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Journal	Anatomia, Histologia, Embryologia, 37(2): 147-152
URL	http://hdl.handle.net/10130/848
Right	The definitive version is available at www.blackwell-synergy.com

Changes in the myosin heavy chain 2a and 2b isoforms of the anterior belly of the digastric muscle before and after weaning in mice

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With 8 figures

Summary

During the process of growth and development, the digastric muscle is subjected to marked functional changes, including the change from suckling to mastication. In particular, because the anterior belly of the digastric muscle, which is one of the suprahyoid muscles, plays an important role in mastication. Therefore, this muscle seems to undergo a marked functional change before and after weaning. However, the details remain unknown. Here, in order to clarify the changes in the muscle fiber characteristics of the anterior belly of the digastric muscle before and after weaning, we examined myosin heavy chain (MyHC) isoforms at the protein (immunohistochemistry) and mRNA (transcription) levels. As a control, the changes in the muscle fiber characteristics of the sternohyoid muscle, which is anatomically aligned in the same direction as the anterior belly of the digastric muscle, were analyzed. The results showed that, in the anterior belly of the digastric muscle that is involved in mandibular movements in mice, the ratio of a fast-contraction isoform with strong contractile force increased after weaning. We believe that this occurred in response to a functional change from suckling to mastication. On the other hand, there was little change in the composition of sternohyoid muscle.

Introduction

Myosin, one of muscle contraction proteins, is a major protein constructing

muscle. In particular, the MyHC most closely reflects muscular function (Pette and Sarton, 1990). It has become clear in recent years that there are different isoforms of MyHC and that based on the speed of contraction, the isoforms can be divided into fast-twitch (MyHC-2b, MyHC-2d and MyHC-2a) and slow-twitch types (MyHC-1). Furthermore, several studies have documented that muscle properties are determined by the composition ratio of MyHC isoforms (Brueckner et al., 1996; Hori et al., 1998; Sakiyama et al., 2005; Schiaffino and Reggiani, 1996).

With regard to the expression of various MyHC isoforms during muscular growth and development, many studies have analyzed limb muscles (Fischman et al., 1985; Pette and Sarton, 1990; Schiaffino and Reggiani, 1996), but recent studies have examined muscles in the head and neck region, such as the masticatory muscles (Abe et al., 2002; Gojo et al., 2002; Maejima et al., 2005; Shida et al., 2005; Usami et al., 2003). Gojo et al. (2002) focused on weaning within growth and development, and observed the changes in muscle fiber characteristics of the murine masseter muscle at the protein level. They reported that the composition ratio of MyHC-2b (fast contraction and high contractile force) increased during weaning, and concluded that masticatory movement after weaning brought about marked functional changes in the masseter muscle (Gojo et al., 2002).

In addition, one study found that, like the masseter muscle, the anterior belly of the digastric muscle, which is found between the mandible and hyoid and is involved

in mastication, particularly mouth opening, and is composed of fast-twitch muscle fibers (Erzen et al., 1999). However, there have been few reports closely examining the functional changes before and after weaning within the process of growth and development. Hence, in order to clarify the changes in muscle fiber characteristics of the anterior belly of the digastric muscle before and after weaning, we immunohistochemically examined the changes in MyHC isoforms in relation to the sternohyoid muscle, which anatomically aligns in the same direction via the hyoid. Furthermore, we measured the expression of genes encoding MyHC proteins at the transcription level. Since it was known that the anterior belly of the digastric muscle consists of only a fast-twitch muscle, we examined only to the fast type in MyHC isoforms (Hartmann et al., 2001). Especially we selected MyHC-2b (fastest contraction) and MyHC-2a (slowest contraction).

Materials and Methods

Materials

Based on a study documenting that mouse pups were weaned an average of 3 weeks after birth (Shida et al., 2005), *ICR* mice at the age of 2 weeks (before weaning), 4 weeks (after weaning) and 9 weeks (adulthood) were used. After the age of 3 weeks, juvenile mice were separated from their mothers and were fed a solid diet. A total of 30 mice were used: 5 mice in each of the three age groups for immunohistochemical tests,

and 5 mice in each of the three age groups for mRNA expression. In line with the animal study guidelines established by the Tokyo Dental College, the mice were euthanized by injecting a lethal dose of pentobarbital. Then, the anterior belly of the digastric muscle and the sternohyoid muscle were excised (Figure 1). These muscles were immediately frozen in liquid nitrogen and stored in isopentane at -80°C until testing.

Immunohistochemical analysis

Using a cryostat (Leica, Nussloch, Germany), 8 µm serial frozen sections of each sample were prepared orthogonal to the long axis of muscle fibers. These sections were immunologically stained as follows: As primary antibodies, SC-71 (anti-MyHC-2a; American Type Tissue Culture, Manassas, VA, USA) and BF-F3 (anti-MyHC-2b) (American type tissue culture), which are anti-mouse monoclonal antibodies extracted from hybridoma cells (Eason et al., 2000; Schiaffino et al., 1989; Tuxen and Kirkeby, 1990), were used. Hybridoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM)(Sigma-Aldrich Co., St. Louis, MO) (10% FBS) for 72 hours at 37°C and 5% CO₂, and the resulting cultured cells were centrifuged to isolate the supernatant, which contained the primary antibodies. As secondary antibodies, Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibody (Novocastra Laboratories, Newcastle, UK) was used for SC-71, while

Rhodamine-Conjugated GOAT F(AB')₂ FRAGMENT TO MOUSE IgM (MP Biomedicals, Solon, OH, USA) was used for BF-F3. Muscle fibers were examined using a confocal laser scanning microscope (MRC-1024/2P; Nippon Bio-Rad Lab. Tokyo, Japan). MyHC-2a-positive muscle fibers were defined as those labeled with FITC, while MyHC-2b-positive muscle fibers were defined as those labeled with RITC. The morphology of muscle fibers was analyzed by modifying the methods of Sartorius et al. (1998) and Wakisaka et al. (1993) Briefly (Sartorius et al., 1998; Wakisaka et al., 1993), a 100 µm square was randomly placed on each section, and the number of MyHC-2a-positive and MyHC-2b-positive muscle fibers were counted to give a ratio of the total number of muscle fibers in the section (Figure 2) (Shida et al., 2005). If a cell was stained both MyHC-2a and -2b, the way stained deeply was made into the positivity. The number of muscle fibers per square and the average cross-sectional software were carried out with NIH Image version 1.61 (Scion Corporation, Frederick, MD, USA). This test was repeated ten times for each mouse and the mean and standard deviation for each group (containing 5 mice each) were calculated. Student's *t*-test was used for statistical analysis in this study.

Analysis of mRNA expression using RNA extraction and LightCycler™

Using the LightCycler™ (Roche Diagnostics, Mannheim, Germany), which is capable of quantifying mRNA, the amounts of MyHC-2a and -2b mRNA expression in

the two muscles at the three different age groups were determined (Sakiyama et al., 2005). After extracting mRNA from each sample using the Quick Prep micro mRNA Purification Kit (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK), cDNA was prepared using Ready-To-Go (Amersham Pharmacia Biotech UK Ltd.). The optimal conditions for all primers were determined and experiments were conducted according to the standard protocols of the LightCycler™. LC Fast Start DNA Master SYBR Green I (Roche Diagnostics) was used as a hot start PCR solution for the LightCycler™. A solution containing 4.0 mg/μl of PCR products was serially diluted, and 10⁶, 10⁷, 10⁸ and 10⁹ dilutions were used. The control PCR mixture for each serial dilution was prepared by adding 1.6 μl of MgCl₂ (25 mM) and 2 μl of LC Fast Start DNA Master SYBR Green I to 10.2 μl of sterile water. Furthermore, after adding 0.6 μl of the forward and reverse primers (10 pmole/μl of each) prepared using Oligo primer design (Nihon Gene Research Laboratories Inc., Sendai, Japan), 5 μl of each diluted PCR product was added to give a final volume of 20 μl. Two primers were made for MyHC-2b and MyHC-2a based on specific areas within their full DNA sequences. The base sequence of each primer was as follows: MyHC-2a (Forward: 5'-CGATGATCTTGCCAGTAATG-3, Reverse: 5'-TGATAACTGAGATAACCAGCG-3', Accession NM_144961) and MyHC-2b (Forward: 5'-ACAGACTAAAGTGAAAGCC-3', Reverse: 5'-CTCTCAACAGAAAGATGGAT-3', Accession XM_126119). With regard to the

PCR mixture, to 14.2 μ l of sterile water, 1.6 μ l of $MgCl_2$ (25 mM), SYBR Green I (1/60,000 dilution), 2 μ l of LC FastStart DNA Master SYBR Green I, and 0.6 μ l of the forward and reverse primers (10 pmole/ μ l of each) were added. Next, 1 μ l of target DNA was added to give a final volume of 20 μ l. The two PCR mixtures (20 μ l) for MyHC-2a and MyHC-2b prepared in the above manner were added to the glass part of the capillary. Cycling conditions were as follows: 95°C for 10 minutes followed by 50 cycles of 95°C for 10 seconds, 62°C for 10 seconds and 72°C for 7 seconds. Gene amplification followed a melting program at 70°C for 15 seconds, and fluorescence was continuously monitored during the transition from 70 to 95°C at a rate of 0.1°C/second. Fluorescence channel F1 (530 nm) was used, and the gain indicated 88.2°C for MHC-2a and 89.9°C for MHC-2b. The amount of each MyHC isoform calculated by the above method was divided by the amount of GAPDH (house-keeping gene) in order to calculate the level of mRNA expression (Maejima et al., 2005; Sakiyama et al., 2005; Shida et al., 2005). The base sequence for GAPDH was as follows: (Forward: 5'- TGAACGGGAAGCTCACTGG-3', Reverse: 5'-TCCACCACCCTGTTGCTGTA-3', Accession: NM_008084). Each PCR fragment was verified as part of a MyHC isoform by ABI PRISM 310 Genetic Analyzer (Perkin- Elmer Japan Applied Biosystem, Tokyo, Japan). Data were treated with the windows SAS System (Version 8.02; SAS Institute Inc., Cary, NC, USA). Student's tukey's HSD test was used for statistical

analysis in this study.

Results

Immunological staining

At the age of 2 weeks, there were many MyHC-2a-positive and MyHC-2b-positive muscle fibers (22.0 ± 2.3 and $26.3 \pm 1.8\%$, respectively) in the anterior belly of the digastric muscle (Figure 3 and 5). In the sternohyoid muscle, there were comparable numbers of MyHC-2a-positive and MyHC-2b-positive muscle fibers (36.2 ± 3.1 and $40.5 \pm 2.9\%$, respectively) (Figure 4 and 6). At the age of 4 weeks, in the anterior belly of the digastric muscle, the ratio of MyHC-2a-positive muscle fibers had decreased to $16.8 \pm 1.9\%$, but the ratio of MyHC-2b-positive muscle fibers had increased to $39.5 \pm 3.5\%$ (Figure 3 and 5). At the age of 4 weeks, the ratio of MyHC-2b-positive muscle fibers was significantly higher than that of MyHC-2a-positive muscle fibers in the anterior belly of the digastric muscle. On the other hand, there was little difference in the ratio of MyHC-2a-positive and MyHC-2b-positive muscle fibers in the sternohyoid muscle between the ages of 2 and 4 weeks (37.8 ± 2.0 and $43.1 \pm 3.5\%$, respectively at the age of 4 weeks) (Figure 3 and 5).

At the age of 9 weeks, very few MyHC-2a-positive muscle fibers were seen in the anterior belly of the digastric muscle in all samples ($8.4 \pm 1.3\%$) (Figure 3 and 5).

However, the ratio of MyHC-2b-positive muscle fibers at the age of 9 weeks was about twice that at the age of 4 weeks ($80.6 \pm 5.1\%$) (Figure 3 and 5), and there was little difference in the ratio of MyHC-2a-positive and MyHC-2b-positive muscle fibers in the sternohyoid muscle between the ages of 4 and 9 weeks (39.9 ± 3.8 and $44.4 \pm 2.8\%$, respectively at the age of 9 weeks) (Figure 4 and 6).

Analysis of mRNA expression using LightCycler™

In the anterior belly of the digastric muscle, mRNA expression of MyHC-2a at the age of 2 weeks was high and subsequently decreased. On the other hand, mRNA expression of MyHC-2b at the age of 2 weeks was initially low but increased at the ages of 4 and 9 weeks (Figure 7 and 8).

In the sternohyoid muscle, there was little change in the mRNA expression of MyHC-2a and -2b at any age (Figure 7 and 8).

Discussion

Among the myosin isoforms belonging to the fast-twitch muscle group, MyHC-2b has been reported to exhibit the fastest contraction, while MyHC-2a has the slowest contraction (Bottinelli et al., 1991). By determining the composition ratio of these two isoforms, it is possible to compare and analyze muscular functions. Since the above report was published, it has been possible to identify functional changes in

greater detail based on the composition ratios of fast-twitch MyHC isoforms. It has been known for some time that the adult murine masseter muscle is composed only of fast-twitch muscle fibers (Doi et al., 2003; Eason et al., 2000; Gojo et al., 2002; Tuxen and Kirkeby, 1990). Gojo et al. (2002) attempted to identify the changes in the composition ratios of these fast-twitch isoforms during the process of growth and development in terms of MyHC-2a and MyHC-2b. They documented that there were marked changes in the composition ratios of MyHC-2a and MyHC-2b before and after weaning, and that these changes were not seen in limb muscles (Usami et al., 2003). Furthermore, Shida et al. (2005) compared the superficial and deep layers of the masseter muscle and documented the functional changes associated with weaning in each layer (Shida et al., 2005). They clarified that the expression of MyHC-2b protein, which has the fastest contraction and strongest contractile force, was increased during weaning, and concluded that a change in mastication caused a functional change in the masseter muscle. Several studies have examined the function of MyHC-2b. Like the murine masseter muscle, the adult rat extensor digitorum longus (EDL) requires high levels of contractile force, and one study showed that the EDL consisted only of MyHC-2b. Furthermore, when the function of the EDL was experimentally reduced by denervation, the expression of MyHC-2a increased, while that of MyHC-2b decreased (Erzen et al., 1999). This suggests that the EDL is composed only of MyHC-2b in adults due to the high contractile force requirement, but when high contractile force is

no longer needed, MyHC-2a is expressed. The above findings show that the contractile force of MyHC-2a is lower than that of MyHC-2b.

Like the masseter muscle, the anterior belly of the digastric muscle has been said to be composed only of fast-twitch muscle fibers. One study found that the different regions of the anterior belly of the digastric muscle possessed different characteristics (Heather et al., 2002). However, to the best of our knowledge, no studies have closely examined the differences in muscle fiber characteristics before and after weaning.

The objective of the present study was to determine the muscle fiber characteristics of the anterior belly of the digastric muscle and the sternohyoid muscle before and after weaning in an attempt to elucidate the functional changes in these muscles. The results of the present study did not show marked differences in the expression of MyHC-2a (immature isoform with slower muscle contraction) and the expression of MyHC-2b (mature isoform with faster muscle contraction) at the age of 2 weeks (before weaning) between the two muscles. In addition, there were no marked differences in MyHC mRNA expression between the two muscles at the age of 2 weeks. However, at the age of 4 weeks, there were marked differences in muscle fiber characteristics between the anterior belly of the digastric muscle and the sternohyoid muscle; the ratio of MyHC-2b in the anterior belly of the digastric muscle increased markedly at the age of 4 weeks, but that in the sternohyoid muscle barely increased.

Furthermore, mRNA expression of MyHC-2b in the anterior belly of the digastric muscle increased markedly at the age of 4 weeks, but that in the sternohyoid muscle barely increased. These findings suggest that, in response to a rapid function change from suckling to mastication, the anterior belly of the digastric muscle underwent changes in order to carry out mouth opening. Although the anatomical alignment of the sternohyoid muscle is the same as that of the anterior belly of the digastric muscle, there were no marked changes before and after weaning, thus suggesting that this muscle is not effect on the functional changes. At the age of 9 weeks, there were few MyHC-2a-positive muscle fibers in the anterior belly of the digastric muscle, and this muscle was mostly composed of MyHC-2b-positive muscle fibers. In addition, the mRNA expression of MyHC-2a was very low. Hence, even when production of MyHC-2a was signaled, little MyHC-2a protein was synthesized due to the functional role of the anterior belly of the digastric muscle following weaning. Because the ratio of MyHC-2b increased after weaning, we deduced that the contractile speed of the anterior belly of the digastric muscle increased after weaning.

The results of the present study suggest that different muscle fiber characteristics are attained in order to carry out target functions for responding to the change in oral function during weaning, while the anatomical alignment of the anterior belly of the digastric muscle and sternohyoid muscle is the same. In other words, like the masticatory muscles, such as the masseter muscle, the anterior belly of the digastric

muscle undergoes functional changes that suit the complexity of masticatory movements.

Acknowledgements

This study was supported by grants-in-aid for scientific research (19592131: Abe S) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, by the Foundation of Japan Medical Association, by Oral Health Science Center Grant HRC7 (Abe S) from Tokyo Dental College, and by a “High-Tech Research Center” Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology) of Japan, 2006-2011.

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Figure 1 Muscle fiber bundles running in the anterior belly of the digastric muscle (A) and sternohyoid muscle (B) of the mouse. The muscle fiber bundles in the anterior belly of the digastric muscle as same as the masseter muscle function in chewing, because the mouse lower jaw moves rapidly in the anteroposterior direction while chewing.

Figure 2 The cells within the hatched black frame in the square section were counted, and the count was divided by the total number of cells in the section^{4, 15, 18, 21}. Cells on the upper transverse line and the left longitudinal line were excluded from counting.

Figure 3 Immunostained image of the anterior belly of the digastric muscle.

An arrow in all figures shows MyHC-2a-positive muscle fibers. Bars: 50 μ m.

Figure 4 Immunostained image of the sternohyoid muscle.

An arrow in all figures shows MyHC-2a-positive muscle fibers. Bars: 50 μ m.

Figure 5 Immunohistochemical staining of the anterior belly of the digastric muscle (mean \pm S.D.). n=5. Statistical analysis: *p<0.05.

Figure 6 Immunohistochemical staining of the sternohyoid muscle (mean \pm S.D.). n=5.

Figure 7 Expression level of MyHC-2a mRNA (mean \pm S.D.). n=5. The amount of each MyHC isoform calculated by the above-mentioned methods was divided by the amount of GAPDH which was one of house keeping genes. Statistical analysis: *p<0.05.

Figure 8 Expression level of MyHC-2b mRNA (mean \pm S.D.). n=5. The amount of each MyHC isoform calculated by the above-mentioned methods was divided by the amount of GAPDH which was one of house keeping genes. Statistical analysis: *p<0.05.

Fig.1

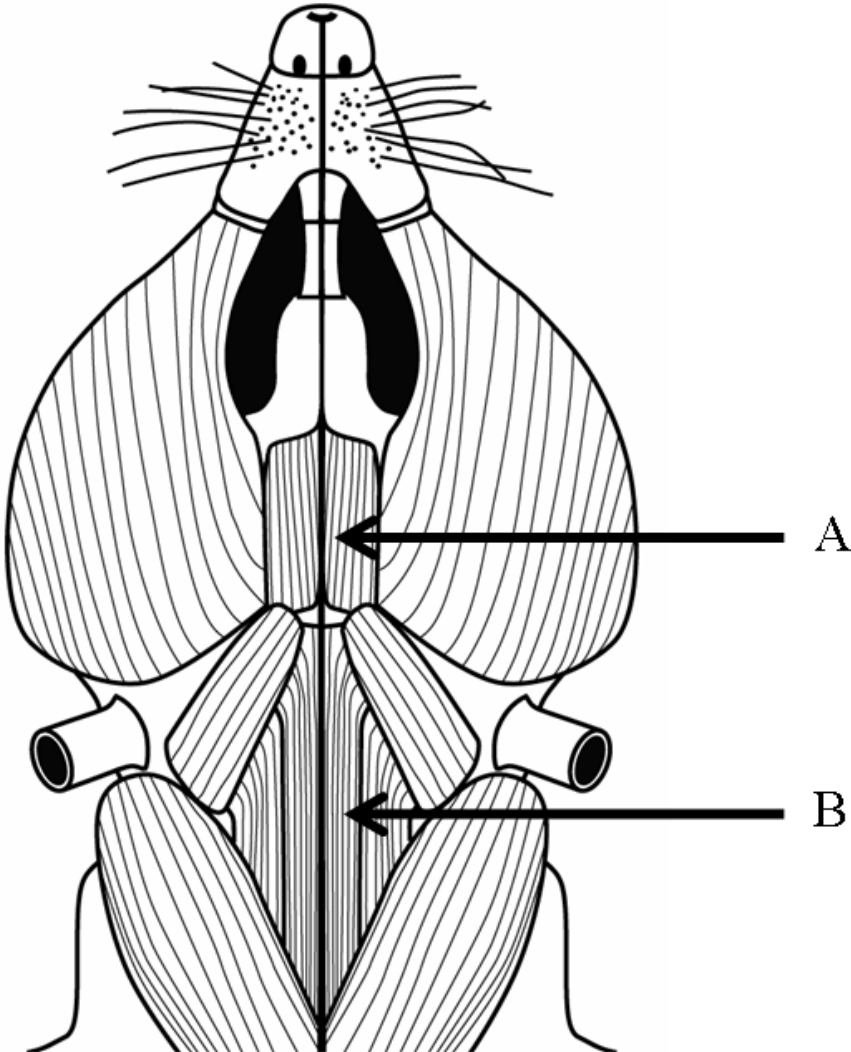


Fig.2

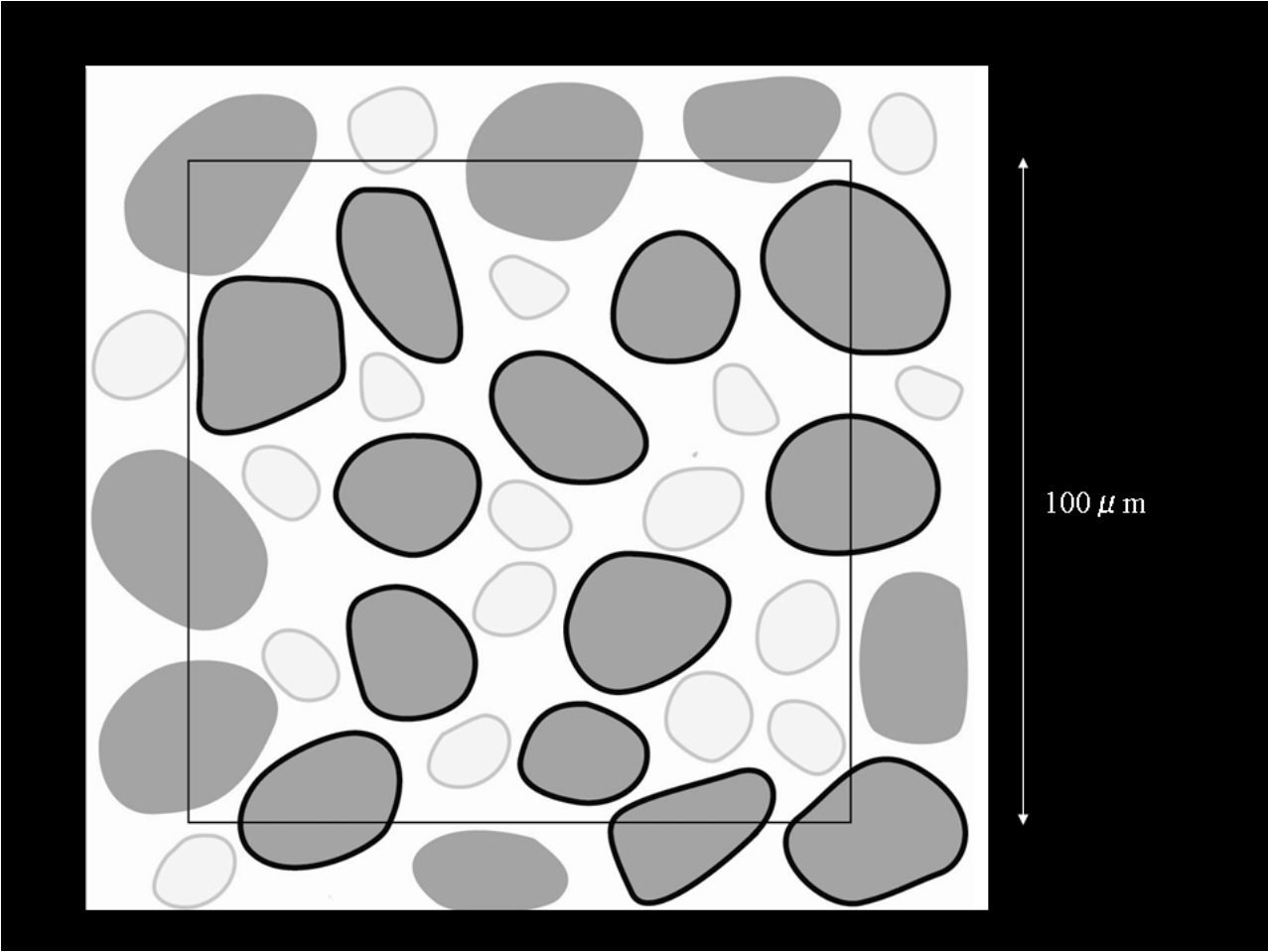
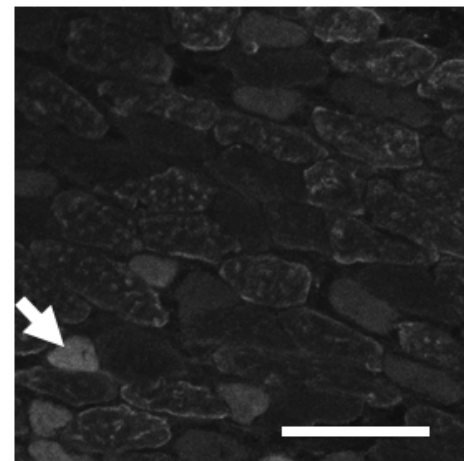
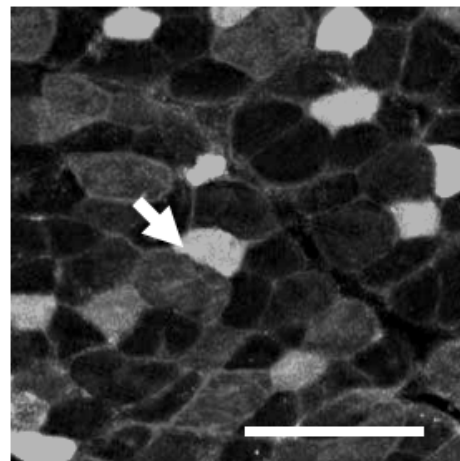
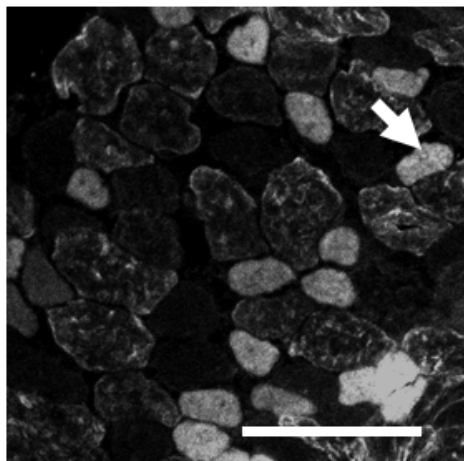
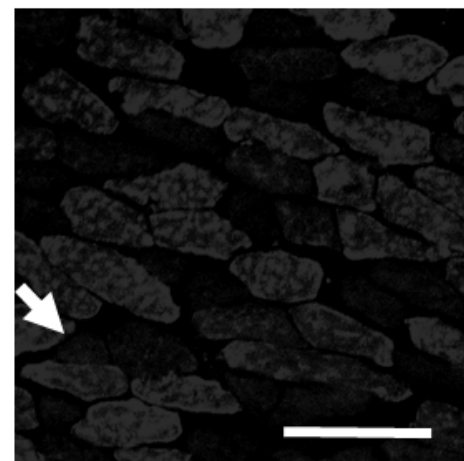
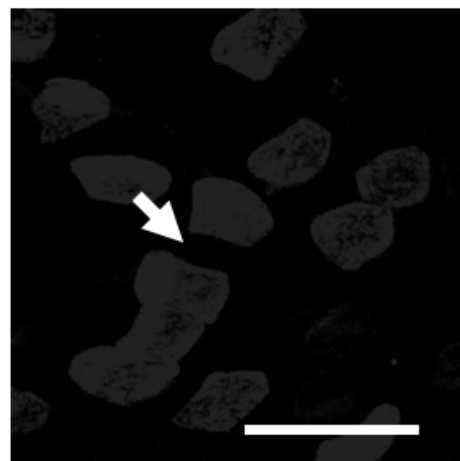
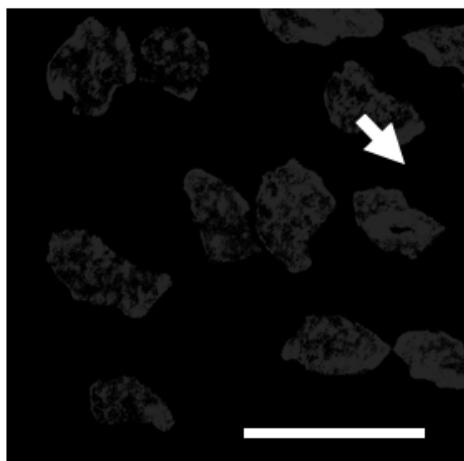


Fig.3

MyHC-2a



MyHC-2b



2 weeks

4 weeks

9 weeks

Fig.4

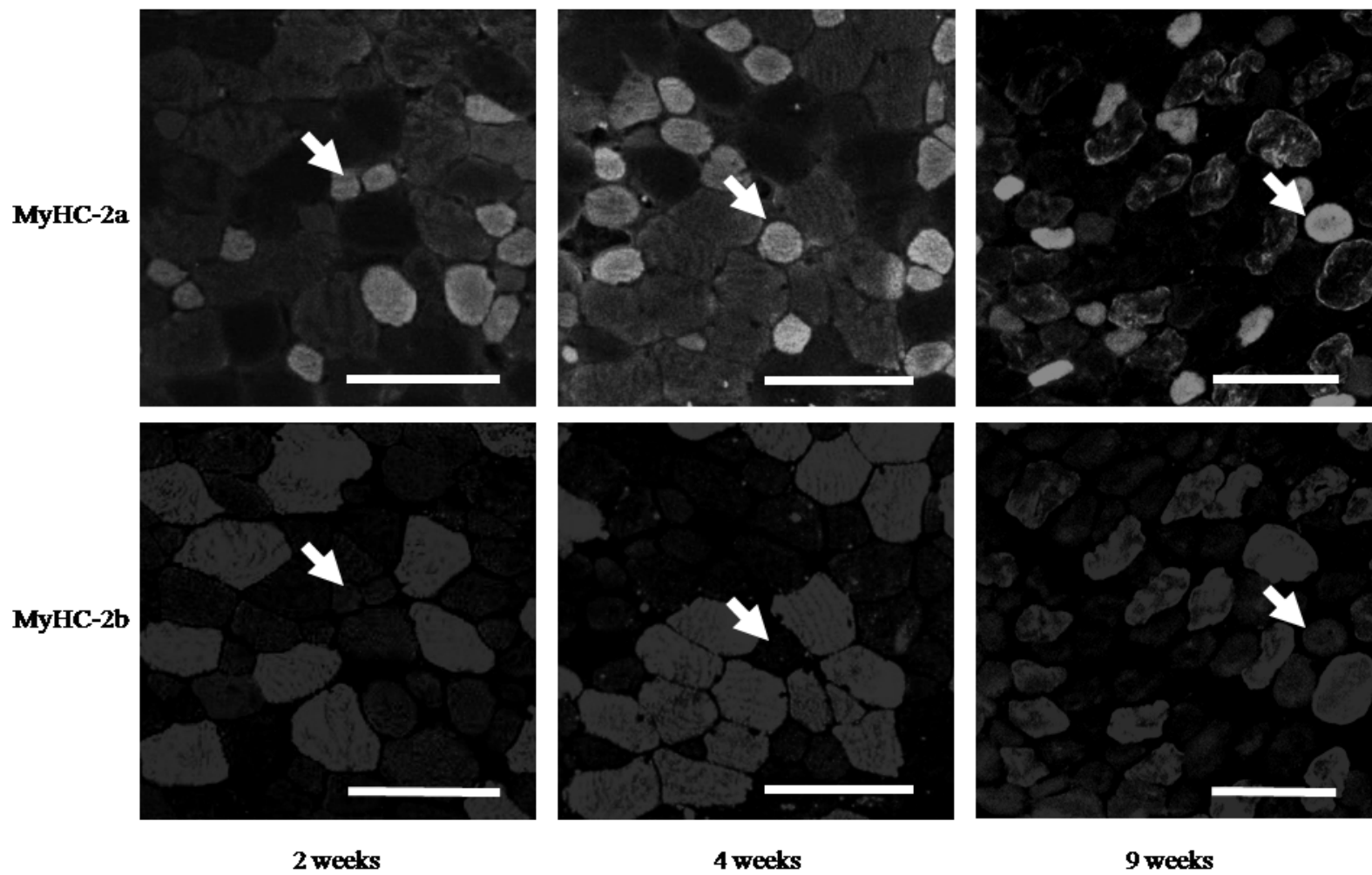


Fig.5

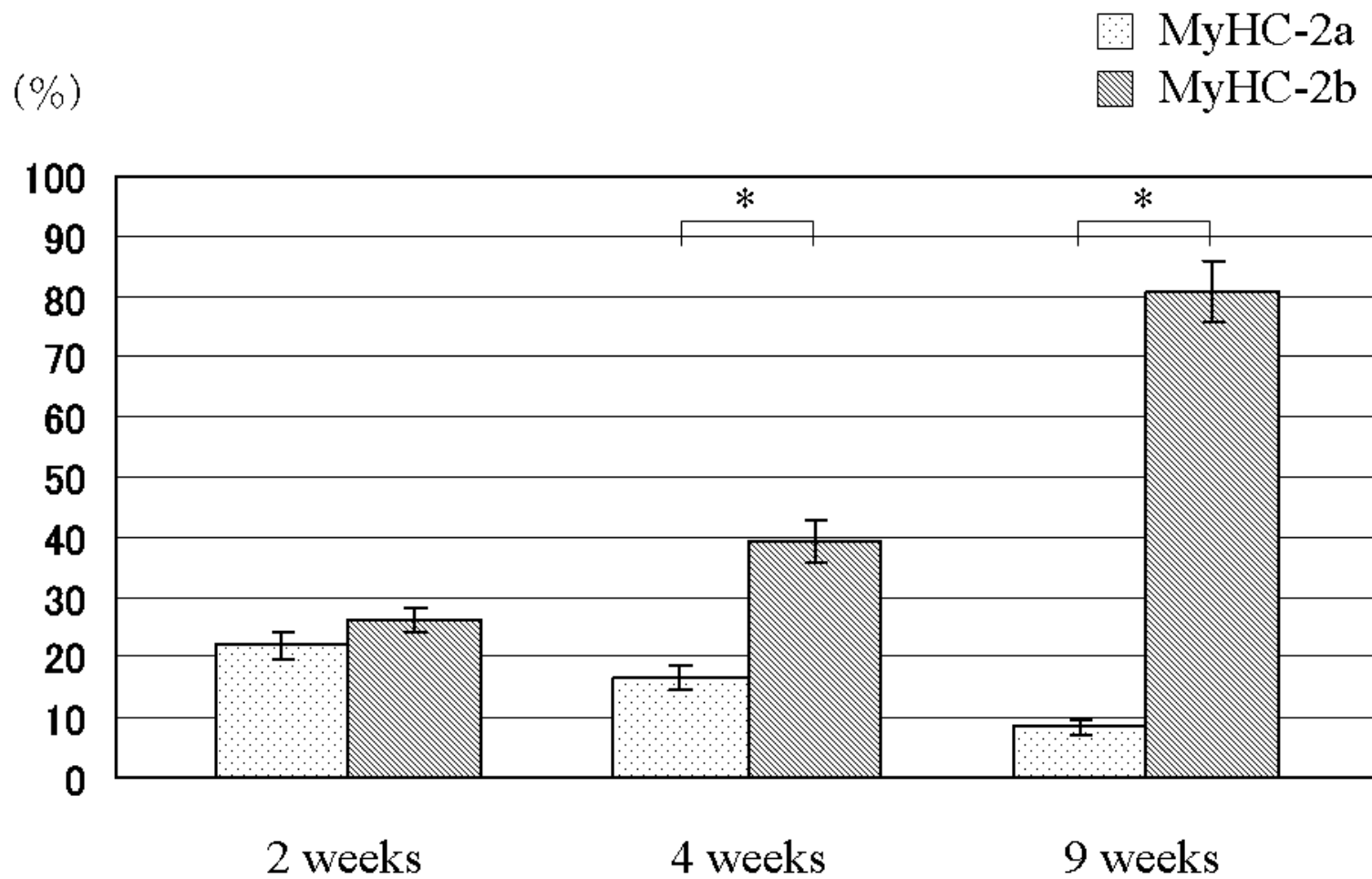


Fig.6

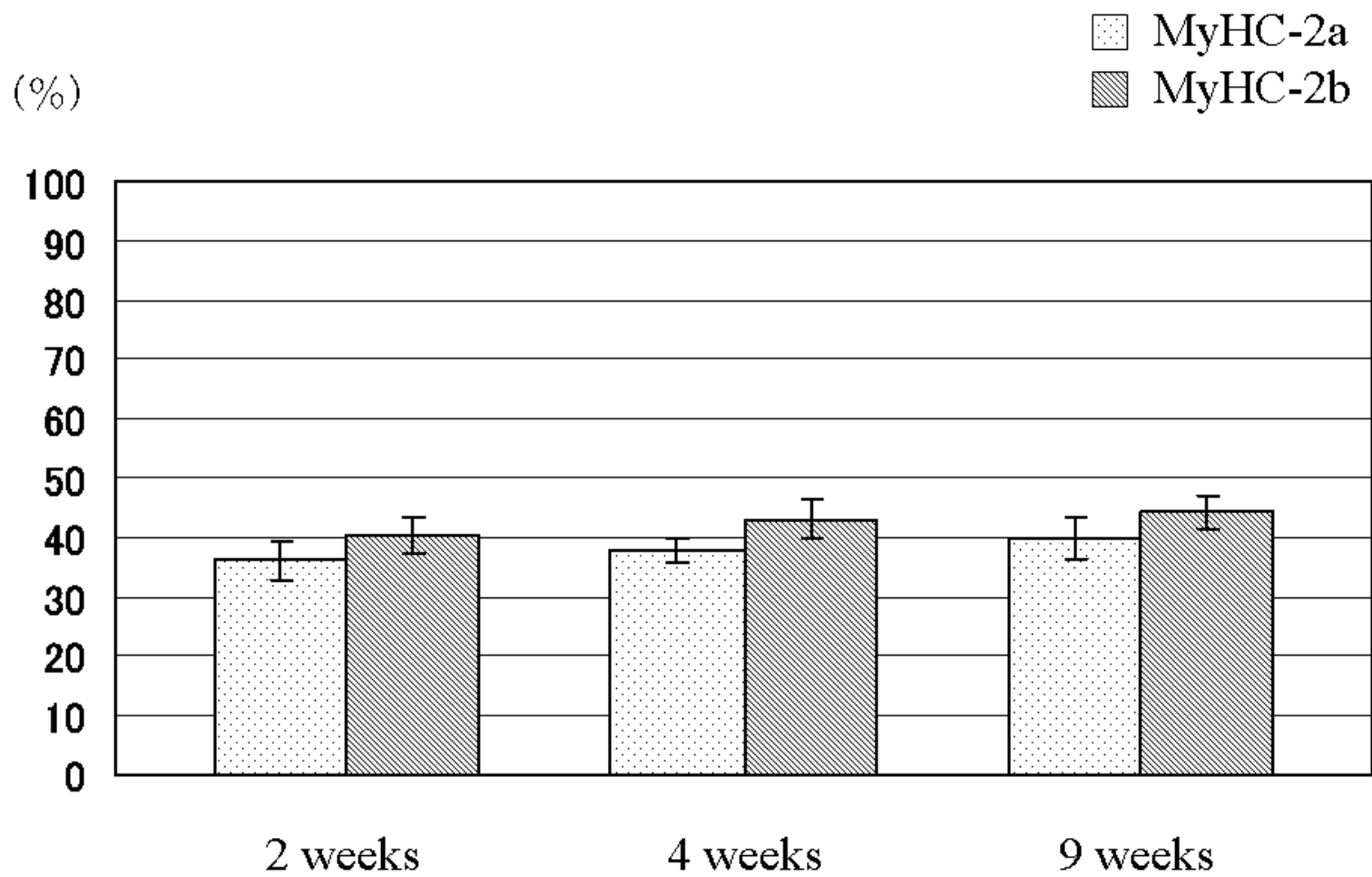


Fig.7

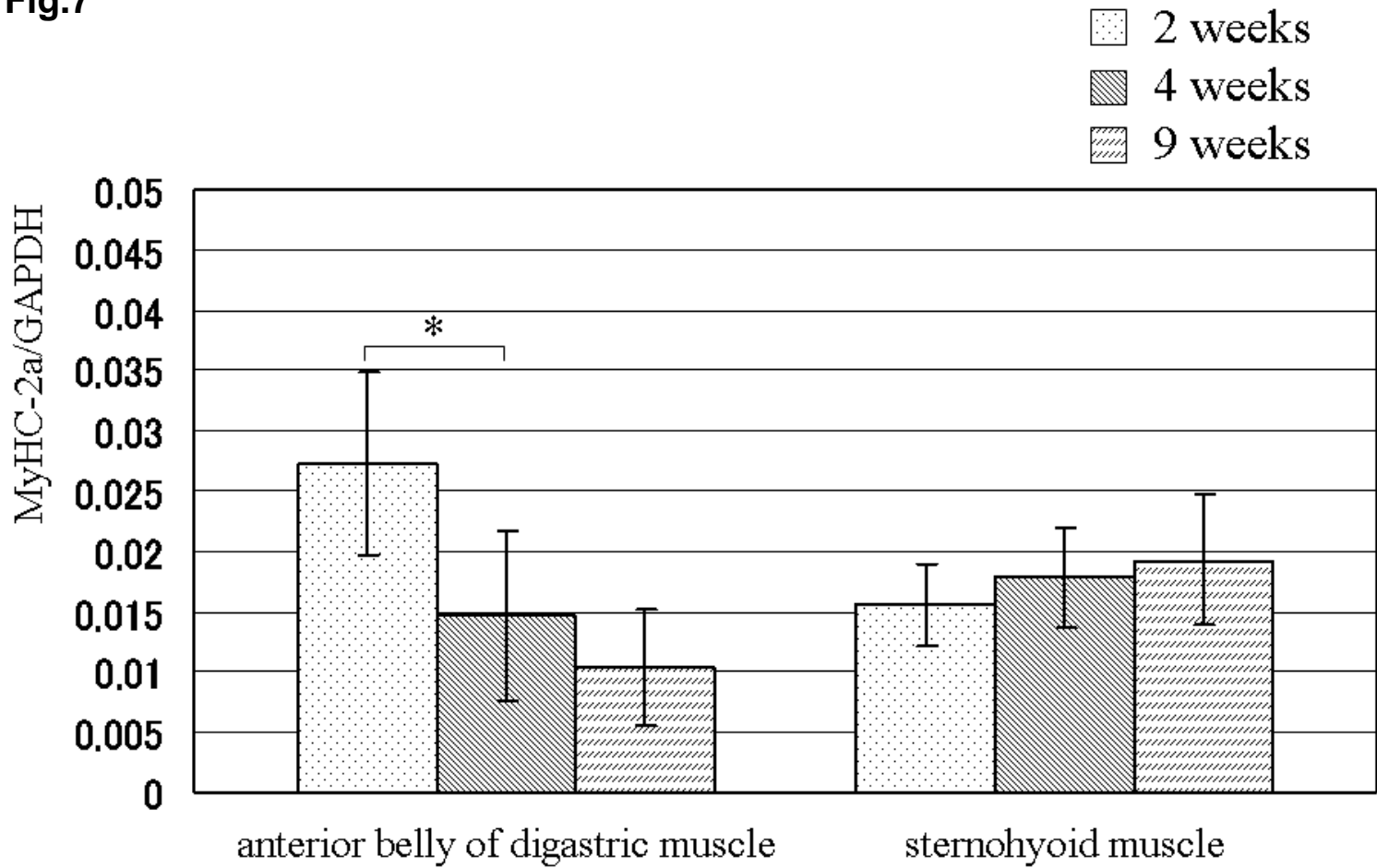


Fig.8

