Morphology and Myosin Isoforms Expression of Regenerating Innervated and Denervated Myotubes of Tibialis Cranialis Muscles in Two Strains of Turkey *(Meleagris Gallopavo)*

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Abstract

The regenerative capacity of *tibialis cranialis* (TC) muscle in absence of the nerve was examined in two Heavy- (HW) and light-weight (LW) strains of 6-week-old male turkeys. The study was focused in the early stages of myotubes differentiation during the process of regeneration. Narasin, an ionophorus antibiotic was used to induce type II (fast-twitch) fibers necrosis following by a regeneration. Myotubes diameter and MHCs isoforms expression were investigated during the experimental period (1 to 10 days after narasin administration) in both innervated and denervated TC muscles. The denervated regenerating myotubes are smaller in size than the innervated regenerating myotubes in the HW strain as well as in the LW strain. The diameter of denervated regenerating myotubes in the HW increases with the time, whereas in the LW strain it does not grow during experimental period. Both innervated and denervated regenerating myotubes express cardiac-ventricular and fast embryonic / adult isoforms of Myosin Heavy Chains (MHCs) during the experimental period. In the innervated TC muscles, some regenerating myotubes express the fast neonatal isoform in both turkey strains from day 3 after Narasin administration. In denervated TC muscles by contrast this isoform was expressed only by certain myotubes from day 4 in HW and day 5 in LW after narasin administration. The slow adult isoform was expressed only in denervated regenerating myotubes from 9 to 10 days in the HW strain. The fast adult isoform was expressed only in HW strain at day 9 and 10 in both innervated and denervated myotubes.

Key words: denervation, muscle regeneration, myosin isoforms, turkey.

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Functional innervation is an obligatory requirement for the growth and maturation of muscle during development [26, 30, 34, 39, 49, 56]. The role of innervation in the regeneration process was studied by many investigators. Most of them agree with the fact that the innervation have the same importance in regeneration than in muscle development (for review see [29]).

Regenerating muscles recapitulate some of the principle events of myogenesis [4]. It is initiated by activation of the reserve myogenic precursor, the satellite cells [38], which proliferate, differentiate into myoblasts, fuse into myotubes, and finally mature into myofibers [29, 46, 54]. This process may be divided in two phases: (i) a reformative phase in which formation of regenerating myotubes occurs to replace degenerated fibers; and (ii) a growth and maturation phase, in which the regenerating myotubes undergo a rapid increase in size and become mature. If all the authors agree with the fact that the first phase (i) has no requirement for innervation, the influence of innervation upon the second phase (ii) produced some conflicting results in the literature. For instance, Sesodia and Cullen [55] assert that regenerating myotubes have an obligatory requirement for innervation to grow normally and to differentiate into mature fibers, even though according to these authors the limited increase size seen in denervated regenerating myotubes in the first week following their formation may reflect the purely myogenic potential of myotubes to grow. Mussini et al. [43] have reported that fibers regenerating in the absence of innervation showed mature morphological characteristics within the first two weeks after injury. Moreover, these authors indicate that the final differentiation of the newly-formed fibers into adult fiber types is not attained. They concluded that

regenerated myofibers devoid of innervation keep their intrinsic ability to regenerate after injury.

The myosin, a major component of myofibrillar proteins in skeletal muscle, exists as numerous isoforms specific for the muscle fiber type [5].

Regulation of MHC isoform expression is quite complex, with neural, hormonal, and myogenic factors involved in determinating which MHC is synthesized [7, 8].

Myosin heavy chain isoforms provide useful markers to analyse the role of intrinsic and extrinsic factors like innervation during muscle development [2, 3, 10, 11, 12, 60] and muscle regeneration [19, 22, 23, 27, 50].

The aim of this work was to study in heavy (HW) and light-weight (LW) turkey strains, the role of the nerve on the growth and the differentiation of regenerating myotubes induced by Narasin administration.

Narasin is an ionophorus antibiotics known for its myotoxic potentialities [21, 25, 51]. We used this antibiotics to generate specific necrosis of fast-twitch (type II) fibers scattered in the mixed part of the hindlimb muscles. The narasin is administered during 3 days and necrosis is observed 1 day after in HW strain and 2 days after in LW strain.

This necrosis is followed by macrophage phagocytosis of damaged cells and regeneration with satellite cells activation and myotubes formation. Regeneration is apparently complete 14 days after narasin administration.

Denervation by complete section of the sciatic nerve generate an atrophy of the *tibialis cranialis* muscle in both strains of turkeys [9]. Satellite cell activation was observed only from day 1 to day 10 after denervation. Then, during this first days period, the influence of denervation on myotube growth and differentiation can be examined with the association of denervation and narasin administration.

The denervation of the *tibialis cranialis* muscle involve an atrophy of type II fibers (fast-twitch fibers) and an hypertrophy of type I fibers (slow-twitch fibers). The type II fiber atrophy was similar in the two strains of turkeys and reach -25% of control fiber at day 10.

Materials and Methods

Animals

Hundred six-week-old male turkeys (*Meleagris gallopavo*) belonging to two strains were used in this study. The HW strain was a commercial BUT-T9 strain (British United Turkeys Limited, Warren Hall, Broughton, Chester, CH4OEW, UK).

The heavy-medium-line turkeys are raised in light-controlled units for 14 weeks before slaughtering and then used as butchered turkey. They were obtained from breeders selected at 15 weeks of age on criteria of weight, muscle mass and absence of leg weakness. The LW strain was a commercial Betina strain (Grimaud Frères, La Corbière, 49450 Roussay, France) exclusively used as whole-roasted Christmas turkeys. No weight selection criterion was applied to these birds.

The animals were purchased from commercial hatcheries and reared on litter in pens. Food and water were provided ad libitum. They were fed on a starter diet with crude protein and metabolizable energy respectively of 28% and 2,800 kcal/kg.

Surgical procedures

The animals were anesthetized by a mixture of ketamin hydrochloride (Imalgene 500ND) and xylazin hydrochloride (RompunND), using doses of 10 mg/kg and 1 mg/kg I.M. respectively. The common branch of the right sciatic nerve was isolated between the *flexor cruris medialis* and *the pubo-ischio-femoralis* muscles, then recut over a length of about 1 cm to avoid any risk of reinnervation.

Twenty four hours after denervation, muscle degeneration was induced in forty animals of each strain by Narasin (Lilly France) poisoning. Narasin (2 x 2 mg/kg per day) was administered *per os* to animals, during 3 days. From day 1 to day 10 after, four animals intoxicated and one control (non-intoxicated) were sacrificed by intravenous injection of pentobarbital (DoléthalND). The *tibialis cranialis* (TC) muscles were removed from right (denervated) and left (non denervated) limbs.

Histology and histochemistry

Transverse sections (about 0.5 cm thick) were performed at the muscle midbelly and frozen for 30 seconds in isopentane previously cooled in liquid nitrogen. Serial sections of 12 µm were cut using a cryostat and then stained with hematoxylin-eosin (HE) and Gomori trichrome (GT) to evaluate changes in muscle morphology.

Sections were treated with histoenzymological techniques. Acid phosphatase reaction (AP) was used to identify macrophages in necrotic fibers, Nicotinamide Adenine Dinucleotide Tetrazolium-Reductase (NADH-TR) reaction to characterize fiber metabolism and the myofibrillar Adenosine Triphosphatase (ATPase) reaction after acid preincubations (pH = 4.35 and 4.6) and basic preincubation (pH = 10.4) to detect ATPase activities within fibers.

Immunocytochemistry

Cryostat-cut sections (10 µm thick) were used for immunocytochemical studies. Sections were treated with a 1:2500 dilution of primary antibodies in phosphate-buffered saline (PBS) for 1 hour at 37°C in a humid box. The primary antibodies were monoclonal antibodies (MAb) raised against myosin heavy chain (MHC) isoforms of chicken muscle. The specificity of HV11 MAb for ventricular MHC, EB165 MAb for embryonic and adult fast MHCs, 2E9 MAb for neonatal MHC, AB8 for adult fast MHC and NA2 for slow isoform, have previously been documented [5, 6, 8, 19]. These chicken specific MAb react with turkey myosin iso-

forms [37]. Sections were washed in PBS, and the secondary antibody (fluorescein-conjugated goat anti-mouse IgG1 (EUROMEDEX, AP 106F), diluted 1:100 in PBS) was laid on sections for 1 h at room temperature.

Histomorphometrical analysis

The diameter of regenerated myotubes were assessed using a VIDS IV semi-automatic image analyzer (Analytical Measuring Systems, London Road, Pampisford, Cambridge CB2 4EF, UK.).

The mean (± S.D.) diameter of myotubes was determined from an average of 100 myotubes randomly chosen in both limb. Myotubes diameter measurements were done on sections of frozen muscles treated with basic ATPase (pH = 10.4).

Diameter of type II fiber was measured in denervated and contralateral muscle. The atrophy of the type II fibers from the right muscle was used as a control of the efficiency of the denervation.

Statistical analysis was performed using ANOVA, Fischer PLSD, linear regression and Student t tests.

Results

Morphology

Fast-twitch (type II) fibers degeneration of the *tibialis cranialis* muscle was observed 24 h and 48 h following Narasin poisoning respectively in the HW and LW strains. One day later (day 2 in HW strain and day 3 in LW strain), immature myotubes reacted strongly with the basic ATPase reaction. These immature myotubes were characterized by central nuclei. By 10 days, regeneration was quite in both strains without residual lesions. The type II fiber of denervated muscle exhibited an gradual atrophy proving the efficiency of the denervation.

Histomorphometrical results

For both strains, morphometrical results showed that innervated myotubes exhibited a similar linear growth pattern. The innervated myotubes diameters, were 12.1 ± 1.2 in LW strain and 14.9 ± 0.2 in HW strain at day 4 and 20.0 ± 1.4 µm in LW strain and 21.6 ± 0.3 µm in HW strain at day 10 (Fig. 1). No difference was observed at day 10 between the innervated myotubes diameter of the two strains.

A comparison of regression line slopes [HW: y=9.408 + 1.216x; r = 0.92 and LW: y = 8.991 + 1.163x; r = 0.92 (y = myotubes diameter, x = number of days)] showed that increase of innervated myotubes diameter was identical in both strains during the 10 days of experimentation (No significant difference at p = 0.05).

Growth of denervated myotubes was different from those observed in the innervated myotubes. In LW strain, denervated myotubes exhibited a very slow growth from 10.5 ± 0.5 µm at day 3 to 12.3 ± 0.7 µm at day 10 (Fig. 1). The LW denervated myotubes did not show an important increase throughout the ten days. A comparison of regression line slopes [LW: y = 9.303 + 0.298x; r = 0.71 and the 0 slope (y = myotubes diameter, x = number of days)] showed that denervated myotubes diameter in LW strain do not increase during the 10 days of experimentation.

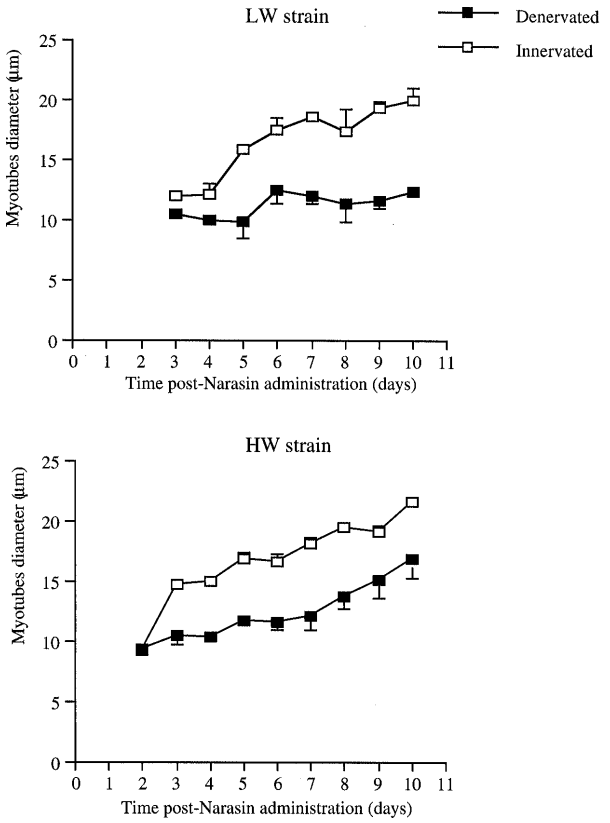

Figure 1. Myotubes diameter (mm)(means ± SD) of denervated and innervated regenerating myotubes following Narasin administration in HW and LW strains of turkey.

In HW strain, growth of denervated myotubes was less intense than growth of contralateral innervated myotubes during the first 5 days of regeneration. From the day 6 to day 10, the increase of denervated myotube diameter presented a similar linear pattern to those of innervated ones. At day 10, the denervated myotube diameter reached 16,8 ± 1,7 µm (Fig. 1). A comparison of regression line slopes [HW denervated: y = 7.190 + 0.856x; r = 0.95 and HW innervated: y = 9.408 + 1.216x; r = 0.92 (y = myotubes diameter, x = number of days)] showed that the increase of innervated and denervated myotubes diameter were identical in HW strains during the 10 days of experimentation.

MHCs isoform expression in myotubes

Tables 1, 2 and 3 shows the MHCs isoform expression in regenerating innervated myotubes. From day 2 in HW strain and day 3 in LW strain to day 10, innervated myotubes reacted strongly with the ventricular isoform [MAb

Table 1. Expression of ventricular (Mab HV11) myosin isoform in regenerating myotubes of innervated and denervated tibialis cranialis muscle after Narasin administration in two strains of turkey.

Time Post-narasin administration (days)	Mab HV11 reactivity HW strain Den	HW strain In	LW strain Den	LW strain In
1				
2	+++	+++		
3	+++	+++	+++	+++
4	+++	+++	+++	+++
5	+++	+++	+++	+++
6	+++	+++	+++	+++
7	+++	+++	+++	+++
8	+++	+++	+++	+++
9	+++	+++	+++	+++
10	+++	+++	+++	+++

-: no reactivity; +: low reactivity; ++: medium reactivity; +++: high reactivity; /f: few myotubes; Den: Denervated; In: Innervated.

HV11 (Table 1)] and the embryonic and adult fast isoform [MAb EB165) (Table 2)]. Few innervated myotubes reacted with antibodies to neonatal (MAb 2E9) isoform at day 2 in HW strain and day 3 in LW strain (Table 2).

From day 5, for both strains, most of the innervated myotubes which express ventricular isoform (MAb HV11) also express neonatal isoform (MAb 2E9). The adult fast (MAb AB8), and adult slow (MAb NA2) isoforms were not expressed by the innervated myotubes during the experimental period (Table 3). The neonatal myosin isoforms expression was different in denervated and innervated myotubes from the same animal. Neonatal myosin isoform expression was observed only at day 4 in denervated myotube (1 day later than in innervated ones) in HW strain and only at day 5 in LW strain (2 days later than in contralateral innervated myotubes) (Fig 2c and d). Adult fast (MAb AB8) isoform was expressed by certain denervated myotubes at day 9 and day 10, only in HW strain (Table 3). Adult slow (MAb NA2) isoform was expressed by some denervated myotubes from day 9 in HW strain, but was not expressed before day 10 in LW strain (Table 3).

Expression of ventricular [MAb HV11, (Fig. 2a and b)] and embryonic/adult fast (EB165) isoforms by denervated myotubes was similar to those observed in innervated myotubes, for both strains.

Discussion

This study has examined the capacity of the regenerating myotubes to differentiate in absence of innervation in *tibialis cranialis* muscles from two strains of turkey.

The morphometric datas showed that the denervated and innervated myotube diameters were similar at the first day of observation in basic ATPase sections, but denervation generated a decrease of myotube growth in both strains during the 3 following days. Then, in LW strain, growth stayed very low, by contrast in HW strain, diameter increased and the sloope of the growth curve was similar to those of innervated myotubes.

In so far the control of the efficiency of the denervation has been positive, this results suggested that the innervation was a less necessary requirement for myotubes growth in HW than in LW strain during the first 10 days.

The capacity of the skeletal muscle to regenerate in absence of the motor innervation has been largely discussed in the literature [8, 14, 15, 16, 19, 22, 23, 24, 32, 42, 55], but the assessment of the differentiation

Table 2. Expression of fast embryonic and adult (Mab EB 165), fast neonatal (Mab 2E9) myosin isoforms in regenerating myotubes of innervated and denervated tibialis cranialis muscle after Narasin administration in two strains of turkey.

Time Post-narasin Administration (days)	Mab EB 165 reactivity HW strain Den	HW strain In	LW strain Den	LW strain In	Mab 2E9 reactivity HW strain Den	HW strain In	LW strain Den	LW strain In
1								
2	++	++						
3	++	++	++	++		++/f		++/f
4	++	++	++	++	++/f	++/f		++/f
5	++	++	++	++	++/f	++/f	++/f	++
6	++	++	++	++	++	++	++/f	++
7	++	++	++	++	++	++	++/f	++
8	++	++	++	++	++	++	++	++
9	++	++	++	++	++	++	++	++
10	++	++	++	++	++	++	++	++

-: no reactivity; +: low reactivity; ++: medium reactivity; +++: high reactivity; /f: few myotubes, Den: Denervated; In: Innervated.

Table 3. Expression of slow adult (Mab NA2), fast adult (Mab AB8) myosin isoforms in regenerating myotubes of innervated and denervated tibialis cranialis muscle after Narasin administration in two strains of turkey.

Time Post-narasin Administration (days)	Mab NA2 reactivity				Mab AB8 reactivity			
	HW strain		LW strain		HW strain		LW strain	
	Den	In	Den	In	Den	In	Den	In
1								
2	-	-			-	-		
3	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-
9	+/f	-	-	-	+/f	+/f	-	-
10	+/f	-	-	-	+/f	+/f	-	-

-: no reactivity; +: low reactivity; ++: medium reactivity; +++: high reactivity; /f: few myotubes; Den: Denervated; In: Innervated.

state of the myotubes differed widely from one study to another. Nevertheless, most authors do agree with the fact that regeneration would not be complete and functional in the absence of innervation.

Muscle/nerve relationships during muscular regeneration, by analogy with the embryonic myogenesis can be divided in two phases.

The first phase corresponds to the development of the myotubes from the activation and proliferation of satellite cells until myoblasts fuse to form myotubes. The second phase corresponds to the differentiation of the myotubes into adult and mature fibers.

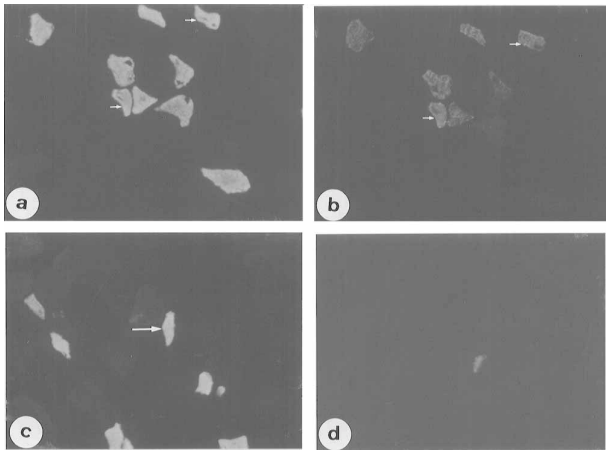

Figure 2. a and b: Serial sections of regenerating denervated myotubes of HW strain, 4 days after narasin administration. Myotubes (arrows) reacted with ventricular HV11 (a) and neonatal 2E9 (b) MHC antibodies. c and d: serial sections of regenerating denervated myotubes of LW strain, 4 days after narasin administration. Myotubes (arrows) reacted with ventricular HV11 (c). No myotubes were observed that reacted with neonatal 2E9 (d) MHC antibody. (Magnification x 250).

All the authors do agree that innervation has no influence on the first phase of the regenerating process. The process of regeneration seems to start more rapidly when the muscles was denervated prior to the injury, as demonstrated by Carlson [13]. The author reported that, in the free graft denervated before, the initial phase of regeneration developed faster than in the free innervated graft. Maybe, the proliferation of satellite cells which following the denervation [36, 40, 41, 42, 44, 45, 48, 53, 57] could explain the differences between innervated and denervated free grafts.

In turkeys, we do not observed the myotubes earlier in the denervated than in the innervated muscles. Proliferation of satellite cells generate by denervation appears too late [9] to cross with the first phase of post necrotic regeneration.

Many observations shows that innervation does not influence the early stage of myotube differentiation [14, 15, 16, 24, 32, 43]. In the denervated limb of the frog, Hsu [32] found large numbers of regenerating myotubes, but soon thereafter a massive degenerative response set in, leaving only a few striated muscle fibers by the end of a month. Carlson and Gutmann [15] have studied the regenerative ability of the sliced grafts *extensor digitorum longus* muscle of the rat in the absence of nerves with physiological and histochemical techniques.

They found no consistent morphological or histochemical differences between sliced grafts muscle regeneration in normal and denervated limbs, during the first postoperative month. By contrast, they found a substantial difference in the contractile properties between innervated and denervated regenerates. At day 30, innervated regenerates are making a rapid transition from slow to fast contractile properties, whereas denervated regenerates continue to contract very slowly like immature muscle. Between 30 and 60 days

after operation, important differences between innervated and denervated regenerates occurs.

The diameter of innervated regenerating muscle fibers increase greatly, the contractile properties of muscles becomes stabilized at near-normal levels, and a heterogeneous pattern of histochemically distinct muscle fiber types emerges, reflecting the differential activities of the motor neurons. By contrast, the muscle fibers of denervated regenerates begin a long period of atrophy and remaining immature in their histochemical and contractile properties. In conclusion, the authors suggested that the absence of innervation does not impose any specific block in the differentiation of regenerating muscle fibers up to the histological stage characterized by presence of cross-striations and peripherally migrated nuclei.

Mussini et al. [43] report, in bupivacaine induced necrosis and regeneration of the fibers of the muscle *soleus* of the rat, that the denervated regenerating myotubes can change and show histological and ultrastructural characteristics of mature fibers during the two first weeks following the necrosis. The authors specify that the terminal histoenzymological differentiation in the fast and slow fiber type of newly developed fibers has never been reached.

In the absence of nerves, regenerating muscle can differentiate to a certain level that represents the inherent myogenic potential for development, characterized by histological differentiation up to cross-striated fibers with peripheral nuclei, slow and weak contractile properties [15, 24, 43].

Sesodia and Cullen [55] using the notexin as a myotoxic agent [31] reports that, in absence of innervation, the regenerating myotubes atrophy and never do reach the morphological stage of mature fiber described by Mussini et al. [43]. The conclusions of Sesodia and Cullen [55] bring forward the principle of the absolute nervous dependence of myotubes during their differentiation into mature muscular fiber. In our study, we only observed myotube growth during 10 days and we don't know the behavior of the cells after this time. We show that the aneural myogenic potential seems to be more important in HW strains than in LW strains.

In HW strain, from day 9 after denervation, some regenerating denervated myotubes express all the tested myosin isoforms (cardiac-ventricular, fast embryonic / adult, fast neonatal, fast and slow adult). The regenerating innervated myotubes express all the isoforms except the slow adult one. In LW strain, innervated and denervated myotubes only express developmental isoforms (cardiac-ventricular, fast embryonic and adult, fast neonatal). In both strains, neonatal isoform appear earlier in regenerating myotubes from innervated than denervated muscle.

The expression of the MHC isoforms in the skeletal muscle in development or in regeneration is under the control of many factors among which the innervation state and the hormonal environment [1, 12, 28].

Denervation of adult fast muscles has been shown to result in the reexpression of isoforms which have been previously repressed during maturation [19, 27]. Similarly, if regenerating muscle is denervated, most of the regenerated muscle fibers continue to express the fast neonatal MHC isoform along with the adult fast twitch MHC isoform [19, 27].

Regeneration has been found to lead the reappearance of SM1 MHC (slow), previously repress in mature muscle [47, 58]. Hemidiaphragm of the rat, a mixed muscle, lose slow myosin and is transformed into fast-like muscle after several months [17, 18]. Carraro et al. [18] reported that all the denervated fibers of hemidiaphragm reacted with anti-fast myosin, while many of them reacted with anti-slow myosin as well.

They have concluded that aneural regeneration events continuously occur and significantly contribute to the increasing uniformity of the myosin gene expression in long-term denervated diaphragm.

Regenerating innervated myotubes in *tibialis cranialis* from 10 to 21 days after denervation does not express the slow adult isoform in HW as well as in LW. By contrast, we observed that some denervated regenerating myotubes express simultaneously the slow and fast adult myosin from 9 to 10 days after narasin administration in HW strain.

When adult fast or slow chicken muscles are injured, the regenerating muscle fibers expressed the same subset of MHCs that were observed during the development of the respective muscles [7, 19, 50]. This seem occur independently of innervation since regeneration induced in previously denervated muscles resulted in the appearance of the same subset of isoforms expressed by regenerating fiber innervated muscle [8]. Bandman et al. [7, 8] suggested that it could have an inherent programs of MHC isoform expression that differ between fast and slow muscles. But they did not know whether this is the result of an intrinsic difference in regenerating myotubes, or the result of external cues other than innervation within the muscles.

We suggest that, during the regeneration of *tibialis cranialis* (mixed muscle) following narasin administration, different subset of satellite cells like fast type and/or the slow/fast type are put into contribution as reported by Stockdale and Miller [59] and could thus explain the accompanying expression by some myotubes in the HW strain of the fast isoforms and slow adult MHCs.

The difference of growth observed in our study between the denervated myotubes in HW and LW strain could be related to an eventual difference in their myogenic potentials.

Cherel et al. [20] have reported in the *anterior latissimus dorsi* (ALD) muscle of HW turkey strain, that

the postnatal growth was conjointly assured by the mechanisms of hypertrophy and hyperplasia of muscular cells, in contrast of the LW strain in which only the hypertrophy contributes.

This observation could suggest differences between the two strains either of myogenic potentialities of the satellite cells or of the environment factors like hormonal levels, in which the satellite cells are located.

Satellite cells of HW strain are more able to fuse into neomyotubes during spontaneous growth. The ability of myotubes of this strain to grow without innervation is one of the expression of these capacity of neomyogenesis and maybe a factor of it.

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