Effects of Roxarsone and Monensin on Digital Flexoral Tendons of Broiler Chickens

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ABSTRACT Roxarsone and monensin are common poultry feed additives that are used alone or in combination with other drugs to improve growth and feed utilization in young birds. The effects of monensin and roxarsone on the physiology of flexoral tendons of broiler chickens were examined to understand their relationships to leg weakness that have been occasionally associated with these drugs. Day-old chickens were fed either roxarsone or monensin for a period of 6 wk with two regimens of each of the drugs (roxarsone, 45.4 or 90.8 g/ton feed; monensin, 100 or 150 g/ton feed). None of the treatments had any adverse effect on the growth of the birds or caused any significant leg problem. Roxarsone at 45.4 g/ton caused a significant gain in body weight. The biomechanical strength of digital flexoral tendons was measured in several ways. There were no statistical differences in load at break, the modulus of elasticity, or stress or strain levels between different treatment groups and birds that received no medication. There were no differences in collagen, proteoglycan, and pyridinoline content of tendons. Sequential extraction of tendons with different solvents revealed a significant increase in the percentage of guanidine HCl extractable collagens in monensin-treated birds, and a decrease in the acid extractible collagen in both roxarsone- and monensin-treated groups. The relative content of collagen in acid extractible collagens were significantly small relative to total collagen content. Majority of collagen (84 to 90%) was extractible with pepsin. About 8 to 11% of total collagen was resistant to pepsin that was extractable with collagenase; this did not differ between treatment groups. Roxarsone treatment had no effect on the guanidine soluble collagen pool. The effect of monensin on the increase in guanidine soluble pool of collagen may relate to its disruptive effects on cellular secretory processes, which may be of significance in modulating connective tissue function in conjunction with other factors. However, in the present study, neither roxarsone nor monensin alone produced any significant leg problems nor caused any significant differences in the physiology of flexoral tendons or altered their biomechanical properties.

(Key words: roxarsone, monensin, tendon, biomechanical strength, collagen)

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INTRODUCTION

Anticoccidial drugs and growth promoting antibiotics are widely used in poultry diets. Monensin and roxarsone (3-nitro-4-hydroxyphenylarsonic acid) are two such drugs that are used alone or in combination with other feed additives to improve growth in broiler chickens. Monensin is a polyether ionophorous antibiotic used for the control of coccidiosis (Reece, 1988), whereas roxarsone improves feed utilization (Roney, 1995; Waldroup et al., 1995). Both of these drugs are considered safe if used according to the manufacturer’s recommendations. However, occasional side effects, some relating to leg problems, such as ataxia, incoordination, and lameness, in poultry have been reported for both of these drugs (Wagner et al., 1983; Dowling, 1992; Bartov, 1994; Roney, 1995). Monensin disrupts intracellular Na+ and H+ gradients and affects cellular transport process (Mollenhauer et al., 1990) and may cause myotoxicity (Reece, 1988), whereas the exact cellular mechanisms of action of roxarsone are sparsely understood. The toxic effects of the drugs can be exacerbated by other factors such as environmental stress, their uneven distribution in the feed, and interactions with other compounds in the feed. Problems of leg weakness can be due to an adverse effect of these drugs upon...
molecules, nerves, and connective tissues such as the bone, cartilage, and tendon. Because tendons are important coordinators of locomotory activities and are responsible for the transfer of forces between muscle and bone (Herzog and Loitz, 1994), it is likely that poultry leg weakness could be related to the changes in the physiology of tendon that may be adversely affected by these drugs. Tendons resemble bone and cartilage in their extracellular matrix composition. Tendon contains collagen, which is the major structural constituent responsible for its biomechanical properties and tensile strength. Proteoglycans are also present in tendon, although they constitute only a small percentage of tendon composition. Proteoglycans are responsible for the tendon’s lubricating properties and possibly play some role in collagen fibrillogenesis (Rooney, 1994). Mature collagen fibrils are reinforced by intermolecular pyridinoline/deoxypyridinoline crosslinks that are formed through the condensation of lysine and hydroxylysine residues of adjacent collagen molecules, which substantially increase tensile strength of collagen (Eyre, 1987; Birk et al., 1991). We have, therefore, investigated the effects of roxarsone and monensin on the biomechanical properties, collagen, proteoglycan, and the pyridinoline-crosslink content of digital flexural tendons of chickens in order to understand the possible basis of leg weakness. Because collagen undergoes several posttranslational and maturational changes, such as fibrillogenesis, fibrillar assembly, and cross-linkings, that affect its solubility and strength (Eyre, 1987; Birk et al., 1991), we have also used differential extraction procedures (Ronziere et al., 1990) to study the distribution of collagen as influenced by these drugs.

**MATERIALS AND METHODS**

**Chemicals**

Heptafluorobutyric acid (HFBA), 1, 19 dimethyl methylene blue was purchased from Aldrich Chemical Company.\(^3\) Roxarsone (20% roxarsone)\(^4\) and monensin sodium (Coban-60)\(^5\) were obtained as premixes from a commercial supplier. Cellulose fiber (CF-1) was purchased from Whatman Inc.,\(^6\) and the pyridinoline and deoxypyridinoline crosslink content of digital flexural tendons of chickens were obtained as a gift from Metra Biosystem.\(^7\) All other chemicals and reagents were purchased from Sigma Chemical Company.\(^8\)

\(^1\)Aldrich Chemical Co., Milwaukee, WI 53223.
\(^2\)Alpharma Inc., Fort Lee, NJ 07024.
\(^3\)Elanco Products Co., Indianapolis, IN 46285.
\(^4\)Whatman Inc., Clifton, NJ 07014.
\(^5\)Metra Biosystem, Mountain View, CA 94043.
\(^6\)Sigma Chemical Co., St. Louis, MO 63178-9916.
\(^7\)Cobb-Vantress Inc., Siloam Springs, AR 72761.
\(^8\)Lixi Inc., Downers Grove, IL 60515.

**Chickens**

Day-old male broiler Cobb-500 chicks\(^9\) were obtained from a local hatchery, and randomly allocated to five pens, each containing 15 birds. Each pen (2.13 m × 1.68 m) was equipped with a bell waterer and tube feeder. The birds were given free access to water and a starter ration that had been formulated to meet the nutritional needs of broiler chickens (National Research Council, 1994). A grower ration was provided when the birds were 3 wk old until the termination of the experiment. Birds were given either roxarsone (45.4 or 90.8 g/ton) or monensin (100 or 150 g ton) in the feed or no medication. The lower of the two concentrations of these drugs are approved for commercial use in broiler chickens. At 6 wk of age, the birds were weighed and then visually examined for leg problems such as reluctance to walk, curled toes, and the presence of obvious deformities. Tibial dyschondroplasia was scored using a Lixiscope,\(^10\) a fluorescence x-ray device that enables identification of this defect based on relative skeletal density. Eight healthy birds selected at random from each pen were killed by cervical dislocation. Flexoral tendons from both left and right legs were dissected and carefully cleaned of adherent tissues and used for the determination of tensile strength and biochemical studies respectively.

**Biomechanical Properties of Tendon**

Ten-centimeter sections were removed from the middle portion of each flexor tendon and used to determine tensile strength. The tendons were held by a rubber grip (part 2710-002) in an Instron 4502 Shear Press\(^11\) and stretched at a rate of 25 mm/min until breakage. The load at break, stress (internal resistance), strain (percentage of deformation), and the modulus of elasticity (measure of stiffness or rigidity) were recorded.

**Extraction of Proteoglycans and Collagen**

Approximately 50 mg of tissue was taken from the middle portion of each flexor tendon for biochemical studies. The tissues were blotted dry and fractionated with different extraction media according to a procedure modified from that described by Ronziere et al. (1990). Individual pieces of tendon were placed in 1 mL of 4 M guanidine hydrochloride/0.05 M sodium acetate pH 5.8 containing protease inhibitors (phenylmethylsulfonyl fluoride 1 mM, N-ethylmaleimide 2 mM, 5 mM benzamidine, EDTA 10 mM, final concentration). The extraction was carried out for 48 h at 4 °C with constant agitation using a Rotamix apparatus.\(^12\) The material was centrifuged at 15,000 × g for 30 min and the supernatant, guanidine HCl soluble collagen, saved for the determination of sulfated proteoglycan and hydroxyproline. The residue was rinsed twice with 2 mL of distilled deionized water, centrifuged to remove water, and lyophilized using
Determination of Sulfated Glycosaminoglycans and Hydroxyproline

The sulfated proteoglycan content of the 4 M guanidine HCl fractions was determined using the dimethyl methylene blue dye binding assay of Chandrasekhar et al. (1987) using whale cartilage chondroitin sulfate as the standard. Proteoglycan was only detected in the guanidine HCl extracted fractions and, therefore, was used as the total tissue content of proteoglycans. Hydroxyproline in each fraction from different extraction procedures was determined following hydrolysis with 6 M HCl using the microplate method of Cawston et al. (1994). Appropriate extraction solvent was hydrolyzed for use as a blank for respective fractions. The hydrolyzed samples were lyophilized using a Speedvac concentrator, and reconstituted in distilled water for hydroxyproline determination. As hydroxyproline represents ~13.4% of collagen, the collagen content was calculated multiplying by a factor of 7.4 (Ronziere et al., 1990). The percentage distribution of collagen in individual fractions was calculated using the sum of all collagen found in different fractions.

Pyridinoline Crosslinks

The pyridinoline content in 6 M HCl hydrolysates of tendons from individual birds was measured by a method described by Petit et al. (1996) with some modifications. Samples were prepared using a 250-µL aliquot of acid hydrolysate (~25 mg equivalent tissue) mixed with 1 mL of acetic acid:butanol:water (1:4:1), which was later added to CF-1 pellet prepared from a 5% suspension made with a mixture of acetic acid:butanol:water (1:4:1, vol/vol) as follows. A slurry of 1.5 mL of CF-1 suspension was pipetted into each microtube and centrifuged for 5 min and the supernatant was removed, leaving the pellet. The samples prepared above were added to the pellet and incubated for 1.5 h with constant agitation, centrifuged to remove the liquid, and the pellet was washed thrice with acetic acid:butanol:water (1:4:1). The pyridinoline compounds adsorbed to CF-1 were then eluted with 0.5 mL of distilled water, recovered by centrifugation, and lyophilized. The samples were reconstituted with 30 mM HFBA prior to HPLC using a C18 reverse phase column (4.6 mm x 150 mm, Ultrasphere 5 µm) and a Waters HPLC system equipped with an autosampler and a fluorescence detector. Pyridinoline/deoxypyridinoline standards and samples were chromatographed in an isocratic condition using 30 mM HFBA:acetonitrile (18:82) as elution buffer and quantified using the measurement fluorescence (295 nm excitation, 400 nm emission). Deoxypyridinoline was not detected in these preparations. The collagen content of the same hydrolysates was measured as described earlier and the results were expressed as nanograms of pyridinoline per microgram of collagen.

Statistical Analysis

The results were evaluated using analysis of variance using SAS software (SAS Institute, 1988). Significant effects of treatment means were separated using Duncan’s multiple range test. Differences were considered to be significant at \( P < 0.05 \).

RESULTS

Body Weight and Leg Problems

Birds given 45.4 g roxarsone/ton gained more weight than birds given other treatments (Figure 1). Body weights of birds given 90.8 g roxarsone/ton and 150 g/ton monensin were not significantly different from birds that received no medication. No significant leg problems were observed in birds in any treatment group (data not
shown). The incidence of tibial dyschondroplasia was 6 to 7% in each group and was not considered consequential for the purpose of this study because of the small population size; however, the birds with TD were excluded from studies using tendons.

### Biomechanical Properties of Tendon

Biomechanical properties of tendons from birds given roxarsone or monensin are shown in Table 1. There were no significant differences in load at break, stress, strain, or modulus of elasticity between the groups.

### Collagen, Glycosaminoglycan, and Pyridinoline Crosslinks of Tendon

There were no significant differences in the collagen, sulfated glycosaminoglycans, or pyridinoline content of tendons from birds given different treatments (Table 2). Only pyridinoline crosslink was detectable in the tendon. Hydroxypyridinoline was not detectable in tendon hydrolysates but was detectable in similarly prepared bone hydrolysates (data not shown).

### Distribution of Collagen in Tendon Fractions

Percentage distribution of collagen in different fractions of extraction media is shown in Table 3. Approximately 1 to 4% of the collagen was extracted in guanidine HCl, < 1% with acetic acid, approximately 84 to 90% with pepsin, and 8 to 11% with collagenase digestion. A higher percentage of guanidine HCl extractable collagen was found in the tendons of chickens treated with monensin than either roxarsone-treated birds or the controls. Tendons of birds given roxarsone or monensin showed a significant decrease in acetic acid soluble collagen content compared with birds that received no medication. There were no differences in pepsin or collagenase extractible collagen between different groups. Less than 0.1% of hydroxyproline was detected in residual pellet and there were no differences between the groups (Table 3).

### DISCUSSION

The objectives of this study were to investigate the effects of roxarsone and monensin on the biomechanical properties, collagen, proteoglycan, and pyridinoline content of flexoral tendons that may relate to the leg problems of broiler chickens; however, no significant leg problems were observed in any treatment groups. These data are in agreement with the observations of Waldrup et al. (1995) who found no leg problems in birds that were given 45.4 g/ton roxarsone. Nevertheless, in field environments such as in poultry houses, crowding may compromise adequate physical activities in these birds and could contribute toward leg weakness. It is well known that tension, loading, and exercise significantly improve the physical and physiological properties of tendons (Herzog and Loitz, 1994). In the present study, however, neither roxarsone nor monensin had any significant effect upon biomechanical properties of the tendons. There were little differences between various treatment groups with respect to collagen, proteoglycan, and pyridinoline content of tendons. The pepsin or collagenase extractible collagen together

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### TABLE 1. Biomechanical properties of flexoral tendons of chickens treated with roxarsone and monensin (n = 13)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Load at break (kg/mm²)</th>
<th>Stress (kg/mm²)</th>
<th>Modulus of elasticity (%)</th>
<th>Strain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (No medication)</td>
<td>4.34</td>
<td>5.53</td>
<td>402.05</td>
<td>9.91</td>
</tr>
<tr>
<td>Roxarsone</td>
<td>3.99</td>
<td>5.07</td>
<td>339.65</td>
<td>12.81</td>
</tr>
<tr>
<td>45.4 g/ton feed</td>
<td>2.51</td>
<td>3.20</td>
<td>260.06</td>
<td>9.45</td>
</tr>
<tr>
<td>90.8 g/ton feed</td>
<td>2.27</td>
<td>2.89</td>
<td>237.79</td>
<td>12.67</td>
</tr>
<tr>
<td>Monensin</td>
<td>2.16</td>
<td>2.75</td>
<td>276.75</td>
<td>15.31</td>
</tr>
<tr>
<td>100 g/ton feed</td>
<td>0.82</td>
<td>1.04</td>
<td>46.69</td>
<td>1.79</td>
</tr>
<tr>
<td>150 g/ton feed</td>
<td>0.18</td>
<td>0.18</td>
<td>0.09</td>
<td>0.13</td>
</tr>
</tbody>
</table>

### TABLE 2. Collagen, glycosaminoglycan, and pyridinoline content of tendons from chickens treated with roxarsone and monensin (n = 8)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Collagen (µg/mg tendon)</th>
<th>Glycosaminoglycan (µg/mg tendon)</th>
<th>Pyridinoline (ng/µg collagen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (No medication)</td>
<td>241.06</td>
<td>1.45</td>
<td>0.21</td>
</tr>
<tr>
<td>Roxarsone</td>
<td>250.56</td>
<td>1.44</td>
<td>0.19</td>
</tr>
<tr>
<td>45.4 g/ton feed</td>
<td>239.19</td>
<td>1.49</td>
<td>0.19</td>
</tr>
<tr>
<td>90.8 g/ton feed</td>
<td>239.19</td>
<td>1.49</td>
<td>0.19</td>
</tr>
<tr>
<td>Monensin</td>
<td>262.22</td>
<td>1.45</td>
<td>0.20</td>
</tr>
<tr>
<td>100 g/ton feed</td>
<td>292.80</td>
<td>1.68</td>
<td>0.21</td>
</tr>
<tr>
<td>150 g/ton feed</td>
<td>14.49</td>
<td>0.09</td>
<td>0.007</td>
</tr>
<tr>
<td>SEM</td>
<td>0.06</td>
<td>0.31</td>
<td>0.20</td>
</tr>
</tbody>
</table>
constituted the major bulk of collagen in tendon and showed no differences between different treatment groups. The collagenase extractable fraction is likely to represent the collagens that are highly crosslinked and are not released by pepsin or any other treatments. Absence of any difference between various treatment groups may indicate that these drugs may not affect collagen crosslinks, which was also evident by the measurement of pyridinoline content of tendons. On the other hand, a significant increase in the guanidine HCl soluble collagen was observed in birds that were treated with monensin. Collagen is secreted as procollagen into extracellular spaces, where it is processed and aggregated in a staggered array to form fibrils that are further stabilized through intermolecular pyridinoline crosslinks in matured fibrils (Buckwalter et al., 1987; Eyre, 1987; Birk et al., 1991). Monensin is known to exert its toxic effect by disrupting the transport process of secretory proteins (Mollenhauer et al., 1990). The effect of monensin on collagen synthesis has been shown to be negligible, but it inhibits collagen secretion by embryonic chicken tendon fibroblasts in vitro, leading to its accumulation in Golgi apparatus (Berg and Neblock, 1985). Similar results have been reported using cultures of chondrocytes (Nishimoto et al., 1982). It is therefore possible that the increased collagen content of guanidine HCl soluble collagen in monensin-fed birds is collagen that has not been secreted to extracellular spaces or has not undergone further assembly to form fibrils. Although similar changes should be expected of proteoglycans, it was not possible to distinguish the effects on proteoglycans as they were not detectable in any other fractions except the guanidine HCl fraction. This change in the collagen distribution in birds fed monensin, however, did not significantly affect the biomechanical properties of tendon or result in any leg problems. The effects of roxarsone were less dramatic in any of the parameters except that the body weight was significantly increased in birds treated with 45.4 g roxarsone/ton. The absence of leg problems or any effect on collagen metabolism or mechanical strengths of tendons may support the argument of Waldroup et al. (1995) that the exacerbation of leg problems in roxarsone-treated birds is perhaps circumstantial and may be related to other factors such environmental and nutritional stress. Peripheral neuropathy has been shown in chickens that were fed roxarsone-supplemented diets and subjected to experimental heat stress, although these birds had no overt clinical signs of leg weakness (Gregory et al., 1995). Toxicological studies of roxarsone in rats and mice have not indicated any gross pathological changes attributable to connective tissue or neuromuscular pathology (NTIS, 1989).

In conclusion, our study showed that neither roxarsone nor monensin alone caused any significant leg problem or had any substantial effect on tendon physiology. However, monensin caused a substantial increase in the levels of guanidine soluble collagen, which could modulate connective tissue physiology and function in conjunction with other factors such as environmental stress or other feed additives.

ACKNOWLEDGMENTS

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REFERENCES


### TABLE 3. Percentage distribution of collagen in different fractions of tendon extracts from chickens treated with roxarsone and monensin (n = 8)

<table>
<thead>
<tr>
<th>Group</th>
<th>Guanidine HCl soluble</th>
<th>Acetic acid soluble</th>
<th>Pepsin soluble</th>
<th>Collagenase soluble</th>
<th>Residual pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (No medication)</td>
<td>1.25±b</td>
<td>0.54±b</td>
<td>89.35</td>
<td>8.83</td>
<td>0.03</td>
</tr>
<tr>
<td>Roxarsone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45.4 g/ton feed</td>
<td>1.19±b</td>
<td>0.31±b</td>
<td>87.44</td>
<td>11.01</td>
<td>0.05</td>
</tr>
<tr>
<td>90.8 g/ton feed</td>
<td>1.96±b</td>
<td>0.10±b</td>
<td>87.37</td>
<td>10.51</td>
<td>0.05</td>
</tr>
<tr>
<td>Monensin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 g/ton feed</td>
<td>3.46±a</td>
<td>0.05±c</td>
<td>84.49</td>
<td>11.97</td>
<td>0.04</td>
</tr>
<tr>
<td>150 g/ton feed</td>
<td>3.88±a</td>
<td>0.01±c</td>
<td>87.24</td>
<td>8.83</td>
<td>0.04</td>
</tr>
<tr>
<td>Pooled SEM</td>
<td>0.48</td>
<td>0.06</td>
<td>1.49</td>
<td>1.58</td>
<td>0.01</td>
</tr>
<tr>
<td>P</td>
<td>0.0004</td>
<td>0.0001</td>
<td>0.27</td>
<td>0.55</td>
<td>0.89</td>
</tr>
</tbody>
</table>

*±Means in a column with no common superscript differ significantly (P < 0.05).


