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Oxidative stress-induced skeletal muscle injury involves in NF-κB/p53-activated immunosuppression and apoptosis response in copper (II) or/and arsenite-exposed chicken

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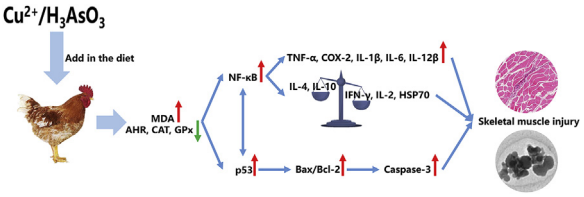
HIGHLIGHTS

- Cu^{2+} or/and arsenite insult results in skeletal muscle injury in chicken model.
- Cu^{2+} and arsenite co-administrated groups induced more deteriorated effects.
- NF-kB/p53 axis might participate in the damage process.

GRAPHICAL ABSTRACT



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ABSTRACT

The adverse effects of environmental toxicants such as copper and arsenic occur due to the generation of reactive oxygen species. Recent study also reported that both copper (Cu) and arsenic (As) may alter muscle regeneration. In order to assess the toxic effects of copper and arsenic on chicken skeletal muscle, chickens were subjected by different toxicologically relevant concentrations of copper or arsenic and their combination in diets for 12 weeks. Upon comparative analysis, a significantly higher malondialdehyde (MDA) and hydroxy radical content were observed in Cu or/and As exposed chicken skeletal muscle, which confirmed the strong lipid peroxidation nature of these two heavy metals. In addition, the depleted activity of catalase and glutathione peroxidase suggested the strong association of copper and arsenic with oxidative stress. Moreover, the higher elevation of pro-inflammatory mediators (NF-κB et al.) and Th1 bias immune system, suggested that exposure to Cu or/and As induces inflammation via NF-κB mediated response pathway. These results further coincided with inflammatory infiltration and nuclear condensation. Further, the execution of apoptosis machinery were characterized by a considerably elevated pro-apoptotic response and apoptotic index. In conclusion, the increased p53 levels detected in Cu or/and As treated chickens suggest the possibility that the NF-kB/p53 axis might lead to the impairment of immune-apoptosis cross talk in the present model.

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1. Introduction

Arsenic is a global-occurring metalloid that is ubiquitous in environment from natural and anthropogenic sources. Due to its impending genotoxicity, immunotoxicity and carcinogenicity (Chen and Ahsan, 2004), arsenic exposure on animal and human is relevant with considerable morbidity and mortality, particularly in areas where the arsenic pollution exceeds the admissible limit of

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10 ppb in drinking water (Bodwell et al., 2006). The physiological and toxicological effects of arsenic depend on its chemical form. As^{3+} possesses higher toxicity, mobility, solubility than As^{5+}. Most inhaled and ingested arsenic tracks through the lungs and gastrointestinal tract into the blood stream and then distributes throughout the body (Akinrinde et al., 2015).

Arsenic toxicity, chiefly known to be mediated through the generation of reactive oxygen species (ROS), leads to oxidative damage of cellular macromolecules (Chowdhury et al., 2010). Thus arsenic is responsible not only for the elevated lipid peroxidation, but also the inhibition of many anti-oxidative enzyme activities, including catalase (CAT) and glutathione peroxidase (GPx) (Sawyer et al., 2002). Arsenic is also immunotoxic and renders the host immunocompromised. Epidemiological and experimental studies have depicted a close connection between decreased proportions of natural T lymphocytes and elevated urinary arsenic levels in peripheral blood (Hernandez-Castro et al., 2009). In addition, As_2O_3 makes chicken immune-compromised evidenced by abnormal expression of Th1 and Th2 (Th, T cell helper) cytokines and upward heat shock protein (HSP) genes in kidney tissue (Wang et al., 2017). In the same scenario, NF-κB, a redox-sensitive transcription factor, is activated by arsenic, thus constitutively initiates inflammation and induces the release of pro-inflammatory mediators (Zhao et al., 2017). On the contrary, after adjustment for a marker of oxidative stress, 8-oxo-7, 8-dihydro-2-deoxyguanosine (8-oxoG), the significant relation between maternal urinary arsenic and placental pro-inflammatory cytokines were no longer exists, suggesting the evident influence of arsenic-induced oxidative stress on cytokine production (Ahmed et al., 2011).

Besides the NF-κB inflammatory pathways, cellular stress signals such as excess ROS also elicit p53 apoptotic pathway, which is responsible for cell cycle regulation, DNA repair and apoptosis (Schneider and Kramer, 2011). The genes induced by p53 are Bax and Bcl-2 which respectively promotes and inhibits apoptosis to protect cell death (Vaseva and Moll, 2009). Intriguingly, NF-κB becomes activated upon activated p53. Even more so, p53-induced apoptosis requires NF-κB (Ryan et al., 2000). It has been observed that arsenite induced p53/NF-κB-regulated apoptosis and inflammation in hepatocytes (Choudhury et al., 2016) in mice, however, few studies on chicken.

Copper is an essential micronutrient and a key catalytic cofactor in a wide range of enzymes (Tisato et al., 2010). Meanwhile, as one of dominant pollution-causing heavy metals (Bopp et al., 2008), copper is leaked into the environment due to the abuse of fungicides, pesticides and industrial wastes (Yruela, 2005), just like arsenical herbicides (Doyle and Spaulding, 1978). Recent study suggested that overexposure of the combinations of copper and cadmium showed additive effects on the biomarker responses of oxidative stress, including CAT and GPx (Bigot et al., 2011). On the other hand, as an anti-inflammatory cytokine, IL-4 exerts its anti-inflammatory function by reducing inflammatory mediators e.g. iNOS, TNF-α and IL-1β (Ledeboer et al., 2000). However, after exposure to aluminum plus copper, the enhanced IL-4 response was not able to regulate the increased pro-inflammatory mediators, which displayed enhanced levels compared to individual exposure (Becaria et al., 2006). We hypothesize that together with other heavy metals, copper might change into a cofactor of various metal toxicity involved in antioxidant and immune defenses and so on. So we designed this study to explore the effects of copper and arsenic on chicken skeletal muscle and whether their combination deteriorates the damage induced by their individuals.

Chicken is the highest consumption of meat in North America on a per capita basis, with an annual supply of 17.7 billion kg (AAFC, 2013; USDA, 2014). However, chicken skeletal muscle is highly sensitive to oxidative damage (Franco et al., 1999). What's worse, the negative effect of intensive breeding and genetic selection makes chicken more vulnerable (Zhang et al., 2011; Sihvo et al., 2014). So, areas bearing cross contaminated with copper and arsenite might be frequently worst-hit. The present study aimed to perform a comprehensive risk assessment of Cu or/and As in chicken skeletal muscle with regard to oxidative damage, immunization disorder and apoptosis.

2. Materials and methods

2.1. Animals and experimental design

Seventy two 1-day-old Hy-line male chickens (Weiwei Co. Ltd., Harbin, China) were housed in the Institutional Animal Care of NEFU. According to our previous study (Wang et al., 2018c), the dosages of As_2O_3 (2.5 mg/kg BW, corresponding 30 mg/kg feed) and $CuSO_4$ (300 mg/kg feed) were used, which were then mixed into food to make supplements (Table S1). Chickens were randomly divided into 4 groups (18 individuals every group), that contains a control group: standard diet, three experimental groups: Cu-group, As-group and Cu + As group. The feed ingredients and rearing condition of experimental animals were referred to our previous study (Zhao et al., 2017). Six individuals in each group were randomly sacrificed at the 4th, 8th and 12th week and sacrificed with sodium pentobarbital. The skeletal muscles (pectoralis) were quickly refrigerated at −80 °C until use.

2.2. Detection of antioxidant system

Skeletal muscles were collected to determine the oxidation state (n = 6/group). According to the manufactures' protocols, the antioxidant indicators presented in the figures were detected by using spectrophotometer (Jiancheng Bioengineering Institute, Nanjing, China) as previously described (Zhang et al., 2017).

2.3. RNA isolation and qPCR

Total RNA of skeletal muscle samples (50 mg tissue; n = 6/group) were isolated by using TRIzol reagent (Takara, Japan). cDNA was created by the PrimeScript™ RT Reagent Kit (Takara, Japan).

Specifc primers used for amplification were shown in Table 1 qPCR was completed by using FastStart Universal SYBR Green Master mix (Roche, Switzerland) on LightCycler® 480 (Roche, Switzerland) as previously described (Wang et al., 2018d). The relative abundance of mRNA for each gene was calculated according to the $2^{-\triangle\triangle Ct}$ method and was normalized to the mean expression of β-actin.

2.4. Western blotting analysis

Protein was extracted by using sodium dodecyl sulfate (SDS) Lysis Buffer (Solarbio, USA) which then were separated by electrophoresis through 15% SDS-polyacrylamide (PAGE) gels. The SDS-PAGE was transferred to polyvinylidene fluoride membranes, which specific reaction products were then detected by horseradish peroxidase-conjugated secondary antibody (Wang et al., 2018b). The protein levels were normalized by β-actin. Antibody information was shown in Table S2.

2.5. Microscopy

The skeletal muscle tissues (size, 1.0 × 1.0 × 1.0 mm) were rapidly collected from all groups at the 12th weeks. Hematoxylin and eosin (H&E) staining was employed in histological analysis. The specimens were subjected to ultrathin sectioning and double

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