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The effect of hyperammonemia on myostatin and myogenic regulatory factor gene expression in broiler embryos

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Abstract

Myogenesis is facilitated by four myogenic regulatory factors and is significantly inhibited by myostatin. The objective of the current study was to examine embryonic gene regulation of myostatin/myogenic regulatory factors, and subsequent manipulations of protein synthesis, in broiler embryos under induced hyperammonemia. Broiler eggs were injected with ammonium acetate solution four times over 48 hours beginning on either embryonic day (ED) 15 or 17. Serum ammonia concentration was significantly higher ($P < 0.05$) in ammonium acetate injected embryos for both ED17 and ED19 collected samples when compared to sham-injected controls. Expression of mRNA, extracted from pectoralis major of experimental and control embryos, was measured using real-time quantitative PCR for myostatin, myogenic regulatory factors myogenic factor 5, myogenic determination factor 1, myogenin, myogenic regulatory factor 4, and paired box 7. A significantly lower ($P < 0.01$) myostatin expression was accompanied by a higher serum ammonia concentration in both ED17 and ED19 collected samples. Myogenic factor 5 expression was higher ($P < 0.05$) in ED17 collected samples administered ammonium acetate. In both ED17 and ED19 collected samples, myogenic regulatory factor 4 was lower ($P = 0.05$) in ammonium acetate injected embryos. No significant difference was seen in myogenic determination factor 1, myogenin, or paired box 7 expression between treatment groups for either age of sample collection. Additionally, there was no significant difference in BrdU staining of histological samples taken from treated and control embryos. Myostatin protein levels were evaluated by Western blot analysis, and also showed lower myostatin expression ($P < 0.05$). Overall, it appears possible to inhibit myostatin expression through hyperammonemia, which is expected to have a positive effect on embryonic myogenesis and postnatal muscle growth.

Keywords

broiler; hyperammonemia; myogenesis; myogenic regulatory factor; myostatin

Introduction

It is widely accepted in both agricultural and human research that embryonic and immediate post-natal environment plays an important role in muscle development. In poultry, it is well established that muscle fiber number is determined during embryonic development and that post hatch growth is dependent on hypertrophy of the existing fibers (Remignon *et al.*, 1995; Mozdziak *et al.*, 1997). Embryonic muscle hyperplasia, or increase in myoblast proliferation, is controlled by a group of four basic helix-loop-helix transcription factors called myogenic regulatory factors (**MRFs**).

Two of the four MRFs, myogenic determination factor 1 (**MyoD**), and myogenic factor 5 (**MyF5**), are regulators of myogenic progenitor specification. These transcription factors are evident early in embryonic development supporting that they play a crucial role in the determination of embryonic stem cells to become committed myogenic cells. Research in *MyoD* and *MyF5* knockout mice has demonstrated the importance of these regulatory factors on myoblast determination. Mice that lack *MyoD* or *MyF5* develop normally, suggesting that there is redundancy in the role of these genes, while mutant mice for both *MyoD* and *MyF5* completely lack skeletal muscle and do not survive (Rudnicki *et al.*, 1992 and 1993). Myogenin (**MYOG**), and myogenic regulatory factor 4 (**MRF4**), also known as MyF6 or herculin, are expressed later in embryonic development. MYOG is the major determinant of myoblast differentiation, while MRF4 is expressed in mature myocytes (Nabeshima *et al.*, 1993). Paired box 7 (**PAX7**) is a transcription factor that is expressed in proliferating myoblasts, but is downregulated during myoblast differentiation (Seale *et al.* 2000). PAX7 is also expressed in quiescent and proliferating satellite cells in mature muscle (Zammit *et al.* 2004).

Myostatin (**MSTN**), a transforming growth factor- β (**TGF- β**) family member, is the most powerful negative regulator of myogenesis, but is also expressed in adult muscles, suggesting it also inhibits postnatal muscle growth (McPherron *et al.*, 1997; Lee and McPherron, 2001; Amthor *et al.*, 2004). *MSTN* knockout mice display an extreme hyperplastic and hypertrophic phenotype termed double muscling (McPherron and Lee, 1997). Mutations in the *MSTN* gene were deemed responsible for the same phenotype observed, as a result of genetic selection for muscle growth, in Belgian Blue and Piedmontese cattle proving that the role of the *MSTN* gene is highly conserved across species (McPherron and Lee, 1997). MSTN has been shown to inhibit myogenesis by downregulating expression of the crucial growth factors MyoD, MyF5, and MYOG (Langley *et al.*, 2002; Amthor *et al.*, 2004; Dasarathy *et al.*, 2004).

In agricultural research, the importance of MSTN and MRF expression in early stages of development is well understood to impact meat quality and ultimate meat yield. Though *MSTN* mutant livestock have significantly increased muscle yield, *MSTN* mutations also presents disadvantages. Increased calving difficulty and reduced reproductive performance are associated with *MSTN* mutant hypermuscular animals preventing the selection for this phenotype in commercial practice (Wiener *et al.*, 2009). There is evidence that performing selective breeding for livestock that carry only one mutant *MSTN* allele may be a more realistic opportunity to benefit from increased hyperplasia and hypertrophy observed with

MSTN mutations (Wiener *et al.*, 2009; Hope *et al.*, 2013). It is therefore of particular interest for agricultural advancement to metabolically adjust the expression of *MSTN*, and subsequently MRFs, without completely eliminating its role in normal regulation of muscle growth.

Increased *MSTN* expression has been noted in the investigation of diseases, such as cancer, heart and kidney failure, and cirrhosis, where muscle wasting is frequently a secondary, but serious, side effect (Dasarathy *et al.*, 2004; Han and Mitch, 2011; Qiu *et al.*, 2013). Regulation of *MSTN* expression is targeted as a potential therapy for sarcopenia and cachexia (Dasarathy *et al.*, 2004, Han and Mitch, 2011). In a recent study, Qiu *et al.* (2013), observed increased *MSTN* expression under induced hyperammonemia using rats as a model for cirrhosis. Additionally, hyperammonemia has been observed in other diseases including chronic obstructive lung disease and heart failure (Andrews *et al.*, 1997; Calvert *et al.*, 2010). These clinical and mechanistic observations suggest that hyperammonemia has an adverse effect on muscle structure and function in a broad range of disorders.

Ammonia is generated in the developing embryos when amino acids are catabolized to generate energy (Terjesen *et al.*, 2002; He *et al.*, 2007). Ewe and murine embryos are adversely affected by hyperammonemia, while bovine embryos have been reported resistant to increased ammonia concentrations in vitro (Bishonga *et al.*, 1996; McEvoy *et al.*, 1997; Hammon *et al.*, 2000). Similar results of the adverse effects of ammonia have been reported in amphibians and echinoderms (Webb and Charbonneau, 1987). Further, previous studies have reported that ammonia inhibits transcription of ribosomal RNA in *Xenopus* embryos, therefore inhibiting protein synthesis (Shiokawa *et al.*, 1986; Shiokawa *et al.*, 2010). Thus, the adverse effects of ammonia on the growing embryo are inconsistent in contrast to the consistent negative effects in adults. There is no data on the role of ammonia toxicity in the developing embryo of broilers. The objective of the present study was to investigate chick embryonic gene manipulation of *MSTN* and MRFs under induced hyperammonemia, and the effect of the gene manipulation on protein synthesis and growth.

Materials and methods

Injection of eggs

Fertilized broiler eggs (Ross 708 x Ross 344) were incubated at 37°C to embryonic day (ED) 15 or 17. In late stage broiler embryos, secondary muscle fiber formation is occurring and a population of adult myoblasts, or satellite cells, is being established, providing a great opportunity for growth enhancement between ED15 and ED19 (Stockdale *et al.*, 1981; Maier, 1993). Ammonium Acetate (Sigma Life Science, St. Louis, MO) was diluted in Hanks' Balanced Salt Solution and blue food coloring (FD&C Blue 1; Food Lion, LLC, Salisbury, NC) was added to the solution (2%). In preparation for injections, the shell surface was disinfected using ethyl alcohol. The amnion was identified by candling, and ammonium acetate injections were delivered using a 25-gauge needle. A total of 200 mg (50 mg per dose) of ammonium acetate was delivered into the amniotic sac, via injection of 0.2 mL (250 mg/mL) every 12 hours, over 2 days. The ammonium acetate dosage and frequency of administration was empirically derived, using non-toxic intraperitoneal dosages from murine models (2.5 mmol/kg) and intraperitoneal LD₅₀ toxicity observed in 4-week-old

chicks as guidelines for an initial low (2 mmol/kg) and high (10 mmol/kg) amount of ammonium acetate to be administered (Wilson *et al.*, 1968; Yonden *et al.*, 2010). The average body weight (12g) of ten ED15 embryos was used to calculate dosages for all injections. Empirically, it was determined that a dose of 50 mmol/kg every twelve hours imparts a significant increase in serum ammonia concentration without any adverse effects on the embryo. Control eggs were injected with a solution consisting of Hanks' Balanced Salt Solution and food coloring only. After each injection, entry holes in the shell were sealed using cellophane tape and eggs were immediately returned to the incubator. Blood and tissue samples were collected 12 hours after the last injection (i.e. for groups beginning injections in ED15, samples were harvested on ED17, and those beginning on ED17, harvesting was performed on ED19). There was no observed toxicity, or marked difference in health, in experimental embryos, as compared to controls, at the time of sampling. Additionally, upon sampling from the ED19 group, internal pipping was noted in both the experimental and control groups, indicating that chicks in each group were preparing for hatch.

Collection of samples

Blood samples were drawn directly from blood vessels using beveled glass capillary tubes and placed in 0.5 mM EDTA treated tubes. Samples were centrifuged at 12,000 rpm for 10 min, the serum was separated, and placed in fresh tubes for storage at -80°C . The embryos were removed from the shell, killed via decapitation and total body weight was recorded. Pectoralis major tissue was dissected and stored in RNAlater (Ambion Inc., Grand Island, NY) at -20°C for total RNA extraction, or snap frozen in liquid nitrogen and stored at -80°C for total protein extraction.

Serum ammonia analysis

Serum samples were analyzed in duplicate for ammonia concentration using an ammonia assay kit (AA0100; Sigma Aldrich, St. Louis, MO) using methods previously reported by Qiu *et al.*, 2013. In brief, serum samples were diluted 1:3 in ultra pure water. The enzyme, L-Glutamate dehydrogenase, was diluted 1:4 with 0.1 M Phosphate buffer (KH_2PO_4 ; pH 7.5) as needed prior to each assay. The working volume of reagent blank, test, and standard reactions were reduced by a factor of 5 to accommodate the working volume of a 96-well plate.

RNA extraction, reverse transcription, and quantitative real time-PCR

Total RNA was isolated using the RNeasy Mini Kit protocol (Qiagen, Venlo, Limburg). Approximately 30 g of RNAlater (Ambion Inc., Grand Island, NY) preserved pectoralis muscle was placed in 600 μL of the provided buffer for homogenization using a Mini-Beadbeater-1 (BioSpec Products, Bartlesville, OK). Total RNA concentration was determined by measuring absorbance at 260 nm. RNA quality was assessed using agarose gel electrophoresis. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Grand Island, NY) using the reverse transcriptase from the murine leukemia virus and random hexamers.

Five samples for each treatment (ED17 AA, ED17 C, ED19 AA, ED19 C) were prepared for qPCR analysis of *MSTN*, *MyoD*, *MYOG*, *MyF5*, *MRF4*, and *PAX7* genes. These genes were chosen because they are known to play a critical regulatory role in muscle development and differentiation. β -actin was used as an internal control for normalization of each sample because our preliminary data showed no change in expression under our experimental conditions (data not shown). Primer sequences are shown in Supplementary Table S1. Real-time PCR for quantification of mRNA was performed on a fluorescent thermocycler (Applied Biosystems Inc., Grand Island, NY) using a SYBR protocol on the fluorescence temperature cyclers. Standard curves for amplification efficiency were produced for each set of primers by performing serial dilutions of pooled cDNA (1:5, 1:25, 1:125, 1:625). At each extension step, fluorescence was measured and the cycle threshold (Ct) was calculated by the StepOne software (version 2.1, Applied Biosystems Inc., Grand Island, NY). All experiments were run in triplicate. Fold changes were calculated by method described by Pfaffl (2001). qPCR products for each gene were visualized by agarose gel electrophoresis and DNA sequence verified to ensure amplification specificity (Eton Bioscience, Research Triangle Park, NC).

Protein extraction, SDS-PAGE, and Western blot analysis

Total protein was extracted using a modification of methods previously described (Dasarathy *et al.*, 2004). In brief, previously weighed muscle samples (25 mg) were homogenized in 250 μ L cold lysis buffer (20 mM Tris-HCL pH 7.5, 10 mM NaCl, 10 mM KCl, 3 mM MgCl₂) using a bead beater and immediately placed on ice. The homogenate was centrifuged at 12,000 rpm for 4 min, returned to ice for 4 min, and centrifuged again for an additional 4 min to remove undissolved tissue debris. Protein concentration in the supernatant was measured using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories Inc., Richmond, CA).

Samples (20 μ g protein) were boiled 1:1 in Laemmli buffer (Bio-Rad Laboratories Inc., Richmond, CA) and separated by SDS-PAGE using a 10% Mini-PROTEAN TGX precast gel (Bio-Rad Laboratories Inc., Richmond, CA) with a 10–245 kDa Prism Ultra Protein Ladder (Abcam, Cambridge, MA). The protein was transferred to a polyvinylidene difluoride membrane (PVDF) via wet transfer, using a 0.02 M Tris/0.15 M Glycine buffer with 18% Methanol, at 400 mA for 1 hour. The membrane was Ponceau S stained to observe loading and transfer accuracy, then destained using ultra pure water.

The membrane was blocked with 5% goat serum (Life Technologies Corporation, Carlsbad, CA) in TBS-T (50mM Tris HCL, 200 mM NaCl, pH 7.5, 0.5% Tween-20) for 1.5 hrs on an orbital shaker at room temperature. The primary antibody for MSTN (Abcam, Cambridge, MA) is a rabbit polyclonal to MSTN C-terminal peptide. The MSTN primary antibody was diluted 1:250 in 5% goat serum and incubated at 4°C overnight. Three washes with TBS-T, 10 min each, followed primary antibody incubation. The secondary antibody used to detect the MSTN primary antibody is a goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP; Abcam, Cambridge, MA). The secondary antibody was diluted 1:500 in 5% goat serum and incubated at room temperature on an orbital shaker for 1.5 hrs. The membrane was washed three times with TBS-T, 10 minutes each wash. HRP activity was

detected using chemiluminescent reagent (Bio-Rad Laboratories Inc., Richmond, CA). The membrane was stripped of the MSTN antibodies using an acidic glycine buffer, as outlined in the Abcam mild stripping protocol (Abcam, Cambridge, MA). Chemiluminescent reagent was used to confirm the antibodies had been removed from the membrane.

After the PVDF membrane was stripped of the antibodies detecting myostatin, the membrane was re-blocked with 5% goat serum, washed, and re-probed with β -actin for a loading control. The primary antibody for β -actin (Thermo Scientific, Rockford, IL) is a mouse monoclonal to β -actin N-terminal peptide. The β -actin antibody was diluted 1:1000 in 5% goat serum and incubated overnight at 4°C. Following primary antibody incubation, the membrane was washed three times with TBS-T, then probed with goat anti-mouse IgG secondary antibody conjugated with HRP (SouthernBiotech, Birmingham, AL), diluted 1:10,000 in 5% goat serum, for 1.5 hours on an orbital shaker at room temperature. Three washes with TBS-T were performed and HRP was detected using Chemiluminescent reagent. Semi-quantitative analysis of protein was determined by band intensity using Kodak 1D Scientific Imaging Systems (v.3.6.2, New Haven, CT). MSTN intensity of each band was normalized to β -actin prior to comparing experimental and control samples.

Statistical Analysis

Statistical analysis was performed using JMP Pro (v.10.0.0, SAS Institute Inc., Cary, NC) and SAS (v. 9.4, SAS Institute Inc., Cary, NC). All experiments were performed in triplicate and data is presented as mean \pm SE. Significance ($P < 0.05$) was determined by oneway ANOVA. PCR results of mRNA content are expressed as mean fold-changes relative to the internal control, *β -actin*, \pm SE.

Results

Serum ammonia concentration

Eggs injected with ammonium acetate had a higher serum ammonia level in both the ED17 and ED19 groups when compared to the controls. The ED17 collected samples (n=6 per treatment) were found to have significantly higher ($P < 0.0001$) serum ammonia concentration in ammonium acetate administered embryos compared to controls (Figure 1A). Similarly, the ED19 group (n=5 per treatment) had higher serum ammonia concentrations ($P < 0.05$) in ammonium acetate administered embryos as compared to control injected embryos (Figure 1B).

mRNA expression

In both ED17 and ED 19 collected groups, the increase in serum ammonia concentration was accompanied by a highly significant reduction in *MSTN* expression (ED17 $P < 0.01$; E19 $P < 0.0001$) (Figure 2). ED17 collected samples showed a significant increase in *MyF5* expression ($P < 0.05$); while there was no significant difference in *MyF5* expression in ED19 collected samples (Figure 2). In both groups, *MRF4* expression was decreased with increased serum ammonia concentration (ED17 $P < 0.05$; ED19 $P = 0.05$) (Figure 2). No significant difference was found in *MyoD*, *MYOG*, or *PAX7* expression in either sample population.

Protein expression

MSTN protein expression was detected by Western blot and normalized to β -actin for relative quantification and statistical significance. The MSTN band assessed is approximately 43 kDa. ED17 ammonium acetate injected samples had a lower amount of MSTN expression when compared to the control injected samples (Figure 3A). Relative intensity measurements confirm the visual observation of decreased MSTN protein ($P < 0.05$) in experimental samples (Figure 3B). Similarly, the ED19 experimental samples showed a lower amount of MSTN expression, when the β -actin loading control showed consistent results between the two treatment groups (Figure 3C). The relative mean intensity of experimental samples was decreased ($P < 0.05$) as compared to controls (Figure 3D).

Discussion

The aim of this study was to investigate changes in chick embryonic myogenesis under conditions of increased serum ammonia concentration, as seen in cirrhotic patients. Based upon previous studies reporting increases in plasma ammonia concentration negatively effecting satellite cell proliferation, via increased MSTN expression and reduced expression of proliferation factors, the present study aimed to understand the effects of hyperammonemia in late stage broiler embryos (Dasarathy *et al.*, 2004; Qiu *et al.*, 2012). During late chick embryonic development a transition from fetal myoblasts to adult myoblasts occurs. By ED18, adult myoblasts, also called satellite cells, become the predominant type of myoblasts, and are virtually exclusive at hatch (Hartley *et al.*, 1992). Altering the proliferative activity of satellite cells, which provide essential nuclei to growing post-hatch myofibers, affects the reservoir of myogenic cells available for myonuclear donation and can have an impact post-hatch growth.

Previous studies, using portacaval anastomosis (PCA) rats as a model, investigating the mechanisms of muscle wasting secondary to cirrhosis have demonstrated a strong correlation between hyperammonemia and increased MSTN expression (Dasarathy *et al.*, 2004; Qiu *et al.*, 2013). Therefore, it was expected that increasing serum ammonia in broiler embryos would upregulate MSTN expression and have a negative effect on muscle growth. The ammonium acetate administration protocol was successful in increasing serum ammonia concentration that is consistent with previous reports of inducing hyperammonemia in murine models (Kosenko *et al.*, 2004; Qiu *et al.*, 2013). These authors induced hyperammonemia in adult animals and reported adverse effects. However, the current results show a significant downregulation of *MSTN* mRNA expression in both ED17 and ED19 collected samples under induced hyperammonemia. Western blot analysis on *MSTN* protein content confirmed the downregulation of mRNA observed by qPCR in pectoralis samples obtained from embryos with increased serum ammonia concentration.

Potential reasons for suppression of MSTN by hyperammonemia include the relatively short term exposure to hyperammonemia compared to the previously reported PCA rat model with a long term hyperammonemic condition or the murine model with induced hyperammonemia (Qiu *et al.* 2012). Also, the primary mechanism of excess ammonia excretion in mammals is producing urea to be eliminated as a urinary waste product. Avian species do not have a developed urea cycle and the principal mode of ammonia disposal is

through uric acid generation (Wiggins *et al.*, 1982). Administration of low levels of ammonium acetate to the embryonic environment may have provided substrate, acetic acid, for acetyl coenzyme A synthesis, which is oxidized via the citric acid cycle for energy production and increased nitrogen availability for protein synthesis. Additionally, expression of glutamine synthetase is high in avian tissue and the glutamine synthesized is used for purine and uric acid biosynthesis (Campbell and Vorhaben, 1976). Glutamine synthesis is driven by the reaction between ammonia and α -ketoglutarate from the tricarboxylic acid cycle to generate glutamate that combines with another molecule of ammonia to generate glutamine (Hod *et al.*, 1982). Glutamine synthesis is potentially a major mechanism of skeletal muscle ammonium detoxification (Thompson and Wu, 1991; He *et al.*, 2010). Glutamine also plays an important role in the metabolic activity of muscle and has been reported to inhibit MSTN expression (Hickson *et al.*, 1995; Salehian *et al.*, 2006; Bonetto *et al.*, 2011). Thus increased skeletal muscle glutamine synthesis in response to hyperammonemia may be responsible for reduced MSTN expression in the broiler embryos. Furthermore, glutamine has been shown to upregulate gluconeogenesis via increased expression of phosphoenolpyruvate carboxykinase (**PEPCK**), a rate limiting enzyme of the gluconeogenesis pathway, providing energy in the form of glucose to the developing embryo (Lavoine *et al.*, 1996). This model needs to be evaluated further since it provides a potential mechanism of MSTN suppression and increased muscle yield in the avian system.

In addition to downregulating *MSTN* expression, a significant increase in *MyF5* was observed in ED17 collected samples. Increased expression of *MyF5* during myogenesis supports myoblast proliferation, which is determined during embryonic development. The downregulation of *MRF4*, which is expressed in mature myocytes, further suggests that ammonium acetate administration creates a proliferative embryonic environment. Increasing myoblast activity, and ultimately increasing myofiber number, during development not only provides a greater potential for postnatal muscle growth, but may be a more efficient manner to increase ultimate meat yield in poultry. Given these interesting results, it would be necessary to examine the functional impact of these transcriptional changes induced by hyperammonemia on skeletal muscle mass, protein content, translational efficiency, rates of protein synthesis and breakdown in the embryos.

There were no significant changes in satellite cell activity between ammonium acetate treated and control embryos (BrdU immunostaining data not shown). Additionally, no significant difference in *PAX7* expression was observed in the ammonium acetate treated embryos as compared to the controls, where increased *PAX7* expression would suggest increased satellite cell specification and proliferation. However, based upon *in vitro* studies, which indicate that significant differences in myoblast proliferation are not apparent until 5 days after initiation, the embryos were not maintained for a sufficient time to reveal impacts on the myoblast proliferative compartment (McCroskery *et al.*, 2003). Further supporting this notion, the rat model that precedes the current model of hyperammonemia was under hyperammonemic conditions for 4 weeks, which resulted in significant changes in muscle mass (Dasarathy *et al.*, 2004). Therefore, it was not expected that the data collected in this study would show a significant changes in the downstream targets of these alterations in gene expression.

In summary, the results of the current study suggest that increased serum ammonia concentration in developing broiler embryos leads to a reduction of MSTN. Muscle mass is positively affected by reductions in MSTN expression. Therefore, it is possible that a transient reduction in MSTN expression in the embryo can increase post hatch muscle mass. An investigation of the downstream effects on growth, an understanding of the biochemical pathway that leads to a transient downregulation of MSTN expression, and the amount of time required for this gene regulation to have an effect on embryonic myogenesis are needed to optimally employ these unanticipated findings as a strategy for improving muscle growth and ultimate meat yield in poultry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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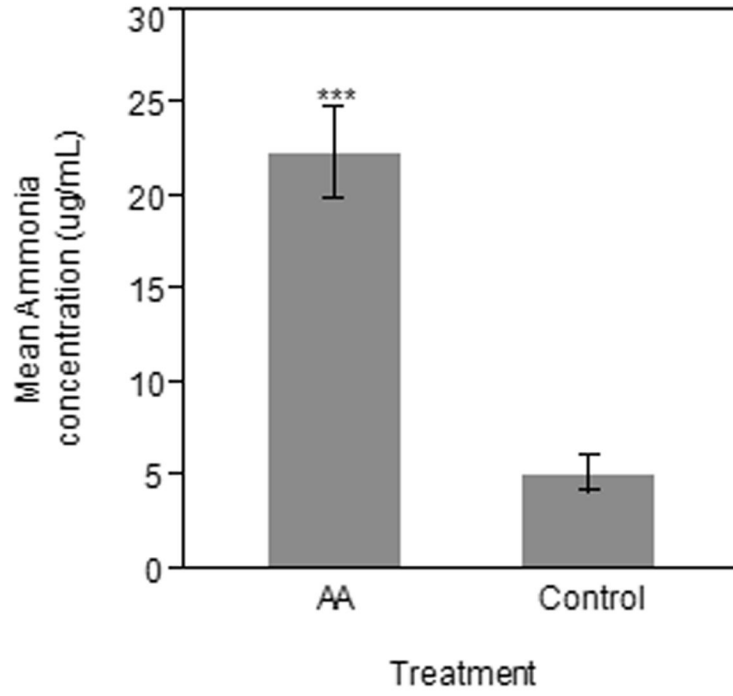
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Implications

This study was designed to observe the effect of induced hyperammonemia on myostatin and myogenic regulatory factor expression in developing broiler embryos. The findings are important because they have the potential to positively impact growth. The results from this research may lead to a novel strategy of improving muscle growth and ultimate meat yield in avian species.

1A



1B

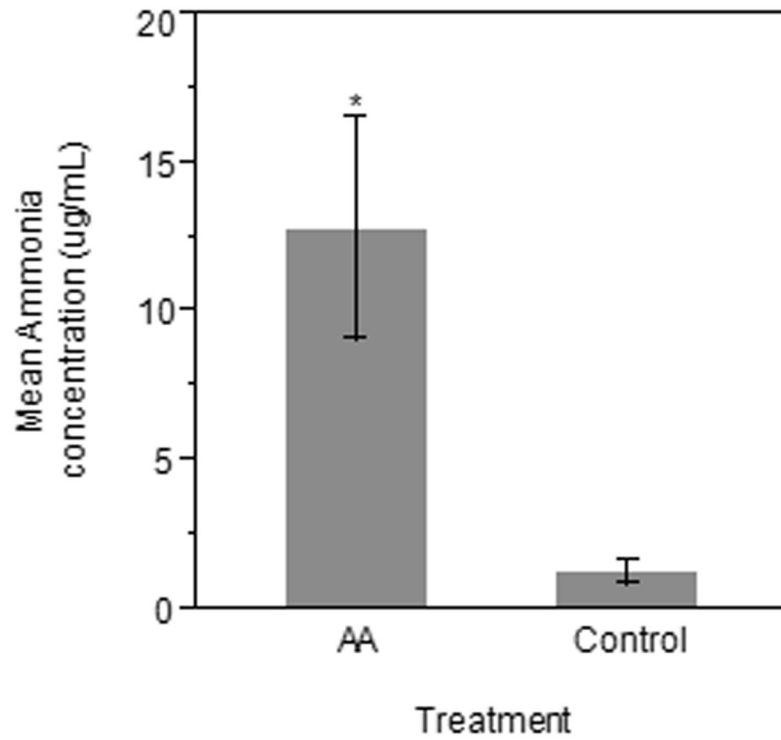


Figure 1.

Serum ammonia concentrations taken from ammonium acetate and control injected embryos 12 hours after the last injection on A) ED17 or B) ED19. Bars represent mean ammonia concentration ($\mu\text{g/mL}$) \pm SE. *** Indicates significance of ($P < 0.0001$); * Indicates significance of ($P < 0.05$).

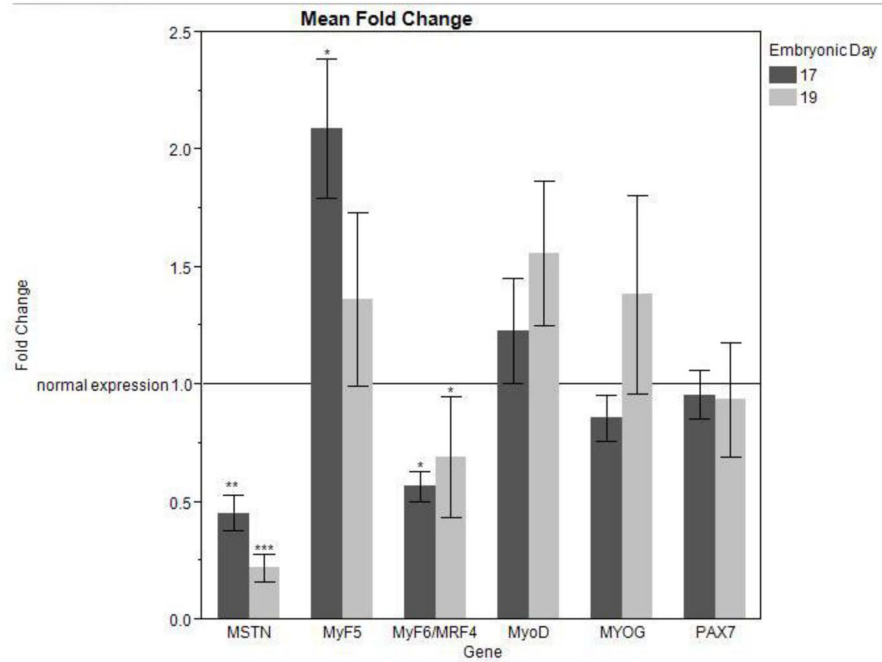
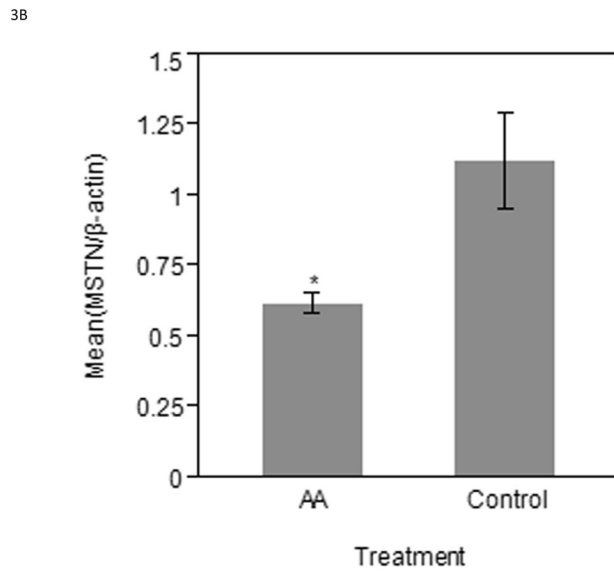
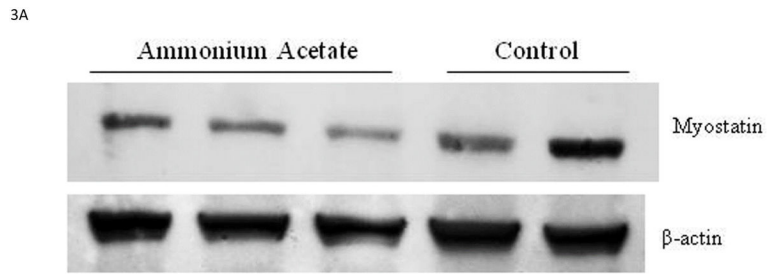


Figure 2. mRNA expression of *MSTN* and MRFs in ED17 and ED19 collected pectoralis major samples after 48 hours of induced hyperammonemia (Four injections of [50 mmol/kg] ammonium acetate) in experimental samples. mRNA expression was measured by quantitative real-time PCR and each sample was normalized using β -actin as an internal control. Bars represent the mean fold change \pm SE. *** indicates significance of ($P < 0.001$); ** indicates significance of ($P < 0.01$); * indicates significance of ($P < 0.05$).



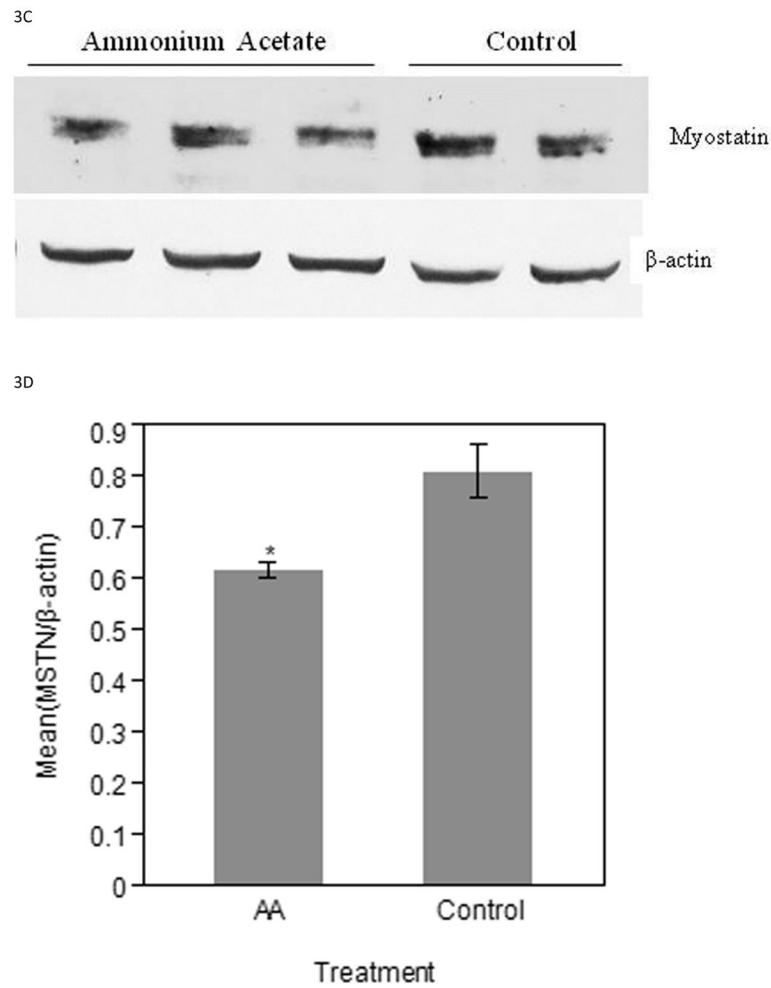


Figure 3. Western blot analysis of MSTN protein in ED17 and ED19 collected samples. A) PVDF membrane images of ED17 MSTN and β -actin bands. B) Relative intensity measurements of ED 17 PVDF membrane images. C) PVDF membrane images of ED19 MSTN and β -actin bands. D) Relative intensity measurements of ED19 PVDF membrane images. Bars represent the mean relative intensity, normalized to β -actin, \pm SE. * indicates a significance of ($P < 0.05$).