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The involvement of the mitochondrial pathway in manganese-induced apoptosis of chicken splenic lymphocytes

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HIGHLIGHTS

- Excess Mn resulted in oxidative stress of chicken splenic lymphocytes.
- Apoptosis occurred in chicken splenic lymphocytes treated with Mn.
- Oxidative stress participated in Mn-induced apoptosis.
- Excess Mn induced apoptosis via mitochondrial pathway.

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ABSTRACT

The purpose of this study was to investigate the effect of excess manganese (Mn)-induced cytotoxicity on apoptosis in chicken splenic lymphocytes. Chicken splenic lymphocytes were cultured in medium in the absence and presence of manganese (II) chloride ($MnCl_2$) (2×10^{-4}, 4×10^{-4}, 6×10^{-4}, 8×10^{-4}, 10×10^{-4}, and 12×10^{-4} mM), in N-acetyl-L-cysteine (NAC) (1 mM), and the combination of $MnCl_2$ and NAC for 12, 24, 36, and 48 h. Tests were performed on morphologic observation, reactive oxygen species (ROS) and malondialdehyde (MDA) content, manganese superoxide dismutase (Mn-SOD) and glutathione peroxidase (GSH-Px) activities, B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax), p53, and calmodulin (CaM) messenger RNA (mRNA) expression, Caspase-3 mRNA and protein expression, intracellular free Ca^{2+} ($[Ca^{2+}]i$), and mitochondrial transmembrane potential ($\Delta\Psi m$). Our research indicated that excess Mn induced ROS and MDA content, inhibited Mn-SOD and GSH-Px activities, induced Bax and p53 mRNA expression, inhibited Bcl-2 and CaM mRNA expression, induced Caspase-3 mRNA and protein expression, upregulated $[Ca^{2+}]i$, inhibited $\Delta\Psi m$, and induced apoptosis in a dose effect. NAC relieved excess Mn-caused the changes of all above factors. Mn-induced oxidative injuries were alleviated by treatment with NAC, an ROS scavenger. The above results demonstrated that excess Mn caused oxidative stress and apoptosis via mitochondrial pathway in chicken splenic lymphocytes.

1. Introduction

Manganese (Mn) is an essential trace metal of normal metabolism in humans and animals. It is also an environmental pollutant. Mn in automobile exhaust caused environment pollution (Crump, 2000). Mn exploitation contaminated the Boubo River and lagoon in Grand-Lahou, Côte d'Ivoire (Mariame et al., 2013). Excess Mn is harmful for health, causing manganism, and has been shown to cause immune suppression in Norway lobsters (Hernroth et al., 2004), affect immune function in rats (Son et al., 2007), and induce apoptosis in Nephrops norvegicus (Oweson et al., 2006) and chicken testes (Du et al., 2015; Liu et al., 2013b, 2013c).

Apoptosis is regulated by many factors. Bcl-2 inhibits apoptosis and Bax induces apoptosis. Caspase-3 activation is required for induction of apoptosis (Dolka et al., 2016). p53 has a direct effect on apoptosis by downregulating Bcl-2 expression and upregulating Bax expression (Chipuk et al., 2004). Heavy metals can induce apoptosis by decreasing Bcl-2 messenger RNA (mRNA) expression, and increasing Bax, Caspase-3, and p53 mRNA expression. Excess lead increased p53 and caused apoptosis in mouse livers (Xu et al., 2008). Excess nickel inhibited Bcl-2, stimulated Bax and Caspase-3,

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and led to apoptosis in broiler cecal tonsils (Wu et al., 2014). Oxidative stress induced apoptosis (Sinha et al., 2013). Reactive oxygen species (ROS), malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) were markers of oxidative stress in chicken splenic lymphocytes (Sharma et al., 2006). Excess Mn increased ROS and MDA, and decreased GSH-Px, resulting in apoptosis. Excess Mn increased ROS content and led to apoptosis in rat brains (Erikson et al., 2004). Excess Mn decreased SOD activity, increased intracellular free Ca^{2+} ($[Ca^{2+}]i$), and led to apoptosis in neonatal Wister rat neurocyte (Xu et al., 2009). Excess Mn increased MDA content, decreased SOD and GSH-Px activities, and caused apoptosis in chicken spleens, thymus, bursa of Fabricius (Liu et al., 2013a), and testes (Liu et al., 2013b). Oxidative stress disrupted calcium homeostasis, increased $[Ca^{2+}]i$, and caused apoptosis (Ermak and Davies, 2002). Calmodulin (CaM) can increase $[Ca^{2+}]i$ (Wayman et al., 2011). Excess cadmium (Cd) increased $[Ca^{2+}]i$ and induced apoptosis in human liver L-02 cells (Ye et al., 2007). Mitochondria are the primary site of origin for the initiation of apoptosis. Mitochondrial dysfunction participated in apoptosis. ROS, $[Ca^{2+}]i$, mitochondrial transmembrane potential ($\Delta\Psi m$), and Caspase participated in salinomycin-induced apoptosis in human prostate cancer cells (Kim et al., 2011).

N-acetyl-L-cysteine (NAC) is an antioxidant of oxidative stress. NAC ameliorates tissue injury induced by oxidative stress. NAC relieved lead-induced oxidative stress in the blood of lead-exposed workers (Kasperczyk et al., 2014). NAC also relieved apoptosis caused by chromium, Cd, and cobalt in human lung cells (Luczak and Zhitkovich, 2013). In recent years, increased research on manganism has focused on nervous and reproductive systems in humans (Xu et al., 2009; Yoon and Ahn, 2015) and rats (Jiang et al., 2014; Wang et al., 2015) as well as the effects of excess Mn on the immune system of chickens. Liu et al. studied Mn-induced oxidative damage and apoptosis in chicken spleens, thymus, and bursa of Fabricius (Liu et al., 2012, 2013a). Our previous studies found that excess Mn changed heat shock protein (Zhu et al., 2013) and cytokine (Lu et al., 2015) mRNA expression in chicken splenic lymphocytes.

However, Mn-induced apoptosis in chicken splenic lymphocytes is still unclear. Therefore, chicken splenic lymphocytes were cultured in medium in the absence and presence of manganese (II) chloride ($MnCl_2$), in NAC, and in the combination of $MnCl_2$ and NAC ($MnCl_2$/NAC) to investigate Mn-induced apoptosis via detecting the content of ROS and MDA, the activities of Mn-SOD and GSH-Px, the mRNA expression of Bcl-2, Bax, p53 and CaM, Caspase-3 mRNA and protein expression, $[Ca^{2+}]i$, $\Delta\Psi m$, and morphological observation.

2. Materials and methods

2.1. Animal model

A totle of 240, 1-day-old healthy Hyline mate chickens were fed and watered ad libitum until they were 60 days old. The preparation, culture, and treatment of chicken splenic lymphocytes followed the method described in our previous study (Zhu et al., 2013). Chicken splenic lymphocytes were cultured in medium in the absence and presence of $MnCl_2$ (2×10^{-4}, 4×10^{-4}, 6×10^{-4}, 8×10^{-4}, 10×10^{-4}, and 12×10^{-4} mM), 1 mM NAC, and $MnCl_2$/NAC for 12, 24, 36, and 48 h.

2.2. Morphology

Morphological observation of chicken splenic lymphocytes was performed using acridine orange (AO, Amrisco, USA) and ethidium bromide (EB, Sigma-Aldrich, USA) double staining. The process followed the method described in our previous study (Li et al., 2013).

2.3. Bcl-2, Bax, p53, Caspase-3, and CaM mRNA expression

Total RNA was extracted using TRIzol reagent, and then RNA was reverse transcribed in 40 μl of reaction mixture according to the manufacturer's instructions (Invitrogen, USA). The reverse transcription products (complementary DNA) were stored at −20 °C for polymerase chain reaction (PCR). The primer sequences of β-actin (L08165.1), Bcl-2 (D11382.2), Bax (NM_001030920.1), p53 (NM_205264.1), Caspase-3 (NM_204725.1), and CaM (NM_205005.1) published in GenBank, were synthesized by the Invitrogen Biotechnology Co. Ltd. in Shanghai, China. β-actin was used as reference gene. Real-time quantitative PCR was performed using SYBR Premix Ex TaqTM (Takara, China) with the Applied Biosystems PRISM 7500 real-time PCR system according to the manufacturer's instructions (Applied Biosystems, Foster, USA). Dissociation curve was analyzed using Dissociation Curves version 1.0 (Applied Biosystems, Foster, California, USA) to verify the specificity of the primers.

2.4. Caspase-3 protein expression

Caspase-3 protein expression was measured by using western blot followed the method described in previous study (Yao et al., 2013). The Caspase-3 first antibody (1:100), secondary antibody (1:1000), monoclonal β-actin antibody (1:1000), and goat anti-mouse IgG (1:1000) were purchased from Santa Cruz Biotechnology, USA. The signal was detected by X-ray films (TransGen Biotech Co., Beijing, China). The optical density of each band was determined by Image VCD gel imaging system.

2.5. ROS and MDA content, Mn-SOD and GSH-Px activities, $[Ca^{2+}]i$, and $\Delta\Psi m$

ROS and MDA content, Mn-SOD and GSH-Px activities, $[Ca^{2+}]i$, and $\Delta\Psi m$ were measured using diagnostic kits produced by Beyotime Institute of Biotechnology (Nantong, China) according to the manufacturer's instructions.

2.6. Statistical analysis

All the data were performed using SPSS for Windows (version 17; SPSS Inc., Chicago, IL, USA) with one-way analysis of variance (ANOVA). Different lowercase letters were significant at $P < 0.05$ among all the groups. Each value was represented as the mean ± standard deviation of five individuals ($n = 5$).

3. Results

3.1. Morphology

The morphology of chicken splenic lymphocytes was shown in Fig. 1. Nuclei in the control group were round, green, and the same size (Fig. 1a). In the 2×10^{-4} mM $MnCl_2$ group (Fig. 1c), chromatin condensation began in a few nuclei, and there were a few irregular nuclei for 12 h. Nuclei appeared crescent-shaped for 24 h (Fig. 1f) and numbers of irregular nuclei for 24 h were higher than those for 12 h. For 36 h (Fig. 1i), nuclei appeared irregular in shape, a few nuclei were yellow, and chromatin condensation increased more than that for 24 h. Yellow nuclei for 48 h (Fig. 1l) were higher than that for 36 h.

Morphology of different concentrations of $MnCl_2$ for 48 h was shown in Fig. 1l–w. Chromatin condensation and fragmented chromatin appeared in nuclei of 2×10^{-4} (Fig. 1l), 4×10^{-4} (Fig. 1n),