Copper (Cu) is a trace element necessary in animals as well as human beings. However, excessive Cu is toxic to immunocytes, but the precise mechanism is largely unclear so far. This work was conducted aiming to examine the Cu-mediated autophagy mechanism together with its role in Cu toxicology in RAW264.7 cells. Here, we demonstrated that CuSO$_4$ reduced the cell viability depending on its dose. CuSO$_4$ could obviously increase autophagy in RAW264.7 cells. According to the obtained results, CuSO$_4$ induced autophagy through Akt/AMPK/mTOR pathway which characterized by down regulation of p-Akt (Ser473)/Akt, p-mTOR/mTOR, p-ULK1 (Ser757)/ULK1 and subsequent up-regulation of p-AMPK/AMPKα and p-ULK1(Ser555)/ULK1. Furthermore, CuSO$_4$ significantly induced the production of mitochondrial reactive oxygen species (mtROS). In addition, CuSO$_4$-mediated autophagy and autophagy might be suppressed through suppressing mtROS generation by exposure to Mito-TEMPO. Intriguingly, autophagy promotion with rapamycin could decrease the apoptosis and inhibition of autophagy with knock down Atg5 could enhance the apoptosis induced by CuSO$_4$. Moreover, our results suggested that mtROS is the original cause in CuSO$_4$-induced autophagy and apoptosis. Additionally, CuSO$_4$ induced autophagy through mtROS-dependent Akt/AMPK/mTOR signalling pathway in RAW264.7 cells. Moreover, autophagy activation might potentially generate a protection mechanism for improving CuSO$_4$-induced RAW264.7 cell apoptosis.

**1. Introduction**

Copper (Cu) has been identified as one of the essential metals in living body [1]. Cu acts as a critical cofactor of vital enzymes responsible for catalyzing electron transfer necessary for iron oxidation, cell respiration, neurotransmitter synthesis, pigmentation, and connective tissue production [2]. Moreover, Cu accounts for an active transition metal for redox reaction, and oxidative stress (OS) is identified as a toxicity mechanism [4]. Patwa et al. [5] have demonstrated that treatment with 20 mg/kg copper sulfide (CuSO$_4$) can increase reactive oxygen species (ROS) production and decrease the levels of antioxidants enzymes in the liver of rats. In addition, CuSO$_4$ induces down-regulation of antioxidant defense enzymes and up-regulation of caspase-3 in the brain of rats [6]. Our previous studies also have indicated that chronic exposure to excess Cu can lead to overproduction of ROS and decrease antioxidant functions, and ROS can promote apoptosis in livers of mice [7]. Results obtained by Wang et al. [5] have revealed that oxidative stress, cell cycle arrest and apoptosis induced by CuCl$_2$ are the important toxicological mechanism in SGC-7901 cells.

Autophagy is recognized as the lysosomal decomposition-related...
pathway, which plays a vital role in cell development, differentiation, as well as homeostasis [9]. In the presence of stressful conditions, like OS, malignancy and serum deprivation, autophagy will be triggered for the sake of adapting to structural remodeling induced by the stresses, which is achieved through the synthesis of greater amounts of energy and nutrients, removal of misfolded and long-lived proteins in cells, and the elimination of redundant or injured organelles together with the invasive microorganisms [10]. It has shown that Cu can induce autophagy [11–13]. Fang et al. [14] have reported that CuSO₄ promotes autophagy occurrence and ROS exert an important role in autophagy induced by CuSO₄ in duck renal tubular epithelial cells. Besides, Liao et al. [15] have found that increased Cu consumption possibly induced autophagy of broiler chicken kidney via the AMPK-mTOR signal transduction pathway. Recently, investigation into the roles of autophagy has increased. Cell death as a result of various stressful conditions represents a complicated process under the joint control by apoptosis and autophagy; in some cases, the cross-talk of these two processes is quite intricate [16,17]. Many articles demonstrate the effect of Cu on inducing autophagy in vitro and in vivo [7,18–20].

Previous studies have demonstrated that over-exposure Cu can induce immunotoxicity including significant pathological damage, oxidative stress, inflammation and apoptosis in immune organs [1–4]. Macrophages are the main immune cells in the immune system. In this study, cytotoxicity of CuSO₄ in the RAW264.7 mouse monocytes was examined. Dysregulation or dysfunction of autophagy has been implicated in immune-related diseases. However, despite extensive studies on the roles of autophagy in Cu toxicity, little is known regarding its involvement in Cd-induced immunotoxic effects. Therefore, the present work aimed to examine the Cu toxicology mechanism in RAW264.7 cells including oxidative stress, autophagy, and apoptosis. Meanwhile, the relationship between autophagy and apoptosis in Cu toxicology is still unclear. The role (promotion or inhibition) of autophagy in CuSO₄-induced apoptosis in RAW264.7 mouse monocytes is also be investigated.

2. Materials and methods

2.1. Cell culture and chemicals

The RAW264.7 monocytes were cultured within the DMEM (Gibco BRL, Grand Island, NY) that contained the 10% fetal bovine serum (FBS, heat-inactivated) and penicillin-streptomycin antibiotics (both 100 μg/ml), followed by incubation within the incubator under 37 °C and 5% CO₂ conditions. The antioxidant Mito-TEMPO specific to mitochondria (CAS 1569257-94-8) was provided by Santa Cruz Biotechnology. CuSO₄ (C1297) was provided by Sigma Aldrich Corporation. Bafilomycin A1 (B1793), 3-MA (M9281) and rapamycin (V900930) were bought from Sigma-Aldrich. Mitosox (M36008, Invitrogen) was utilized to treat RAW264.7 cells with 150 μL cell suspension to incubate for 24 h. Afterwards, CuSO₄ was added to treat the cells, later, 0.5 mg/ml MTT solution was used to incubate cells for 4 h. Therefore, we used dimethyl sulfoxide to dissolve the resultant formazan crystals, and the microplate reader (PerkinElmer) was used to measure the absorbance (OD) value at 540 nm. All results were presented in the manner of mean ± SD from 3 independent assays.

2.4. Western blotting

Cells were treated with CuSO₄ and lysed using the pre-chilled RIPA buffer. Then the cell lysate was subjected to 15 min centrifugation at 15,000g and 4 °C, and 12% SDS-PAGE was performed to separate the proteins, followed by transfer to the PVDF membranes. Later, 5% non-fat milk dissolved within TBST was used to block the membranes, followed by incubation with primary antibodies and HRP-labeled secondary antibodies in succession. Cells were then visualized by using the ECL detection kit (GE Healthcare, Piscataway, NJ, USA). Besides, the Bio-Rad ChemiDoc XRS + System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was utilized to visualize protein bands. The ImageJ2x software was utilized to determine the significant difference in protein expression.

2.5. Mitochondrial ROS detection

The ROS contents in mitochondria were measured using Mitosox (M36008, Invitrogen). Briefly, CuSO₄ was used to treat RAW264.7 cells for 24 h. After washing by PBS twice, cells were subjected to 10 min incubation using the 5 μM Mitosox. Then, cells were rinsed by PBS twice, and the Synergy 2 multimode plate reader (BioTek Instruments) was used to determine fluorescence intensity. All results were standardized based on PBS controls.

2.6. Apoptosis analysis by flow cytometry

Cells were treated with CuSO₄ and washed by the pre-chilled PBS (pH 7.2–7.4), followed by suspension within PBS to 1×10⁶ cells/ml. Briefly, the 5 ml tube was added with 100 μL cell suspension, followed by 15 min Annexin V-FITC (5 μL, Cat: 51-65874X, BD, USA) and PI (5 μL, Cat: 51-66211E, BD, USA) staining under 25 °C in dark. After the addition of 1× binding buffer (400 μL), the flow cytometry (BD FACSCIru) was used to assess stained cells in 40 min after they were prepared. In addition, the ModFit LT v3.0 software was employed to analyze the flow cytometric data.

2.7. Statistical analysis

Data were represented as mean ± SD. Significant differences between control and experimental groups were compared through one-way ANOVA by using SPSS17.0. A difference of P < 0.05 indicated statistical significance.

3. Results

3.1. CuSO₄ induces cytotoxicity in RAW264.7 cells

To evaluate the RAW264.7 cytotoxicity of CuSO₄, RAW264.7 were treated with CuSO₄ (0, 10, 20, 50, 100, 200 and 500 μM) for 24 h. As presented in Fig. 1, CuSO₄ inhibited cell viability, cell viability significantly (p < 0.05 or p < 0.01) decreased when the CuSO₄ concentration exceeded 50 μM.

3.2. CuSO₄ induces autophagy in RAW264.7 cells

Thus, the response of RAW264.7 cells to Cu-induced autophagy was analyzed. Firstly, we test the LC3 conversion and p62 degradation. In
3.3. CuSO₄ induces autophagy through the Akt/AMPK/mTOR signaling

In the presence of external stimuli, the Akt/AMPK/mTOR signal transduction pathway exerts a vital part in cell autophagy. For exploring the effect of Akt/AMPK/mTOR/ULK1 on the CuSO₄-mediated RAW264.7 cells autophagy, this study performed Western blotting to measure the critical proteins related to the Akt/AMPK/mTOR signal transduction pathway. As demonstrated in Fig. 3a and b, CuSO₄ exposure markedly up-regulated the protein levels of p-ULK1 (Ser555) and p-AMPKα within RAW264.7 cells, but down-regulated those of p-Akt (Ser473), p-ULK1 (Ser757) and p-mTOR (P < 0.01). Meanwhile, proteins involved in the autophagy flux were also detected. The results showed that the Beclin1, Atg5-Atg12, Atg7 Atg3 and Atg16L1 were not changed in CuSO₄-treated RAW264.7 cells (Fig. 3c and d).

3.4. CuSO₄ induces apoptosis in RAW264.7 cells

According to Fig. 4, CuSO₄ treatments at 50 and 200 μM significantly elevated the apoptosis rates (P < 0.01). In addition, we also measured the expression of apoptotic proteins. The protein levels of cleaved-caspase-3/-8/-9 and cleaved-PARP significantly (P < 0.01) increased in CuSO₄-treated RAW264.7 cells.

3.5. CuSO₄ induces autophagy and apoptosis through mtROS in RAW264.7 cells

We detected the fluorescent intensity related to the CuSO₄-induced mtROS level (Fig. 5a). Following CuSO₄ treatment, mtROS level
Fig. 2. CuSO₄ induces autophagy in RAW264.7 cells. (a and b) Cells were treated with CuSO₄ (0, 50 and 200 μM) for 24 h, and immunoblotted for the whole cell lysis LC3 and p62 protein expression. (c and d) Cells were transiently transfected with GFP-LC3B and then treated with CuSO₄ for 24 h. Fluorescence microscopy images show diffuse green staining in control cells, but GFP-LC3B fluorescence puncta after CuSO₄ exposure was observed. (e and f) Cells were treated with 200 μM CuSO₄ for 0 h, 12 h and 24 h, and immunoblotted for the whole cell lysis LC3 and p62 protein expression. (g) Cells were pretreated with BaF1 (100 nM) 6 h and 3-MA (5 mM) for 3 h, and followed by CuSO₄ (200 μM) treatment. After 12 h, the LC3 protein expression were detected. Data are presented with the means ± standard deviation. *p < 0.05, compared with the control group; **p < 0.01, compared with the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
markedly elevated ($P < 0.01$) (Fig. 5b).

To determine the role of mtROS in CuSO$_4$-induced autophagy and apoptosis, we used Mito-TEMPO to suppress mtTOS generation. It was illustrated from Fig. 5c that, pretreatment with Mito-TEMPO suppressed the CuSO$_4$-mediated mtROS generation (Fig. 6c), while suppressing ROS enhanced the CuSO$_4$-mediated cell viability. Mito-TEMPO attenuated autophagy induced by CuSO$_4$ (Figure e and f). Mito-TEMPO also can suppress apoptosis induced by CuSO$_4$ (Figure g and h). The related protein of autophagy and apoptosis including LC3-II/LC3-I, p-AMPK, p-Akt (Ser473), p-mTOR, p-ULK1 (Ser 757), p-ULK1 (Ser 555), cleaved-caspase-3 and cleaved-PARP was dramatically reversed by Mito-TEMPO. According to these findings, CuSO$_4$-induced autophagy and apoptosis within RAW264.7 cells may be induced by mtROS.

3.6. Autophagy inhibits apoptosis in Raw264.7 cells

More and more studies have indicated the important roles of autophagy, including cytoprotection and cytotoxicity. For investigating the relationship of CuSO$_4$-induced autophagy with the apoptosis of RAW264.7 cells, we used an autophagy promotor (rapamycin) to increase autophagy induced by CuSO$_4$. As a result, combining CuSO$_4$ with rapamycin markedly increase cell viability and inhibit apoptosis (Fig. 6a and b). Meanwhile, LC3-II/LC3-I, the autophagic protein, was up-regulated, and apoptosis-related protein cleaved-caspase-3 and cleaved-PARP were dramatically reversed by Mito-TEMPO. According to these findings, CuSO$_4$-induced autophagy and apoptosis within RAW264.7 cells may be induced by mtROS.
combination of CuSO₄ and autophagy inhibition (Atg5 knock down) group (Fig. 6d and e). In comparison with CuSO₄ treatment group, CuSO₄ treatment combined with autophagy suppression (Atg5 deletion) markedly suppressed the expression of autophagic protein LC3-II/LC3-I while increasing that of apoptotic proteins cleaved-PARP and cleaved-caspase-3, as revealed by Western blotting analysis (Fig. 6f).

4. Discussion

More attention should be paid to macrophages in terms of toxicology because they have diverse activities. Results in this work suggested that CuSO₄ showed high toxicity to macrophages (RAW264.7 cells) in vitro. Our data are in consistence with the findings of Triboulet et al. [21], who reported that copper nanoparticles can alter macrophage function and toxic to macrophage. Then, the molecular mechanism of Cu-induced RAW264.7 cells toxicology is explored, including oxidative stress, autophagy, and apoptosis.

Autophagy, a form of cell decomposition, is related to material transfer from cytoplasm to lysosomes [17]. It has been reported that Cu is a novel stimulator of autophagy [11,22]. However, it is still unknown about the involvement of autophagy in the Cu-induced toxicity to RAW264.7 cells. In the case of autophagy, LC3 will be increasingly cleaved into LC3-I and LC3-II, while LC3-II relates to the amount of autophagosomes formed. The findings showed that CuSO₄ overexposure can increase the LC3 puncta and ratio of LC3-II/LC3-I as well as decrease p62 protein expression, which indicated that autophagy level is the up-regulation in RAW264.7 cells. In consistence with our results, Cu compounds can increase autophagy in male germ cells [19] and in the kidney as well as brain of broiler chickens [15,23]. Furthermore, our results demonstrated that down regulation of p-Akt (Ser473)/Akt, p-mTOR/mTOR p-ULK1 (Ser757)/ULK1 and subsequent up-regulation of p-AMPKα/AMPK and p-ULK1 (Ser555)/ULK1 were observed in CuSO₄-treated RAW264.7 cells. The canonical PI3K/Akt-mTOR signal transduction pathway has been identified to be the critical factor to adversely regulate autophagosome formation [24]. The PI3K/Akt signal transduction pathway controls mTOR activity [25]. Moreover, the activation of AMPK is recently suggested to result in autophagy by negatively modulating mTOR [26]. Besides, AMPK can directly phosphorylate ULK1 to induce autophagy [26]. These results demonstrate that CuSO₄ induces autophagy through Akt/AMPK/mTOR/ULK1 signaling. The above results conform to findings from Liao et al. [15], in which CuSO₄ induces autophagy in kidney of broiler chickens through activation of AMPK-mTOR pathway. Meanwhile, proteins involved in the autophagy flux also were detected. According to the results, Beclin1, Atg5-Atg12, Atg7 Atg3 and Atg16L1 were not changed in CuSO₄-treated RAW264.7 cells. In contrary, the result of Fang et al. [14] showed that CuSO₄ treatment can increase Beclin-1, ATG7, ATG5, ATG3 expression in duck renal tubular epithelial cells. The inconsistency of the findings is
Fig. 5. CuSO$_4$ induces autophagy and apoptosis through mtROS. (a and b) Relative mtROS amounts determined by MitoSOX-red staining of CuSO$_4$-primed RAW264.7 cells. Scale bar 50 μm. (c) mtROS changes in CuSO$_4$-treated (200 μM, 24 h) RAW264.7 cells in the presence/absence of Mito-TEMPO (500 μM, 1 h) pre-treatment. Changes of cell viability (d), GFP-LC3 fluorescence puncta (e), autophagy proteins (f), apoptosis (g) and apoptotic proteins (h) in CuSO$_4$-treated (200 μM, 24 h) RAW264.7 cells in the presence/absence of Mito-TEMPO (500 μM, 1 h) pre-treatment. Data are presented with the means ± standard deviation. *p < 0.05, compared with the control group; **p < 0.01, compared with the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
possibly a result of cell type. Autophagy usually occurs concurrently with apoptosis, and it represents the well-aligned and autonomous cell death process, which mainly functions to maintain the balance of certain cell populations within the tissues. Findings in the present work revealed that CuSO$_4$ treatment promoted RAW264.7 cell apoptosis while up-regulating cleaved-caspase-3/-8/-9 and cleaved-PARP.

It has confirmed oxidative stress in the basic mechanism of Cu toxicology [4]. Here, we find that CuSO$_4$ treatment increased the mtROS production. Many studies indicate that ROS synthesis induces autophagy and apoptosis [17]. However, how mtROS affects the CuSO$_4$-induced autophagy and apoptosis of RAW264.7 cells has not been reported. Afterwards, this study examined whether mtROS production was an upstream event in the CuSO$_4$-mediated autophagy and apoptosis. Mito-TEMPO (mtROS scavenger) treatment evidently mitigated apoptosis, autophagy and mtROS formation induced by CuSO$_4$ in RAW264.7 cells. Besides, Mito-TEMPO pretreatment abolished the effects of CuSO$_4$ treatment on the Akt/AMPK/mTOR/ULK1 signal transduction pathway. Consistently, Fang and colleagues [14] discovered that Cu activated the ROS/HO-1/NQO1 signal transduction pathway to cause autophagy. Collectively, findings in this work indicated the vital part of mtROS in the CuSO$_4$-mediated autophagy and apoptosis. Additionally, the mtROS-induced interference with the Akt/AMPK/mTOR/ULK1 signal transduction pathway was related to the CuSO$_4$-mediated RAW264.7 cell apoptosis.

Autophagy plays a role of a double-edged sword, which can regulate cell death and survival. The autophagy level is low under physiological condition, and this contributes to cell survival. In the presence of some chemicals, autophagy can be significantly activated, resulting in cell death. It has been widely indicated that apoptosis and autophagy are both critical for cell death. The association of autophagy with apoptosis is generally categorized as three types, namely, interdependence, mutual transformation and mutual antagonism. Besides, autophagy may interact with apoptosis in diverse manners. Autophagy acts as an antagonist to resist apoptosis and facilitate cell apoptosis at diverse conditions. For investigating how autophagy affected RAW264.7 apoptosis induced by CuSO$_4$, autophagy promotion (rapamycin) and inhibition (Atg5 knock down) were used. Noteworthily, findings in this work indicated that, rapamycin pretreatment suppressed the CuSO$_4$-mediated RAW264.7 cell apoptosis; by contrast, Atg5 deletion had the opposite effect. These observations suggested the possible cytoprotective effect of autophagy on CuSO$_4$-mediated RAW264.7 cell apoptosis.

5. Conclusions

To conclude, our results suggested that mtROS is the original cause in CuSO$_4$-induced apoptosis and autophagy. Moreover, autophagy activation may protect RAW264.7 cells from CuSO$_4$-induced apoptosis.

Author contributions

Hongrui Guo, Qin Luo and Yuzhen Song designed the experiments. Qin Luo, Yuzhen Song, Jingjing Kang, Xuchen Wu and Fengsun Wu carried out the experiments. Qin Luo, Yuzhen Song, Yueqin Li, Qing Dong, Jun Wang and Chao Song analyzed and interpreted the data. Hongrui Guo, Qin Luo and Yuzhen Song wrote and revised the
manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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