

# The Histochemical and Ultrastructural Structures of Avian Latissimus Dorsi Muscle Fiber Types and Changes in Them Caused by Water Copper Level

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**Abstract:** The histochemical and ultrastructural effects of water Cu on the anterior latissimus dorsi (ALD) muscle of 120 newly hatched broilers were investigated in a 45-day experiment. Two doses of water Cu (250 mg Cu/kg water and 500 mg Cu/kg water) were given to the poults. Two distinct categories (IIIA and IIIB) of slow tonic fibers in the control groups were detected by enzyme reactions. The ultrastructure of the Z-line, mitochondrial content and sarcotubular content differed between the 2 fiber types.

In the application of 250 mg Cu/kg water, the histochemical characteristics of the IIIB fibers were clearly different from those observed in the control. In addition, a few IIIB fibers transformed to fast fiber types in their ultrastructure. No transformation of IIIA fiber type occurred, but a significant increase in fiber percentages (86.7%) was observed in the application of 500 mg Cu/kg water. In the application of 500 mg Cu/kg water, there was degeneration in IIIB fibers. Mitochondrial degeneration also occurred in both applications.

**Key Words:** Anterior latissimus dorsi, histochemistry, ultrastructure, copper, chicken.

## Kuş Latissimus Dorsi Kas Lifli Tiplerinin Histokimyasal ve Ultrastrüktürel Yapılarının İncelenmesi ve Su Bakır Seviyesinin Etkisi ile Değişmeleri

**Özet:** Yumurtadan yeni çıkmış 120 broilerin anterior latissimus dorsi (ALD) kası üzerine su bakırının histokimyasal ve ultrastrüktürel etkileri 45 günlük bir deneyde araştırıldı. Kümes hayvanlarına su bakırının iki dozu (250 mg Cu/kg su ve 500 mg Cu/kg su) verildi. Kontrol gruplarında enzim reaksiyonlarıyla yavaş tonik liflerin iki ana kategorisi (IIIA ve IIIB) tespit edildi. İki lif tipi arasında Z-çizgisinin ultrastrüktürü, mitokondri içeriği ve sarkotübüler içerik farklı oldu.

250 mg Cu/kg'lı su uygulamasında, IIIB liflerinin histokimyasal özellikleri kontrolde gözlenenlerdekinden açıkça farklı oldu. Aynı zamanda, birkaç IIIB lifli ultrastrüktürlerinde hızlı lif tipine dönüştü. IIIA lif tipinde hiçbir transformasyon olmadı, ancak 500 mg Cu/kg su uygulamasında lif yüzdelerinde belirgin bir artış (% 86,7) gözlemlendi. 500 mg Cu/kg su uygulamasında IIIB liflerinde dejenerasyon görüldü. Mitokondri dejenerasyonu her iki uygulamada da meydana geldi.

**Anahtar Sözcükler:** Anterior latissimus dorsi, histokimya, ultrastrüktür, bakır, piliç.

## Introduction

The analysis of trace biologically essential or toxic ionic compounds found in the environment is very important. Copper has been identified as an essential trace element for living from bacterium to man. However, the addition of dietary Cu in excess of the nutritional requirement to poultry diets has been

common practice for many years. The excess supplemental Cu has been reported to have growth-promoting effects (1). The data regarding Cu supplementation of turkey diets have yielded variable results. Weeks and Sullivan (2) reported positive growth responses in poults fed 500 and 1000 ppm Cu in practical rearing conditions, but decreased growth in poults reared

in battery cages. Guenther et al. (3) reported a positive growth response at 120 ppm Cu, but Kashani et al. (4) found decreased growth to 8 weeks of age at 120 and 240 ppm Cu. Negative responses were observed at both 500 and 750 ppm Cu in studies conducted by Christmas and Harms (5); however, Harms and Buresh (6) found a positive growth response at 500 ppm Cu. It was reported that much of this variation may be due to the level of Cu supplementation, growing conditions, type of diet, and length of treatment (6).

The avian latissimus dorsi (ALD) is important for muscle biologists and investigators working in the areas of muscular dystrophy and neurotrophic interactions (7). It is a red, true slow (or tonic) muscle and various investigators have reported that it is composed of extrafusal fibers exhibiting exclusive homogeneity in its structural and functional properties (8). All of its extrafusal fibers are said to possess ultrastructural and physiological features typical of slow muscle fibers (9) and they are all thought to be innervated by multiple "en grappe" nerve terminals (10). Some studies also indicated that this muscle might be more complex than was originally assumed (11).

Although there is growing interest in the ALD as a unique experimental preparation for direct structure-function correlation, virtually nothing is known about the histochemical and ultrastructural effect of copper on the extrafusal fibers in this muscle of chickens. For this reason, the present report analyzed the histochemical and ultrastructural properties of muscle fibers in the ALD muscle of normal and application (fed with 250 mg Cu/kg water and 500 mg Cu/kg water) groups.

## Materials and Methods

One hundred twenty newly hatched broiler toms were obtained from the Rural Services Directorate, Erzurum, Turkey. They were separated into 3 groups as a control group and 2 experimental groups. These were randomly assigned to treatments. Each group had 40 animals. The average initial weight was 80 g. Poults were provided with continuous fluorescent lighting and caged in heated, thermostatically controlled (mean temperature was 35 °C) starter batteries with a raised wire floor. All poults

were fed a basal diet to meet the nutrient requirements of the growing poult. Tap water was provided for ad libitum consumption in plastic water containers. The water containers were filled with a known weight of water and weighed daily (0700) to determine water loss. Evaporative loss (35.7 g water/day) was determined using an additional water container placed in the same room as the batteries.

Water Cu treatments were obtained by adding acidified  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (21.97% Cu) to tap water on a mass basis (i.e. milligrams of Cu per kilogram of water). The water Cu levels of 250, 500 mg Cu/kg water represent 2 and 4 times the recommended dosage<sup>1</sup>. Water Cu concentrations were determined by atomic absorption spectrophotometric analysis<sup>2</sup>. The basal diet and tap water contained 16.5 mg Cu/kg and 0.02 Cu/kg water, respectively. Water Cu treatments were imposed from hatching to 45 days posthatching. Each treatment was replicated 10 times with 4 poults each.

## Histochemistry

The chickens were killed under ether anesthesia. ALD muscles were carefully excised, pinned to corkboard and rapidly quenched for 30 s in isopentane cooled to -150 °C in liquid nitrogen. Muscles were then transferred to a cryostat (-20 °C) placed on mounting chucks, and serial transverse frozen sections (10 µm) were cut through mid-belly portions of each muscle. The sections were then placed on glass coverslips and allowed to dry at room temperature for 1 h. A modification (12) of the Brooke and Kaiser method for the demonstration of myosin ATPase activity (mATPase) after preincubations at differing pH was used. This method can be directly correlated with the speed of muscle contraction. Consecutive sections were either alkaline-preincubated at pH 9.4 or acid-preincubated at pH 4.6 or pH 4.3 as outlined in the modified procedure. For the alkaline-preincubation series, sections were first preincubated in 0.2 M barbital-acetate buffer for 5 min at pH 4.6 or pH 4.3, and then washed in the barbital-calcium chloride solution for several seconds. All sections were subsequently incubated at pH 9.4 for 40 min in the substrate solution. Further treatment of sections was undertaken in a manner similar to that described in the modified procedure (12).

<sup>1</sup> I.D. Russell Co. Laboratories, Longmont, CO 80501, USA.

<sup>2</sup> Model 3030B, Perkin-Elmer Corp., Norwalk, CT 06856, USA.

Succinic dehydrogenase (SDH) was assayed according to Bancroft and Stevens (13) and was used to assess the oxidative capacity of the fibers. In these reactions, as succinate is oxidized, nitroblue tetrazolium accepts electrons forming diformazan, a blue precipitate. Fibers with a dark reaction product were inferred to be those of high-oxidative capacity.

The glycolytic capacity of muscle fibers was indicated by a dark precipitation reaction catalyzed by the enzyme  $\alpha$ -glycerophosphate dehydrogenase ( $\alpha$ GPDH). Rather than an aqueous incubation medium (14), muscle sections were applied to an agarose gel surface containing all necessary reagents in concentrations equal to those referred to above. This technique, adapted from Sigel and Pette (15), sharpened the differences in color intensity for this soluble enzyme.

For lipid staining, Sudan black B was used. Sudan black stains triglycerides blue-black (13).

The distribution and diameters of extrafusal fibers were determined in 60 whole muscle cross sections between the control and experimental groups. The fibers were counted on a photographic montage of the muscle in cross section stained for SDH. Fiber diameters were measured with a calibrated ocular micrometer fitted to the microscope. The shortest diameter of the fibers was measured to avoid errors due to fiber obliquity. Significant differences among 200 fibers of each type were tested for by Student's t test ( $P < 0.05$ ). The data are presented as means  $\pm$  one standard error of the mean (SEM) (16).

### Electron microscopy

Conventional electron microscopic processing procedures were used (13). A small portion of muscle

was removed from the center of the muscle length, oriented on a wooden stick, and fixed in phosphate-buffered (0.1 M, pH 7.2-7.3) glutaraldehyde- (3%) formaldehyde for 3 h, with mincing after 30 min. The tissues were washed with phosphate-buffered (0.1 M, pH 7.2-7.3) sucrose (0.15 M), postfixed in buffered osmium (1%), dehydrated in ethanol, rinsed in propylene oxide, and embedded in Epon-Araldite. Thin sections were made on a Reichert OmU2 ultramicrotome, stained with saturated aqueous uranyl acetate (5%) and lead citrate (4%), and examined with a Siemens Elmiskop I electron microscope.

### Results

The findings summarized in the Table based on the mATPase reaction at different pH-preincubations show that 2 populations of slow fiber are demonstrated in the ALD muscle. One type had a higher activity than the other after acid preincubation and often showed a higher activity after alkaline preincubation. On the other hand, a characteristic feature of these fibers was that neither type showed a reversal in relative staining intensity after acid versus alkaline preincubation. We thought that identification of these types could be confirmed by additional histochemical criteria (Table). Thus, serial sections of the same muscle fibers were compared. A direct correlation between oxidative enzyme activity and mATPase staining (pH 9.4) was seen. Furthermore, type IIIB fibers contained many lipid droplets and appeared to have greater glycolytic capacity. Therefore, we designated them as types IIIA and IIIB. However, in the application of 250 mg Cu/kg water, the levels of histochemical reactions of IIIB fibers were generally lower

Table. Histochemical characterization of ALD muscle fibers in control and experimental chicks.

Histochemical reaction	Control		250 mg Cu/kg water		500 mg Cu/kg water	
	IIIA	IIIB	IIIA	IIIB	IIIA	IIIB
mATPase (pH 9.4)	●	●	●	●	●	●
mATPase (pH 4.6)	⊙	●	⊙	⊙	⊙	●
mATPase (pH 4.3)	⊙	●	⊙	⊙	⊙	●
SDH	●	●	●	●	●	●
Sudan black B	●	●	●	●	●	●
$\alpha$ GPDH	⊙	●	⊙	⊙	⊙	●

Relative stain intensities: ⊙ light staining, ● moderate staining, ● dark staining.

than those found in the controls. Furthermore, these fibers were stained as IIIA fibers. On the other hand, in the treatment of 500 mg Cu/kg water, ALD muscle showed histochemical characteristics similar to those of the controls (Table).

In control ALD, the average diameter of IIIB fibers ( $45.4 \pm 5.1$ ) was significantly smaller than that of the IIIA fibers ( $62.4 \pm 4.9$ ). The percentage of IIIA fibers ranged from 70.50% to 74.12% (mean,  $72.27 \pm 2.32$ ). The percentage of IIIB fibers ranged from 25.91% to 29.71% (mean,  $27.86 \pm 2.37$ ). On the other hand, IIIB fibers have a homogeneous pattern of succinic dehydrogenase in the application of 250 mg Cu/kg water (Table). This last situation may cause misleading measurements in both the diameter and percentages of fibers. Therefore, these fibers were not counted. Similarly, in the application of 500 mg Cu/kg water, because of quite variable muscle fiber sizes, the fiber diameters could not be quantified. In this application, the IIIA fibers had percentages ranging from 86.18% to 87.75% (mean,  $86.76 \pm 0.37$ ). The IIIB fibers made up 12.24% to 14.30% (mean,  $12.64 \pm 2.07$ ) of ALD. In conclusion, a significant difference was observed for the extrafusil fiber percentages between the control and experimental groups ( $P < 0.01$ ). In addition, the ultrastructural differences between the slow fibers in the control ALD were observed. IIIA fibers had irregular and poorly developed separation of myofibrils, and Z-lines appeared as narrow amorphous structures in longitudinal sections (Figure 1a). However, the sarcoplasmic

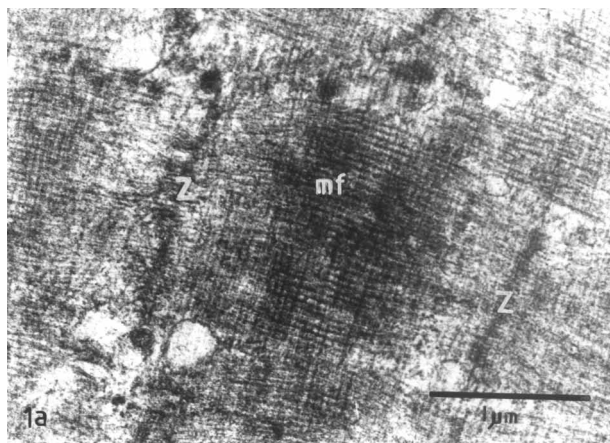


Figure 1a. Longitudinal section of the ALD from control chick. The IIIA fibers have irregular myofibrils (mf). The Z-lines (Z) are narrow amorphous structures, and M-lines are absent. x25,000.

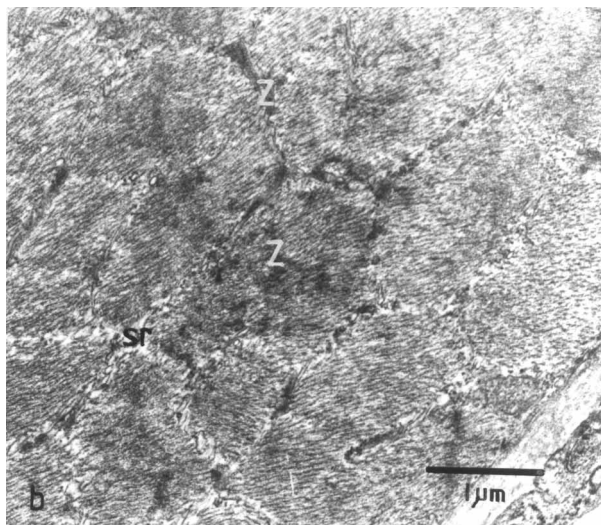


Figure 1b. Cross section of the ALD from control chick. The IIIA fibers have a slow-fiber morphology. Sarcoplasmic reticulum (sr) is sparse. The Z-lines (Z) are narrow amorphous structures. x15,000.

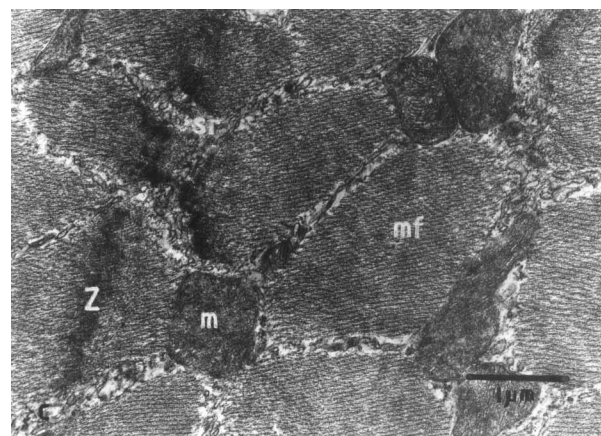


Figure 1c. Cross section of the ALD from control chick. The IIIB fibers have broad and nonpatterned Z-lines (Z). Note abundance of mitochondria (m) and sarcoplasmic reticulum (sr). Myofibrils are irregular (mf). x15,000.

reticulum was sparse in cross sections (Figure 1b). IIIB fibers had an irregular and developed sarcoplasmic reticulum, rich mitochondrial content and Z-lines appeared in a thick nonpatterned structure in cross sections (Figure 1c).

In the treatment of 250 mg Cu/kg water, IIIA fibers had slow fiber morphology. However, minor percentages of IIIB fibers showed more regularly arranged myofibrils despite slow Z-line morphology. The degeneration of the inside of the mitochondria was also noted (Figure 2a).

Furthermore, the actin filaments were grouped (Figure 2b) and some muscle fibers were characterized by triads at most of the A-I junctions, well-developed M-lines and a zigzag substructure of the Z-lines at the sites associated with these regions (Figure 2c).

In the application of 500 mg Cu/kg water, the IIIA fibers of ALD showed ultrastructural characteristics similar to those of the controls. However, degeneration of the myofibrils was observed in some of the IIIB fibers and in most cases degeneration of the membranes of the mitochondria occurred (Figure 2d).

## Discussion

While the notion of the chicken ALD as a true slow muscle had not been refuted, the results of several reports (11,17) as well as those of the present study strongly indicated that its extrafusal fiber type composition was not homogeneous. The fact that the extrafusal fibers in the ALD did not react like typical fast fibers in their pH-lability of myosin ATPase suggested that they might actually represent 2 varieties (IIIA and IIIB) of slow fiber. IIIB fibers appeared to have greater SDH and ATPase concentrations than IIIA as recorded by

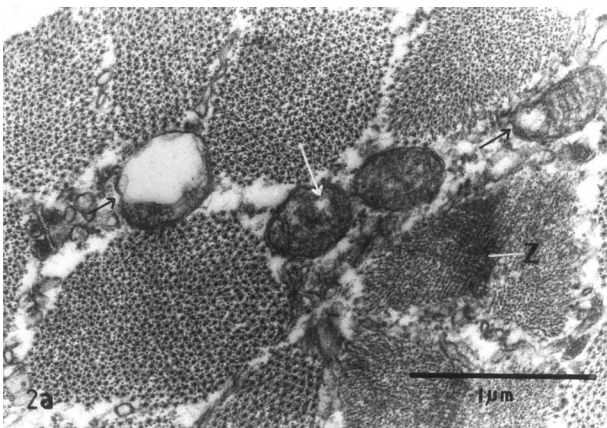


Figure 2a. Cross section of the ALD in application of 250 mg Cu/kg water. The fast fiber morphology is indicated by regular myofibrils, despite slow Z-line (Z) morphology. Note the degeneration of the mitochondria (arrows). x30,000.

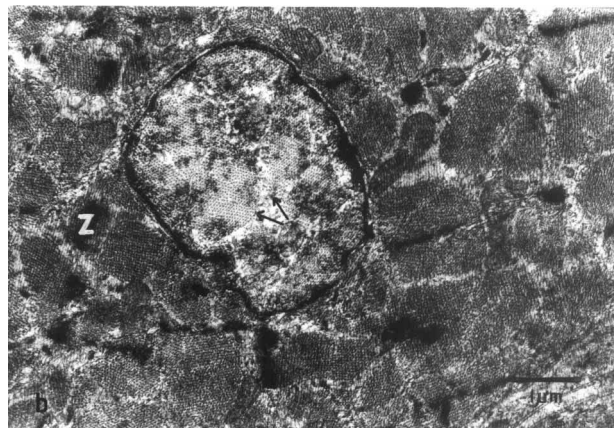


Figure 2b. Cross section of the ALD in application of 250 mg Cu/kg water. The IIIB fibers have slow Z-line morphology (Z). Myofibrils are well-delineated. This area contains actin filaments (arrows) which may be precursors to I bands. x 10,000.

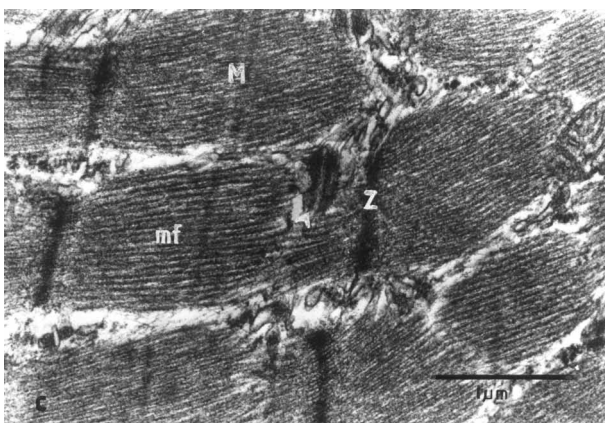


Figure 2c. Longitudinal section of the ALD in application of 250 mg Cu/kg water. Myofibrils (MF) are well delineated. The fast-fiber morphology is indicated by zigzag substructure of the Z-line (Z); typical of a fast Z-line. Triads (arrowhead) are present at each A-I junction, the M-line prominent. x25,000.

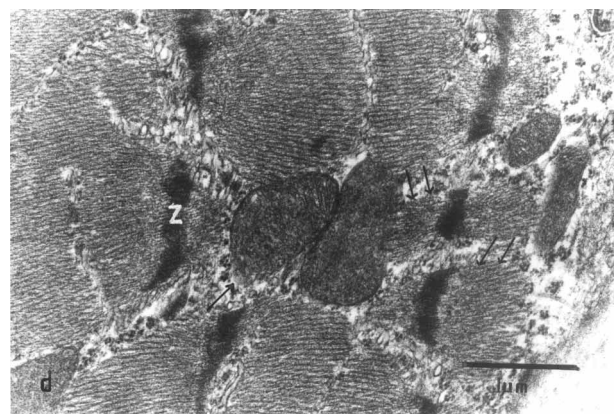


Figure 2d. Cross section of the ALD in application of 500 mg Cu/kg water. The IIIB fibers (double arrows) are degenerated. Note the degeneration of membranes of the mitochondria (arrow). These fibers contain a slow Z-line morphology. x20,000.

Barnard et al. (18). Furthermore, preliminary findings based on an attempt to directly correlate histochemical staining with ultrastructure suggested that the 2 extrafusal fiber types in the ALD might be in fact structural varieties of slow fiber (17). As in amphibian muscles, where variants of true slow fibers were commonly known to occur (19), the 2 slow fibers in the chick's ALD appeared to differ in their mitochondrial content, Z-lines' appearance and sarcofibrillar content.

Shear and Goldspink (20) showed that during early stages of embryological development extrafusal fibers in the ALD at first possessed morphological features typical of twitch fibers. During this early period their myofibrils were "fibrillenstruktur" (the twitch type, has relatively small punctate fibrils, each separated from its neighbour by sarcoplasmic reticulum) in appearance. During early postnatal growth the muscle fiber morphology was then gradually transformed into a slow "felderstruktur" (larger fibrils more irregularly arranged) type by an incomplete splitting of myofibrils. Physiological assessment of this muscle during development also indicated that the rate of contraction in the ALD increased very rapidly just prior to hatching and then decreased during the first postnatal month as it differentiated into a tonic muscle (20).

Conversely, it was of interest that, with experimental manipulation, extrafusal fibers in the slow ALD could be transformed morphologically in a reversible direction to muscle cells displaying twitch (or fast) fiber type characteristics. This was shown to occur after nerve cross-union to the ALD using the nerve supplying the adjacent fast PLD muscle in newly hatched chicks (21) or after tenotomy of the ALD muscle in the adult (22).

Remignon et al. (23) reported that the histochemical muscle fiber profiles were similar without changing their typing among male chickens during growth from hatching to adulthood. Another study (24) tracing the early cytochemical differentiation of extrafusal fibers correlated well with the normal developmental pattern set out by Shear and Goldspink (20). Gordon et al. (24) showed that, prior to hatching, virtually all fibers in the ALD exhibited high mATPase activity. During early postnatal development there was then a gradual decrease in the number of fibers with high ATPase activity and a concomitant increase in fibers with low ATPase activity. In the present study of the ALD in chickens a fairly consistent population (27.86%) of extrafusal fibers

exhibiting high mATPase activity (IIIB) was noted at a time when the muscle and the animal were developed. It was also noted that the mATPase activity of IIIB fibers was reduced by the application of 250 mg Cu/kg water. As a matter of fact, copper (240 mg Cu/kg water) affected the peroxidation of lipids in chick liver (25). The peroxidation of lipids was the reason for the decrease in ATPase activity in rat liver (26). On the other hand, the IIIB fiber population (12.64%) significantly decreased upon the application of 500 mgCu/kg water. This fact, taken together with the findings noted above, led us to interpret this set of extrafusal fibers as ones that had not in all respects undergone complete maturation into typical slow fibers during development. Moreover, it was obvious that the maturation of IIIB fibers was especially increased with the effect of copper. Ultrastructural evidence of a gradual change in the character of the IIIB fibers was seen due to the effect of 250 mg Cu/kg water. One of the first manifestations was the presence of actin myofilaments, interpreted to be the formation of I bands. The formation of a distinct M-line followed the fibrillar formation of fast fiber. The M-line forms by the addition of M-line bridges that connect adjacent thick filaments. These bridges were not found in most slow muscle fibers (27). Finally, triads formed at the A-I junction, and the Z-line changed its structure from a thick, nonpatterned structure to one with a zigzag substructure in longitudinal sections. This is the typical morphology of the Z-line in the fast fiber (28). Therefore, the conversion of the IIIB fibers was monitored ultrastructurally and it was not surprising that histochemical evidence of conversion was observed. However, this was not accompanied by normal mitochondrial morphology between the fibrils. Similarly, the changes in these organelles in the experimental pathological conditions were presented (29). Furthermore, it was reported that the quantity of glutamate and succinate substrates decreased in the mitochondria due to the effect of copper (30). On the other hand, this study indicated that in the application of 500 mg Cu/kg water most of the muscle fibers (IIIA) were still slow tonic in their histochemical and ultrastructural characteristics. This suggested a proliferation of this fiber type (86.76%) in response to atrophic changes in neighboring type IIIB fibers. Conversely, the degeneration of myofibrils of IIIB fibers was seen together with the degeneration of the membranes of mitochondria. It has been widely accepted that a major part of the energy capacitance of mitochondria resides in its membranes (31).

In conclusion, 2 populations of slow fiber (IIIA and IIIB) were histochemically and ultrastructurally demonstrated in the ALD of chickens. IIIB fibers were also affected by 2 levels of water Cu. However, in the application of 250 mg Cu/kg water, whether IIIB fiber

types in the ALD actually represent discrete functional units (from slow tonic to fast fiber type) or not remains to be elucidated. One possible approach to this question might be to examine closely the electrophysiological properties and kinds of motor units.

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