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Liver Damage Induced by Cadmium via mTOR-Mediated Autophagy in Hepatocytes

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Abstract:

Cadmium (Cd) is one of the environmental pollutants that induces hepatotoxicity, but the mechanism remains unknown. In this study, we aimed to probe whether mTOR-dependent autophagy was involved in Cd-induced liver damage in vivo and in vitro. AML12 cells were exposed to various concentrations of $CdCl_2$ for 6, 12, 24hr, or C57BL/6 mice were given $CdCl_2$ solution for 3 days via oral administration. Cell viability was measured by CCK-8 assay. The expression of p-mTOR or LC3 protein was measured with western blotting or immunofluorescence, as well as liver damage via H&E staining. This study focused on the effect of Cd on liver injury, including the mTOR pathway and autophagy, which provides new ideas for further study on the damage mechanism of environmental pollutants.

Keywords: Cadmium; Liver damage; mTOR; Autophagy; Hepatocytes

1. Introduction

Cadmium (Cd) is considered a toxic metal for humans and the ecosystem, which has been classified as a category I human carcinogen by the International Agency for Research on Cancer (IARC). Cd became the mainstream of heavy metal toxicity research when it was identified as the key pathogenic factor of *itai itai* disease[1]. Cd in the air, soil and water may be spread by human activities such as industrial production, waste plastic incineration, and electronic waste recycling[2]. Human exposure to Cd mainly occurs via inhalation, cigarette smoking, and the ingestion of contaminated food and water. Cd hardly undergoes metabolic degradation and has an extremely long biological half-life of about 25-30 years, so it would be deposited in organs: 30% was deposited in the liver, 30% in the kidney, and the rest was distributed in other organs[3].

Studies have indicated that Cd exposure has a variety of toxic effects, including hepatotoxicity, immunotoxicity, neurotoxicity, reproductive toxicity, and even cancer[4]. In recent years, the liver damage mediated by Cd has received extensive attention. Researchers have reported that hepatocytes are involved in liver injury by Cd. Hepatocytes are the basic unit of the liver, which accounts for about 60%-70% of the total volume of the liver. Hepatic cells are responsible for carrying out most of the functions of the liver, such as metabolism, detoxification, storage of glycogen, etc. In addition, liver cells perform a variety of functions, including synthesizing and secreting bile, storing vitamins and minerals, and participating in the metabolism of protein, fat, and sugar. Cd is closely related to damage of hepatic cells; however,

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the mechanism of hepatotoxicity is not totally clear yet.

Some literature report that autophagy is closely associated with cytotoxicity. Autophagy, originally discovered in liver experiments, is a cellular process that degrades damaged organelles or protein aggregation. Autophagy not only plays an important role in cell homeostasis maintenance, but also involves in activities through sequestration in double-membrane vesicles and subsequent fusion with lysosomes for acidic degradation[5]. Exposure of Cd regulated the expression of autophagy-related proteins such as LC3 and p62, then induced autophagosome formation in livers[4].

The mechanistic target of rapamycin (mTOR) is a major regulator of cell growth and metabolism, which inhibits the process of catabolism, including autophagy. The activation of mTOR can directly inhibit the initiation of autophagy. By regulating autophagic-related genes (Atgs) and phosphorylating Atg13, it cannot bind to Atg1, thus blocking autophagy. Rapamycin activates autophagy by inhibiting mTOR[6]. Whether mTOR could be involved in mediating hepatocellular autophagy induced by Cd is still unknown. This work may demonstrate a clearer understanding of the regulation of hepacellular autophagy, which would lead to more advanced therapeutic strategies to treat chronic Cd toxicity.

It is predicted that Cd can trigger liver damage via mTOR-activated autophagy. In this experiment, in vivo and in vitro were used to verify whether the prediction is solid. These will provide clues for further study on the mechanism of liver toxicity of heavy metals.

2. Material & Methods

2.1 Cell culture and treatment

The mouse AML12 hepatocytes line was purchased from ATCC. AML-12 cells were cultured in DMEM media supplemented with 10% fetal bovine serum, containing 1% penicillin (10 kU/mL) together with streptomycin (10 mg/mL), then were cultured at 37 ℃ in a humidified 5% CO_2 incubator[7]. Cells were exposed to cadmium chloride ($CdCl_2$: IC50 of 26.01mg/ml) at the concentrations of 1, 3.3, 10, 33.3, 100μM for 0, 6, 12, 24hr. The cells in the negative control group were only treated with a culture medium with PBS/DMSO, and the positive control group for the vitro experiment was exposed to rapamycin (RAPA), which would cause cell death via mTOR-mediated autophagy. Mice were purchased in Stantec. Each C57BL/6 mice were given $CdCl_2$ solution (Cd content 1, 10, 100 mg/10g body weight) for 3 days via oral administration. The positive control group for the vivo experiment was exposed to carbon tetrachloride (CCl4), which is a widely used reagent for liver damage mice models.

2.2 CCK-8 assay

Cell viability is measured by CCK-8 assay. Cells were cultured in 96-well plates at a density of 5×10^3/well, which was maintained at 37℃ and 5% CO_2 for 12h until the cells were adherent. Discard the old medium and add the pre-configured CCK-8 working solution, adding 100 μl/well at a ratio of 9:1 from the volume of culture medium to the volume of CCK-8[8]. After incubation for 2 h at 37℃ in the dark, the absorbance at 450 nm was measured using a microplate reader.

2.3 Western Blotting (WB)

The protein expression of mTOR and p-mTOR was measured by Western blotting. Cells were harvested, and total proteins from cells were lysed using RIPA lysis buffer containing PMSF. Then, the protein content was determined using a BCA and was equally separated by 8-12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes[7]. Protein bands were cut and incubated with diluted primary antibodies mTOR or p-mTOR overnight at 4 °C and then incubated with secondary antibody for 1hr at room temperature. Blots were visualized by the Enhanced Chemiluminescence (ECL) reagent (Sevenbio, Beijing, China). β-actin acted as the reference protein, and the gray value of each protein band was measured by ImageJ software.

2.4 Immunofluorescence (IF)

The protein expression of LC3 is measured by immunofluorescence. Cells were fixed for 20 min in 4% paraformaldehyde and then permeabilized in phosphate-buffered saline (PBS) with 0.2% Triton X-100 for 15 min. Samples were blocked with 5% bovine serum albumin (BSA) diluted in PBS for 30 min at room temperature. Primary antibody LC3 was used and incubated in PBS-T solution with 1% BSA overnight at 4 °C. Then, the samples were further incubated with CoraLite 594-conjugated goat anti-rabbit IgG or FITC-conjugated goat anti-rabbit-IgG fluorescent secondary antibody at 1:100 dilutions for 1hr at 37 ℃ and avoided light. Eventually, the nuclei were counterstained with DAPI and the images were observed by fluorescence microscope[9].

2.5 H&E Staining

Liver damage in vivo experiment is measured with H&E staining. First, stain with hematoxylin for 3 to 5 min. Then wash under running water until the section turns ‘blue’ for no more than 5 min. Selectively remove the dye from the pieces. A few seconds in 1% acid alcohol. Rinse with

running tap water. Immerse in ammonia until the section turns blue, and then rinse with running tap water. Stain in 10% Eosin Y for 1 minute. Rinse with tap water for 1 to 5 min. Dehydrate as alcohol level rises. To remove slides, place them in two xylene baths and mount them in DPX or another mounting format. At last, observe under a compound microscope[10]special stains can provide additional contrast to different tissue components. Here, we demonstrate the utility of supervised learning-based computational stain transformation from H&E to special stains (Masson's Trichrome, periodic acid-Schiff and Jones silver stain.

2.6 Statistical analysis

All data were analyzed using the SPSS v.26.0 statistical analysis software, and the results were expressed as the mean ± SD for three independent experiments. Statistical significance was assessed using the Student's t-test or one-way analysis of variance (ANOVA) followed by least significant difference (LSD) tests or Dunnett's post hoc test, and $P<0.05$ was considered to be statistically significant. All experiments were repeated three times as independent experiments.

3. Results

Table. 1 Combination of Results

Combination Result # (CR#)	Cd decreases cell viability measured by CCK-8?	Cd decreases phospho-mTOR measured by WB?	Cd increases LC3 expression measured by IF?	Cd increases liver damage observed with H&E?	Support of hypothesis
1	+	+	+	+	Full
2	+	+	+	-	Partial
3	+	+	-	+	Partial
4	+	-	+	+	Partial
5	-	+	+	+	Partial
6	+	+	-	-	Partial
7	+	-	-	+	Partial
8	-	-	+	+	Partial
9	+	-	+	-	Partial
10	-	+	-	+	Partial
11	-	+	+	-	Partial
12	+	-	-	-	Partial
13	-	+	-	-	Partial
14	-	-	+	-	Partial
15	-	-	-	+	Partial
16	-	-	-	-	Fully Contradicts

The "+" sign represents the phenomenon in each column heading is observed significantly in the experiments, and it is similar to the positive control groups (RAPA). The "-" sign means that the phenomenon is not observed in the experiments, which may be due to the observation contradicting the hypothesis or not statistically significant compared to the negative control groups (PBS).

CR1: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show a significant decrease in cell viability. The Western analysis for p-mTOR decreases significantly across the treatment period. The immunofluorescence shows a significant increase in the LC3 expression across the treatment period. Liver damage is observed with microscopy.

CR2: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show a significant decrease in cell viability. The Western analysis for p-mTOR decreases significantly across the treatment period. The immunofluorescence shows a significant increase in the LC3 expression across the treatment period. Liver damage is not observed with microscopy.

CR3: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show a significant decrease in cell viability. The Western analysis for p-mTOR decreases significantly across the treatment period. The immunofluorescence does not show an observant increase in the LC3 expression

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across the treatment period. Liver damage is observed with microscopy.

CR4: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show a significant decrease in cell viability. The Western analysis for p-mTOR decreases insignificantly across the treatment period. The immunofluorescence shows an observant increase in the LC3 expression across the treatment period. Liver damage is observed with microscopy.

CR5: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show no observant decrease in cell viability. The Western analysis for p-mTOR decreases significantly across the treatment period. The immunofluorescence shows a significant increase in the LC3 expression across the treatment period. Liver damage is observed with microscopy.

CR6: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show a significant decrease in cell viability. The Western analysis for p-mTOR decreases significantly across the treatment period. The immunofluorescence does not show an observant increase in the LC3 expression across the treatment period. Liver damage is not observed with microscopy.

CR7: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show a significant decrease in cell viability. The Western analysis for p-mTOR decreases insignificantly across the treatment period. The immunofluorescence does not show an observant increase in the LC3 expression across the treatment period. Liver damage is observed with microscopy.

CR8: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show no observant decrease in cell viability. The Western analysis for p-mTOR decreases insignificantly across the treatment period. The immunofluorescence shows a significant increase in the LC3 expression across the treatment period. Liver damage is observed with microscopy.

CR9: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show a significant decrease in cell viability. The Western analysis for p-mTOR decreases insignificantly across the treatment period. The immunofluorescence shows a signficant increase in the LC3 expression across the treatment period. Liver damage is not observed with microscopy.

CR10: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show no observant decrease in cell viability. The Western analysis for p-mTOR decreases significantly across the treatment period. The immunofluorescence does not show an observant increase in the LC3 expression across the treatment period. Liver damage is observed with microscopy.

CR11: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show no observant decrease in cell viability. The Western analysis for p-mTOR decreases significantly across the treatment period. The immunofluorescence shows a significant increase in the LC3 expression across the treatment period. Liver damage is not observed with microscopy.

CR12: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show a significant decrease in cell viability. The Western analysis for p-mTOR decreases insignificantly across the treatment period. The immunofluorescence does not show an observant increase in the LC3 expression across the treatment period. Liver damage is not observed with microscopy.

CR13: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show no observant decrease in cell viability. The Western analysis for p-mTOR decreases significantly across the treatment period. The immunofluorescence does not show an observant increase in the LC3 expression across the treatment period. Liver damage is not observed with microscopy.

CR14: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show no observant decrease in cell viability. The Western analysis for p-mTOR decreases insignificantly across the treatment period. The immunofluorescence shows a significant increase in the LC3 expression across the treatment period. Liver damage is not observed with microscopy.

CR15: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show no observant decrease in cell viability. The Western analysis for p-mTOR decreases insignificantly across the treatment period. The immunofluorescence does not show an observant increase in the LC3 expression across the treatment period. Liver damage is observed by microscopy.

CR16: The CCK-8 assays for the AML12 cells treated by $CdCl_2$ show no observant decrease in cell viability. The Western analysis for p-mTOR decreases insignificantly across the treatment period. The immunofluorescence does not show an observant increase in the LC3 expression across the treatment period. Liver damage is not observed by microscopy.

With the increasing concentration (0, 3.3, 10, 33.3, 50, 100μM), cell viability decreases, mTOR decreases, LC3 expression increases, and liver damage increases more significantly. With the expanding time duration (0, 1, 2, 6, 12, 24hr), cell viability decreases, mTOR decreases, LC3 expression increases, and liver damage increases more significantly.

4. Discussion

CR1: Cd leads to decreased liver cell viability. According

to research, autophagy plays an important role in cell survival[5]. Cd increases LC3 expression, which is similar to the results in the work of Zou et al. [11]. Hence, it is highly possible that Cd damage liver cells and autophagy mediates cell survival negatively. According to the study, mTOR inhibits autophagy; in this work, Cd decreases mTOR expression significantly, which enhances autophagy, so Cd influences autophagy positively. In addition, Cd induces liver damage. Therefore, the hypothesis that Cd causes liver damage via mTOR-dependent autophagy is supported (Fig1).

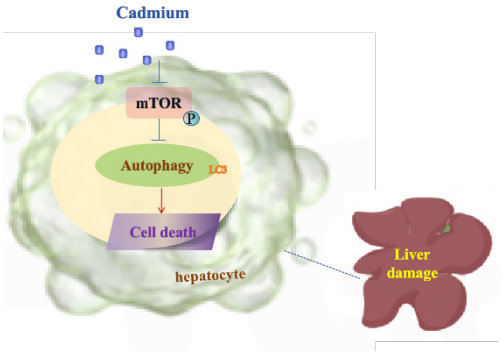

Fig.1 The mechanism diagram of Cd inducing liver damage via mTOR-dependent autophagy

CR2: Cd decreased mTOR, increased LC3 expression, and decreased cell viability. Hence, Cd causes cell death by mTOR-mediated autophagy. However, there is no significant liver damage. This may be because, in vivo experiments, absorption, distribution, metabolism, and excretion happen in the body, which may weaken the toxicity. When further research increases the dose and the duration of toxicity, cadmium will cause liver damage to the organism. CR2 partially advocates the hypothesis, but the effectiveness of the vivo environment also needs further research, such as changing food consumption to injection or inhalation. In the case that liver damage decreases, Cd may activate some mechanisms that cure the liver, which is an unexpected result. Further research can focus on this cure ability of cadmium with specific dose.

CR3: Cd did not increase LC3 expression. This can be attributed to mTOR-dependent mechanism other than autophagy, such as apoptosis and ferroptosis. CR3 also partially supports the hypothesis, but the mTOR-dependent mechanism that killed the cells needs further research. Further experiments can test other potential mechanisms that caused cell death by Western blotting different proteins.

CR4: Cd did not decrease p-mTOR expression. Hence, there may be some other signaling pathways involved, which protected the cells. Specifically, Cd activated autophagy but not by the mTOR pathway, and autophagy was detrimental to the cells. This partially supports the hypothesis, and further experiments can find out the alternative pathways, such as SIRT3-SOD2-mROS-dependent autophagy induced by Cd [12]. This can be done by Western analysis of adhesion molecules over the experiment period to see if downregulations occurred.

CR5: Cd did not decrease cell viability in this study. This contrasted with the results of Pi et al., in which Cd can decrease cell viability in HepG2 cells [12]. The reason behind this might be the difference between cell line, dose and duration. However, Cd inhibited p-mTOR, which means mTOR was involved in Cd-mediated autophagy. Hence, the increase of autophagy may protect the cells. This partially support the hypothesis, and further conclusion can be drawn based on protein analysis.

CR6: Cd failed to promote LC3 expression and cause liver damage. According to this, Cd may induce other mTOR-dependent mechanism, which killed the cells. In addition, absorption, distribution, metabolism, and excretion happen in vivo environment, which weakens the toxicity. This partially supports the hypothesis, and further research can focus on other larger doses and the durations of toxicity, then cadmium will cause liver damage to the organism.

CR7: Cd did not decrease p-mTOR expression and increase LC3 expression. This might because Cd damaged the liver via other mechanisms instead of mTOR-dependent autophagy. This partially supports the hypothesis, and further experiments are required to investigate other signal pathways, which will provide insights into the detailed mechanism.

CR8: There is no observant change in both cell viability and p-mTOR expression, which means that the toxin in this time length and dose have no toxic effect. Thus, the damage mechanism cannot be researched. This partially support the hypothesis. Further experiments should test more of other signaling pathway to establish a concrete hypothesis for the mechanism of resistance.

CR9: Cd did not decrease p-mTOR and cause liver damage. This might be because Cd triggers off autophagy and then cell death by pathways other than mTOR. Besides, absorption, distribution, metabolism, and excretion may happen in vivo environment, which weakens the toxicity. When the dose and the duration of toxicity increase, cadmium will cause liver damage to the organism. This partially supports the hypothesis. Future research should focus on the dose of cadmium that can lead to liver damage in body.

CR10: Cd did not cause cells death obviously and increase LC3 expression. Hence, Cd toxicity did pass the mTOR

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pathway, but it failed to cause autophagy and decrease cell proliferation. It might instead lead to other mechanisms, such as apoptosis and ferroptosis, which are able to damage the liver. This partially support the hypothesis. Future research should focus on the specific dose and duration of cadmium exposure that can lead to liver damage in the body.

CR11: Cd failed to cause cell death and liver damage. To be more specific, Cd triggered off autophagy via mTOR signaling pathway, and autophagy may protect or did not influence cell viability. This partially support the hypothesis. Further experiments should test the mTOR-regulating autophagy pathway [13].

CR12: Only cell viability decrease has been observed. Cd killed the cells with mechanisms other than mTOR-mediated autophagy. In addition, absorption, distribution, metabolism, and excretion may happen in the environment, which weakens the toxicity, or maybe the cells' death was because of the contaminated water system. This partially support the hypothesis. Further experiments with a larger dose and duration of toxicity increase, cadmium may cause liver damage to the organism.

CR13: Only p-mTOR expression decrease has been observed. Specifically, Cd has influenced mTOR somehow, but no other consequence has been observed. This partially support the hypothesis. Further study should focus on discovering an alternative mechanism other than autophagy, which can lead to lower cell viability.

CR14: Only LC3 increase has been observed. That means Cd-induced autophagy with proteins other than mTOR, and the autophagy should be protective or have no effect on the liver cells. This partially support the hypothesis. Future studies should research more on the signaling pathway that induced autophagy in response to cadmium.

CR15: Only liver damage has been observed. Cd causes liver damage with mechanisms other than mTOR-mediated autophagy, and it should be protective of the cells. This partially supports the hypothesis. Future research should focus on other signaling pathways and mechanisms like PPAR-γ/NF-κB axis and ferroptosis.

CR16: The consequences do not support the hypothesis at all. The damage model is not well-constructed. There should be occasions like contaminated cell culture dishes so that the positive control group may not be able to demonstrate the expected consequences. This contradicts the hypothesis. Future studies should discover other toxic metals, signaling pathways, and mechanisms that may be attributed to the liver damage caused by cadmium.

Higher concentrations and longer exposure times of cadmium express more toxicity, which causes more cell death. This indicates cell death is dependent on concentration and exposure duration, which is consistent with the hypothesis.

5. Conclusion

This study reveals that Cd exposure could induce autophagy in the liver by mediating the mTOR pathway. The elucidation of this damage form and mechanism not only enriches the cause and the way of liver toxicity caused by Cd exposure but also provides a theoretical basis for the diagnosis and treatment of Cd poisoning.

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