Caffeic acid phenethyl ester mitigates cadmium-induced damage via the Hsa_circ_0010039/miR-661/Caspase9 axis–mediated apoptosis

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Abstract
Caffeic acid phenethyl ester (CAPE) is a bioactive component of honeybee propolis, which has protective effect against heavy metal induced toxicology. Cadmium is a kind of heavy metal pollutant that is hazardous to human health especially liver, and apoptosis is an important mechanism related to cadmium injury. Circular RNAs (circRNAs) are involved in the regulation of apoptosis. However, the protective effects of CAPE on cadmium-induced cell apoptosis through circRNA related mechanisms remains unclear at the current stage. In the present research, cell-based studies revealed that treating HepG2 cells with CdCl2 (0–30 μM; 24 h) caused a dose-dependent decrease in cell viability, and CAPE at 10 μM could reverse such a decrease. The circular RNA hsa_circ_0010039 may function as a ceRNA for the microRNA miR-661 to enhance caspase 9 expression and regulate apoptosis. The anti-apoptotic effects of CAPE against CdCl2-induced injury may be achieved through inhibiting the expression of hsa_circ_0010039 and caspase 9 while up-regulating miR-661 expression. This is the first report on the involvement of circRNA in CAPE’s alleviating effect on CdCl2-induced injury and apoptosis. The hsa_circ_0010039/miR-661/caspase9 axis deserves further research efforts especially in search of dietary interventions for cadmium injury.

KEYWORDS cadmium, hsa_circ_0010039, Caffeic acid phenethyl ester, miR-661, caspase9

1 INTRODUCTION

Cadmium (Cd) is a hepatotoxic heavy metal and has long been recognized as an environmental pollutant. Humans are exposed to Cd commonly through inhalation, cigarette smoke, ingestion of contaminated foods or drinks, and direct contact with contaminated industrial/agricultural waste (Trouiller-Gerfaux et al., 2019). Cd has a long half-life and can accumulate in organs such as the brain, liver, and kidneys (Zhang et al., 2019b). A number of studies revealed that Cd induces apoptosis in different cell types including neuronal cells (Nguyen et al., 2013). Protective effects of some dietary therapeutics such as flavonoids like quercetin and anthocyanins against Cd-induced injury and apoptosis have also been demonstrated (Abdalla et al., 2013). However, few reports have shown the links between the...
cytotoxicity of Cd and protective function of dietary therapeutics using noncoding RNAs as biomarkers. Our recent review indicates the possibility of some common signaling pathways involved in both the regulation of noncoding RNAs in mycotoxin-induced injury and the protection offered by dietary therapeutics against the toxins (Rong et al., 2019). This study was motivated to examine the roles of noncoding RNAs in the Cd-caused apoptosis and therapies-initiated protective processes.

Caffeic acid phenethyl ester (CAPE) is a biologically active compound of honeybee propolis, possessing antimicrobial, immunomodulatory, antifibrotic, and anti-inflammatory properties (Sun et al., 2017). CAPE can ameliorate Cd-induced injury to the liver, kidney, and heart (Mollaoglu et al., 2006), and such a function has been associated closely with the roles of CAPE in regulating apoptosis (Firat et al., 2019). CAPE can exert its anti-apoptotic effect to against Cd-induced injury through NF-κB signaling pathway (Kobroob et al., 2012). In addition, CAPE and its derivatives can be used as selective inhibitors of acetylcholinesterase, which may be attributed to the presence of two benzylic or two 2-phenylethanol groups in its molecule (Gießel et al., 2019). Furthermore, they can effectively inhibit aggregation of tau-derived hexapeptide and alleviate Alzheimer's disease due to the nitrocatechol scaffold structure (Silva et al., 2019). Excessive exposure to heavy metals like Cd has been linked to cellular injury and even apoptosis (Lin et al. 2017). Apoptosis can be initiated through one of two pathways: intrinsic apoptotic pathway (mitochondrial route), and extrinsic apoptotic pathway (death receptor–mediated route). Caspase 9 and BCL-2 play crucial roles in the initiation of apoptotic signaling and process of apoptosis. Apoptosis can be triggered by the activation of caspases, and caspase 9 functions as an initiator caspase to initiate the caspase cascade through cleaving downstream executioner caspases for apoptosis (McIlwain, Berger, & Mak, 2013). The BCL-2 protein family includes anti-apoptotic members (to prevent apoptosis) and proapoptotic members (to induce apoptosis like the effector protein Bax), and activates intrinsic apoptotic pathway that responded to cellular stresses associated with heat, high level of hydrogen peroxide, DNA damage, and mitochondrial membrane disruption (Oltval et al. 1993). BCL-2 can regulate apoptosis via regulating caspase 9 and caspase 3 (Golovchenko et al. 2008). Bax (pro-apoptotic) can activate caspase 9 via inducing the release of cytochrome c (Yang et al. 2002).

The importance of noncoding RNAs in biological events has attracted increasing attention, and their important roles in apoptosis were recently confirmed (Li et al., 2020; Wang et al. 2019). Most recently, some studies using cell and animal models reported the involvements of noncoding RNAs in the regulation of apoptosis and heavy metal-induced cell injury including circRNAs like CircRar1 (Nan et al., 2017), and miRNAs like miR-661 (Kang & Choi, 2019). CircRar1 promotes lead-induced neuronal apoptosis by targeting miR-671, leading to the up-regulation of caspase 8 and p38 at mRNA and protein levels (Nan et al., 2017). In myelodysplastic syndromes models (MDS), miR-661 induced apoptosis by activating p53 and inhibited cell growth (Kang & Choi, 2019). MiR-661 inhibited cell growth, promoted apoptosis, and induced caspase-9 activation by targeting Cyc1 (Fan et al., 2017). Furthermore, hsa_circ_0010039 is an apoptosis-related circRNA, which expressed in HepG2 cells (Salzman et al., 2013), and we predicted that hsa_circ_0010039 has miR-661 target binding through Starbase and Circbase software.

However, whether or not CAPE can prevent Cd-induced apoptosis especially along the circRNA–miRNA axis and whether hsa_circ_0010039/miR-661 axis is involved in the process of CAPE against Cd-induced apoptosis remains unclear. This study’s purpose on fill this knowledge gap and investigations were performed using HepG2 cells models. The obtained results would not only validate potential benefits of CAPE in the prevention and treatment of Cd-induced apoptosis, but also demonstrate an effective non-coding RNA-related approach for improving the accuracy and specificity of dietary therapeutics to treat heavy metal-induced injury.

2 | MATERIALS AND METHODS

2.1 | Materials

Cadmium chloride (CdCl2) and CAPE were obtained from Sigma Chemical Co., USA. CdCl2 was dissolved in DMEM. CAPE was dissolved in dimethyl sulfoxide (DMSO) and diluted using Dulbecco modified eagle medium (DMEM) before use.

2.2 | Cell culture

HepG2 cells were purchased from Shanghai Academy of Sciences (passage number of HepG2 cells: 5-20) and maintained in DMEM (Gibco, USA) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), before being cultured in a cell incubator (QP-160, Biobase, Jinan, China) at 37°C in 5% CO2 and 95% normal air. Cell experiment was designed for four treatment groups: Group 1, the control; Group 2, the CAPE (10 μM) alone-treated; Group 3, the CdCl2 (25 μM) alone-treated; Group 4, the CAPE (10 μM)-CdCl2 (25 μM) treated combination.

2.3 | RT-PCR experiment

Extracted total RNA from HepG2 cells using TRizol (Invitrogen) were quantified by spectrophotometry using a spectrophotometer (Invitrogen; Carlsbad, CA, USA). The Reverse Transcription Kit (TaKaRa, Dalian, China) was used to carry out reverse transcription. Premix Ex Taq II (TaKaRa, Beijing, China) was used to quantitate polymerase chain reactions. The IQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used for the reactions. The ΔΔCt method was used for the assessment of CircRNA, miRNA, and mRNA relative expression levels (Wei et al., 2019).

2.4 | Cell transfection

The pcDNA 3.1 circRNA mini vector and pcDNA 3.1 vector were used for ectopic hsa_circ_0010039 and caspase9 expression, respectively.
The negative control (si-NC), hsa_circ_0010039 si-RNA, caspase 9 si-RNA, miR-661 mimics, and associated inhibitors were procured from Promega (Beijing, China). Lipofectamine 2000 (Invitrogen) was used for transfection (Feng et al., 2019).

2.5 Cell viability/growth

Cell viability was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay with the assay kit obtained from Sigma-Aldrich (St. Louis, MO, USA). The different groups of HepG2 cells were seeded in wells of the 96-well dishes and maintained in a complete medium (DMEM, Gibco, Shanghai, China). In the incubator culture, the cells of different treatment groups were treated with drugs for 24 h. Then, an aliquot (20 μl) of MTT (5 mg MTT/ml medium) was added to each well, and the cells were incubated at 37°C for 4 h. Epoch ELISA Reader (BioTek, Winooski, VT, USA) was used for the measure of optical density at 570 nm.

2.6 Crystal violet assay

The cells were seeded into 6-well plates at a density of 1 x 10^3, before an incubation at 37°C with 5% CO2. Then, PBS washed the cells three times, 4% paraformaldehyde fixed for 15 min, and 0.5% crystal violet stained (Beyotime, Shanghai, China) for 10 min. The morphology of these cells was observed under phase contrast microscope.

2.7 Reactive oxygen species (ROS) determination

Intracellular ROS was measured using fluorescent microscope and using 2',7'-dichlorodihydro-fluorescein diacetate (DCFH-DA). Nonpolar DCFH-DA can diffuse through into the cytoplasm and then be cleaved to polar DCFH, and in the presence of peroxidase, DCFH is converted to DCF by intracellular ROS (King & Weber, 2013). Therefore, the fluorescence of DCF allows the assessment of the ROS level. In this study, cells added 10 μM DCFH-DA (200 μl) for 30 min at 37°C (in dark), and the resulting fluorescence was recorded.

2.8 Caspase 9 activity

Caspase 9 activity was determined using the Caspase 9 Activity Assay Kit (KeyGen, NanJing, China). The proteins of HepG2 cell lysates and supernatants were tested by the Binchinonic acid (BCA) assay. The absorbance at 405 nm was measured using a microplate reader (Hynenergy HT, BIOTEK, Winooski, VT, USA), and corrected based on those from the control cells.

2.9 Dual luciferase reporter assay

Luciferase assays were performed based on a previously described method (Gould & Subramani, 1988). Briefly, 40 ng of the caspase 9 or hsa_circ_001039 luciferase reporter plasmid was cotransfected with 100 nM miR-661 mimic into HepG2 cells using Lipofectamine 2000. After transfection, we collected the cells for the determination of Renilla and firefly luciferase activities using the Dual Luciferase Reporter assay system (Promega Corp., Beijing, China). The signal intensity of Renilla luciferase was normalized to that of firefly luciferase, and the results were expressed as “the fold-induction relative to the basal activity.”

2.10 Western blot analysis

After treating cells and mice according to the above steps, proteins were extracted from cells and liver tissues, and protein concentration was measured by BCA method. The specific western blot experimental operation is modeled on our previous experimental method (Hao et al., 2020). The primary anti-bodies used were: Bcl-2 (Abcam, ab196495, 1/500), Bax (Servicebio, GB12690, 1/500), and β-actin (Abcam, ab179467,1/5000).

2.11 Mitochondrial membrane potential

Mitochondrial membrane potential (Δψ) was measured using mitochondrial specific lipophilic cationic fluorescent dye JC-1 Kit (Beyotime Biotechnology, China) according to the instructions, and results were observed using the r fluorescence microscope (Nikon Ds-Ri2).

2.12 Statistical analysis

The results are expressed as "mean ± SD." SPSS 2.0 software was used for statistical analysis. The data were analyzed by one-way ANOVA, and compared using Duncan’s test. The p-values < 0.05 was deemed as statistically significant.

3 RESULTS AND DISCUSSION

3.1 The attenuating effect of CAPE on CdCl2-caused injury in HepG2 cells

HepG2 cells are the most common liver cancer cells and widely used as a cellular model for in vitro studies to simulate toxicological changes in vivo. In this study, treating HepG2 cells with CdCl2 alone at 0, 5, 10, 15, 20, 25, and 30 μM for 24 h led to a decrease in cell viability (Figure 1a). Results from MTT assay showed that treating HepG2 cells with CAPE at 0–10 μM for 24 h caused no cytotoxicity and there was no significant difference in the cell viability. When treating HepG2 cells with CAPE at 10–20 μM for 24 h the cell viability was decreased to 94% (Figure 1b). Therefore, we choose a CAPE concentration of 10 μM for subsequent experiments. The treatment of CdCl2-pretreated HepG2 cells with 10 μM CAPE for 24 h could reverse cell viability (Figure 1c).
FIGURE 1 CAPE mitigated CdCl₂-caused injury in HepG2 cells. (a) Effects of CdCl₂ concentration (5–30 μM) on HepG2 cell viability over 24 h; (b) Effects of CAPE concentration (2–20 μM) on HepG2 cell viability over 24 h; (c) Effects of CAPE on the viability of CdCl₂ injured HepG2 cells; (d) Images obtained by crystal violet staining; (e) Images show ROS generation by 2′,7′-dichlorodihydrofluorescein diacetate staining; (f) Mitochondrial membrane potential (Δψ); (g) Relative activity of caspase9 affected by CdCl₂ and CAPE; (h) Relative activity of caspase9 mRNA; (i) Representative immunoblots; (j) Relative Caspase9 expression and Bcl-2/Bax affected by CdCl₂ and CAPE. CAPE (2.5–10 μM) pretreated the cells for 24 h, followed by the treatment with CdCl₂ (25 μM) and CAPE for 24 h. The viability of the treated cells was expressed as a percentage of the viability of the untreated cells. Data are given as mean ± SD of three independent experiments, p < 0.05.
FIGURE 2  Participation of Hsa_circ-0010039 and miR-661 in the conservation offered by CAPE mitigate CdCl2-caused injury in HepG2 cells: (a) Binding sites of caspase 9 to miR-661; (b) Binding sites of hsa_circ_0010039 to miR-661; (c) qPCR revealed the increase initiated by CAPE treatment in miR-661 expression in CdCl2-treated HepG2 cells; (d) qPCR revealed the decrease initiated by CAPE treatment in hsa_circ_0010039 expression in CdCl2-treated HepG2 cells. Data are given as mean ± SD of three independent experiments, p < 0.05

Imaging by crystal violet staining demonstrated that CdCl2 injured the cells, while CAPE preserved/protected cells (Figure 1d). Experiments related to cellular ROS (Figure 1e) revealed that the secretion of ROS induced by CdCl2 could be largely inhibited by CAPE. Experiments related to mitochondrial membrane potential (Figure 1f) showed that CAPE could inhibit the increase of mitochondrial membrane potential. A pretreatment with CAPE suppressed CdCl2-induced rise of activity and mRNA expression of caspase 9 (Figure 1g and h). CdCl2 increased the level of caspase 9 protein but decreased the ratio of Bcl-2/Bax, whereas CAPE pretreatment could combat these changes (Figure 1i and j). All these findings indicate that CAPE can inhibit apoptosis in HepG2 cells. Chen et al. (2017) pointed out that CdCl2 could increase the expression of caspase 9 and alter the ratio of Bcl-2/Bax, whereas CAPE pretreatment could combat these changes (Figure 1g and h). All these findings indicate that CAPE can inhibit apoptosis in HepG2 cells.

3.2  The participation of hsa_circ-0010039 and miR-661 in the conservation provided by CAPE mitigate CdCl2-caused injury in HepG2 cells

As revealed by the StarBase, CircInteratome, and mirBase Databases, hsa_circ_0010039 is a caspase 9-related circRNA, and miR-661 has binding sites for hsa_circ_0010039 and caspase 9 (Figure 2a and b). In this study, the expression of miR-661 for the CdCl2-alone treated group was decreased (Figure 2c). In comparison, CAPE pretreatment could preserve the expression of miR-661. Moreover, CdCl2 could cause the increase of the expression of hsa_circ_0010039, whereas CAPE decreased such CdCl2-induced increase. These results indicated that both hsa_circ_0010039 and miR-661 participated in the conservation offered by CAPE mitigate CdCl2-caused injury (Figure 2d).

Increasing evidence has demonstrated that circRNAs are involved in apoptosis by sponging miRNA (Nan et al., 2017). Circ_0008450 was found to regulate cell proliferation, cell invasion, and apoptosis through functioning as an miR-548p sponge (Zhang et al. 2019). In colorectal cancer cells, hsa_circ_0000523 may regulate cell proliferation and apoptosis via acting as a miR-31 sponge (Jin et al. 2018). Under heavy metal-induced stress, LncRpa, circRar1, and miR-671 were found capable of regulating lead-induced apoptosis (Nan et al., 2017). This paper is the first report on the involvement of circRNA in CAPE’s alleviating effect on CdCl2-induced apoptosis.

3.3  The participation of hsa-circ-0010039 in the regulation of apoptosis

In order to explore the mechanism underlying the protection against CdCl2-induced injury offered by CAPE, hsa_circ_0010039 was silenced or overexpressed, respectively, before qRT-PCR and Western blotting characterization. The results of qRT-PCR showed that the expression of hsa_circ_0010039 were decreased in hsa_circ_0010039-silenced HepG2 cells, compared with the corresponding controls (Figure 3a). The efficacy of si-hsa_circ_0010039-1 was greater than
FIGURE 3 Participation of Hsa-circ-0010039 in the regulation of apoptosis: (a and b) qPCR revealed hsa_circ_0010039 that was silenced or overexpressed in HepG2 cells by transfecting si-RNA or pcDNA, respectively; (c and d) qPCR revealed miR-661 that was silenced or overexpressed in HepG2 cells by transfecting si-RNA or pcDNA, respectively; (e and f) Western blot analysis revealed that caspase 9 protein expression and the ratio of Bcl-2/Bax in hsa_circ_0010039 silenced and overexpressed HepG2 cells.

3.4 The participation of miR-661 in the regulation of apoptosis

To examine further the regulatory effect of miR-661 on apoptosis, miR-661 was overexpressed and silenced before qRT-PCR and Western blotting characterization. The results of qRT-PCR analysis showed that miR-661 expression was increased in overexpressed HepG2 cells but decreased in silenced HepG2 cells, compared with the corresponding controls. These results showed that hsa_circ_0010039 was involved in apoptosis, and silencing of hsa_circ_0010039 inhibited apoptosis whilst overexpressing of hsa_circ_0010039 enhanced apoptosis.
FIGURE 4  (a) qPCR revealed miR-661 that was overexpressed or silenced in HepG2 cells by transfecting the mimic or inhibitor, respectively; (b & c) Western blot analysis revealed that caspase 9 protein expression and the ratio of Bcl-2/Bax in miR-661 overexpressed or inhibited HepG2 cells controls (Figure 4a). Western blot results showed that transfection of the miR-661 mimics decreased the level of caspase 9 protein but increased the ratio of Bcl-2/Bax (Figure 4b), with the knockdown of miR-661 leading to the rise of the ratio of Bcl-2/Bax (Figure 4c). These results showed that miR-661 participated in the regulation of apoptosis, with silencing of miR-661 promoting apoptosis while overexpressing of miR-661 inhibiting apoptosis. Interestingly, the effect of silencing miR-661 and the effect of overexpressing hsa_circ_0010039 resembled.

3.5  The participation of caspase 9 in the regulation of apoptosis

Caspase 9 was silenced in HepG2 cells with si-caspase 9-1, si-caspase 9-2, and si-caspase 9-3. Thereafter, the expression of caspase 9 was examined by qPCR, and using Western blot to investigate apoptosis-related proteins expression. It was found that caspase 9 expression were decreased in caspase 9-silenced HepG2 cells, compared with the control (Figure 5a). The efficacy of si-caspase 9-1 was the highest, thus, si-caspase 9-1 was chosen for further experiments. Results of Western blot showed that caspase 9 expression level was decreased in caspase 9-silenced HepG2 cells, whereas the ratio of Bcl-2/Bax was increased (Figure 5b and c). Taken together caspase 9 was in association with apoptosis, and silencing of caspase 9 inhibited apoptosis. Interestingly, silencing of caspase 9 or hsa_circ_0010039 and overexpressing miR-661 exhibited similar effects of inhibiting apoptosis, and overexpressing hsa_circ_0010039 or silencing miR-661 exhibited similar effects of promoting apoptosis. These results indicate that hsa_circ_0010039 may monitor miR-661 through a “sponge” mechanism to regulate the process of apoptosis.

3.6  The role of hsa_circ_0010039 as a ceRNA for miR-661 to enhance caspase 9 expression

CircRNAs can regulate multiple biological processes and may act as ceRNAs to regulate the expression of target genes of miRNAs (Yu et al. 2019). During hepatic cell carcinoma (HCC) progression, circRNA-104718 may behave as a ceRNA to activate miR-218-5p/TXNDC5 (Thioredoxin domain-containing protein 5) signaling (Yu et al., 2019). Similarly, circ_0020123 can act as an a miR-182 sponge to raise ADAM9 (A disintegrin and metallopeptidase domain 9) expression, and regulate cell growth and apoptosis through ADAM9 signaling (Wan et al. 2019). In this study, hsa_circ_0010039 was likely involved in the process of CAPE’s conservation mitigate CdCl2-caused apoptosis, and exerted a regulatory effect through ceRNA regulatory mechanism. It was possible that hsa_circ_0010039/miR-661/caspase 9 axis regulated CAPE to offer protection against apoptosis induced by CdCl2. Caspase 9 and hsa_circ_0010039 contained miR-661 binding sites, and...
FIGURE 5  (a) qPCR revealed caspase 9 that was silenced in HepG2 cells by transfecting si-RNA; (b & c) Western blot analysis revealed that caspase 9 protein expression and the ratio of Bcl-2/Bax in silenced HepG2 cells. Data are given as mean ± SD of three independent experiments, *p* < 0.05

FIGURE 6  The role of hsa_circ_0010039 as a ceRNA for miR-661 to enhance caspase 9 expression: (a and b) Schematic diagrams showing wild-type and mutant hsa_circ_0010039, as well as caspase 9 3′-UTR reporter vectors containing the wild-type or mutated miR-661 binding site. (c and d) Luciferase activity in HepG2 cells co-transfected with the vectors and miR-661 mimics. (e) Schematic representation showing the possible roles of hsa_circ_0010039 in the regulation of CAPE-mediated protection of CdCl2-treated HepG2 cells. Data are given as mean ± SD of three independent experiments, *p* < 0.05
luciferase reporters were constructed accordingly for caspase 9 and hsa_circ_0010039. The wild-type reporter contained the 3’-UTR of caspase 9 and hsa_circ_0010039 (Figure 6a and b). The mutant-type reporter contained the mutated 3’-UTR, which was designed according to a mutated miR-661 binding site. The results indicated that overexpression of miR-661 markedly decreased the luciferase activity in the wild-type group, but not in the mutant-type group (Figure 6c and d). Hsa_circ_0010039 might act upstream of miR-661 and inhibit the targeting of caspase 9 by miR-661.

This study may help answer the question on whether a circRNA plays a regulatory part in the process of CAPE’s conservation mitigate CdCl2-caused injury (Figure 6e). This study has shown the regulatory function of hsa_circ_0010039 involved in the process of CAPE mitigate CdCl2-induced apoptosis. It can be used as a research point for future thoroughly studies on the function of circular RNA(s) in the process of heavy metal-caused injury and the intervention process initiated by bioactive food ingredients.

In conclusion, hsa_circ_0010039 is a key player and may act as a miR-661 sponge to enhance caspase 9 expression during the process of protection exerted by CAPE against CdCl2-induced injury. The hsa_circ_0010039/miR-661/caspase9 axis likely plays a key role in the fight of CAPE against CdCl2 although much work would be needed in order to confirm the clinical effectiveness of CAPE. This study provided insights on the mechanism of food ingredients to intervent heavy metal induced damage, and provided new approaches for the development of strategies to anti-cadmium injury.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

ETHICAL APPROVAL
The manuscript does not contain clinical studies or data.

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