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Copper sulfate pentahydrate reduced epithelial cytotoxicity induced by lipopolysaccharide from enterogenic bacteria

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ABSTRACT

The over usage of multiple antibiotics contributes to the emergence of a whole range of antibiotic-resistant strains of bacteria causing enterogenic infections in poultry science. Therefore, finding an appropriate alternative natural substance carrying an antibacterial capacity would be immensely beneficial. It has been previously discovered that the different types of cupric salts, especially copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), to carry a potent bactericidal capacity.

We investigated the neutralizing effect of $CuSO_4 \cdot 5H_2O$ (6.25 μg/ml) on the reactive oxygen species generation, and expression of MyD88, an essential adaptor protein of Toll-like receptor, and NF-κB in three intestinal epithelial cell lines exposed to 50 ng/ml lipopolysaccharide. In order to find the optimal cupric sulfate concentration without enteritis-inducing toxicity, broiler chickens were initially fed with water containing 0.4, 0.5, and 1 mg/l during a period of 4 days. After determination of appropriate dosage, two broiler chickens and turkey flocks with enteritis were fed with cupric compound for 4 days.

We found that cupric sulfate can lessen the cytotoxic effect of lipopolysaccharide by reducing the reactive oxygen species content ($p < 0.05$). Additionally, the expression of MyD88 and NF-κB was remarkably down-regulated in the presence of lipopolysaccharide and cupric sulfate. The copper sulfate in doses lower than 0.4 mg/ml expressed no cytotoxic effect on the liver, kidney, and the intestinal tract while a concentration of 0.5 and 1 mg/ml contributed to a moderate to severe tissue injuries. Pearson Chi-Square analysis revealed the copper cation significantly diminished the rate of mortality during 4-day feeding of broiler chicken and turkey with enteritis ($p = 0.000$).

Thus, the results briefed above all confirm the potent anti-bactericidal feature of cupric sulfate during the course of enteritis.

1. Introduction

The maintenance of normal and consistent function of the immune system *via* different tissues is crucial against the ambient bacterial milieu, both in human and animal species. In the poultry industry, the gastrointestinal tract is touted as the main frontier to

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hold the lumen content insulting the body from the bacteria surrounding *via* a synergic collaboration between the immune system and the bio-physical barrier; the intestinal epithelium, submucosa and immune cell pyres [1,2]. But peculiarly, they also seem to possess a secretory epithelial characteristic *via* either the juxtacrine or paracrine manner by separating a great extent of commensal gut flora from the underlying layers of the body [3].

Of note, any irregularity in the dynamic function of intestinal barrier and gut kinetics, induced by the predisposing factors, could hamper the steady-state reciprocal equilibrium of gut flora and the epithelium juxtaposition *per se*, which can facilitate the passing of commensal and non-pathogenic microorganisms to the epithelium basolateral surface [4]. According to the figures, avian enteritis-causing agents, particularly bacterial gastroenteritis, led to a profound economic loss caused by stock mortality, and accordingly a diminished production rate [5,6]. It was previously hypothesized that Toll-like receptors (TLRs) played a crucial role in the commensal- and pathogen-associated molecular sensing and the recognition of intestinal flora. Different types of TRLs including TLR1, TLR2, TLR3, TLR4, TLR5 and TLR9 have been detected on the luminal surface of intestinal epithelial cells [3]. An inflammatory response against the bacterial LPS, TLR4 and with a lesser degree of importance, TLR2, TLR3, TLR5 and TLR9 will be the underlying pathophysiological process occurring in the luminal cells, and the tissue damage which follows [7]. TLR signaling was prompted by an adaptor myeloid differentiation primary-response protein 88 (MyD88) which subsequently led to an epithelial-derived response to the injury [3]. Lack of MyD88 function or generation of MyD88 deficient mice was suspected in impaired TLR signaling [8]. Despite the physiological role of TLR signaling in normal cell function, it was declared that the TLR system overactivity could augment the intracellular generation of reactive oxygen species in different cell types [9]. The integral relationship between the activated status of TLR signaling and regulation of network downstream of nuclear factor κB (NF-κB) factors has been elucidated previously [10].

For several decades, subtherapeutic and therapeutic levels of different antibiotics have been extensively prescribed in poultry livestock feeds to promote the growth as well as containing the incidence of a potential gastrointestinal inflammation derived from a commensal and pathogenic bacteria [11]. Nevertheless, a consistent subtherapeutic or therapeutic administration of in-feed antibiotics could likely contribute to the emergence of resistant bacteria as well as antibiotic residues in the poultry industry products [12,13]. Given that a global attempt has been initiated to limit the inclusion of all types of antibiotics in animal feeds, finding a whole slew of alternative natural substances possessing antibacterial capacity would be crucial [14]. On the same token, a vast array of mineral compounds containing metal derivatives, notably copper sulfate ($CuSO_4$), have been reported to possess a potential antibacterial and antifungal features which can be widely used against the microbial organisms [15,16]. On the other hand, copper accumulation is suspected of invading mucous membranes and inactivates enzymes such as glucose-6-phosphate dehydrogenase and glutathione reductase [17]. Given the dose-dependent activity, it seems logical to be cautious in possible *in vivo* administration of copper ion.

In the following lines, we will investigate the biological activities and the inhibitory impact of copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) against the LPS-induced cytotoxicity in intestinal epithelial cells both in *in vitro* and *in vivo*. The potency of $CuSO_4{\cdot}5H_2O$ in reducing LPS-associated cytotoxicity was studied in terms of reactive oxygen species generation, the modulation of TLR signaling and NF-κB expression. For *in vivo* comparison, we also administrated the $CuSO_4{\cdot}5H_2O$ in poultry flocks with bacterial enteritis.

2. Material and methods

2.1. Ethical issues

The bird experimentation was done in compliance with the approval of National Institute of Health guidelines (NIH Publication No. 85-23, revised 1996) and the local ethics committee of Tabriz's Islamic Azad University and Tabriz University of Medical Sciences.

2.2. In vitro *cell culture and treatment procedure*

Three different human intestinal cell lines, including HCT116, HT-29 and SW480 were purchased from the National Cell Bank of Iran (Pasteur Institute) and were used in the experiment. All cell lines were cultivated in RPMI-1640 medium (Gibco) containing 10% Fetal Bovine Serum (FBS; Gibco), 100 IU/ml Penicillin and 100 μg/ml Streptomycin (Biowest) and maintained in humidified atmosphere adjusted to 7% CO_2 and 37 °C [18]. The exhausted media were regularly changed every 3–4 days and cells of passage 3 were subjected to current experiment.

2.3. Determination and screening of $CuSO_4$ concentration by MTT assay

To establish cytotoxic and sub-lethal (non-cytotoxic) concentrations of copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$), an initial cell density of 1×10^4 from the cell lines were seeded into each well of 96-well plates (SPL). After reaching to a 70–80% degree of confluency, the supernatant media was discarded and cells were starved in RPIMI1640-free FBS for 2 h. Thereafter, cells were primed with a serial two-fold dilution of $CuSO_4$ comprising of 0, 1.6, 3.125, 6.25, 12.5, 25, 50, 100 and 200 μg per ml of medium. In short, 200 μl of media supplemented with 2% FBS and different concentration of $CuSO_4$ added to each well. After a 24-h incubation time, the media was removed and the cells were exposed to the 200 μl of MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) solution (5 mg/ml), incubated for 4 h and then 100 μl Dimethyl Sulfoxide (DMSO; Merck) were overlaid and kept for 15 min [19]. OD absorbance of samples was measured at 570 nm by using an ELISA microplate reader system (ELx808, BioTek). Finally, the cell viability of the control group was set to 100% and we obtained data from the $CuSO_4$-treated cells laid as the percentage of control. Three independent experiment sets in octuplicate wells dedicated to each group were performed.

2.4. Assessment of $CuSO_4$ on cell viability using by LDH leakage method

Following our preliminary results obtained from MTT assay, it was elucidated that the $CuSO_4$ concentration of more than 6.25 μg/ml caused a prominent decrease in the cell viability. Therefore, the solution containing 6.25 μg/ml $CuSO_4$ was chosen for the following analysis. Additionally, the LDH-based cytotoxicity assay was also performed to confirm the extent which the $CuSO_4$-primed cells died after exposure to baseline $CuSO_4$ levels. No significant differences were observed in comparison to dose-matched controls. Therefore, the supernatant of cells under treatment of 6.25 μg/ml $CuSO_4$ were collected, and the level of LDH analyzed by solution kit (Pars Azmun Co, Iran) was measured following the manufacturer's instructions [20].

2.5. Measurement of reactive oxygen species

Change in a total content of endogenous reactive oxygen species (ROS) mediated by $CuSO_4$ solution was also assessed. Cells belonging to different lines were exposed to the non-toxic $CuSO_4$