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Pathway underlying small intestine apoptosis by dietary nickel chloride in broiler chickens

Bangyuan Wu [a, 1], Hongrui Guo [a, 1], Hengmin Cui [a, b, *], Xi Peng [a, b], Jing Fang [a, b], Zhicai Zuo [a, b], Junliang Deng [a, b], Xun Wang [a], Jianying Huang [a]

[a] Key Laboratory of Animal Diseases and Environmental Hazards of Sichuan Province, Sichuan, China
[b] College of Veterinary Medicine, Sichuan Agricultural University, Ya'an, 625014, China

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ABSTRACT

The aims of this study were to investigate the pathways which dietary nickel chloride ($NiCl_2$) affects small intestine apoptosis in broiler chickens by observing the ultrastructure, and bcl-2, bax, and caspase-3 protein expression and mRNA expression, and cytochrome C, bak and caspase-9 mRNA expression of the small intestine. A total of 240 one-day-old avian broilers were divided into four groups and fed a corn-soybean basal diet as the control diet or three experimental diets supplemented with 300, 600, and 900 mg/kg of $NiCl_2$ for 42 days. Ultrastructurally, the microvilli were apparently exfoliated, and the mitochondria were swollen and the number of lysosomes increased in the intestinal cells of three experimental groups. As measured by TUNEL and flow cytometry (FCM), the percentage of apoptotic cells in the small intestine and the lymphocytes in the ileum were significantly increased in three experimental groups when compared with those of the control group. Meanwhile, immunohistochemistry, quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immuno-sorbent assay (ELISA) tests showed that the protein expression, mRNA expression levels were decreased in the bcl-2, whereas those of bax and caspase-3, and the cytochrome C, bak and caspase-9 mRNA expression levels were increased in three experimental groups. The abovementioned results show that pathway of dietary $NiCl_2$-induced small intestine apoptosis is related to the mitochondrial damage and promotion of the cytochrome C release from mitochondria, which activates the mitochondrion-mediated apoptosis pathway.

1. Introduction

With the development of modern industries, there has been a great increase in the introduction of heavy metal leading to the environmental changes, of which nickel (Ni) represents a good example. Ni or Ni compounds can spread over a large area through wind and rain, and their soil concentrations are rapidly increasing in different parts of the world, which ultimately leads to uptaken by plants [1]. Now, Ni and Ni compounds can be easily found in the natural environment. Human exposure derives from a variety of sources including air, drinking water, and food, and therefore they have received considerable attention [2]. Accordingly, Ni accumulation in the environment may present a serious hazard to human health or the health of other living organisms [3–6]. It had been reported that Ni exposure can cause skin allergies, lung fibrosis, variable degrees of kidney and cardiovascular system poisoning and stimulation of neoplastic transformation [3]. Ni is also considered a carcinogenic trace element [2]. Ni can also affect reproductive performance in higher animals [7]. For example, dietary Ni has negative effects on laying hens [8], and diets supplemented with Ni above 300 mg/kg are toxic to 3-wk-old male chicks [9]. Our previous study has also documented that dietary nickel chloride ($NiCl_2$) in excess of 300 mg/kg could induce the intestinal oxidative damages in broilers [10]. Moreover, exposure to Ni (II) has multiple effects on the immune system including thymic involution, decreased T cell numbers in the spleen, and decreased activity of natural killer cells in mice [11]. Ni is also a redox active metal and can cause damage to the tissues or cells due to the generation of reactive oxygen species (ROS) [12]. ROS or oxidative damage can

* Corresponding author. College of Veterinary Medicine, Sichuan Agricultural University, Ya'an, Sichuan, 625014, China.
E-mail addresses: cuihengmin2008@sina.com, cui580420@sicau.edu.cn (H. Cui).
[1] Contributed equally to this work.

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induce molecular lesions and apoptosis [12–16].

The small intestine serves as the principal site for digestion and absorption, and plays a very important role in the digestive system [17]. The integrity of the intestinal wall is of great importance for preventing the leakage of unwanted substances (such as large feed components, microorganisms, or microbial toxins) from the intestinal lumen into the submucosal tissue [18]. Besides, the small intestine has another important function – intestinal immunity, which consists of innate and specific (adaptive) defenses against dietary antigens and infections with pathogens [19,20]. The intestinal lymphocyte-mediated adaptive immune functions compartment the humoral immunity via B cell-mediated release of secretary IgA which traverses the epithelium to the gut lumen [21–23], and cell-mediated immunity via the intraepithelial T cells of the intestinal villi [24] or other organized lymphs [25]. The intestinal mucosa displays complex defense mechanisms [26] and occupies a unique situation in mucosal immunity [27,28].

It is well known that the gastrointestinal tract (especially the small intestine) is directly exposed to Ni or Ni compounds from daily food consumption, which are rapidly absorbed and high levels of dietary Ni or Ni compounds may have negative effect on the intestine. Our previous studies have proved that dietary $NiCl_2$ induces intestinal oxidative damage, inhibits intestinal development, and decreases serum and intestinal cytokine content in broiler chickens [10,29–31]. These findings warrant further investigations, because, thus far, there have been no studies of intestine apoptosis induced by $NiCl_2$ in animals and human beings. In the present study, small intestine apoptosis and the pathway of $NiCl_2$-induced apoptosis were determined using ultrastructural observations, immunohistochemistry, enzyme-linked immuno-sorbent assay (ELISA), quantitative real-time polymerase chain reaction (qRT-PCR), terminal deoxynucleotidyl transferase 2′-deoxyuridine 5′-triphosphate dUTP nick end-labeling (TUNEL) assay and flow cytometer (FCM) methods, which clarifies the mechanisms by which $NiCl_2$ affects small intestine apoptosis. Our results help elucidate intestinal digestive and absorptive functions and mucosal immune functions.

2. Materials and methods

2.1. Broilers and diets

Two hundred and forty one-day-old healthy avian broilers were randomly divided into four groups with 70 broilers in each group. Broilers were housed in cages with electrically heated units and provided with water as well as undermentioned experimental diets *ad libitum* for 42 days, as were shown in the Wu et al. [10].

A corn-soybean basal diet formulated by the National Research Council [32] was the control diet. $NiCl_2 \cdot 6H_2O$ (ChengDu Kelong Chemical Co., Ltd., Chengdu, China) was mixed into the corn-soybean basal diet to produce experimental diets with 300 mg/kg, 600 mg/kg, and 900 mg/kg of $NiCl_2$, respectively.

All experimental procedures involving animals were approved by the Animal Care and Use Committee, Sichuan Agricultural University.

2.2. Ultrastructural observations

At the end of the experiment (42 days of age), three broilers in each group were euthanized and the total duodenum, jejunum, and ileum in each subject were dissected and fixed in 2.5% glutaraldehyde and postfixed in 2% Veronal acetate-buffered OsO_4. After dehydration in graded alcohol, they were embedded in Araldite. The blocks were sectioned in a microtome with a glass knife into 65–75 nm thick fragments, which were first placed in uncoated copper grids, and then stained with uranyl acetate, and poststained with 0.2% lead citrate, and finally examined with an H-600 electron microscope.

2.3. Detection of intestine apoptosis by TUNEL and FCM

2.3.1. TUNEL method

Five chickens in each group were humanely sacrificed for gross examination at 14, 28, and 42 days of age. The total duodenum, jejunum, and ileum were collected and fixed in 10% neutral buffered formalin after postmortem examination, and then processed and trimmed, and embedded in paraffin. TUNEL assay was performed in dewaxed sections (5 μm thick) with an Apoptosis Detection Kit (Cat#QIA33, Merck, Germany) according to the instructions, as described by Peng et al. [33] Briefly, slices were rehydrated in a series of xylene and ethanol solutions and then incubated in a humidified chamber at room temperature for 20 min with proteinase K (Cat. No: JA 1477). Slices were then rinsed with tris-buffered saline (TBS). The entire specimens were covered with 3% H_2O_2 and then incubated at room temperature for 5 min. Slices were rinsed with TBS. TUNEL enzyme (Cat. No. JA 1559) and label solution (Cat. No. JA 1560) were mixed and applied to slices, which were incubated again in the humidified chamber for 1 h at 37 °C. Slices were thoroughly rinsed with TBS. Stop buffer, block buffer, and conjugate were applied in turn. Diaminobenzidine solution was applied for 10–15 min to stain the nuclei of apoptotic cells. The methyl green solution was used to counterstain the nuclei of normal cells. Slices were dehydrated in a series of three ethanol baths and twice xylene baths, 5 min for each.

The TUNEL positive cells (apoptotic cells) were counted using a computer-supported imaging system connected to a light microscope (OlympusAX70) with an objective magnification of ×400. Then apoptotic cells were quantified by Image-Pro Plus 5.1 (USA) image analysis software. Five sections in each group and five fields in each section were measured and averaged.

2.3.2. FCM method

Isolation of intraepithelial lymphocytes (IELs): The methods described by Montufar-Solis et al. [34] and Todd et al. [35] were used in the present study. After five broilers in each group were humanely killed at 42 days, each ileum was collected and was cut into three pieces, and opened longitudinally and washed with D-Hank's solution by shaking in the plate, and finally placed in a 100 mm tissue culture dish containing supplemented RPMI-1640 (CatNo: SH4007-13, LOT: MXL0747; Hyclone, USA). Then ileac pieces and RPMI-1640 were transferred to a 50 mL plastic conical centrifuge tube. After ileac pieces had settled, RPMI-1640 was removed by suctioning and replaced with 15–20 mL of Ca^{2+}, Mg^{2+} free phosphate buffered saline (PBS), followed by 50 mL of Ca^{2+}, Mg^{2+} free PBS containing 2 mM DTT (CAS NO: 3483-12-3) and 5 mM EDTA (CAS NO:64-02-8; 13235-36-4). The tissue slurry was transferred to a 100 mL beaker and stirred gently at 37 °C for 30 min. The tissue slurry was passed successively through two 10 cc syringe barrels filled to 5 mL with wetted nylon wool in order to remove undigested tissue pieces. The cell suspension was separated equally into two 50 mL tubes and centrifuged for 10 min at 400 × *g*. After centrifuged, the cell suspension were mixed in 3 mL of 40% isotonic percoll (4 parts 100% percoll and 6 parts 10 × DPBS) and layered onto 4 mL of 70% isotonic percoll (7 parts 100% percoll and 3 parts 10 × DPBS) and then centrifuged at 400 × *g* for 30 min. Cells were collected from the two 40/70% interface areas, and washed by centrifugation in supplemented RPMI-1640. The cell suspension was resuspended in 3 mL of 40% isotonic percoll and overlayed onto 4 mL of isotonic 70% percoll, and centrifuged a second time at 400 × *g* for 30 min. IELs were collected from the 40/70% percoll