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Effects of monensin on Na^+/K^+-ATPase and Ca^{++}-ATPase activities in chick skeletal muscle and myocardium after subacute treatment

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SUMMARY

Chicks were treated at 2 weeks of age with 4, l5, 40, 100 and 150 mg/kg of monensin, an ionophore used for its anticoccidial and growth-promoting properties. In the present immunohistochemical study, the expressions and distribution of Na^+/K^+-ATPase and Ca^{++}-ATPase were studied in myocardium and skeletal muscles (pectoral and quadriceps femoris). We detected an increase of Na^+/K^+-ATPase immunostaining with prominent staining of the sarcolemma and a slight increase of Ca^{++}-ATPase with prominent staining of the sarcoplasma.

INTRODUCTION

Monensin is a monovalent polyether ionophore antibiotic, obtained as a fermentation product of *Streptomyces cinnamonensis*. It is used extensively in veterinary medicine as a coccidiostatic in poultry and as a growth promotor in cattle. In particular, this antibiotic is used for the prevention of coccidiosis caused by *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, *E. tenella* and *E. mivati* (Adams H.R., 1995; Atef *et al*., 1993; Brander & Pugh, 1971; Mc Dougald *et al*., 1978; Muto *et al*., 2000; Prescott, 1997; Reid *et al*., 1972, Reid, 1975; Shumard, 1979; Stockdale, 1981).

Monensin contains a carboxylic acid and two alcohol and five cyclic ether functions which form reversible coordination complexes with monovalent cations (Na^+, K^+, Rb^+, Li^+, Cs^+) (Mollenhauer *et al*., 1990; Reed, 1982; Pressman, 1976; Pressman and Fahim, 1982). The complexes are believed to form in such a way that the cation interacts with oxygen functions at the center of the complex, orienting relatively hydrophobic non-coordinating functions to the outside, so that the complex can partition into the lipid phases of biological membranes. When the complex partitions out of the lipid phase of a membrane into the cytoplasm and dissociates, the result is a net flux of the cation through the membrane, regenerating the ionophore which passes back through the membrane as either the free acid or carrying a different cation.

Monensin has been shown to result in a Na^+ influx with a corresponding efflux of H^+ or K^+ in numerous cell types. (Shier *et al*., 1992; Taormino *et al*., 1990; Teubl *et al*., 1999) The transport of specific ions, Na^+, K^+, and Ca^{++} across membranes accounts for the

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pharmacological and toxicological effects of the ionophores. Numerous reports have reported on the monensin toxicity in cattle, sheep, horses, pigs, dogs, guinea fowl, turkey and chickens that have received excessive amounts in feed (Sutko *et al*., 1977; Van Vleet an Ferrans, 1986). The major morphological alterations in monensin-induced myocyte necrosis were contraction bands and accumulation of mitochondrial matrix densities (Sandercock *et al*., 1995, 1999). Smith and Galloway (1983) reported that monensin stimulated sporozoite Na^+/K^+-ATPase but that the Na^+ levels increased in the sporozoites, indicating that monensin caused a Na^+ influx at a rate that exceeded the capabilities of the Na^+/K^+-pump to remove the excess Na^+. Consequently, coccidia could not make ATP to drive their Na^+/K^+-ATPase pumps and lost the ability to osmoregulate, and died.

Neurotoxic effects of monensin (anorexia, thirst, dyspnea, motor incoordination, ataxia, flaccid paralysis, convulsions, increase in mortality rate, etc.), have been reported by several authors, especially at the highest doses, on guinea-hen and turkey (Bassett *et al*., 1978; Senatorov *et al*., 2000; Stuart, 1978; Sutko *et al*., 1977; Naccari *et al*., 1996; Lehel & Laczay, 1995; Matsuoka *et al*., 1996; Prescott, 1997).

In a previous study, we used HPLC to measure noradrenaline (NA) and dopamine (DA) levels in various cerebral areas in chickens which had been given varying doses of monensin in their feed**,** over a period of a fortnight. Our results showed significant alterations in the NA content in the cerebral areas examined.

The aims of the present study were to evaluate the effectiveness of monensin on the expression and distribution of Na^+/K^+-ATPase and Ca^{++}-ATPase in myocardium and skeletal muscles (pectoral and quadriceps femoris) of chicks after subacute treatment of this antibiotic in order to elucidate the mechanisms controlling the toxicological effects.

MATERIALS AND METHODS

Experimental Procedure

Sixty male chicks in the weight range of 100 to 120 g at two weeks of age were divided into six groups of 10 chicks each. Chicks in groups I were treated by oral gavage with the vehicle only and served as nontreated controls. Chicks in groups II, III, VI, V, VI received a single dose by oral gavage of monensin sodium at 4, 15, 20, 40, 100, 150 mg/kg of body weight, respectively, for one week (monensin, 40% in rice lining was a kind gift from Lilly Italia, Sesto Fiorentino, Italy). All animals were examined daily for physical signs of toxicity. Individual animal body weight and food consumption were measured daily.

The animals were necropsied and pathologic examinations were made of all major organs and tissues. Specimens of skeletal (pectoral, quadriceps femoris) and cardiac muscles were fixed in 4% paraformaldehyde solution for 4 hours and consequently embedded in paraffin for staining with hematoxylin & eosin (H & E) and other sections were taken for immunohistochemistry.

Indirect immunoperoxidase method

Sections were subsequently incubated with:

- 0.3% H_2O_2 in PBS for 30 min. to abolish endogenous peroxidase activity;
- Normal goat serum (1:20 Sigma, St.Louis MO, U.S.A.) for 60 min;
- Primary monoclonal antibodies for 12 h in a moist chamber at 4°C: mouse anti-Na^*/K^* ATPase and mouse anti-Ca^{++}ATPase (prediluted; Developmental Studies Hybridoma Bank, Department of Biological Sciences, University-Iowa);
- Goat anti-mouse IgG (1:100; Sigma, St. Louis MO, U.S.A) peroxidase conjugates for 2h at room temperature.

Finally, sections were stained with a freshly made solution of 3, 3-diamino-benzidine (30 mg/ml) and H_20_2 (0.005%) in PBS for 10 min.

RESULTS

Behavioural activity

During the experiment, no significant signs of monensin toxicity were observed in animals treated with the 4 or 15 mg/kg body wt dose in comparison to the controls. Locomotion was increased after monensin 40 mg/kg, whereas after 100 mg/kg it was decreased until sedation. Higher doses of monensin (150 mg/kg body wt) caused death of 50% of animals within 24 hrs. All the animals receiving this dose died within 48 hours.

Histology

The histopathologic lesions (Figs. 2, 4, 6, 7 and 8) were represented by edema and degenerative

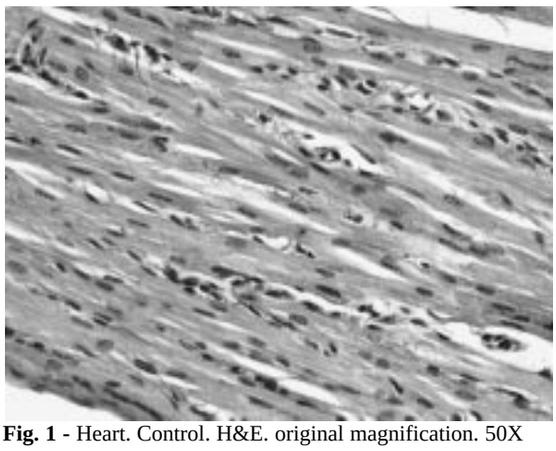

Fig. 1 - Heart. Control. H&E. original magnification. 50X

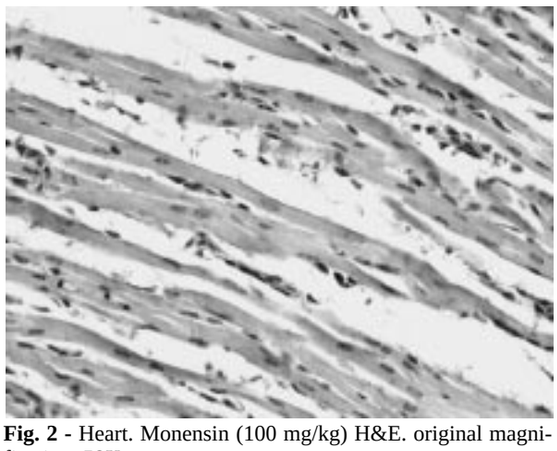

Fig. 2 - Heart. Monensin (100 mg/kg) H&E. original magnification. 50X

changes marked by *homogenization* of fibres, focal loss of cross striations and nuclear pycnosis in heart and in skeletal muscles at the highest doses (100-150 mg/kg).

Immunohistochemistry

The staining pattern of Na^+/K^+-ATPase and Ca^{++}-ATPase antibodies in all the muscle specimens ((heart, pectoral and quadriceps femoris), showed differences concerning the dose used (4, 15 and 40 mg/kg body wt) (Fig. 9 B, C, D, H, I, L, P, Q, R and Fig. 10 B, C, D, H, I, L, P, Q, R). 40 mg/kg body wt produced a higher response, indicating an increase in both enzyme activities (Fig. 9 D, L, R and Fig. 10 D, L, R). Higher doses (100-150 mg/kg body wt) resulted in a reduction of both enzyme activity staining patterns (Fig. 9 E, F, M, N, S and Fig. 10 E, F, M, N, S). The staining patterns obtained with Na^+/K^+-ATPase antibody in all the tissues studied were generally higher (in particular at the subsarcolemma level) than those observed in the specimens processed for the demonstration of Ca^{++}-ATPase enzyme (higher at the sarcoplasmatic level).

DISCUSSION

Our data are in agreement with those previously reported (Sandercock *et al*; 1995; Smith and Galloway, 1983) on the toxic effects of monensin**,**

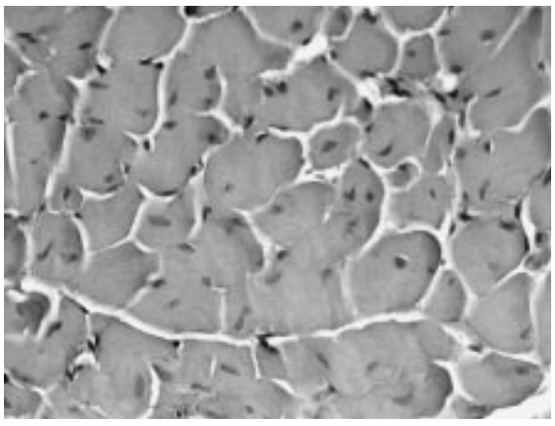

Fig. 3 - Pectoral muscle. Control. H&E. original magnification. 50X

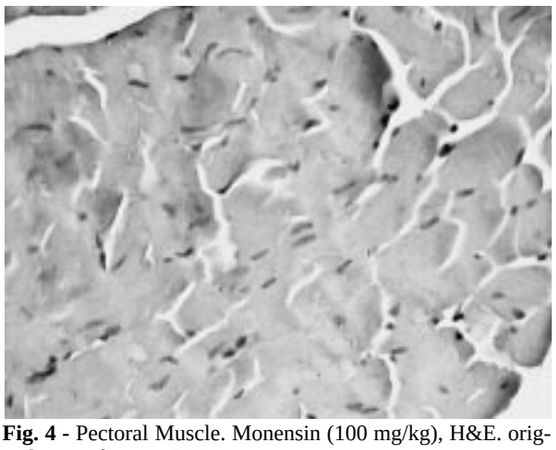

Fig. 4 - Pectoral Muscle. Monensin (100 mg/kg), H&E. original magnification.50X.

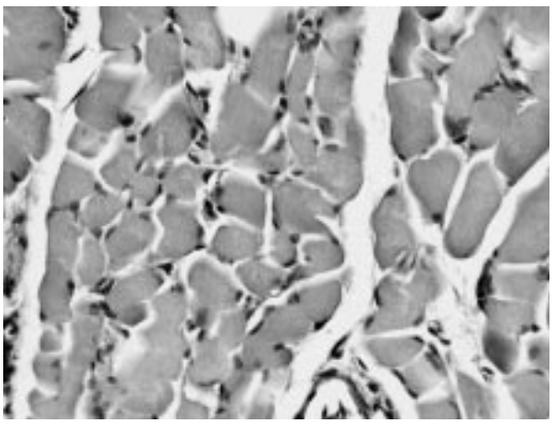

Fig 5 - Quadriceps femoris. Control. H&E. original magnification. 50X.

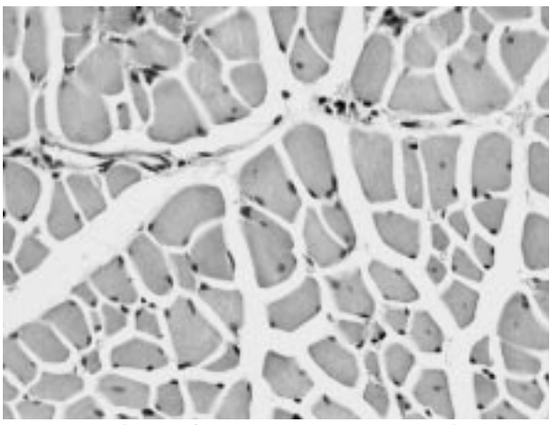

Fig. 6 - Quadriceps femoris. Monensin (100 mg/kg), H&E. original magnification. 50X

which have been associated with the impairment of Na^+/K^+-ATPase and Ca^{++}-ATPase enzyme activities. These effects caused sodium and calcium ions imbalance and thus a loss of functional integrity of the cell membranes. In fact, Sandercock *et al*. (1999) have shown that monensin-induced myopathy may be caused in part by Na^+-mediated disturbances in muscle intracellular Ca^{++} homeostasis. These disturbances lead to an elevation in myoplasmic Ca^{++} concentration and to the activation of several Ca^+-dependent degradative processes, resulting in tissue degeneration and the loss of intracellular constituents such as creatine kinase, a recognized indicator of myopathy (Sandercock *et al*., 2000).

Smith and Galloway (1983) reported that monensin stimulated sporozoite Na^+/K^+-ATPase, but that the Na^+ level increased in the sporozoites, indicating that monensin caused a Na^+ influx at a rate that exceeded the capacity of the Na^+/K^+pump to remove the excess Na^+. In our study, we found that the Na^+/K^+-ATPase and Ca^{++}-ATPase play a central role in maintaining the viability of avian cells subjected to an increased membrane permeability to $Na^+$and Ca^{++} induced by monensin. Similarly, we have observed an increase of Ca^{++}-ATPase enzymatic activity, and a comparatively weaker response of Na^+/K^+-ATPase, when upregulation of Ca^{++}-ATPase apparently surpasses the Ca^{++}extrusion capacity of the cells.

Further immunohistochemical studies may be extended to various organs including the central ner-

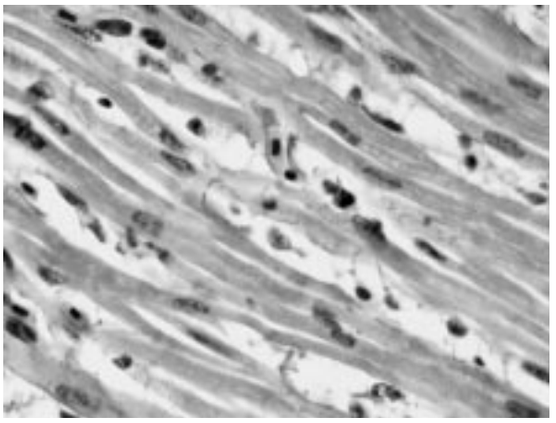

Fig. 7 - Heart. Monensin (100 mg/kg), H&E. original magnification. 100X.

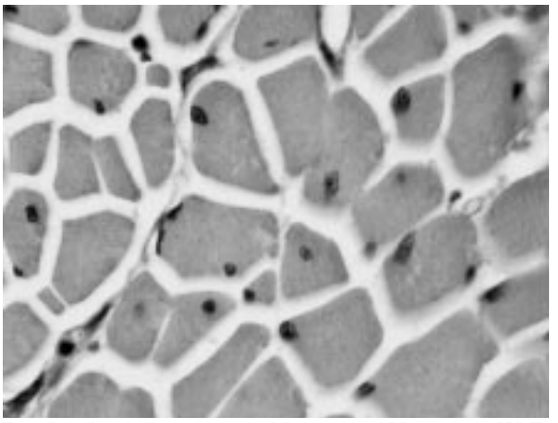

Fig. 8 - Quadriceps femoris Monensin (100 mg/kg), H&E. original magnification. 100X.

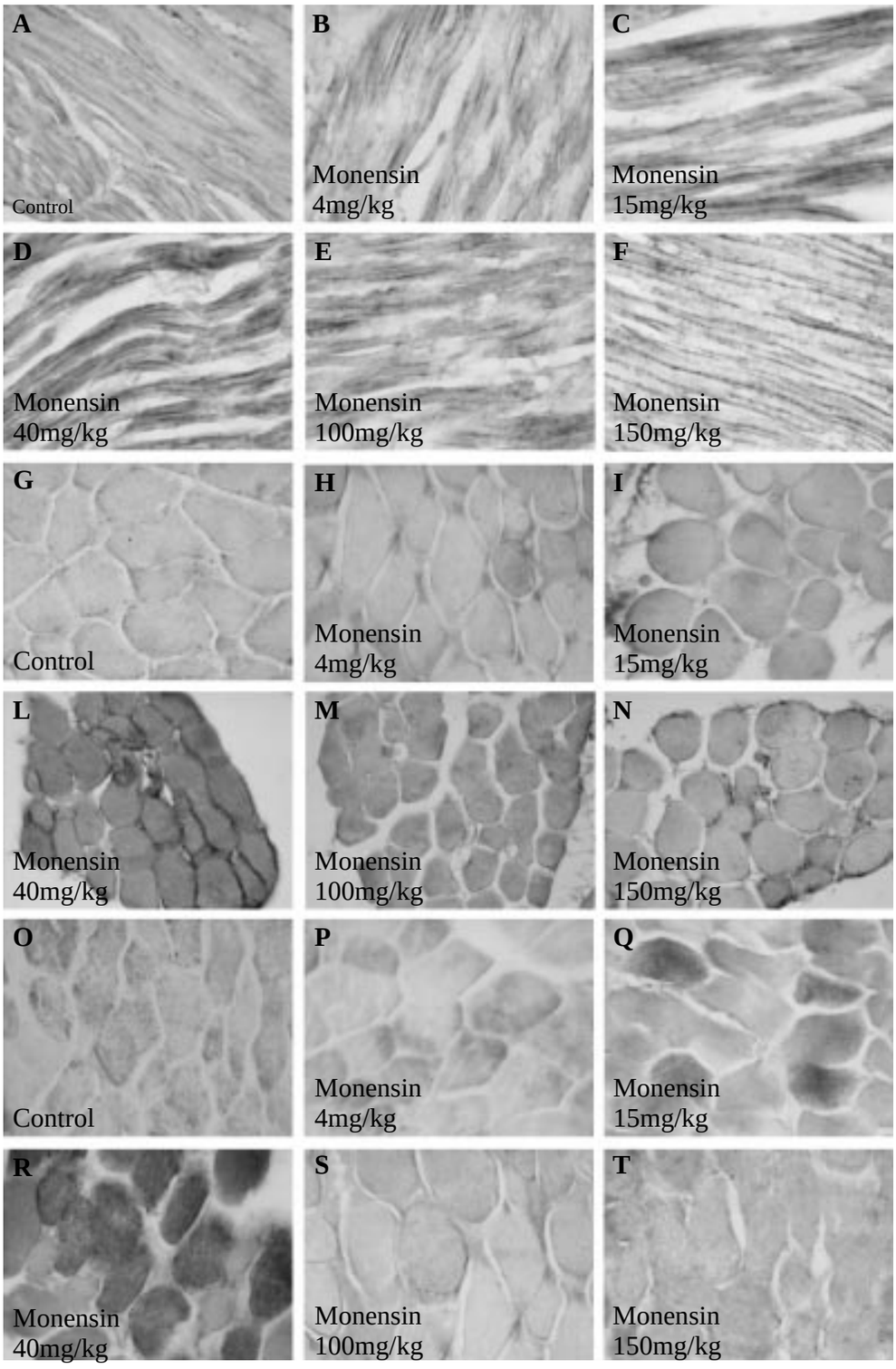

Fig. 9 – Heart A-F; Pectoral muscle G-N; Quadriceps femoris O-T. A strong Na^+/K^+-ATPase immunoreactivity is noticed in muscle specimens taken from chickens treated with 4, 15 and 40mg of monensin/kg body weight. A loss of the immunoreactivity is found using an oral dose of 100 and 150 mg/kg body weight (original magnification 100X).

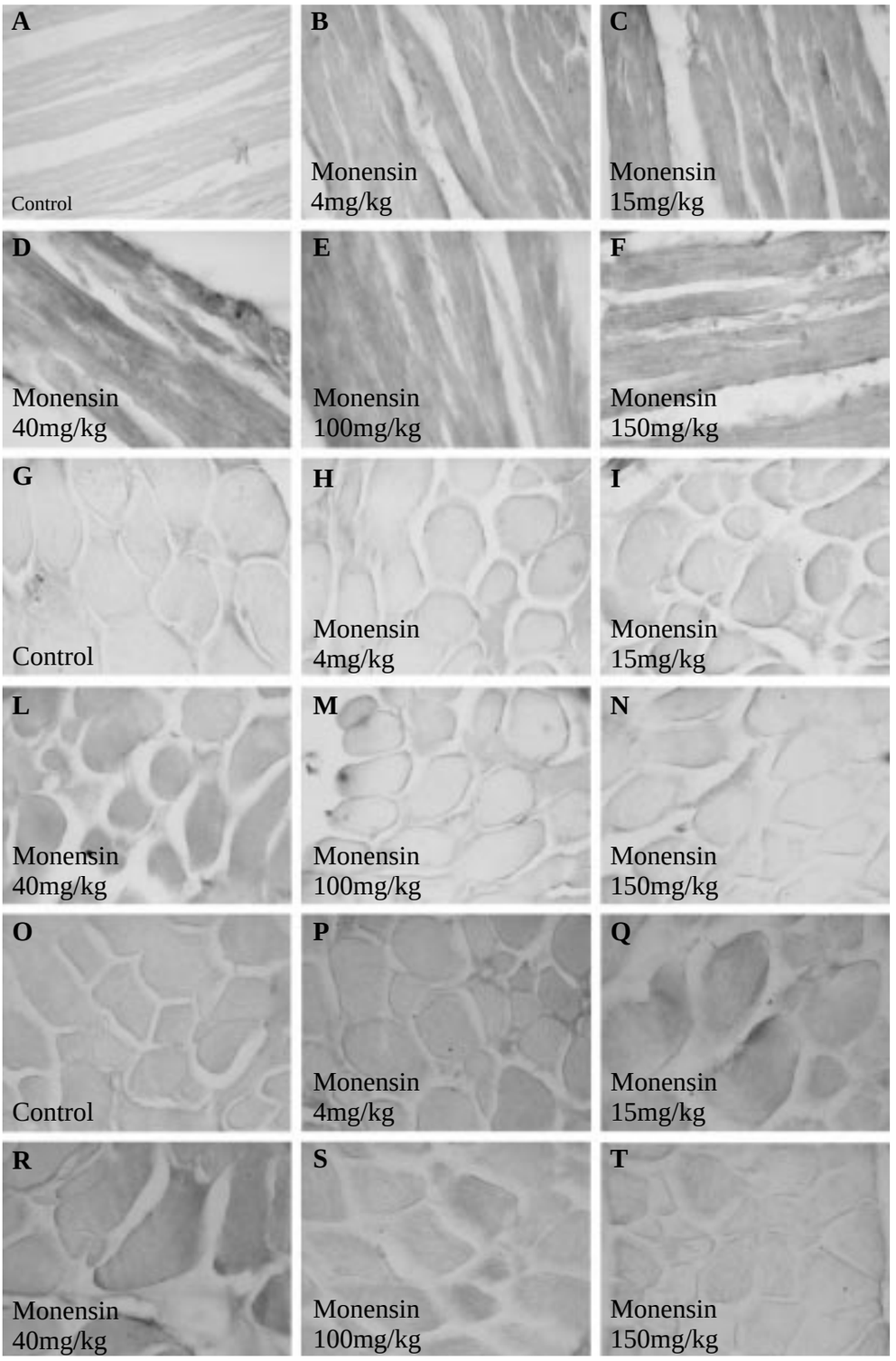

Fig. 10 - Heart A-F; pectoral muscle G-N; quadriceps femoris O-T. Ca^{++}-ATPase immunoreactivity is increased using a dose of monensin at 4, 15 and 40 mg/kg body weight. The doses of 100 and 150mg/kg body weight causes a loss of the enzyme immunoreactivity (original magnification 100X).

vous system. A subacute exposure to this ionophore antibiotic would allow to better investigate the impairment of the cell membrane components.

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