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The influence of oral copper-methionine on matrix metalloproteinase-2 gene expression and activation in right-sided heart failure induced by cold temperature: A broiler chicken perspective

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ABSTRACT

This study was designed to investigate the expression, activation and activity of matrix metalloproteinase-2 (MMP-2) in the heart of broiler chickens reared in cold conditions and fed with copper-methionine supplement at different levels. The chickens (n = 480) were randomly allotted to six treatments and four replicates. Treatments included two rearing temperatures (i.e. normal and cold temperatures) each combined with three levels of supplemental copper-methionine (i.e. 0, 100 and 200 mg/kg). On d 38 and 45 of age, four broilers from each treatment were sacrificed and their hearts were stored at −80 °C. Right-sided heart failure, as evident from abdominal and pericardial fluid accumulation, was observed in broilers under cold stress and not receiving supplemental copper. This clinical observation was confirmed at molecular level through increased MMP-2 expression, activation and activity in this group. Birds reared under normal temperature, however, were not involved in right-sided heart failure nor benefitted from copper-methionine supplementation. In contrast, gelatin zymography and real-time PCR demonstrated that dietary supplementation with copper-methionine decreased pro-MMP-2 and MMP-2 in the heart of chickens reared in cold conditions. However, gelatin reverse zymography did not show any difference between treatments in tissue inhibitor of metalloproteinase-2. Level of supplementation showed similar effects on parameters determined. It is concluded that dietary supplementation with copper-methionine reduced right-sided heart failure at clinical and molecular levels in cold-stressed chickens.

1. Introduction

Right-sided heart failure is a clinical syndrome in which heart is unable to pump the blood effectively [1]. Fast growth of broiler chickens makes them more susceptible to right-sided heart failure because these birds genetically need much higher oxygen levels while their cardiorespiratory system fails to supply enough oxygen to meet their demand. The condition is exacerbated in chickens grown under cold temperature [2,3]. During heart failure, matrix metalloproteinases (MMPs) are increased [4]. MMPs are classified to subgroups of gelatinases, e.g. MMP-2 and MMP-9, that act as key enzymes for collagen degradation [5] ultimately resulting in ventricular remodeling [6]. Li et al. demonstrated that up-regulation of MMP-2 plays a predominant role in collagen hydrolysis, right ventricle (RV) dilation and ultimately, heart failure [7]. In contrast, tissue inhibitors of metalloproteinases (TIMPs) are a family of endogenous protease inhibitors, which assist in preventing excessive matrix degradation by MMPs [4].

Abbreviations: MMPs, matrix metalloproteinases; ROS, reactive oxygen species; RV, right ventricle; SDS, Sodium dodecyl sulfate; SOD, superoxide dismutase; TIMPs, Tissue inhibitors of metalloproteinases.

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Lysyl oxidase is a copper-dependent amine oxidase that is necessary for stabilization of the extracellular matrix proteins (e.g. collagen and elastin). Increased dietary copper concentration has been shown to increase lysyl oxidase activity, though it may have no effect on its cellular concentration [8]. In addition, copper acts as a cofactor of superoxide dismutase (SOD), thereby scavenging reactive oxygen species (ROS) [9]. The latter also could be involved in heart failure pathophysiology [10]. Effects of copper supplementation on various aspects of metabolism and immune performance have been studied in length [11], however, to our knowledge, there is no study investigating the effect of copper (in the form of copper-methionine chelate) on MMP-2 expression, activation, and activity in heart. The aim of this study, therefore, was to determine effects of copper-methionine on MMP-2 expression in the heart of cold-stressed broilers. Further, TIMP-2 was investigated as a MMP-2 regulator in heart cells.

2. Materials and methods

2.1. Experimental design

A total of 480 broiler chickens (Ross 308) were used. Chickens were allocated randomly to three groups and received supplemental copper as copper-methionine (Zinpro, corp., USA, US patent no. 5698724) at 0, 100, and 200 mg/kg of diet from d 1 until d 45. The basal diet contained 8 mg/kg copper as estimated by nutrient requirements of poultry [12]. On d 28, broilers in each group were divided into two groups where one half was kept in normal temperatures (25–28 °C), whereas the other half was exposed cold temperatures (15–19 °C). The study conforms the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publications No. 8023, revised 1978).

Clinical right-sided heart failure, also referred to as ascites in broilers, was diagnosed from abdominal and pericardial fluid accumulation [13]. On d 38 and 45, four broilers per treatment were sacrificed and their hearts were immediately excised. The RV were then dissected and immediately stored at −80 °C until biological analysis. On d 38 no clinical case of ascites was evident in chickens, however, cold-stressed chickens not receiving copper showed clinical signs of ascites on d 45. These birds were selected for sampling from this treatment assuming that clinically-ascitic birds would have already been involved in right-sided heart failure.

2.2. Gelatin zymography and gelatin reverse zymography

Frozen RVs were homogenized in a lysis buffer (50 mmol/L Tris base, 1% Triton X-100 and 100 mmol/L NaCl). The homogenates were centrifuged at 14,000 rpm in 4 °C for 10 min. The protein concentration of the supernatants was estimated using Bradford Protein Assay Kit. Gelatin zymography was performed, as explained in Kleiner and Stetler-Stevenson [14], with some modification. Samples were mixed with a sample loading buffer (30% (v/v) glycerol, 250 mmol/L Tris base, 0.25% (v/v) bromophenol blue, and 10% (w/v) sodium dodecyl sulfate, at pH 6.8), followed by loading on 8% Sodium dodecyl sulfate (SDS) polyacrylamide gels containing 0.1% gelatin. Electrophoresis was performed at 4 °C for 1–2 h in the migration buffer (190 mmol/L Glycine, 24 mmol/L Tris base, 5 mL SDS 20%, pH 8.5). Following electrophoresis, gels were soaked in 2.5%Triton X-100 for 30 min to remove SDS and protein renaturation. Gels were then incubated at 37 °C overnight in a developing buffer (5 mmol/L $CaCl_2$ ($2H_2O$), 200 mmol/L NaCl, 50 mmol/L Tris base, pH 7.6). After incubation, the gels were stained with Coomassie blue G-250 (50 mL acetic acid, 200 mL methanol, 0.5 g brilliant Blue, 250 mL dH_2O) for detection of gelatinase bands [15].

Detection of the activity of tissue inhibitors of metalloproteinase-2 in protein extracts was performed by gelatin reverse zymography. For this method, the same supernatant used for MMP determination was used for TIMP-2 detection. Gelatin reverse zymography was similar to gelatin zymography with some changes. Proteins were separated by 15% SDS-PAGE supplemented with 0.1% SDS, 0.1% gelatin, and pro-MMP-2 [16]. HT-1080 fibrosarcoma cells conditioned medium was finally used as positive control for both zymography and reverse zymography to detect pro-MMP-2 and TIMP-2.

The gels were scanned and analyzed by ChemiDoc MP using Image Lab Software version 4.0 (BioRad) and the MMP-2/TIMP-2 results were reported in arbitrary units.

2.3. RNA isolation, reverse transcription PCR, and real-time PCR

The total RNA content of the frozen RV was isolated using chloroform and Trizol (TRI Reagent® TR 118). RNA quantity was measured using spectrophotometer (NanoVue Plus, USA). The complementary strand of DNA (cDNA) was synthesized by the Maxima first strand cDNA synthesis kit for RT-qPCR with dsDNase (Thermo Scientific). Polymerase chain reaction (PCR) was performed to produce a DNA fragment of MMP-2 gene. The primer sequences of MMP-2 (forward), 5′-AAACTCACCAGCCTGGGACTAC-3′; MMP-2 (reverse), 5′-TCCATTCCAAGAATCCGCAATG-3′; actin (forward), 5′-ACGTCGCACTGGATTTCGAG-3′; and actin (reverse), 5′-TGTCAGCAATGCCAGGGTAC-3′ were used for PCR amplification in 35 cycles. The resulting PCR products were run on 2% agarose gel and stained with ethidium bromide. Real-time PCR was implemented using the Maxima SYBR Green ROX qPCR Master Mix (2X) and the Mx3005P QPCR System for the quantification of MMP-2 mRNA expression. Equal concentrations of cDNA were used in duplicate. MMP-2 mRNA expression was calculated according to the comparative CT method [15].

2.4. The MMP-2 activity assay

Specific MMP-2 activity in the RV extracts was measured using SensoLyte® 520 MMP-2 Fluorimetric Assay Kit (AnaSpec., San Jose, USA). It allowed the researchers to quantify the protease activity by a 5-FAM/QXL™ 520 FRET peptide. Upon cleavage into two separate fragments by MMP-2, the fluorescence of 5-FAM was monitored at Ex/Em = 490 and 520 nm. MMP-2 activity was measured with a spectrofluorometer (Mithras LB940, Berthold Technologies, Thoiry, France).

2.5. Statistical analysis

Statistical analysis was performed using a completely randomized block design with a 2×3 factorial arrangement of treatments with three levels of dietary copper-methionine and two levels of temperature as main effects. General Linear Model procedure of SAS software (1998) was used. The Least Significant Difference test was used to detect the significant differences among treatments and $P < 0.05$ was considered significant.

3. Results

3.1. Alterations in pro-matrix metalloproteinase-2 levels and matrix metalloproteinase-2 activity in RV

On d 38 and 45 of age, pro-MMP-2 levels were significantly higher in the RV of the broilers reared under cold temperature compared to those reared under normal temperature (Figs. 1 and 2) so that the highest pro-MMP-2 level and MMP-2 activity were observed in broilers experiencing the right-sided heart failure.