protective effects of theaflavins and epigallocatechin gallate against ZnO-NP-induced apoptosis in rat tracheal epithelial cells

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Research Article

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Abstract

Zinc oxide nanoparticles (ZnO-NPs) can affect human health primarily via inhalation. This study evaluated the protective effects of theaflavins (TFs) and epigallocatechin gallate (EGCG) against ZnO-NP-induced cytotoxicity in rat tracheal epithelial (RTE) cells. After exposure to ZnO-NPs (100 µg/L), treatment with TFs and EGCG (10, 100 and 1000 µg/L) significantly inhibited the levels of reactive oxygen species (ROS), and the content of malondialdehyde (MDA). Treatment also alleviated apoptosis induced by oxidative stress, which was achieved by inhibiting cytochrome C (*CytoC*) and *Caspase 3/8/9* mRNA expression. Upon treatment with the highest concentrations of TFs and EGCG (1000 µg/L), *CytoC* gene expression was downregulated by 59.10% and 77.27%, *Caspase 3* gene expression by 50.03% and 60.01%, *Caspase 8* gene expression by 45.11% and 55.57%, and *Caspase 9* gene expression by 51.33% and 66.67%. In addition, about the interleukin family as interleukin 1β (*IL-1β*) and interleukin 6 (*IL-6*), tumor necrosis factor-α (*TNF-α*) and the other inflammatory chemokines like C-C motif chemokine 2 (*CCL2*), C-X-C motif chemokine 8 (*CXCL8*) were upregulated in RTE cells in the presence of ZnO-NPs. All factors were gradually rescued after the addition of TFs and EGCG. These results showed that TFs and EGCG could effectively protect RTE cells from oxidative damage induced by ZnO-NPs through antiapoptotic and antioxidant effects.

1. Introduction

Zinc oxide nanoparticles (ZnO-NPs) are a novel multifunctional inorganic material used in a variety of commercial applications (Ji et al. 2008). They can enter the body and stimulate respiratory epithelial cells, promoting lung diseases such as asthma (Zuyderduyn et al. 2006). Patron-Romero et al. (Patron-Romero et al. 2020) exposed C2C12 mouse fibroblasts, L929 myoblasts and HLPB human peripheral blood lymphocytes to 20–30 µg/L ZnO-NPs with particle sizes of 16–24 nm for 6 h, 12 h, and 24 h, after which the cells exhibited varying degrees of DNA damage accompanied by severe cell death. Goma et al. (Goma et al. 2021) found that 50 nm ZnO-NPs induced oxidative stress damage in male rats, which triggered an inflammatory response. ZnO-NPs induced inflammatory and apoptosis in the lung cells of female mice (Rossner et al. 2019). Therefore, the respiratory health risk of ZnO-NPs deserves attention, and the threat to human health needs to be taken seriously. However, in the medical field, where nanomaterials can be used for drug delivery, targeted therapy of tumors, etc., it is particularly important to reduce its negative effects. So it is essential to find the necessary mitigation measures.

As evidenced by the above studies, respiratory diseases induced by ZnO-NPs are often caused by oxidative stress damage. Therefore, regulating the balance of the intracellular redox system is an important tool for the treatment of such diseases. It has been found that bioactive substances from plants have good efficacy in alleviating intracellular oxidative stress. For example, chemicals derived from plants can have a potential therapeutic effect on cellular stress induced by NPs (Margaret et al. 2000). Theaflavins (TFs) are a class of bioactive polyphenols with antioxidant properties and anticancer effects on the skin and esophagus of rodents (Z Y et al. 1994). In addition, TFs have antioxidant properties, free radical scavenging abilities, and high bioavailability in different biological tissues and

play an important role in cell protection (Henning et al. 2008). Twelve hours of treatment with 100, 200 and 400 mg/mL TFs was effective in slowing apoptosis in rat hippocampal nerve cells triggered by sevoflurane (Zhou et al. 2020). Wang et al. (Wang et al. 2018) found that treatment of GES-1 gastric mucosal epithelial cells with 20, 40, and 80 µg/mL TFs effectively alleviated apoptosis induced by ethanol exposure. Benzo(a)pyrene, a typical polycyclic aromatic hydrocarbon, induced abnormal apoptosis in mouse embryonic proliferating cells, and Banerjee et al. (Banerjee et al. 2005) found that apoptosis in proliferating cells gradually normalized after treatment with TFs.

Epigallocatechin gallate (EGCG) in green tea has the same biological activity as TFs in black tea. Studies have shown that EGCG appears to be the most biologically active polyphenol at the cellular level (Dale G et al. 2006), with antibacterial, anti-inflammatory, anticancer and cardiovascular disease prevention properties (Yang et al. 2009; Gordon et al. 2010; Singh et al. 2011). Moreover, EGCG can also inhibit oxidative stress (Hye-Kyung et al. 2008), induce apoptosis in cancer cells (Coyle et al. 2008), abrogate cellular DNA damage and improve the antioxidant effect of mitochondria (Xianglin et al. 2000). Xie et al. (Xie et al. 2020) found that EGCG had a significant protective effect against DNA damage and apoptosis in mouse intestinal stem cells under the influence of radiation. Liang et al. (Liang et al. 2019) found that EGCG was effective in alleviating oxidative stress and inflammation in human AC16 cardiomyocytes under the influence of cigarette smoke and had a significant effect on abnormal apoptosis. Microcystin-LR was found to induce abnormal apoptosis in umbilical vein endothelial cells, which was alleviated by EGCG treatment and gradually normalized (Shi et al. 2018). As with TFs, EGCG has been reported to have good therapeutic effects on cellular oxidative stress, inflammatory responses and apoptosis, but there have been few reports on the alleviation of redox imbalance, inflammation and apoptosis induced by ZnO-NPs.

As the basic unit of structure and function of organisms, rat tracheal epithelial cells (RTE) cells are persistent, universal, stable and irreversible. In general, fully differentiated RTE cells will always remain in the post-differentiated state, and their stability and irreversibility as cellular materials for experiments are the basis for ensuring the scientific validity of experimental results. In the previous study, we found that ZnO-NPs can induce apoptosis in many types of cells, and as the susceptible cells of NPs, it is especially important to study the apoptotic response of respiratory epithelial cells and the related mechanism of action. To provide a research basis for the treatment of respiratory tract diseases caused by NPs.

This paper analyzed the correlation between oxidative stress and the expression of inflammatory cytokines, as well as chemokines and apoptosis-related genes, in rat tracheal epithelial (RTE) cells after ZnO-NP exposure in combination with TF and EGCG treatment. Our study contributes to the understanding of the mitigating effects and mechanisms of action of TFs and EGCG on respiratory diseases.

2. Materials And Methods

2.1 Materials

The average particle size of ZnO-NPs was 30 nm and 90 nm, and the purity was 99.99%, and the NPs were purchased from Aladdin (Shanghai, China). TF (95.0%) was obtained from Sigma Aldrich (St. Louis, USA). The natural antioxidant EGCG (98%) was purchased from Hetian Biotechnology (Hangzhou, China). Penicillin and streptomycin were purchased from Sinopharm Chemical Reagent (Shanghai, China). Kits for reactive oxygen species (ROS) and malondialdehyde (MDA) were obtained from Jiancheng Biotechnology (Nanjing, China). RNA extraction kits were purchased from Invitrogen (Carlsbad, USA). The RTE cell line was obtained from Bena Biotechnology (Beijing, China).

2.2 Preparation of ZnO-NPs working solution

The ZnO-NPs was dissolved using DMEM (Dulbecco's modified Eagle's medium, Gibco, USA) by sonicate, and was diluted to concentration of 100 µg/L as the working solution. The working solution were stored at 4°C and no longer than 2 weeks.

2.3 Cell culture and ZnO-NP exposure

RTE cells were cultured in DMEM medium with incubation environment of 37°C and 5% CO₂. Thawed RTE cells were inoculated into cell culture plates, and when the cells grew to approximately 80% confluence, the medium was discarded. The RTE cells were washed with DPBS and exposed to 100 µg/L ZnO-NPs of different sizes (30 nm and 90 nm). After 12 h of incubation, different concentrations of TFs and EGCG (10, 100, 1000 µg/L) were treated with the RTE cells and continue to culture for an additional 12 h.

2.4 Change in cell proliferation inhibition efficiency

RTE cells need to be incubated for 24 h prior to used, with an incubation volume of 200 µL/well and an incubation density of 4×10^4 cells/mL. After 24 h of incubation, the cells were washed with DPBS and the medium was washed away. Add 200 µL of NPs working solution to the wells and incubate at 37°C with 5% CO₂ for 12 h. Cells counting was performed according to the CCK-8 kit instructions.

2.5 ICP detection of Zn in RTE cells

Zinc concentration was determined used microwave ablation-inductively coupled plasma spectrometry. The cells were inoculated in 96-well plate, and the density of each well was maintained at 5×10^3 /well. The standard solutions of 0.5, 1, 10 and 20 mg/L Zn were prepared, and four parallel solutions were set up for each concentration. The sampling method was manual, and the operating conditions were set as follows: cooling gas of 18 LPM, peristaltic pump lift rate of 25 RPM, RF power of 1200 kW, improvement time of 18 sec, nebulizer of 33 PSI, and integration time of 10 sec. The elemental zinc spectrum was 206.200 (Ahamed et al. 2020).

2.6 Intracellular ROS and MDA content detection

Intracellular ROS changed were measured by flow cytometry. 5×10^3 /well cells were inoculated and incubated for 24 h, then that were exposed to ZnO-NPs for 12 h. After exposure, treated cells with TFs and EGCG, respectively, and incubated for 12 h. After completion of incubation, removed the top layer of medium, rinsed three times with PBS buffer, digested cells with trypsin protein, and resuspended cells in

PBS buffer. The RTE cells were collected by centrifugation, passed through a 40 µm mesh sieve, suspended in pre-cooled PBS, followed by the addition of DCFH-DA dye to a final concentration of 10 µM, and incubated for 20 min at room temperature and protected from light. The detection wavelength range was 488nm-525nm (fluorescence) (Khader et al. 2020).

RTE cells were inoculated into plate at 5.0×10^3 cells/well then incubated for 24 h prior to exposure. Cells were exposed with NP suspension at 100 µg/L for 12 h. The cells were treated with different concentrations of TFs and EGCG after exposure and incubated for 12 h. After treatment and completion of 12 h of incubation, the cells were measured for MDA content at an absorbance of 532 nm according to the instructions of the MDA assay kit. The specific operation was performed according to the instructions of the kit (Zhao et al. 2021).

2.7 RNA isolation and qRT-PCR

RTE cells were inoculated into plate at 5.0×10^3 cells/well and incubated for 24 h. And then the cells were exposed for 12 h using nanosuspension. After exposure, the cells were treated with different concentrations of TFs and EGCG for 12 h. RNA was extracted using the TRIzol method, and used the NanoDrop to measure about the concentration and purity. RNA was reverse transcribed used the Invitrogen SuperScript™ III Reverse Transcriptase Kit (Carlsbad, USA). Then 300 ng cDNA in each PCR reactions for 40 cycles were detected by Real-Time PCR CFX384 system (Bio-Rad, USA) in triplicate with SYBR Green PCR master mix. The design and synthesis of primers were done by Bioengineering Co. (Shanghai, China). The primer sequences are detailed in (S Table 1).

2.8 Statistical analysis

All data were analyzed using Original 8.0 Statistics software. The results were expressed as the mean standard deviation (SD). The level of significance was set at * $P < 0.05$ or ** $P < 0.01$. Correlation data analyses with the R statistical software R 3.1.1 and the packages "leaps", "lars", "caret", "psych", and "relaimpo".

3. Results

3.1 Uptake of zinc by RTE cells and the inhibition efficiency of RTE cells

The zinc content in RTE cells was negatively correlated with the particle size of ZnO-NPs and positively correlated with the NP concentration, and all exposed groups showed a significant increase in Zn content compared to the control group ($P < 0.05$, Fig. 1). When RTE cells were exposed to the ZnO-NPs (1000 µg/L), there was a significant size effect of intracellular zinc content, i.e., 30 nm > 90 nm. It implied that ZnO-NPs could enter the cell membrane and accumulate within cell. Meanwhile smaller ZnO-NPs could enter RTE cells more easily than larger ZnO-NPs.

The highest inhibition efficiency was observed, while the cells were exposed to NPs, and the inhibition efficiency of 90 nm was higher than 30 nm as shown in Fig. 2. When TFs and EGCG were applied to ZnO-NP-induced RTE cells, the cell inhibition efficiency was reduced in a dose-dependent manner significantly ($P < 0.01$). The proliferation inhibition efficiency of RTE cells was reduced by 91.4% and 28.6% in the 30 nm exposure group (0 µg/L) after treatment with TFs and EGCG, respectively (1000 µg/L). In the ZnO-NP-90 nm exposure group (0 µg/L), the proliferation inhibition efficiency of RTE cells was reduced by 80.0% and 66.0% after treatment with TFs and EGCG, respectively.

3.2 The regulation effects of ROS and MDA generation

While exposure with 100 µg/L ZnO-NPs, ROS levels in RTE cells were increased compared with the control group significantly (Fig. 3a). After treatment with TFs and EGCG, ROS levels decreased with increasing concentrations of TFs and EGCG. Moreover, the intracellular MDA level of RTE cells in the 100 µg/L ZnO-NP exposure group was increased compared with the control group significantly (Fig. 3b). About the MDA level decreased with increasing concentrations of the antioxidant's TFs and EGCG. TFs (1000 µg/L) decreased the MDA level by 46.7% and 76.0% in the TF-treated group and 71.0% and 83.3% in the EGCG-treated group (1000 µg/L) compared with the exposed group (0 µg/L).

3.3 mRNA expression levels of *IL-1β*, *IL-6* and *TNF-α*

The expression levels of *IL-1β*, *IL-6* and *TNF-α* were significantly upregulated in RTE cells after exposure to 100 µg/L ZnO-NPs compared with the blank control (Fig. 4). However, after treatment with TFs and EGCG, all genes were down-regulated with increased concentrations of TFs and EGCG compared to the group exposed with ZnO-NPs only. As Fig. 4 shows, the expression of genes was most significantly down-regulated after 1000 µg/L TFs and EGCG treatment.

3.4 mRNA level expression of C-C motif chemokine 2 (*CCL2*) and *CXCL8*

To investigate the intracellular inflammatory response induced by ZnO-NP exposure in the cells, the expression level of *CCL2* and *CXCL8* were examined and were significantly increased compared to the control group (Fig. 5). The relative mRNA expression levels of *CCL2* and *CXCL8* showed significant dose-response effect after treatment with TFs and EGCG. While the treatment with TFs and EGCG (1000 µg/L), the relative mRNA expression levels of *CCL2* induced by 30 nm exposure (0 µg/L) decreased by 56.7% and 65.2% (Fig. 5a). In the same way, the relative mRNA expression levels of *CXCL8* decreased by 40.0% and 60.0% (Fig. 5b), respectively. In contrast, in the ZnO-NP-90 nm exposure group (0 µg/L), the relative mRNA expression levels of *CCL2* and *CXCL8* decreased by 50.0% and 60.0% (Fig. 5a) and 47.8% and 65.2% (Fig. 5b), respectively, after treatment with 1000 µg/L TFs and EGCG.

3.5 mRNA level expression of cytochrome C (*CytoC*) and *Caspase 3/8/9*

As shown in Fig. 6, the mRNA expression level of *CytoC* was raised in RTE cells after exposure to ZnO-NPs compared to the control group significantly. But the relative mRNA expression levels of *CytoC* decreased with increasing concentrations after treatment with TFs and EGCG. In response to 1000 µg/L TFs and EGCG, the relative mRNA expression levels of *CytoC* in 30 nm exposure group (0 µg/L) decreased by 59.1% and 77.3%, respectively, and the relative mRNA expression levels of *CytoC* in 90 nm exposure group (0 µg/L) decreased by 55.0% and 75.0%, respectively.

About the gene's level like the *Caspase 3*, *Caspase 8* and *Caspase 9* were significantly upregulated in RTE cells exposed to 100 µg/L ZnO-NPs compared with the control group (Fig. 7). More interestingly, the genes level about the *Caspase 3*, *Caspase 8* and *Caspase 9* were decreased significantly after 1000 µg/L TFs and EGCG treatment.

3.6 Correlation analysis of oxidative stress, inflammation and apoptosis

The correlation analysis results confirmed a high correlation between oxidative stress, the inflammatory response and apoptosis. The correlation between oxidative stress, inflammation and apoptosis response variables in the TF-treated ZnO-NP-90 nm-treated group was highly significant and showed a high positive correlation (Fig. 8a). However, the correlation analysis of the TF-treated ZnO-NP-90 nm-treated group showed a decrease in the Pearson correlation between ROS and the *IL-1β*, *IL-6*, *TNF-α*, *CCL2*, and *CXCL8* (Fig. 8b). Similarly, correlation analysis of the EGCG-treated ZnO-NPs-30 nm-treated group showed a significant decrease in the Pearson correlation between ROS and the inflammatory genes related to the control of apoptosis (Fig. 8c). In contrast to the above correlation analysis of the EGCG-treated ZnO-NP-90 nm-treated group, there was a slight decrease in the Pearson correlation between *Caspase 9* and response variables such as ROS and inflammatory genes (Fig. 8d).

4 Discussion

With the widespread use of nanomaterials, the exposure pathways of NPs are also increasing. It has been shown that small-sized particles of similar nanomaterials are more detrimental to the health of the respiratory system than large-sized particles and can induce inflammation (Oberdorster et al. 2005). Figure 1 shows that higher concentrations of ZnO-NPs resulted in higher Zn levels in RTE cells, and size had an effect, demonstrating that smaller particles are more likely to enter RTE cells. Thus, implied that small particle size may had more toxic effects, validated the previous point to some extent.

Fine nanoparticles often exist as aerosols, which can increase the risk of respiratory disease when they entered the respiratory tract. Currently, various antioxidants are widely used in the treatment of diseases. Among them, EGCG, the most abundant catechin, has been shown to have ideal antioxidant effects similar to TFs, such as scavenging superoxide anions and inhibiting lipid peroxidation damage (Ziyin et al. 2008; Halder et al. 1998; Leung et al. 2001). We found that the intracellular ROS and MDA levels of RTE cells treated with TFs and EGCG showed significant decreases compared with those in the ZnO-NP exposure group. In the group treated with high concentrations of TFs and EGCG, the efficiency of ROS

scavenging was up to 55.56% for TFs and 38.89% for EGCG. MDA decreased by up to 33.3% and 83.33% compared to the exposed group without TFs and EGCG treatment. Combined with the powerful proton-donating properties conferred by phenolic hydroxyl groups in TFs (Paganga et al. 1996), active hydrogen can deactivate free radicals and terminate the free radical chain, reflecting the ability of TFs to act as free radical scavengers and reduce intracellular ROS and MDA levels by scavenging various types of free radicals generated in RTE cells after exposure to ZnO-NPs. In addition, TFs can be used as activators to increase cellular enzymatic activity to inhibit lipid peroxidation (Saha et al. 2003). The antioxidant activity of EGCG is related to its specific structure. Structurally, EGCG has hydroxyl groups at the 3', 4' and 5' carbon atoms of the B ring and shows major antioxidant properties in the epigallocatechin ester fraction, which is esterified at carbon 3 of the C ring (Juan et al. 2018). However, it has been suggested that EGCG may produce a certain amount of ROS in cells (Mario et al. 2013), and combined with the results of this study, the mitigating effect of EGCG is not as good as TFs in terms of effects on ROS, most likely because EGCG releases a certain amount of ROS while scavenging ROS in RTE cells.

When ROS and MDA levels are constantly increasing in RTE cells, the intracellular redox balance is disrupted, resulting in an inflammatory cellular response. Gossiau (Gossiau et al. 2011) found that TFs significantly inhibited LPS-induced inflammatory cytokine secretion and the expression of related genes by establishing an in vitro model of cellular inflammation. Treatment for macrophages with TFs and the stimulation of the cells with *Clostridium perfringens* nuclei revealed that the *IL-1 β* , *IL-6* and *TNF- α* were significantly inhibited. Crouvezier (Crouvezier et al. 2000) also established an in vivo animal model and found that TFs could inhibit the secretion of inflammatory cytokines and the expression of related proteases, demonstrating the anti-inflammatory role of TFs. Similarly, EGCG has shown anti-inflammatory effects on animal and in vitro models (Crouvezier et al. 2000; Irfan et al. 2006). Similar results were obtained in this study using RTE cells as an experimental model for in vitro experiments, demonstrating that TFs and EGCG can eliminate the negative effects of inflammatory responses to some extent. Figure 3 shows that EGCG inhibits the expression of *IL-1 β* , *IL-6* and *TNF- α* , thereby suppressing inflammation (Shen et al. 2009; Fujiki et al. 2003). In this study, based on the analysis of *IL-1 β* , *IL-6* and *TNF- α* , it is evident that RTE cells experienced a severe inflammatory response when exposed to ZnO-NPs. When TFs and EGCG were added, inflammation was alleviated, and EGCG had a more pronounced alleviating effect than TFs.

In addition to inflammatory cytokines, chemokines are also important players in the intracellular inflammatory response. *CCL2* plays an important role in inflammatory diseases by binding to the corresponding receptors to induce cytokine synthesis. *CXCL8*, which is produced by lung macrophages and airway epithelial cells, also very important about the development and progression of inflammatory diseases (Lei et al. 2013; Khazali et al. 2017). Gram-negative bacteria have high inflammatory potential and can effectively promote the inflammatory process in organisms. TFs have significant alleviating effects on *Fusobacterium nucleatum*-induced inflammation and can effectively reduce the expression of chemokines such as *CXCL8* (Ben Lagha et al. 2016). Yamagata et al. (2015) found that EGCG could effectively alleviate TNF- α -induced human endothelial cell apoptosis, which is mainly achieved by inhibiting the expression of genes such as *CCL2*. Similarly, EGCG has a positive therapeutic effect on

human cystic fibrosis caused by the F508del-CFTR mutation, which is mediated mainly by reducing the abundance of *CXCL8* transcript and protein levels in nasal epithelial cells (De Stefano et al. 2014). In this study, RTE cells treated with TFs and EGCG showed significantly lower levels of the intracellular inflammatory chemokines *CCL2* and *CXCL8* than cells in the ZnO-NP-exposed group. The results showed that the highest decrease of 56.68% and 60.04% in *CCL2* and 47.83% and 65.21% in *CXCL8* in RTE cells after treatment with the highest concentration of TFs and EGCG, demonstrating the alleviating effect of TFs and EGCG on cellular inflammation. In addition, there was a difference in the therapeutic effect of TFs and EGCG in the high concentration treatment group. According to the results shown in Fig. 5, it is clear that the therapeutic effect of EGCG is better than that of TFs.

It has been shown that the cellular inflammatory response is closely related to the occurrence of aberrant apoptosis. The current study shows that the onset of apoptosis is highly regulated by the caspase cascade. When cells are stimulated by ROS, *CytoC* in mitochondria is released into the cytoplasm to bind to *Caspase 9* and control the mitochondrial apoptotic pathway by activating *Caspase 3* (Zhang et al. 2005). It was shown that TFs alleviate abnormal apoptosis by inhibiting the biological activity of caspases. In contrast, EGCG reduced the genes level of *Caspase 3* and *Caspase 9* by cleavage, thereby alleviating apoptosis (Michelle A et al. 2015). It has been shown that sevoflurane induces apoptosis in rat hippocampal neurons, while the gene's level about the *Caspase 3* and *Caspase 9* in rat hippocampal neurons is downregulated after treatment with TFs, which act to mitigate apoptosis (Zhou et al. 2020). Similarly, EGCG was significantly effective in treating endogenous stress-induced apoptosis in rat hippocampal neurons, and *Caspase 3* and *Caspase 9* mRNA expression was inhibited in hippocampal tissues after EGCG treatment (Wang et al. 2020). It showed that the genes regulating apoptosis were downregulated significantly in RTE cells which were treated with TFs and EGCG compared with cells in the ZnO-NP-exposed group (Fig. 7). It observed that *Caspase 3* gene expression was downregulated by up to 50.03% and 60.01%, *Caspase 8* gene's expression level was downregulated by up to 45.11% and 55.57%, and *Caspase 9* gene expression was downregulated by up to 51.33% and 66.67% after treatment with the highest concentration of TFs and EGCG. In RTE cells, these findings indicated that TFs and EGCG had a significant inhibitory effect on the apoptosis-related genes induced by ZnO-NPs.

According to the correlation analysis, the alleviating effect of TFs and EGCG was mainly mediated by rescuing the intracellular redox balance by scavenging peroxy radicals in RTE cells; thus, the inflammatory response induced by oxidative stress was alleviated. When the inflammatory response was alleviated, the CytoC-induced mitochondrial apoptotic pathway is not activated at high levels, and abnormal RTE cell apoptosis is alleviated. Details about the mechanism by which TFs and EGCG alleviate the apoptosis of RTE cells induced by ZnO-NPs are shown in Fig. 9.

5 Conclusions

When ZnO-NPs enter RTE cells, cell proliferation is inhibited and oxidative stress is induced, resulting in the production of large amounts of ROS and MDA. With the disruption of the redox balance in RTE cells, the cells undergo an inflammatory response, resulting in the expression of inflammation-related cytokines

and chemokines. Therewith the inflammatory response is triggered in RTE cells, genes associated with the mitochondrial apoptotic pathway are activated, like *CytoC*, *Caspase 3*, *Caspase 8* and *Caspase 9*. When TFs and EGCG are added, the ROS and MDA levels in RTE cells are controlled and the intracellular redox balance is stabilized. After the intracellular redox system is stabilized, the inflammatory response of RTE cells is also effectively alleviated. Along with the elimination of intracellular inflammation, the expression of genes relates to the control of apoptosis in RTE cells start to be down-regulated and approached to normal levels.

Declarations

Authors Contributions Yan Li and Xiaodong Shan contributed to the conception of the study; Xiaodong Shan, Feifei Chen and Huikang Lin performed the experiment; Xiaodong Shan and Yan Li contributed significantly to analysis and manuscript preparation; Hangjun Zhang, Yuchi Zhong, Xiaodong Shan and Yan Li performed the data analyses and wrote the manuscript; Zhiquan Liu and Yan Li helped perform the analysis with constructive discussions. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that there are no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Not applicable

Ethical Approval, Consent to Participate & Consent to Publish are not applicable in this manuscript.

References

- Ahamed, M.; Akhtar, M.J.; Alaizeri, Z.M.; Alhadlaq, H.A (2020) TiO₂ nanoparticles potentiated the cytotoxicity, oxidative stress and apoptosis response of cadmium in two different human cells. *Environ Sci Pollut R* 27(10):10425-10435
- Banerjee, S.; Manna, S.; Saha, P.; Panda, C.K.; Das, S (2005) Black tea polyphenols suppress cell proliferation and induce apoptosis during benzo(a)pyrene-induced lung carcinogenesis. *Eur J Cancer Prev: the official journal of the European Cancer Prevention Organisation (ECP)* 14(3):215-221

Ben Lagha, A.; Grenier, D (2016) Tea polyphenols inhibit the activation of NF-kappa B and the secretion of cytokines and matrix metalloproteinases by macrophages stimulated with *Fusobacterium nucleatum*. *Sci Rep* 6:34520

Coyle, C.H.; Philips, B.J.; Morrisroe, S.N.; Chancellor, M.B.; Yoshimura, N (2008) Antioxidant effects of green tea and its polyphenols on bladder cells. *Life Sci* 83(1-2):12-18

Crouvezier; Powell; Keir; Yaqoob (2001) The effects of phenolic components of tea on the production of pro- and anti-inflammatory cytokines by human leukocytes in vitro. *Cytokine* 13(5):280-286

Dale G, N (2001) Epigallocatechin-3-gallate (EGCG): chemical and biomedical perspectives. *Phytochemistry* 67(17):1849-1855

De Stefano, D.; Villella, V.R.; Esposito, S.; Tosco, A.; Sepe, A.; De Gregorio, F.; Salvadori, L.; Grassia, R.; Leone, C.A.; De Rosa, G.; Maiuri, M.C.; Pettoello-Mantovani, M.; Guido, S.; Bossi, A.; Zolin, A.; Venerando, A.; Pinna, L.A.; Mehta, A.; Bona, G.; Kroemer, G.; Maiuri, L.; Raia, V (2014) Restoration of CFTR function in patients with cystic fibrosis carrying the F508del-CFTR mutation. *Autophagy* 10(11):2053-2074

Fujiki, H.; Suganuma, M.; Kurusu, M.; Okabe, S.; Imayoshi, Y.; Taniguchi, S.; Yoshida, T.; Fujiki, H.; Suganuma, M.; Kurusu, M (2003) New TNF-alpha releasing inhibitors as cancer preventive agents from traditional herbal medicine and combination cancer prevention study with EGCG and sulindac or tamoxifen. *Mutat Res-Fund Mol M* 523(1):119-125

Goma, A.A.; Tohamy, H.G.; El-Kazaz, S.E.; Soliman, M.M.; Shukry, M.; Elgazzar, A.M.; Rashed, R.R (2021) Insight Study on the Comparison between Zinc Oxide Nanoparticles and Its Bulk Impact on Reproductive Performance, Antioxidant Levels, Gene Expression, and Histopathology of Testes in Male Rats. *Antioxidants* 10(1):41

Gordon, N.C (2010) Antimicrobial activity of the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) against clinical isolates of *Stenotrophomonas maltophilia*. *Int J Antimicrob Ag* 36(2):129-131

Gossiau, A.; Jao, D.L.E.; Huang, M.T.; Ho, C.T.; Evans, D.; Rawson, N.E.; Chen, K.Y (2011) Effects of the black tea polyphenol theaflavin-2 on apoptotic and inflammatory pathways in vitro and in vivo. *Mol Nutr Food Res* 55(2):198-208

Halder, J.; Bhaduri, A.N (1998) Protective Role of Black Tea against Oxidative Damage of Human Red Blood Cells. *Biochem Bioph Res Co* 244(3):903

Henning, S.M.; Choo, J.J.; Heber, D (2008) Nongallated Compared with Gallated Flavan-3-ols in Green and Black Tea Are More Bioavailable. *J Nutr* 138(8):1529-1534

Hye-Kyung, N (2008) Modulation of Nrf2-mediated antioxidant and detoxifying enzyme induction by the green tea polyphenol EGCG. *Food Chem Toxicol: an international journal published for the British Industrial Biological Research Association* 46(4):1271-1278

- Irfan, R (2006) Regulation of inflammation and redox signaling by dietary polyphenols. *Biochem Pharmacol* 72(11):1439-1452
- Ji, S.L.; Ye, C.H (2008) Synthesis, growth mechanism, and applications of zinc oxide nanomaterials. *J Mater Sci Technol* 24(4):457-472
- Juan, N.; Xihan, G.; Han, W.; Tao, Z.; Xu, W (2018) Differences in the Effects of EGCG on Chromosomal Stability and Cell Growth between Normal and Colon Cancer Cells. *Molecules* 23(4):788
- Khader, S.Z.A.; Syed Zameer Ahmed, S.; Ganesan, G.M.; Mahboob, M.R.; Vetrivel, M.; Sankarappan, M.; Manickam, P (2020) *Rhynchosia rufescens* AgNPs enhance cytotoxicity by ROS-mediated apoptosis in MCF-7 cell lines. *Environ Sci Pollut R* 27(2):2155-2164
- Leung, L.K.; Su, Y.; Chen, R.; Zhang, Z.; Huang, Y.; Chen, Z.-Y (2001) Theaflavins in Black Tea and Catechins in Green Tea Are Equally Effective Antioxidants. *J Nutr* 131(9):2248-2251
- Liang, Y.M.; Ip, M.S.M.; Mak, J.C.W (2019) (-)-Epigallocatechin-3-gallate suppresses cigarette smoke-induced inflammation in human cardiomyocytes via ROS-mediated MAPK and NF-kappa B pathways. *Phytomedicine* 58:10
- Margaret; Manson; and; Andreas; Gescher; and; Ann, E.; Hudson (2000) Blocking and suppressing mechanisms of chemoprevention by dietary constituents. *Toxicol Lett* 112(3):499-505
- Mario; Lorenz (2013) Cellular targets for the beneficial actions of tea polyphenols. *Am J Clin Nutr* 98(6):1642S-1650S
- Michelle A, K (2015) Vascular and metabolic actions of the green tea polyphenol epigallocatechin gallate. *Curr Med Chem* 22(1):59-69
- Nandhini, J.T.; Ezhilarasan, D.; Rajeshkumar, S (2021) An ecofriendly synthesized gold nanoparticles induces cytotoxicity via apoptosis in HepG2 cells. *Environ Toxicol* 36(1):24-32
- Oberdorster, G.; Oberdorster, E.; Oberdorster, J (2005) Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ Health Persp* 113(7):823-839
- Paganga, M.G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Bio Med* 20(7):933-56
- Patron-Romero, L.; Luque, P.A.; Soto-Robles, C.A.; Nava, O.; Vilchis-Nestor, A.R.; Barajas-Carrillo, V.W.; Martinez-Ramirez, C.E.; Chavez-Mendez, J.R.; Alvelais Palacios, J.A.; Leal Avila, M.A.; Almanza-Reyes, H (2020) Synthesis, characterization and cytotoxicity of zinc oxide nanoparticles by green synthesis method. *J Drug Deliv Sci Tec* 60

- Rossner, P.; Vrbova, K.; Strapacova, S.; Rossnerova, A.; Topinka, J (2019) Inhalation of ZnO Nanoparticles: Splice Junction Expression and Alternative Splicing in Mice. *Toxicol Sci* 168(1):190-200
- Saha, P.; Das, S (2003) Regulation of hazardous exposure by protective exposure: Modulation of phase II detoxification and lipid peroxidation by *Camellia sinensis* and *Swertia chirata*. *Teratog Carcinog Mutagen* 23(S1)
- Shen, Y.J.; Li, W.G.; Zhang, H.; Li, B.; Zhang, J.J (2009) [Effects of (-)-epigallocatechin-3-gallate on interleukin-1 β expression in mouse wound healing]. *J Mol Cell Biol* 42(3-4):179-185
- Shi, J.; Zhang, M.; Zhang, L.B.; Deng, H.P (2018) Epigallocatechin-3-gallate attenuates microcystin-LR-induced apoptosis in human umbilical vein endothelial cells through activation of the NRF2/HO-1 pathway. *Environ Pollut* 239:466-472
- Singh, B.N.; Shankar, S.; Srivastava, R.K (2011) Green tea catechin, Epigallocatechin-3-gallate (EGCG): Mechanisms, perspectives and clinical applications. *Biochem Pharmacol* 82(12):1807-1821
- Wang, J.P.; Xu, S.; Chen, X.H.; Wang, L.; Li, J.Y.; Li, G.J.; Zhang, B.L (2020) Antidepressant effect of EGCG through the inhibition of hippocampal neuroinflammation in chronic unpredictable mild stress-induced depression rat model. *J Funct Foods* 73:104106
- Wang, Z.; Luo, H.S.; Xia, H (2018) Theaflavins attenuate ethanol-induced oxidative stress and cell apoptosis in gastric mucosa epithelial cells via downregulation of the mitogen-activated protein kinase pathway. *Mol Med Rep* 18(4):3791-3799
- Xianglin; Shi; Jianping; Ye; Stephen; Leonard; Min; Ding; Val; Vallyathan (2000) Antioxidant properties of (-)-epicatechin-3-gallate and its inhibition of Cr (VI)-induced DNA damage and Cr (IV)- or TPA-stimulated NF- κ B activation. *Mol Cell Biochem* 206(1-2):125-32
- Xie, L.-W.; Cai, S.; Zhao, T.-S.; Li, M.; Tian, Y (2020) Green tea derivative (-)-epigallocatechin-3-gallate (EGCG) confers protection against ionizing radiation-induced intestinal epithelial cell death both in vitro and in vivo. *Free Radical Bio Med* 161:175-186
- Yamagata, K.; Xie, Y.J.; Suzuki, S.; Tagami, M (2015) Epigallocatechin-3-gallate inhibits VCAM-1 expression and apoptosis induction associated with LC3 expressions in TNF α -stimulated human endothelial cells. *Phytomedicine* 22(4):431-437
- Yang; Chung, S.; Wang; Xin; Gang; Picinich; Sonia, C (2009) Cancer prevention by tea: animal studies, molecular mechanisms and human relevance. *Nat Rev Cancer* 9(6):429-39
- Z Y, W (1994) Inhibitory effects of black tea, green tea, decaffeinated black tea, and decaffeinated green tea on ultraviolet B light-induced skin carcinogenesis in 7,12-dimethylbenz[a]anthracene-initiated SKH-1 mice. *Cancer Res* 54(13):3428-3435

Zhang, W.; Shi, H.Y.; Zhang, M (2005) Maspin overexpression modulates tumor cell apoptosis through the regulation of Bcl-2 family proteins. *Bmc Cancer* 5(1):1-11

Zhao, Z.L.; Xu, L.M.; Wang, Y.; Li, B.H.; Zhang, W.M.; Li, X.C (2021) Toxicity mechanism of silver nanoparticles to *Chlamydomonas reinhardtii*: photosynthesis, oxidative stress, membrane permeability, and ultrastructure analysis. *Environ Sci Pollut R* 28(12):15032-15042

Zhou, R.S.; Li, X.G.; Li, L.; Zhang, H (2020) Theaflavins alleviate sevoflurane-induced neurocytotoxicity via Nrf2 signaling pathway. *Int J Neurosci* 130(2):1-11

Ziyin, Y (2008) Radical-scavenging abilities and antioxidant properties of theaflavins and their gallate esters in H₂O₂-mediated oxidative damage system in the HPF-1 cells. *Toxicol In Vitro:an international journal published in association with BIBRA* 22(5):1250-1256

Zuyderduyn, S.; Ninaber, D.K.; Hiemstra, P.S.; Rabe, K.F (2006) The antimicrobial peptide LL-37 enhances IL-8 release by human airway smooth muscle cells. *J Allergy Clin Immun* 117(6):1328-1335

Figures

Figure 1

The detection of the zine content in RTE cells induced by 30 nm ZnO-NPs and 90 nm ZnO-NPs for 12 h by ICP. The data are represented as the means $\pm$ standard deviation. The asterisks denote responses that were significantly different from those of the control (*P < 0.05, **P < 0.01).

Figure 2

Effects of different size (30 nm and 90 nm) and concentration (100 µg/L) of ZnO-NPs and treatment with different concentration TFs and EGCG (10,100 and 1000 µg/L) on the cell proliferation inhibition efficiency in RTE cells for 12 h. The data are represented as the means $\pm$ standard deviation. The asterisks denote responses that were significantly different from those of the control (*P < 0.05, **P < 0.01).

Figure 3

Effects of different size (30 nm and 90 nm) and concentration (100 µg/L) of ZnO-NPs and treatment with different concentration TFs and EGCG (10, 100 and 1000 µg/L) on the generation of ROS (a) and MDA (b) in RTE cells for 12 h. The data are represented as the means ± standard deviation. The asterisks denote responses that were significantly different from those of the control (*P < 0.05, **P < 0.01).

Figure 4

Effects of different size (30 nm and 90 nm) and concentration (100 µg/L) of ZnO-NPs and treatment with different concentration TFs and EGCG (10, 100 and 1000 µg/L) on the IL-1β (a), IL-6 (b) and TNF-α (c) in RTE cells for 12 h. The data are represented as the means ± standard deviation. The asterisks denote responses that were significantly different from those of the control (*P < 0.05, **P < 0.01).

Figure 5

Effects of different size (30 nm and 90 nm) and concentration (100 μg/L) of ZnO-NPs and treatment with different concentration TFs and EGCG (10, 100 and 1000 μg/L) on the relative mRNA expression of CCL2 (a) and CXCL8 (b) in RTE cells for 12 h. The data are represented as the means ± standard deviation. The asterisks denote responses that were significantly different from those of the control (*P < 0.05, **P < 0.01).

Figure 6

Effects of different size (30 nm and 90 nm) and concentration (100 µg/L) of ZnO-NPs and treatment with different concentration TFs and EGCG (10, 100 and 1000 µg/L) on the relative mRNA expression of CytoC in RTE cells for 12 h. The data are represented as the means ± standard deviation. The asterisks denote responses that were significantly different from those of the control (*P < 0.05, **P < 0.01).

Figure 7

Effects of different size (30 nm and 90 nm) and concentration (100 µg/L) of ZnO-NPs and treatment with different concentration TFs and EGCG (10, 100 and 1000 µg/L) on the relative mRNA expression of Caspase 3 (a), Caspase 8 (b), Caspase 9 (c) in RTE cells for 12 h. The data are represented as the means ± standard deviation. The asterisks denote responses that were significantly different from those of the control (*P < 0.05, **P < 0.01).

Figure 8

Correlation between oxidative stress indicators ROS, MDA and inflammatory factors IL-1 β , IL-6, TNF- α , CCL2, CXCL8 and CytoC and mitochondrial apoptosis-related indicators Caspase 3, Caspase 8 and Caspase 9 in TFs-treated RTE cells exposed to ZnO-NPs-30 nm (a), ZnO-NPs-90 nm (b) and EGCG-treated RTE cells exposed to ZnO-NPs-30 nm (c), ZnO-NPs-90 nm (d).

Figure 9

The mechanism of TFs and EGCG treatment alleviating the apoptosis of RTE cells induced by ZnO-NPs exposure.

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