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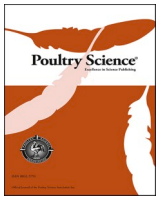

Chlorogenic acid alleviates cadmium-induced neuronal injury in chicken cerebral cortex by inhibiting incomplete autophagy mediated by AMPK-ULK1 pathway

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ABSTRACT

Cadmium **(Cd)** is an environmental pollutant that has neurotoxic properties, which poses serious threats to human health and the development of poultry farming. Chlorogenic acid **(CGA)** is a dietary polyphenol exhibit various biological activities such as antioxidant, anti-inflammatory, and autophagy regulation. In addition, CGA can penetrate the blood-brain barrier and exert neuroprotective effects. This study explored the mechanism of CGA in alleviating Cd-induced cerebral cortical neuron injury in chickens. The results showed that in vivo, CGA reduced the Cd level and alleviated Cd-induced histopathological and ultrastructural damages in the chicken cerebral cortex. Further research has found that CGA alleviated Cd-induced incomplete autophagy and activation of the AMPK-ULK1 pathway. In vitro, AMPK inhibitors (Compound C) could alleviate Cd-induced incomplete autophagy in chicken cerebral cortical neurons. In addition, CGA alleviated the decreased viability, incomplete autophagy, and activation of the AMPK-ULK1 pathway induced by Cd in chicken cerebral cortical neurons. In summary, CGA can alleviate Cd-induced cerebral cortical neuron injury in chickens, which is related to CGA alleviating Cd-induced incomplete autophagy by inhibiting the AMPK-ULK1 pathway.

Introduction

Cadmium **(Cd)** is an important occupational toxicant and environmental pollutant. It can enter in the body through the soil, water bodies, and atmosphere through naturally and anthropogenically, and be ingested by animals or humans through the food chain, posing serious harm to their bodies. To assess environmental pollution, avian species have been suggested and used as biological monitors(Cizdziel, et al., 2013; Kribi-Boukhris, et al., 2020; Nam and Lee, 2006). Chicken (*Gallus* domesticus) is also used as a good biological indicator for monitoring industrial pollution(Li, et al., 2013). In poultry, feed and water are the primary sources of Cd, after absorption it can accumulate in several vital organs of the body including the brain, lead to multiple organ damage (Kar and Patra, 2021). The brain is one of the important target organs of Cd toxicity. Studies have shown that Cd can damage the blood-brain barrier, allowing it to enter in the brain tissue and induce neuronal damage by affecting multiple signaling pathways and/or signaling molecules, causing behavioral and cognitive disorders, and memory deficits in animals and humans(Aljohani, 2023; Arruebarrena, et al., 2023; Namgyal, et al., 2021). Therefore, understanding the neurotoxic mechanisms of Cd and identifying effective preventive treatments is a critical area of future research for both human health and animal welfare.

Macroautophagy, referred as autophagy, is a cellular catabolic process that relies on lysosomes in which there is degradation and recycling of cellular components. In this process, autophagosomes wrap the damaged organelles, protein aggregates and pathogens, and then deliver the wrapped goods to lysosomes to form autophagosomes and degrade and recycle them to maintain autophagy flux(Zhao, et al., 2021). However, cells exhibit defective autophagy due to autophagosome

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Table 1
Antibodies used in the present study.

Antibody	Dilution	Catalog number	Company
AMPK	1:500	2532	Cell Signaling Technology
p-AMPK	1:1000	2535	Cell Signaling Technology
LC3B	1:1000	83506	Cell Signaling Technology
p-ULK1	1:500	14202	Cell Signaling Technology
ATG5	1:1000	12994	Cell Signaling Technology
β-actin	1:1000	4970	Cell Signaling Technology
P62	1:1000	P0067	Sigma-Aldrich
ULK1	1:500	bs-3602R	BIOSS Biotechnology
NeuN	1:500	GB11138-100	Servicebio Technology
HRP-conjugated Goat Anti-Mouse IgG	1:10000	115-005-146	Jackson ImmunoResearch
HRP-conjugated Goat Anti-Rabbit IgG	1:10000	111-005-045	Jackson ImmunoResearch

accumulation, impaired fusion, defective degradation, blockage of lysosome, and flux impairment known as incomplete autophagy(Guo, et al., 2024; Jin, et al., 2024; Liu, et al., 2017). Moreover, flux impairment is a fundamental distinction between incomplete and other forms of autophagy(Zhang, et al., 2022). The signaling molecules and pathways that regulate incomplete autophagy include oxidative stress, endoplasmic reticulum stress, Beclin1, the mammalian rapamycin target **(mTOR)**, and AMP-activated protein kinase **(AMPK)**(Zhang, Cao, Qiu and Kang, 2022). AMPK is an essential cellular energy sensor and a major kinase regulating autophagy. In the case of incomplete autophagy, AMPK activation induces autophagy via two distinct mechanisms: the negative regulation of mTOR protein kinase complex and the direct phosphorylation to activate ULK1 (Unc-51-like kinase 1, the mammalian ortholog of Atg1)(Wang, et al., 2022). AMPK, as the up regulator of ULK1, directly phosphorylates at least four sites (Ser467, Ser555, Ser637, and Thr574) to enhance the activity of ULK1 and recruit more autophagy-related proteins (ATG proteins) to the membrane domain of the autophagosome, thereby affecting autophagy in the initial stage(Egan, et al., 2011). Studies have revealed that the Duck Tembusu virus **(DTMUV)** induces incomplete autophagy in mouse brain tissue and Neuro-2a cells by activating the AMPK/mTOR pathway, which promotes virus replication and leads to neurotoxicity of DTMUV(Wang, et al., 2023b). Incomplete autophagy is also a special mechanism of metal ion toxicity response. For instance, under Cd exposure, skin epidermal cells(Son, et al., 2011), mesenchymal stem cells(Yang, et al., 2016), and pig heart tissue(Zhao, et al., 2022) exhibit defective autophagy flux with AMPK serving as a significant target in this process. Based on the above results, incomplete autophagy plays an important role in destroying cell homeostasis. Therefore, it is necessary to pay attention to the toxicological mechanism of incomplete autophagy induced by Cd, especially by regulating the AMPK pathway, which may be a potential therapeutic strategy to alleviate Cd-induced neurotoxicity.

Chlorogenic acid **(CGA)** is a dietary phenolic acid widely present in plants has gained a significant attention due to its diverse and potent biological effects and making it a promising candidate for anti-inflammatory, antioxidant, neuroprotective, liver-protective, blood pressure-lowering, and diabetes-relieving therapies(Nguyen, et al., 2024). Interestingly, evidence suggests that CGA exhibits autophagy-regulating effects in cells and tissues. For instance, CGA can alleviate incomplete autophagy and cognitive impairment in SH-SY5Y neurons induced by $A\beta_{25-35}$ in mice by regulating the mTOR/TFEB signaling pathway(Gao, et al., 2020). CGA can also restore incomplete autophagy in rat gastric tissue induced by indomethacin, thereby alleviating gastric ulcers(Ahmed, et al., 2021). Notably, the structure of CGA, containing multiple hydroxyl groups, endows the ability to chelate metal ions, thus reducing the harmful effects of lead, aluminum, and Cd in the body(Cheng, et al., 2019; Ding, et al., 2021; Zhang, et al., 2019). However, the mechanism of CGA in Cd-induced incomplete autophagy in chicken cerebral cortical neurons remains unclear, particularly the role of the AMPK-ULK1 pathway in this process. Therefore, this study is designed to investigate the protective role of CGA in Cd-induced damage to chicken cerebral cortical neurons and the involvement of the AMPK-ULK1 pathway in mediating incomplete autophagy, using both in vivo and in vitro experiments. The findings give a scientific foundation for the identification of drug targets for treatment and preventive strategies against Cd poisoning.

Table 3
Primer sequences of target genes.

Gene name	Primer sequence (5′-3)
AMPK	F: GCTGGATTATGAATGGAAGGTTGTA R: TTGCAGTCCCAGACTTCGT
ULK1	F: GTTCACCGATGTACATGGCAC R: TGGGGATGTTTGGCATCAGT
LC3I	F: TTACACCCATATCAGATTCTTG R: ATTCCAACCTGTCCCTCA
LC3II	F: AGTGAAGTGTAGCAGGATGA R: AAGCCTTGTGAACGAGAT
Beclin 1	F: TGGGGATGTTTGGCATCAGT R: ACAGTACAAGGGTAGCTCCTTC
P62	F: AAGAAGAATCGGTGTGCTAA R: GTTAATCCTCTGCGTGTGA
β-actin	F: CCGCTCTATGAAGGCTACGC R: CTCTCGGCTGTGGTGGTGAA

Table 4
The determination of Cd level in chicken cerebral cortex.

Groups	Cd (μg/g)
Control	0.0074 ± 0.001^{a}
Cd	0.0700 ± 0.044^{b}
CGA200	0.0094 ± 0.002^{a}
CGA400	0.0068 ± 0.005^{a}
CGA200+Cd	0.0180 ± 0.003^{c}
CGA400+Cd	0.0210 ± 0.008^{c}

Completely different letters mean significant differences ($P<0.05$), the same letters mean no significant differences ($P>0.05$). The same as below

Table 2
The grouping and processing of animal experiments.

Group	Control group	Positive Control	CGA200 group	CGA400 group	CGA200+ Cd group	CGA400+ Cd group
No. of birds	12	12	12	12	12	12
Feed	Basal Diet	Basal Diet	Basal Diet	Basal Diet	Basal Diet	Basal Diet
$CdCl_2$(mg/Kg)	–	70	–	–	70	70
CGA(mg/Kg)	–	–	200	400	200	400

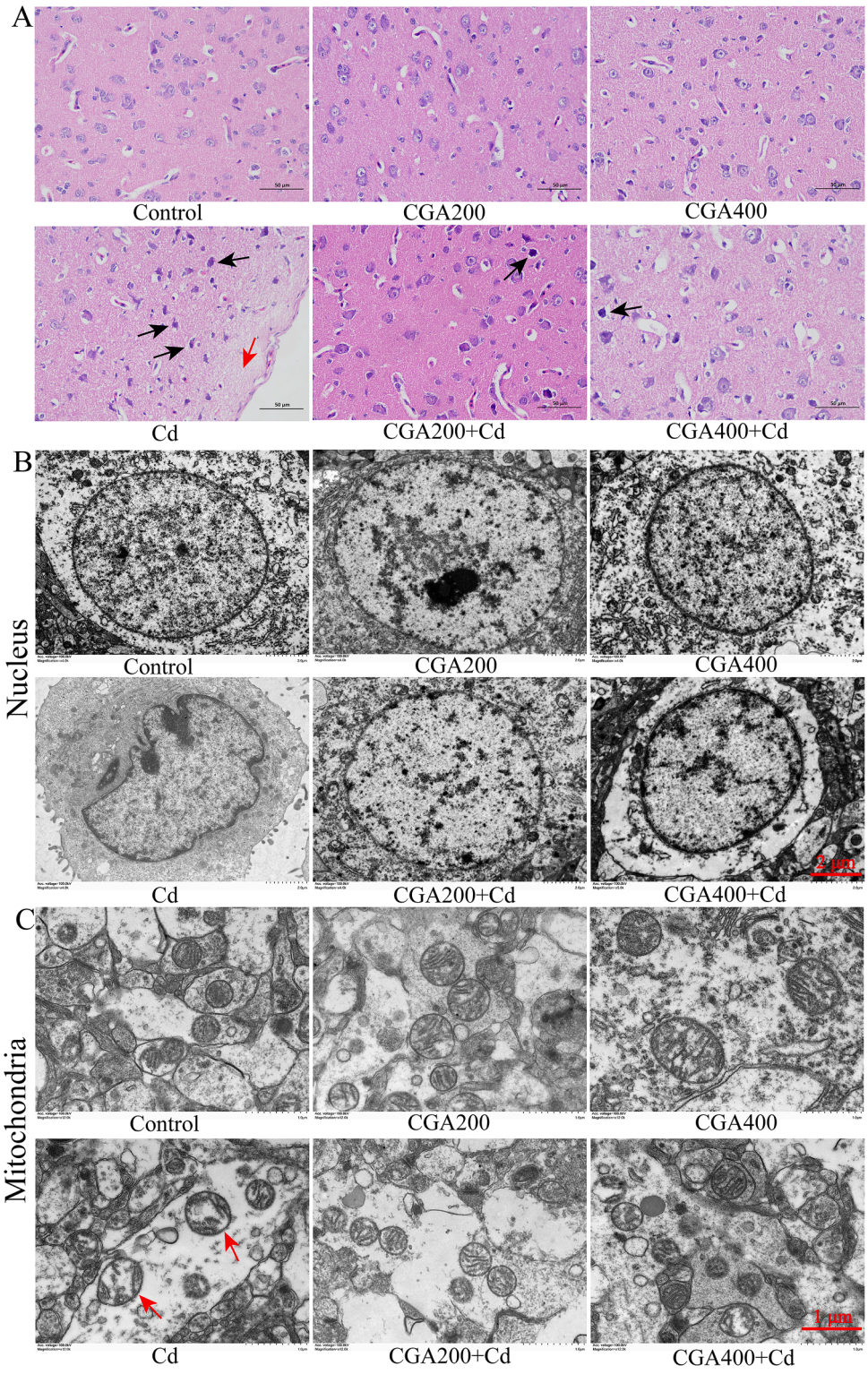

Fig. 1. CGA alleviates Cd-induced cerebral cortex injury in chickens. Chickens were treated with CGA (200 and 400 mg/kg) and/or Cd (70 mg/L) for 30 days. (A) Histopathology changes of the chicken cerebral cortex. Scale bars: 50 μm. (B) Ultrastructural observation of the nuclei in chicken cerebral cortex. Scale bars: 2 μm. (C) Ultrastructural observation of the mitochondria in chicken cerebral cortex. Scale bars: 1μm.

Materials and methods

Main reagents

Cadmium chloride **($CdCl_2$)** and Compound C were supplied by Sigma-Aldrich (St. Louis, MO, USA). CGA was purchased from Yuanye Biotechnology Co., Ltd (Shanghai, China). Neurobasal medium, B-27 supplement, and Trizol were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Hifair III reverse transcriptase kit and Hieff qPCR SYBR Green Master Mix kit were purchased from Yeasen Biotechnology Co., Ltd (Shanghai, China). Antibodies used in the present study are shown in Table 1.

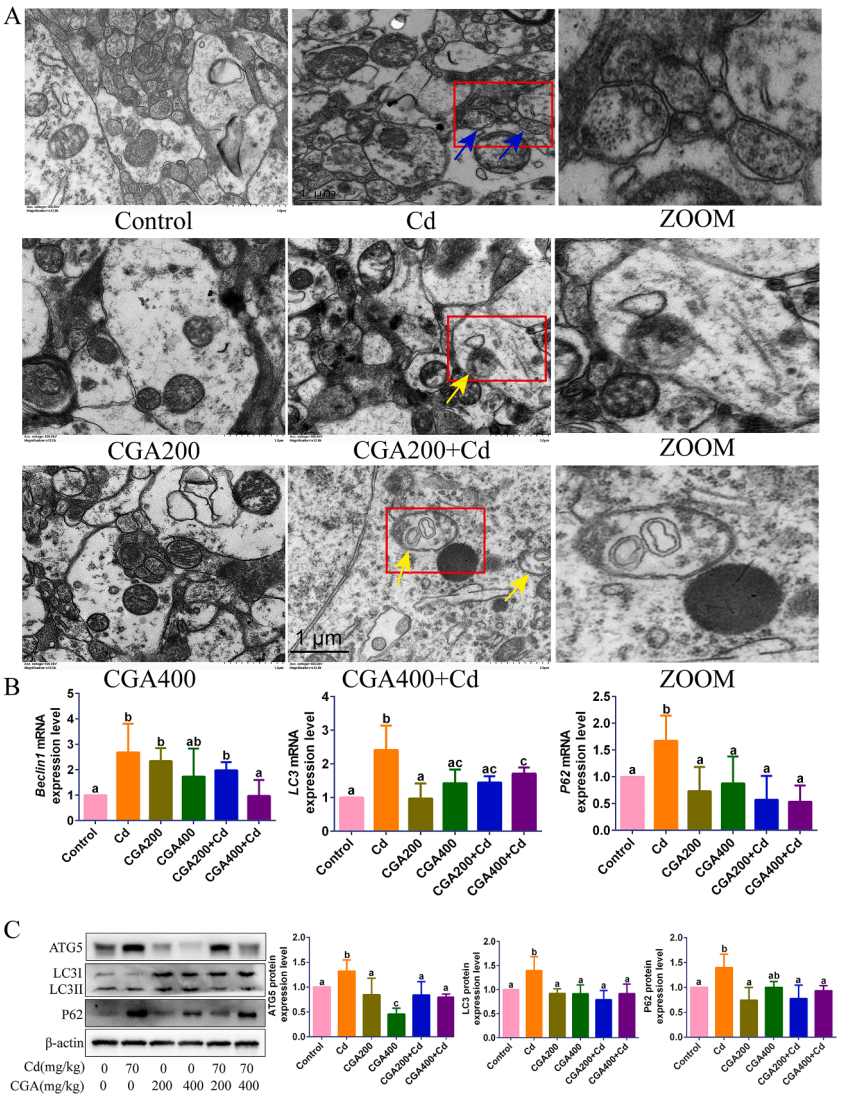

Fig. 2. CGA inhibits Cd-induced activation of the AMPK-ULK1 pathway in the chicken cerebral cortex. Chickens were treated with CGA (200 and 400 mg/kg) and/or Cd (70 mg/L) for 30 days. (A) qRT-PCR was used to detect the mRNA levels of AMPK and ULK1. (B) Western blot was used to detect the phosphorylation level of AMPK and ULK1 protein.

Animals and treatment

Seventy-two one-day-old female Sanhuang chickens were obtained from Jiangsu Poultry Research Institute. According to previous research, this study established an animal model by supplementing the basic diet with Cd and CGA(Abaidullah, et al., 2021; Talukder, et al., 2021; Talukder, et al., 2023). Chicks were pre-fed for a week and then randomly divided into 6 groups as shown in Table 2. On the first day of the experiment, the temperature in the chicken house was set at 34°C. Subsequently, the temperature in the chicken house was decreased by 1°C every three days until it reached 23°C while maintaining a relative humidity of 60%-70%. The chickens were provided with adequate drinking water and a basal diet. The experiment lasted for 30 days. After the experiment, all chickens were fasted for 12 h and then humanely euthanized. The cerebral cortex tissue of the chickens was collected for further experiments.

Cd measurement

The chicken cerebral cortex samples were dried and ground into powder. A 100 mg sample was taken 5 mL concentrated nitric acid was added for digestion by using a microwave digester. Subsequently, the digested sample was put into an acid-removing instrument to remove acid, and then the Cd level was determined by flame atomic absorption spectrometry.

Hematoxylin and Eosin (H&E) staining

The chicken brain tissue was surgically removed and trimmed, then added to 4% paraformaldehyde fixed solution and fixed for 24 h. After discarding the fixative solution, the tissue was washed and dehydrated using a gradient of ethanol. Subsequently, the dehydrated tissue was transparent with xylene, embedded with paraffin, and the tissue was sliced by microtome and attached to the glass slide. Finally, the cell nucleus and cytoplasm were stained with H&E staining solution for 10 min, washed twice with running water, and air-dried. The histopathological changes of the chicken cerebral cortex were observed under the optical microscope.

Transmission Electron Microscopy (TEM)

The $1mm^3$ size of chicken cerebral cortex tissue was sequentially fixed with 2.5% glutaraldehyde and 1% osmium acid. Subsequently, the sample was sequentially dehydrated with ethanol gradient, soaked in

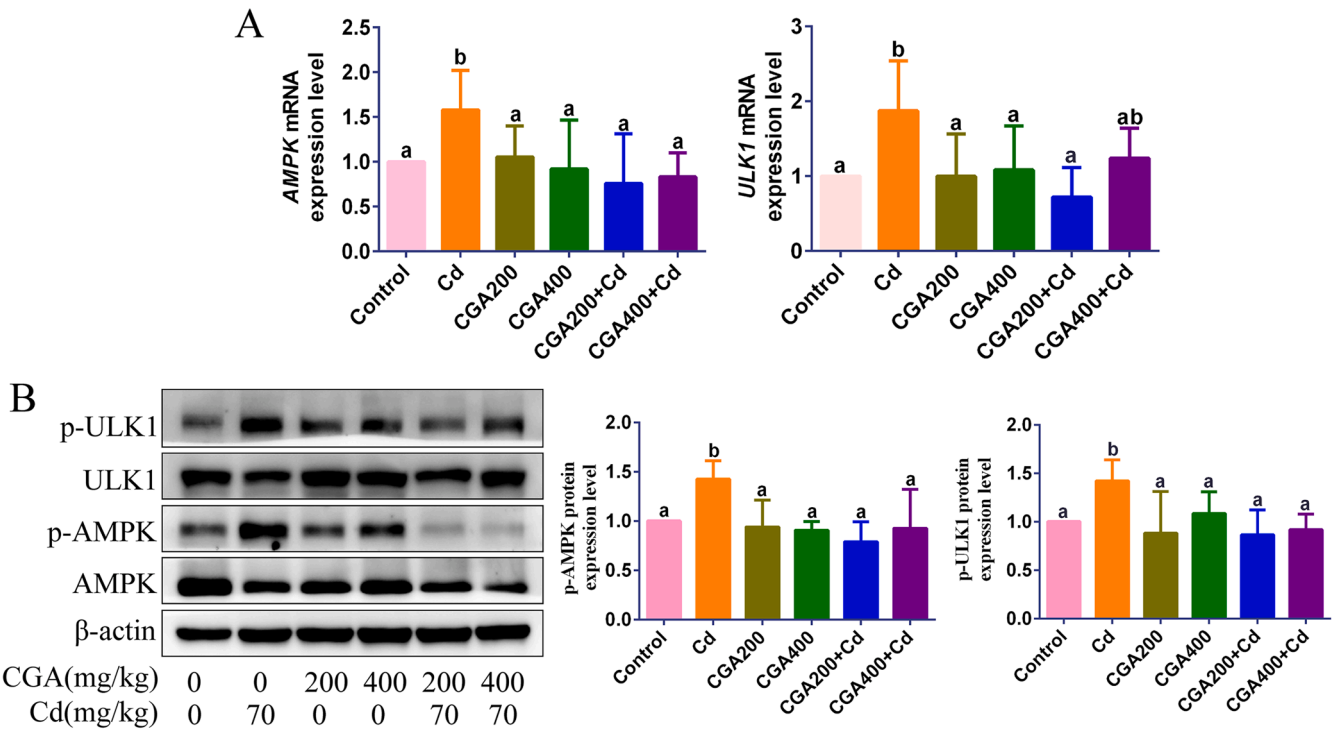

Fig. 3. CGA alleviates Cd-induced incomplete autophagy in the chicken cerebral cortex. Chickens were treated with CGA (200 and 400 mg/kg) and/or Cd (70 mg/L) for 30 days. (A) Ultrastructural observation of the autophagosomes (blue arrow) and autophagolysosome (yellow arrow) in chicken cerebral cortex. The selected areas were magnified. Scale bars: 1 μm. (B) The mRNA transcription levels of Beclin1, LC3, and P62 in chicken cerebral cortex. (C) The protein levels of ATG5, LC3, and P62 in chicken cerebral cortex.

acetone, and embedded in resin. Finally, the ultrathin sections were stained with uranium acetate and lead citrate, and observed and photographed under TEM.

Culture and treatment of cerebral cortical neurons

Specific Pathogen Free **(SPF)** chicken embryos were provided by Zhejiang Lihua Agricultural Technology Co., Ltd. (Zhejiang, China). Cerebral cortical neurons were obtained from 9-day-old SPF chicken embryos followed by culture and seperation of chicken embryo neurons and rat cerebral cortical neurons, with minor modifications(Yan, et al., 2012; Yuan, et al., 2013; Zhu, et al., 2017). Briefly, chicken cerebral cortical neurons were cultured in a neural basal medium containing 2% B-27 supplement and 1mM L-glutamine, and incubated at 37°C in 5% CO_2. On the third day of culture, neurons formed a dense neural network, and the chicken cerebral cortical neurons were identified by NeuN immunofluorescence staining. The neurons were treated with 10 μM Cd and 75 μM CGA alone or in combination for 12 h, or with 10 μM Compound C and 10 μM Cd alone or in combination for 12 h. Cd and CGA were dissolved in ultrapure water and dimethyl sulfoxide **(DMSO)** respectively. The final concentration of DMSO in the culture medium is less than 0.1%.

Immunofluorescence staining

The chicken cerebral cortical neurons were inoculated on 0.01% polylysine-coated sterile cover glass at a density of 2×10^5 cells per well. On the third day of culture, neurons were fixed with 4% paraformaldehyde for 20 min, permeated with 0.1% Triton-100 for 20 min, and blocked with 5% BSA for 1 h. Subsequently, the samples were incubated with rabbit anti-NeuN antibody overnight at 4°C. The next day, the fluorescent-labeled secondary antibody was added and incubated for 2 h in the dark. The nucleus was stained with DAPI and observed and photographed by laser confocal microscope.

Cell viability assay

Chicken cerebral cortical neurons were inoculated into a 96-well plate (3×10^4 cells per well) and treated with different concentrations of Cd (0, 5, 10, 20, 40 μM) for 6 h or 12 h, or treated with different concentrations of Compound C (0, 5, 10 μM) and 10 μM Cd alone or in combination for 12 h. Subsequently, 10 μL CCK-8 kit was added to each well of the 96-well plates 2 h before the end of incubation. The absorbance at 450 nm was measured by enzyme-labeled instrument (Biotek Instruments, Winooski, VT, USA).

Western blot

Total protein was extracted from chicken cerebral cortex tissue and cell samples by using RIPA lysate containing protease inhibitor and BCA kit. Protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis **(SDS-PAGE)** and transferred to a polyvinylidene difluoride **(PVDF)** membrane. Subsequently, the membrane was sealed with 5% skimmed milk powder for 1.5 h at room temperature, and the membrane was incubated with the corresponding primary antibody and secondary antibody. Using Image Lab software to analyze the gray values of stripes. The ratio of the target protein to the β-actin gray value is the relative expression of the target protein.

Quantitative Real-Time PCR (qRT-PCR) analysis

Total RNA was extracted from chicken cerebral cortex tissue and cell samples by Trizol reagent. Then the concentration of RNA was determined by Nanodrop2000 spectrophotometer, and the RNA was reverse transcribed into cDNA by Hifair III reverse transcriptase kit. Subsequently, the CT value of the target gene was detected by Hieff qPCR SYBR Green Master Mix kit combined with a 7500 real-time PCR system. Using β-actin transcription level as an internal reference, the transcription level of the target gene was analyzed by $2^{-\triangle\triangle CT}$ strategy. Primer

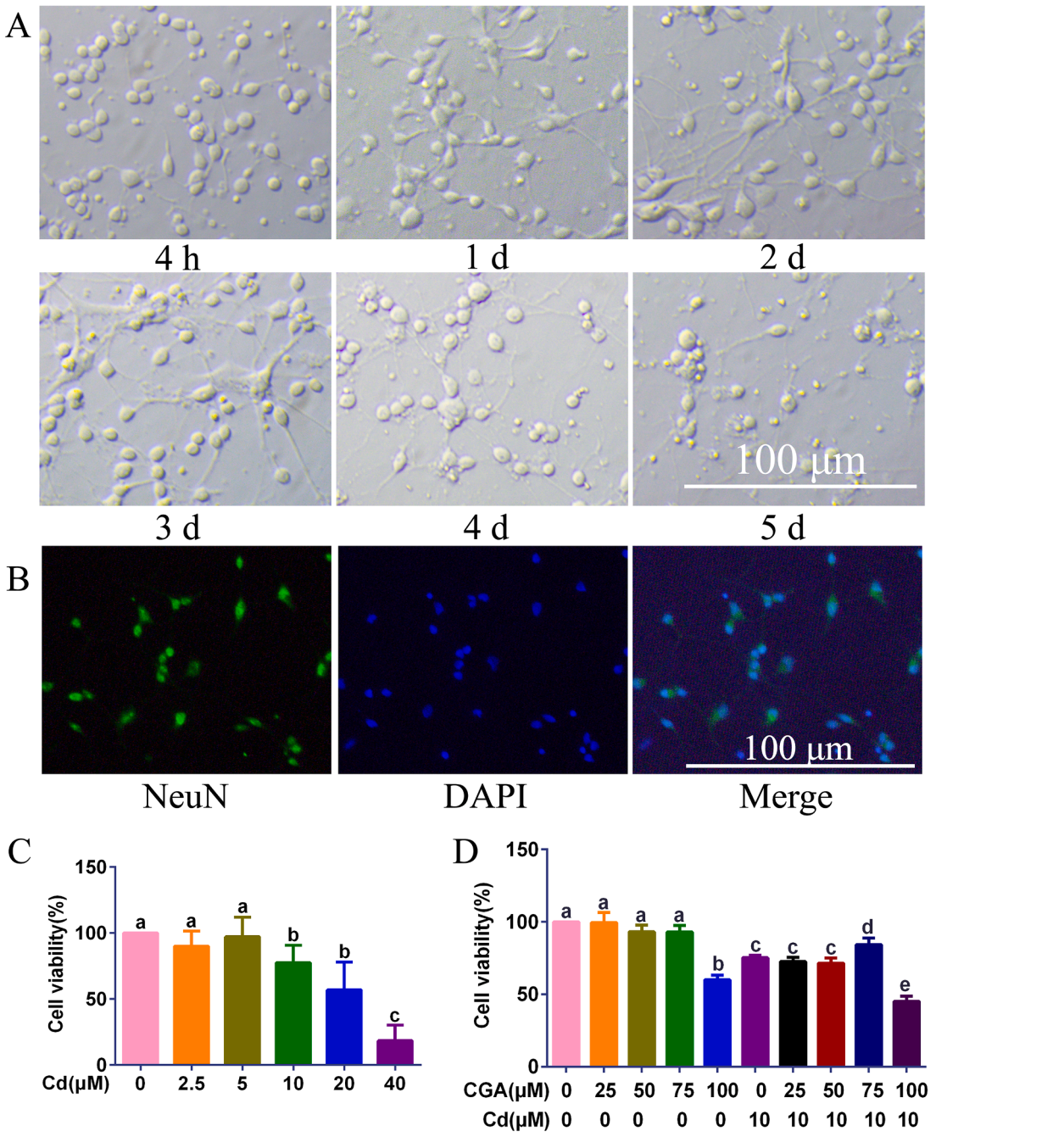

Fig. 4. CGA alleviates Cd-induced injury of cerebral cortical neurons in chickens. (A) The growth state of chicken cerebral cortical neurons was observed by an optical microscope. Scale: 100 μm. (B) NEUN (green) and DAPI (blue) immunofluorescence staining were used to identify the neurons in the chicken cerebral cortex. Scale: 100 μm. (C) Different concentrations of Cd (0, 2.5, 5, 10, 20, and 40 μM) were used to treat chicken cerebral cortical neurons for 12 h, and the cell viability was detected by CCK-8 kit. (D) Chicken cerebral cortical neurons were pretreated with CGA (0, 25, 50, 75, and 100 μM) for 1h, and then treated with 10 μM Cd alone or in combination for 12 h, and the cell viability was detected by CCK-8 kit.

sequences of target genes are shown in Table 3.

Statistical analysis

Each experiment was repeated at least three times for statistical analysis. The data were statistically analyzed by SPSS 26.0 software, and the differences were compared by one-way analysis of variance **(ANOVA)**, and then Tukey multiple comparison tests were used to test the differences between each group. Data were presented as the mean ± standard deviation. Completely different letters mean significant differences ($P<0.05$), the same letters mean no significant differences ($P>0.05$).

Results

CGA alleviates Cd-induced injury of chicken cerebral cortex

To evaluate the effect of CGA on Cd accumulation, the Cd levels in the chicken cerebral cortex were detected using flame atomic absorption spectrometry. The results showed that 200 and 400 mg/kg CGA treatments significantly alleviated the accumulation of Cd in the chicken cerebral cortex (Table 4).

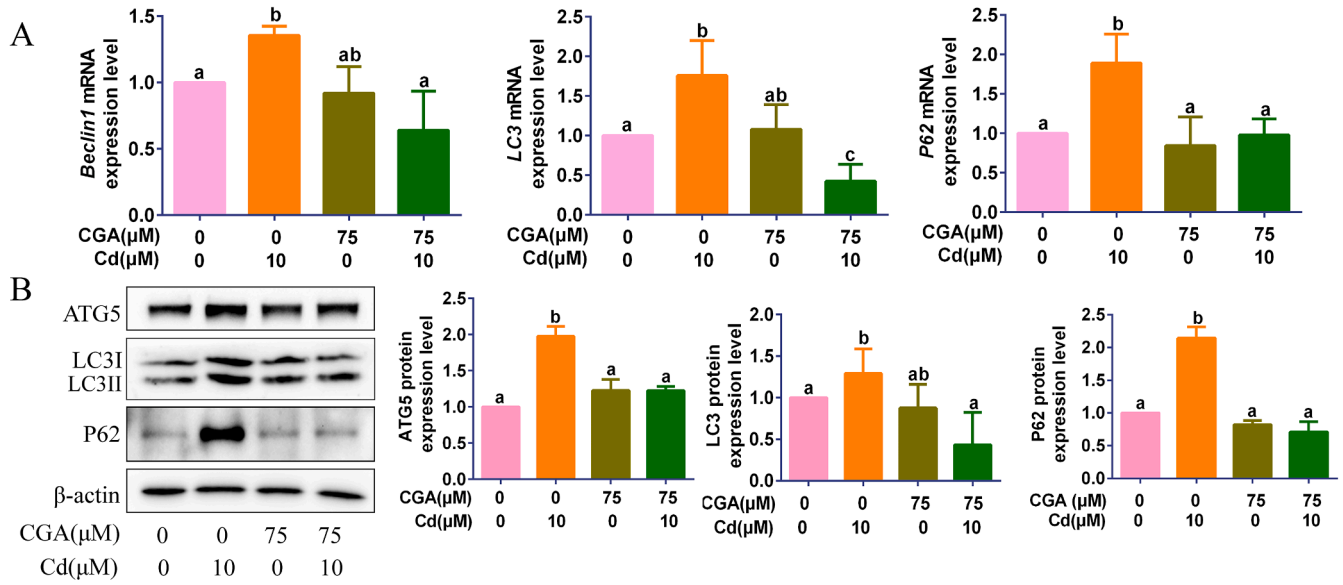

Fig. 5. CGA alleviates Cd-induced incomplete autophagy of chicken cerebral cortical neurons. Chicken cerebral cortical neurons were pretreated with 75 μM CGA for 1 h, followed by 10 μM Cd exposure for 12 h. (A) The mRNA transcription levels of Beclin 1, LC3, and P62 were detected by qRT-PCR. (B) The protein levels of ATG5, LC3, and P62 were detected by Western blot.

To investigate the role of CGA in Cd-induced injury of the chicken cerebral cortex, we examined the histological and ultrastructural changes in the chicken cerebral cortex. The number of neurons in the cerebral cortex was abundant with regular morphology and clear nuclei in the control, 200 mg/kg, and 400 mg/kg CGA samples. However, the neuronal nuclei of the Cd-exposed chicken cerebral cortex were shriveled and deeply stained (black arrows) and the nerve fibers were loosely arranged (red arrows). It is noteworthy that the co-administration of 200 mg/kg or 400 mg/kg CGA significantly alleviated the histological injury of chicken cerebral cortex induced by Cd (Fig. 1 A). Furthermore, Cd also induced ultrastructural changes in the nuclei and mitochondria of the chicken cerebral cortex, which showed uneven chromatin, severe shrinkage and fragmentation of nuclear membrane (Fig. 1 B), mitochondrial vacuolation, mitochondrial cristae disruption, and uneven matrix (red arrows, Fig. 1 C). However, 200 mg/kg and 400 mg/kg CGA significantly alleviated the ultrastructure injury of the chicken cerebral cortex induced by Cd. The above results showed that CGA alleviated the histological and ultrastructural injury of the chicken cerebral cortex induced by Cd.

CGA inhibits Cd-induced activation of AMPK-ULK1 pathway in chicken cerebral cortex

To further investigate the role of CGA in the Cd-induced AMPK-ULK1 pathway in the chicken cerebral cortex, the levels of AMPK, ULK1 mRNA and protein phosphorylation in the chicken cerebral cortex were analyzed by qRT-PCR and western blot. Cd exposure increased the levels of AMPK, ULK1 mRNA and protein phosphorylation in the chicken cerebral cortex, which was significantly reversed by co-administration of 200 mg/kg or 400 mg/kg CGA ($P<0.05$). However, 400 mg/kg CGA had no significant effect on the level of ULK1 mRNA increased by Cd in the chicken cerebral cortex ($P>0.05$). The results showed that CGA inhibited the activation of the AMPK-ULK1 pathway induced by Cd (Fig. 2 A and 2 B).

CGA alleviates Cd-induced incomplete autophagy in chicken cerebral cortex

To investigate the role of autophagy in alleviating Cd-induced injury of the chicken cerebral cortex, the changes of autophagosomes and autophagolysosome in the chicken cerebral cortex were observed by TEM. Cd increased the autophagosomes in the chicken cerebral cortex, while the number of autophagosomes decreased and the number of autophagolysosome increased after 200 mg/kg and 400 mg/kg CGA combined treatment with Cd (Fig. 3 A). The results of qRT-PCR and western blot showed that the mRNA transcription levels of Beclin1, LC3 and P62, as well as the protein levels of LC3, P62 and ATG5 in the chicken cerebral cortex were significantly increased by Cd exposure, which was significantly reduced by the co-administration of 200 mg/kg or 400 mg/kg CGA ($P<0.05$). However, 200 mg/kg CGA had no significant effect on Beclin1 mRNA transcription level increased by Cd in the chicken cerebral cortex (Fig. 3 B and 3 C). These results indicate that CGA can alleviate Cd-induced incomplete autophagy in the chicken cerebral cortex.

CGA alleviates Cd-induced injury of cerebral cortical neurons in chickens

To further confirm the neuroprotective effects of CGA treatment, chicken cerebral cortical neurons were isolated and cultured from 9-day-old SPF chicken embryos. The isolated chicken cerebral cortical neurons formed a dense neural network at 3 d, the neurons clustered at 4 d, and the synapses of the neurons gradually broke at 5 d (Fig. 4 A). Therefore, chicken cerebral cortical neurons cultured for 3 days were selected for subsequent experiments in this study. The isolated and cultured cells were identified as chicken cerebral cortical neurons by NeuN immunofluorescence staining (Fig. 4 B). Subsequently, CCK-8 kit was used to detect neuronal viability. The results showed that, the neuronal viability at 10 μM, 20 μM, and 40 μM Cd was significantly decreased ($P<0.05$) (Fig. 4 C). Based on these results, 10 μM Cd was selected to treat neurons for 12 h for a follow-up study. The results showed that 25 μM, 50 μM and 75 μM CGA have no significant effect on neuronal viability, and 75 μM CGA significantly alleviated the decrease of neuronal viability induced by 10 μM Cd ($P<0.05$). Therefore, in this study, 75 μM CGA and 10 μM Cd were combined to treat chicken cerebral cortical neurons for 12 h for subsequent research (Fig. 4 D).

CGA alleviates Cd-induced incomplete autophagy of chicken cerebral cortical neurons by inhibiting AMPK-ULK1 pathway

To further study the role of CGA in Cd-induced incomplete

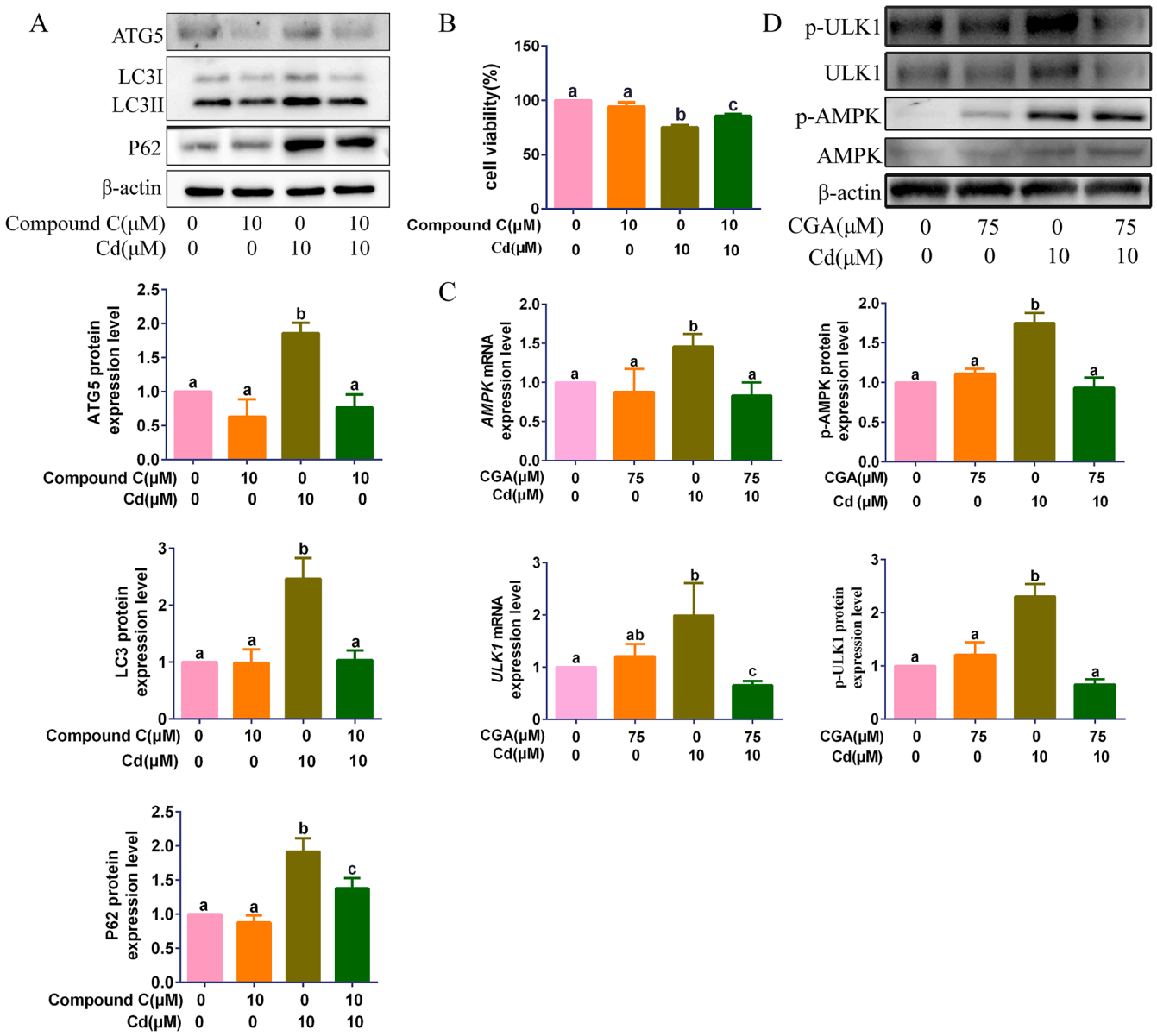

Fig. 6. CGA alleviates Cd-induced incomplete autophagy of chicken cerebral cortical neurons by inhibiting the AMPK-ULK1 pathway. Chicken cerebral cortical neurons were pretreated with 75 μM CGA or 10 μM Compound C for 1 h, followed by 10 μM Cd exposure for 12 h. (A) The protein levels of ATG5, LC3, and P62 were detected by Western blot. (B) The neuron viability was detected by the CCK-8 kit. (C) The mRNA transcription levels of AMPK, and ULK1 were detected by qRT-PCR. (D) The protein levels of p-AMPK and p-ULK1were detected by Western blot.

autophagy of chicken cerebral cortical neurons, the mRNA transcription level and protein level of autophagy-related indicators were detected by qRT-PCR and western blot respectively. Data revealed that Cd significantly increased the mRNA transcription levels of Beclin 1, LC3, and P62, as well as the protein levels of ATG5, LC3, and P62 in chicken cerebral cortical neurons ($P<0.05$), indicating that Cd induced incomplete autophagy of neurons (Fig. 5 A and 5 B). Interestingly, this was significantly reversed by the co-administration of CGA. It shows that CGA alleviated Cd-induced incomplete autophagy in chicken cerebral cortical neurons ($P<0.05$), consistent with in vivo results.

To further verify the role of the AMPK-ULK1 pathway in Cd-induced incomplete autophagy of chicken cerebral cortical neurons. The levels of autophagy-related proteins in neurons were determined by western blot. The viability of neurons was detected by CCK-8 kit. The mRNA transcription level and protein phosphorylation level of AMPK and ULK1 were determined by qRT-PCR and western blot in neurons. The results showed that Compound C significantly inhibited the increase of ATG5, P62, and LC3 protein levels and the decrease of viability induced by Cd in chicken cerebral cortical neurons ($P<0.05$) (Fig. 6 A and 6 B). It is suggested that Cd-induced incomplete autophagy and reduced viability by activating the AMPK-ULK1 signaling pathway in chicken cerebral cortical neurons. Subsequently, the role of CGA in the Cd-induced AMPK-ULK1 pathway in chicken cerebral cortical neurons was further studied. The results showed that Cd exposure increased the mRNA transcription level and protein phosphorylation level of AMPK, ULK1 in chicken cerebral cortical neurons, which was significantly reversed by co-administration of CGA, consistent with the in vivo results ($P<0.05$) (Fig. 6 C and 6 D). These results indicate that CGA can alleviate Cd-induced incomplete autophagy of chicken cerebral cortical neurons by inhibiting the AMPK-ULK1 pathway.

Fig 7

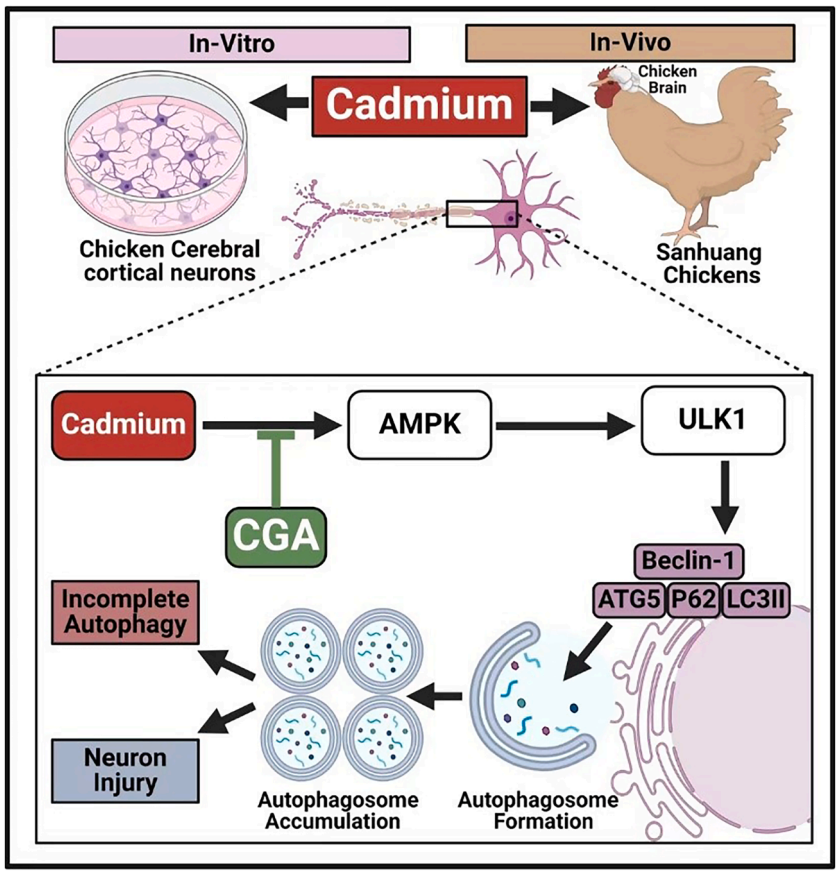

Fig. 7. Mechanism of CGA in alleviating Cd-induced injury of cerebral cortical neurons in chickens.

Discussion

Cd is a toxic environmental pollutant that can harm the health of humans, animals, and poultry throughout the food chain. The investigation revealed that high concentrations of Cd were detected in the liver, kidney, femur, and gizzard samples of chickens fed with feeds containing soil collected near a lead smelter(Li, et al., 2023). In addition, some studies show that Cd is seriously accumulated in the chicken brain and leads to possible toxic mechanisms(Liu, et al., 2021; Talukder, Bi, Jin, Ge, Zhang, Lv and Li, 2021). In this study, the Cd level in the cerebral cortex of chickens exposed to Cd increased significantly, consistent with the above research results. This indicates that Cd can enhance the permeability of the blood-brain barrier and accumulate in the cerebral cortex. Interestingly, CGA significantly reduced the accumulation of Cd in the chicken cerebral cortex, which may be related to the ability of natural plant polyphenol CGA to chelate with heavy metals(Khafaga, et al., 2019). The changes in histopathology and ultrastructure directly reflect the degree of injury to various tissues. In terms of the brain, it is reported that Cd can cause injury to all layers of the rat cerebral cortex after penetrating the blood-brain barrier(Afifi and Embaby, 2016). In this study, Cd exposure induced pyknosis (severe shrinkage), deep staining, loose arrangement of nerve fibers, uneven chromatin in the nucleus, severe shrinkage and fragmentation (karyorrhexis) of the nuclear membrane, vacuolation of mitochondria, disruption of mitochondrial cristae, and uneven matrix in a small number of cerebral cortical neurons in chickens. Similar results were also reported in the previous study. Cd causes severe histopathological and ultrastructural injury, including neuronal necrosis, atrophy, prominent axonal and dendritic loss, nucleolus loss, nuclear atrophy, almost complete mitochondrial cristae loss, and mitochondrial outer membranes fusion in the cerebral cortex tissue of poultry(Liu, et al., 2014). However these histopathological and ultrastructural changes were not as severe as those observed in the above studies due to difference in the dose and Cd exposure time. However, CGA significantly improved these changes and it is suggested that CGA has a protective effect on Cd-induced histopathological and ultrastructural injury in the chicken cerebral cortex, and the neuroprotective effect of CGA may be due to reduce the accumulation of Cd in the cerebral cortex. Our in vitro experiments also confirmed that 75 μM CGA significantly inhibited the viability induced by 10 μM Cd in chicken cerebral cortical neurons. However, when the concentration of CGA is 100 μM, the viability of chicken cerebral cortical neurons is significantly reduced, resulting in the loss of its protective effect. This may be related to different cell types. Prasad et al., showed that the viability of U-937 cells was more than 70% when the concentration of CGA was 10 μM, however, when the concentration of CGA was higher than 12.5 μM, the viability of U-937 cells decreased obviously (Prasad, et al., 2022). Another study showed that 80 μM CGA significantly inhibited the proliferation of A549 cells, but had little effect on the proliferation of JB6 cells(Feng, et al., 2005). These studies also show that the safe range of CGA is relatively narrow, and the dosage of CGA should be strictly controlled when using it. In addition, it is necessary to further study the potential molecular mechanism of CGA neuroprotection in vitro and in vivo.

Autophagy is a complex process composed of multiple stages, and the destruction of any link in the process of autophagy may lead to incomplete autophagy. The accumulation of large amounts of autophagosomes in cells is a typical feature of incomplete autophagy(Zhang, Cao, Qiu and Kang, 2022). Cumulative studies show that, incomplete autophagy is closely related to Cd-induced damage to important organs(Guo, Ruan, Li, Fu, Li, Gong, Gu, Gu and Shi, 2024; Wang, et al., 2023a; Wang-, et al., 2023c). However, the entire process from autophagosome formation to final degradation involves numerous autophagy-related genes and

proteins, including ATG5, Beclin1, LC3, and P62(Schmitz, et al., 2016). ATG5 and Beclin1 are involved in the regulation of autophagosome formation. LC3 mediates the growth, closure and formation of autophagosomes, participates in the degradation of autophagosomes, and is a landmark protein of autophagy(Matsuzawa-Ishimoto, et al., 2018). In addition, autophagic linker protein P62 interacts with LC3, targeting the goods to be degraded and finally degrading them in autolysosomes(Yin, et al., 2017). Therefore, the expression of P62 can reflect the whole autophagy degradation. Previous studies has shown that Cd exposure induces the accumulation of autophagosomes in pig myocardial tissue, upregulating the levels of ATG5, ATG7, ATG12, Beclin1, LC3II, and P62 proteins, resulting in a blockage of autophagy flux(Zhao, Shi, Yao, Li and Xu, 2022). In our study, Cd exposure led to the accumulation of autophagosomes in the chicken cerebral cortex. Furthermore, Cd upregulated the expression levels of Beclin1, LC3, and P62 mRNA, as well as LC3, P62, and ATG5 proteins, in chicken cerebral cortical neurons both in vitro and in vivo. Additionally, a similar mechanism has been reported in Cd-induced kidney injury in chickens(Zhang, et al., 2024). It is worth noting that CGA significantly reversed the mRNA transcription and protein level of autophagy-related indicators as well as autophagosomes accumulation induced by Cd in vitro and in vivo. This finding suggests that one of the critical mechanisms by which CGA alleviates Cd-induced injury to chicken cerebral cortical neurons is by relieving the autophagy flux blockade. Similarly, CGA has been shown to alleviate the autophagy flux blockade triggered by H_2O_2 in SH-SY5Y cells(Gao, et al., 2021). It shows that CGA plays an active role in incomplete autophagy induced by toxic substances.

AMPK is the main regulatory factor that triggers autophagy. When cells are subjected to adverse stimuli, AMPK is activated and directly phosphorylate ULK1 and promote autophagy(Li and Chen, 2019). The anti-tumor drug Tubeimoside I activates AMPK to initiate incomplete autophagy, thereby enhancing Tubeimoside I-induced cervical cancer cell death(Feng, et al., 2018). Similarly, ebastine also promotes incomplete autophagy and induces apoptosis by activating the AMPK/ULK1 pathway of osteosarcoma cells(Pan, et al., 2023). It is suggested that activating AMPK/ULK1 pathway can trigger incomplete autophagy ultimately leading to cell death. In this study, Cd exposure led to activation of the the AMPK-ULK1 pathway and incomplete autophagy in chicken cerebral cortical neurons. Furthermore, AMPK inhibitor (Compound C) could alleviate incomplete autophagy induced by Cd in chicken cerebral cortical neurons. The results showed that the incomplete autophagy of chicken cerebral cortical neurons induced by Cd was related to the activation of AMPK-ULK1 pathway. CGA exerts diverse pharmacological effects through multiple mechanisms. Studies have found that CGA activates the AMPK pathway to maintain metabolic homeostasis of lipids and glucose(Nguyen et al., 2024). Additionally, CGA regulates autophagy of AML12 cells by activating AXL/ERK/LKB1/AMPK/ULK1 signaling pathway(Meng and Song, 2023). The above research shows that the AMPK pathway is one of the important pathways for CGA to play its pharmacological effects. In this study, both in vivo and in vitro results showed that CGA could inhibit Cd-induced activation of the AMPK-ULK1 pathway in chicken cerebral cortical neurons. It is suggested that CGA alleviates Cd-induced neuronal injury in chicken cerebral cortex by inhibiting incomplete autophagy mediated by the AMPK-ULK1 pathway. However, in order to find more effective therapeutic targets of CGA in the future, it is necessary to further study the toxicological molecular mechanism of late autophagy (autophagy-lysosomal fusion and lysosomal degradation) in vitro and in vivo.

Conclusion

In summary, CGA attenuated Cd-induced incomplete autophagy by inhibiting the AMPK-ULK1 pathway, thereby alleviating the injury caused by Cd to chicken cerebral cortical neurons. Therefore, CGA has the potential to become a promising therapy for preventing and treating neurological diseases related to heavy metal Cd pollution.

Ethics approval and consent to participate

Every procedure and protocol involving animals were permitted by the Yangzhou University Comparative Medical Center (Jiangsu Province, China) (approval ID: SYXK (Su) 2021-0027). The study was based on the Guide to moral Control and Supervision in Animal Conservation and use.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare no conflicts of interest.

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