Macroscopic–microscopic characterization of the passive mechanical properties in rat soleus muscle

Sabine F Bensamoun, L. Stevens, M.J. Fleury, G. Bellon, F. Goubel, M.C. Ho Ba Tho

Elsevier Editorial(tm) for Journal of Biomechanics
Manuscript Draft

Manuscript Number:

Title: MACROSCOPIC-MICROSCOPIC CHARACTERIZATION OF THE PASSIVE MECHANICAL PROPERTIES IN RAT SOLEUS MUSCLE

Article Type: Full Length Article (FLA)

Keywords: Passive stiffness , Morphological properties , S; leus muscle , Skinned isolated fibers , Collage;

Corresponding Author: Dr sabine Fanny bensamoun, PhD, UTC Compiegne

Other Authors: sabine F bensamoun, laurence Stevens, Marie-Joe Fleury, George Bellon, Francis Goubel, Marie-Christine Ho Ba Tho

*** Manuscript**

MACROSCOPIC-MICROSCOPIC CHARACTERIZATION OF THE PASSIVE MECHANICAL PROPERTIES IN RAT SOLEUS MUSCLE

S. Bensamoun [a], L. Stevens [b], M.J Fleury [a], G. Bellon[c], F. Goubel [a], M.C Ho Ba Tho [a]

[a] Laboratoire de Biomécanique et Génie Biomédical, CNRS-UMR 6600, Université de Technologie de Compiègne, BP 20529, F-60205 Compiègne cedex, France

[b] Laboratoire de Plasticité Neuromusculaire, Université des Sciences et Technologies de Lille. 59655 Villeneuve d'Ascq cedex, France

[c] Laboratoire de Biochimie et Biologie Moléculaire, CNRS UMR 6198, IFR 53 iomolécules Université de Reims Champagne-Ardenne, 51 095 cedex, France

Corresponding author:

M.C. Ho Ba Tho
Laboratoire de Biomécanique et Génie Biomédical
CNRS UMR 6600
BP 20529, F-60205 Compiègne cedex, France
Tel: (33) 3 44 23 49 18, Fax: (33) 3 44 20 48 13
Email: hobatho@utc.fr

ABSTRACT

The purpose of the study was to investigate changes in passive mechanical properties of the soleus muscle of the rat during the first year of life. These mechanical changes were quantified at a macroscopic (whole muscle) and a microscopic level (fiber) and were correlated with biochemical and morphological properties.

Three passive mechanical tests (a relaxation test, a ramp stretch test and a stretch release cycle test) with different amplitudes and velocities were performed on isolated soleus muscles and fibers in rats aged 1 (R1), 4 (R4) and 12 (R12) months. Mechanical parameters (dynamic and static forces, stresses and Young modulus) were recorded and measured. The morphological properties (size of fibers and muscles) for the three categories of rats were determined with light microscopy and staining, which allowed us to observe the evolution of the fiber type (I, IIc and IIa) in the belly region and along the longitudinal axis of the muscle. In addition, biochemical analyses were performed at the level of the whole muscle in order to determine the collagen content.

The results of the passive mechanical properties between the macroscopic (muscle) and microscopic (fiber) levels showed a similar evolution. Thus, an increase of the dynamic and static forces appeared between 1 and 4 months while a decrease of the passive tension occurred between 4 and 12 months. These mechanical changes were correlated to the morphological properties. Besides, the size of the three fibers type which grew with age could explain the increase of forces between 1 and 4 months. Furthermore, the biochemical analysis showed an increase of the collagen content during the same period which could also be associated with the increase of the passive forces. After 4 months, the passive tension decreased while the size of the fiber continued to increase. The biochemical analysis showed a decrease of the collagen content after 4 months, which could explain the loss of passive tension in the whole muscle. Concerning the similar loss at the fiber level, other assumptions

are required such as a myofibril loss process and an increase of intermyofibrillar spaces. The originality of this present study was to compare the passive mechanical properties between two different levels of anatomical organization within the soleus muscle of the rat and to explain these mechanical changes in terms of biochemical and morphological properties.

Keywords :

Passive stiffness - Morphological properties - Soleus muscle - Skinned isolated fibers - Collagen

1. INTRODUCTION

The originality of this present work was to assess the passive mechanical properties of the muscular tissue at different scales. According to our knowledge no multiscale measurements were performed in order to quantify the relationship between the macroscopic (whole muscle) and the microscopic (muscle fibers) passive mechanical behaviour of soleus muscle.

At the whole muscle level, Kovanen et al. (1984) is one of the major references related to the evolution of the passive mechanical properties (stress– strain relationship) in function of age. Their study consisted in applying passive mechanical tests until the yield point on soleus muscles (rats) aged of 1, 2, 4, 10 and 24 months. Two categories of rats were used: trained and untrained rats. The passive mechanical results showed for the trained rats an increase of the mechanical strength until 10 months followed by a tendency to rise during the remaining period. As to the untrained rats, the passive mechanical properties (ultimate tensile strength) showed an increase between 1 and 2 months while a decrease occurs after 2 months.

The passive elastic component (source of the stiffness) is mainly composed of collagen substances located in epimysium, perimysium, endomysium and sarcolemma. Besides, there are different types of collagen (type I, III, IV and V) which are differently distributed within the muscle and which intervene in the passive strength. Many studies (Kovanen et al. 1984, Kovanen and Suominen, 1987, 1989, Alnaqeed et al., 1984, Ducomps et al., 2003) analysed the role of collagen in the mechanical and physiological properties of the muscle. These studies led to the conclusion that the passive mechanical properties of muscle are dependent on the content of collagen.

In addition to the connective tissue, titin filaments are a source of passive tension at the muscle fiber level. The passive mechanical properties of titin were characterised by different passive mechanical tests on isolated fibers (Anderson et al., 2002, Colomo et al., 1997, Joumaa et al., 2002, Mutungi et al., 1996). Moreover, the stiffness of the muscle fibers is due to an interplay between endosarcomeric and exosarcomeric protein networks (Bartoo et al., 1997, Wang et al., 1993). For instance the desmin network adds passive resistance to stretch at high sarcomere lengths (Salviati et al., 1990). A recent study (Toursel et al., 2002) observed changes in passive elastic properties of rat soleus muscle fibers subjected to hindlimb unloading conditions. It was concluded that this experimental condition did not change titin isoform expression in the soleus muscle but rather indicated a loss of titin content which decreased the stiffness of the muscle. Moreover, Horowitz et al. (1986) concluded that the developed passive forces were related proportionally to the content of titin.

The purpose of this study was to measure the passive mechanical properties of single slow fibers and muscles (soleus) in 1, 4 and 12 months old rats. In addition biochemical (content of collagen) and morphological (fiber characteristics) analyses were performed in order to correlate the mechanical and morphological properties at each level of anatomical organisation. Furthermore, a comparison between the two scales will be made in order to see how the microscopic level influences the macroscopic one.

2. MATERIALS AND METHODS

2.1 Animals

Male Wistar rats aged 1month (R1) (n = 16, average body weight = 104 ± 4g), 4 months (R4) (n = 16, average weight = 395 ± 8g) and 12 months (R12) (n = 10, average weight = 631 ± 46g) lived in a controlled environment, a 12h light and 12h dark cycle, and at constant temperature (23°C). They were housed in 42×42×18 cm cages grouped by age (3-4 rats per cage). Food (Teklab global 18% protein rodent diet, Harlan) and water were provided ad libitum.

2.2 Muscles and fibers preparation

The rats were anaesthetised with an intraperitoneal injection of pentobarbital sodium (30mg/kg) and the soleus muscles were removed from the right and left hindlimbs, weighted and subjected to the following preparation procedures.

The soleus muscles from the right hindlimb were fastened at the proximal and distal extremities of the muscle with a thread separating the muscle from its tendon. The length of the muscle was measured in situ with a micrometric tools by placing the knee and ankle joints at 90°. Then the removed muscles were mechanically tested.

The soleus muscle from the other hindlimb was removed and cut longitudinally into different strips. After this, the rats were killed with a lethal intraperitoneal injection of pentobarbital sodium. The muscle strips were then chemically skinned in an EGTA skinning solution (2.5mM ATP, 20mM MOPS, 170mM potassium propionate, 2.5mM magnesium acetate, 5mM K_2EGTA, pH = 7.0) for 28h at 4°C and stored at –18°C in a 50-50% glycerol skinning solution for one week.

Under a binocular microscope, strips from the different-age muscles (four muscles per age group) were dissected into isolated fibers (1-3mm length). Approximately 5 fibers per muscle were extracted. A total of 19, 18 and 24 fibers were dissected from the group of 1month, 4 months and 12 months, respectively.

Solutions. All reagents were obtained from Sigma (St Louis, U.S.A.). The composition of all solutions was calculated by the Fabiato computer program (10), with a final ionic strength of 200 mM. pH was adjusted to 7.0 and ATP (2.5 mM) was added to each solution. The skinning solution was made up of (mM) MOPS, 10 ; K Propionate, 170 ; Mg Acetate, 2.5 ; and K_2 EGTA, 5. The following solutions were used for the experimental procedure: a washing solution (W) composed of (mM) MOPS, 10 ; K Propionate, 185 ; and Mg Acetate, 2.5 ; a relaxing solution (R) identical to the skinning solution ; pCa or pSr activating solutions consisting of W solution with various concentrations of free Ca^{2+} or Sr^{2+} from $CaCO_3$ or $SrCl_2$, respectively, buffered with EGTA and added in proportion to obtain the different pCa values (5.0 and 4.2) or pSr values (5.0 and 3.4).

Furthermore the titin is very sensitive to proteolysis (Neagoe et al. 2000); thus, to avoid titin degradation, the skinning, the storing and the mechanical experimenting were done with protease inhibitors (leupeptin 20µg/ml, aprotinin 5µg/ml and pepstatin 1µg/ml).

2.3 Experimental set up

The soleus muscle was mounted horizontally in a chamber containing a buffered oxygenated physiological salt solution (95% O_2, 5% CO_2) maintained at pH 7.3 and constant temperature of 25±1°C. The distal extremity of the muscle was connected to a force transducer (2.5N, frequency resonance 1kHz) while the proximal extremity was connected to a displacement transducer (EX58 PRODERA, France) linked to the moving part of a servo-controlled

ergometer (figure 1). The displacement of the transducer and the developed forces of the muscle under stresses were visualized and recorded through an oscilloscope (Hewlett Packard 54645 100MHz) and computer respectively.

The single fiber segment (1-3mm length) was mounted under the binocular microscope in an experimental chamber (2cm x 1cm) containing the relaxing solution and maintained at 19 ± 1°C. The fiber was glued with cellulose polyacetate dissolved in acetone between the hook of a force transducer (AE 801, AME, Horten, Norway, frequency resonance 4,52kHz) and the arm of a feedback-controlled stepping motor (Cambridge Technology, model 6350, USA) (figure 2). Tension and motor position were recorded on a graph recorder (Gould, model, Windograph).

The different steps allowing to measure the passive mechanical properties of the fibers are the following:

2.3.1 Measurement of the slack length

The slack length (Ls), which is defined as the muscle or fiber length when the beginning of the resting tension is developed, was measured before applying the passive mechanical tests.

The slack length of the muscle was manually measured with a graduated micrometric screw.

As to the fiber, the slack length (Ls) and the diameter of the fiber segments were measured with a micrometer (magnification ×60) through a binocular microscope. Then, the slack sarcomere length (SLs) was measured by means of a Helium/Neon laser beam (Spectra Physics) directed perpendicular to the long axis of the fiber. The fibers were then stretched to 120% of their slack length before starting the passive mechanical tests.

2.3.2 Identification of the slow fibers

The following technique was only used to select the slow fibers and did not aim at classifying the different fiber types.

The criterion for functional fiber identification was based on the difference in Ca^{2+} and Sr^{2+} activation characteristics between slow and fast fibers. It is well known that fast muscle fibers are less sensitive to Sr^{2+} than slow ones (Stephenson et al. 1981, Takagi et al. 1977). As previously described (Stevens et al. 1993), the following protocol was performed: first, the fiber was bathed in a pCa4.2 (pCa=- log [Ca^{2+}]) solution which induced maximal active tension. Then, the fiber was activated submaximally with a pCa5.0 solution followed by pCa4.2 solution. Now, the same solutions, but containing strontium ions instead of Ca^{2+}, were infused: pSr5.0 and pSr3.4 (inducing maximal Sr^{2+} tension). Fibers with a pSr5.0/pSr3.4 ratio close to 95% of the pCa5.0/pCa4.2 ratio were identified as slow fibers, whereas fibers with a 0-10% pSr5.0/pSr3.4 ratio were identified as fast ones. In this study only the soleus fibers classified as “slow” were mechanically tested.

2.3.3 Passive mechanical tests

Three different mechanical tests of passive tension were performed according to Anderson et al. (2002).

- Relaxation test

The muscles were stretched with a velocity of 600mm/s for the rats aged of 4 (R4) and 12 (R12) months and 250mm/s for the rats aged of 1 (R1) month. As to the fibers, the three different groups were stretched to 150%Ls with a velocity of 10Ls/s. The length of the stretch applied to muscles and fibers was maintained respectively for 180s and 60s before releasing them back to their slack length. This relaxation test enabled us to measure the dynamic force (Fd) which corresponded to the maximal force developed by the muscle or fiber and the relaxed (Fs) force at the end of the 180s or 60s (figure 3a). Then, Fd and Fs are divided by the

cross-sectional area, which yields to the expression of the dynamic (σd) and the relaxed (σs) tension, respectively. The cross section of the fiber was assumed to be circular and was directly measured under the binocular microscope (x80). The muscle cross section was calculated thanks to the equation (1) (Ranatunga et al., 1982):

$$S = \frac{m}{0.72 \times L_m} \quad \text{Equation (1)}$$

S: section of the muscle (mm), m: weight (g) of the muscle, L_m length of the fiber (mm) which corresponds to 72% of the total length of the muscle.

- Ramp stretch

The second test stretched the muscles and fibers with a slow velocity of 1mm/s and 0.005Ls/s, respectively and with an amplitude up to 120% (R1, R4 and R12 were stretched at 3mm, 5mm and 6mm) for the muscles and 150%Ls for the fibers. At this maximal amplitude the developed force (Fs) and tension (σs) were measured and the muscles and fibers were released with the same velocity to their slack lengths (figure 3b). This test enabled to calculate for both materials the Young's modulus as developed force (Fs) divided by the cross-sectional area.

- Stretch Release cycle

The third test was a repeated stretch applied to muscles and fibers with an amplitude of 105%Ls and 110%Ls respectively, followed by a hold period of 60s in both cases; at the end of the fourth and fifth cycles for the muscles and fibers respectively, they were relaxed to their slack lengths and the relaxed force (Fs) and tension (σs) were measured (figure 3c).

2.4 Light microscopy

When the passive mechanical test performed on isolated muscle was finished, the muscle was frozen in liquid nitrogen and stored at –80°C. Eight muscles by age (R1, R4 and R12) were analyzed in order to study their morphological properties. Each muscle was subdivided into

five regions located near the distal, belly and proximal areas of the muscle (figure 4). Then, the muscles were cut transversally along the longitudinal axis in 10µm sections with a cryostat (jung frogocut 2800E, Leica Instrument, Germany). The sections were stained with the method of Brooke and Kaiser (1970) at pH=4.3 in order to see the ATPase activity and to identify the slow (type I), intermediary (IIC) and fast fibers (types IIA). The glass slides which contained the muscle sections were disposed on a motorized support under an optical microscope in transmission. Then, a software (QWIN Leica) controlled the displacement of the support which was translated in the X and Y directions with an accuracy of 2mm. This process enabled to obtain a total reconstruction of the entire section of the muscle in each region. According to the localization of the regions and age of the muscle, the reconstruction could be composed of 25 to 90 images (pixel surface= 0.99µm^2). Furthermore, thanks to an edge detection of the grey level, the three different types of fibers it were automatically detected (figure 5). The morphological parameters measured through this technique were the average surface of the fibers (I, IIC and IIA) and muscles. Moreover, the evolution of the fiber type with age and within the five regions of the muscle was analyzed. The calibration of the optical microscope in transmission was effected with a Thomas cell.

2.5 Biochemical analyses

Soleus muscles, frozen after the passive mechanical tests, were subjected to a collagen analysis focusing on the determination of the hydroxyprolin content. Besides, hydroxyprolin is composed of the different types of collagen which can not be dissolved due to the strong cross-linked nature of collagen and is therefore related to the mechanical properties (Kovanen et al., 1984). A total of 12, 13 and 7 soleus muscles were lyophilized 24h under a vacuum from the 1, 4, and 12 month groups respectively. This method enabled to obtain 20mg of lyophilized tissue for each sample, which was added to 3ml of HCL 6N. Then it was

hydrolysed for 24h at 110°C after centrifugation. A calorimetric method (Szymanowicz and Laurain, 1981) allowed to assess hydroxyprolin content contained in each muscle (R1, R4 and R12). The content of collagen was then calculated with the content of hydroxyprolin multiplied by the factor 7.25 (Woessner 1961).

2.6 Statistical analysis

The results on fibers and muscles were analyzed statistically using a Kruskall-Wallis and ANOVA tests respectively with the software Statgraphics 5.0 (Sigma Plus) in order to study the variations between the three experimental groups (table 3 and table 4).

3. RESULTS

3.1 Passive mechanical properties of isolated muscles and fibers

Table1 and table 2 summarize all the different parameters recorded during the mechanical tests where the isolated muscles and fibers are in dynamic and static conditions.

The dynamic and static forces of isolated muscles and fibers increased significantly ($P < 0.001$) between the groups of 1 and 4 months. In the muscle, a significant ($P < 0.001$) decrease of these forces was observed between 4 and 12 months while relatively small differences were found between the groups of 4 and 12 months for isolated fibers.

The dynamic and static tensions (normalised forces) and Young's moduli developed by the muscles and fibers of rats from 1 and 4 months exhibited values which were in the same range. The developed tension in the 12 months group decreased significantly ($P < 0.001$) compared to that of the two other groups for both levels (muscle and fiber).

3.2 Morphological properties

3.2.1 Evolution of the muscle and fibers types surfaces with age in the belly region

In the belly region, the total surface of the muscle increased significantly ($P < 0.001$) with age. As to the surface fibers, the slow ones increased significantly ($P < 0.001$) (factor 2) between 1 and 4 months and continued to significantly ($P < 0.001$) increase (factor 1.5) between 4 and 12 months (figure 6). These results follow the same evolution of the fibers diameters measurements found on isolated slow fibers (figure 7). The surfaces of the fast fibers (type IIA) follow the same evolution as the slow fibers compared to the type IIC which also significantly increased ($P < 0.001$) (factor 2.5) between 1 and 4 months, but reached to a constant value between 4 and 12 months.

3.2.2 Changes of fibers types in the belly region and along the soleus muscle (R1, R4 and R12)

The total surface of the belly region was composed of a total number of fibers for the three groups of muscles R1, R4 and R12 of 1901 ± 163, 1906 ± 200 and 2115 ± 111 respectively. A significant increase of the total fibers number occured between R1 and R12 ($P < 0.001$) and between R4 and R12 ($P < 0.05$). Furthermore, an increase of the slow fibers (type I) percentage (13%) with age and a decrease of the fast fibers percentage (10% for type IIA and 3% for type IIC) was observed (figure 8).

Along the longitudinal axis, from the distal to the proximal region of each muscle, (R1, R4 and R12) no variation of fiber types was found (I, IIA and IIC) for the soleus muscles R12 and R4 compared to group R1 which presented a significant increase in the slow fibers and a decrease in the fast fibers from the belly region to the other regions (figure 8).

3.3 Evolution of the collagen content and hydroxyprolin with age

The hydroxyprolin content calculated for 1mg of weight dry decreased significantly ($P < 0.05$, $P < 0.001$) with the increase of age. The average values with standard deviation (SD) of the hydroxyprolin content for soleus muscle age 1 (R1), 4 (R4) and 12 (R12) months are 0.49 ± 0.19µg, 0.30 ± 0.15µg and 0.14 ± 0.08µg, respectively. The reproducibility which was tested on soleus muscles aged of 12 month was 4%. The collagen content calculated from hydroxyprolin content increase significantly ($P < 0.001$) from R1 to R4 and then decreased from R4 to R12 month (figure 9).

4. DISCUSSION

4.1 Comparison with the literature

In this present study, three different passive tests (relaxation test, ramp stretch, stretch release cycle) were performed with different velocities on fibers (10Ls/s and 0.005Ls/s) and muscles (600mm/s and 250mm/s). The response of the skinned fibers and isolated soleus muscles to the passive mechanical tests exhibited a viscoelastic behaviour which is in agreement with other studies as well on soleus muscle fibers from rabbits (Bartoo et al. 1997, Wang et al. 1993) and rats (Mutungi and Ranatunga, 1996) as on isolated soleus muscle of mice (Anderson et al., 2001).

At the microscopic level (skinned fiber), when we examined the forces for a given group of age, our mechanical results were in agreement with those of the literature. A study by Toursel et al. (2002) indicated a Young's modulus of 93.4 ± 2.17 kN/m^2 for the control group composed of soleus fibers from rats aged 4 months compared to 103 ± 53 kN/m^2 of the present study. The values of dynamic and static tensions obtained by Anderson et al. (2002) were 103 ± 10 kN/m^2 and 38 ± 3 kN/m^2, respectively, for soleus fibers of 5 month old mice compared to 124 ± 32 kN/m^2 and 35 ± 15 kN/m^2, respectively, of this study.

At the macroscopic level (isolated muscle), the passive mechanical properties in function of age were mainly studied by Kovanen et al., 1984 who performed passive mechanical tests (ramp stretch with velocity of 1mm/min) on soleus (rat) aged of 1, 2, 4, 10 and 24 months. Kovanen's study found an increase of the resting tension between 1 and 4 months ($0,2N/mm^2$) and then a decrease between 4 and 12 months. Our study which used two additional passive mechanical tests (relaxation test, and stretch release) showed the same resting tension evolution as in this previous work.

4.2 Mechanical properties of muscles and fibers between 1 and 4 months

The dynamic and static forces developed by the skinned fibers and muscles of 4 month old rats were significantly higher than the forces developed by those of 1 month. These passive mechanical properties were correlated with the biochemical and morphological properties performed at the macroscopic (muscle) and microscopic (fiber) levels.

4.2.1 Biochemical analyses

As to the collagen, the study of Kovanen and Suominen (1989) showed a strong enzymatic activity ("PH: Prolyl-4-hydrolase" and "GGT:Galactosylhydroxylysyl Glucosyl Transferase") at the age of 1 month (soleus) indicating the biosynthesis of the collagen. This enzymatic activity decreases strongly at 2 months indicating the cross-linked of collagen. Our study showed an increase of the passive mechanical properties of whole muscles between 1 and 4 months, which at the same time showed an increase in the collagen content. These two phenomena were strongly linked and according to the study of Kovanen and Suominen (1989) the degree of cross-linked collagen fibers were increased during this period of growth. Other studies (Ducomps et al., 2003) observed the evolution of hydroxyprolin content with age. Our study showed a significant decrease of hydroxyprolin content with age. This result is in agreement with Kovanen and Suominen, 1989 who also found a strong decrease of hydroxyprolin content between 2 and 10 months on soleus (rat). The same observation was also made by Ducomps et al., 2003 who obtained the same decrease of hydroxyprolin content in the rabbit (Semimembranous Proprius) aged of 50, 90 and 140 days. On the other hand, these increases in passive force cannot be related to changes in the isoform of titin and secondly not due to the content of titin which have been shown to remain unaltered during the first year of life (Bensamoun et al., 2004).

4.2.2 Evolution of the fiber surface

The increase of forces between 1 and 4 months is related to the growth of the muscles (Baldwin 1984, Goldspink 1970), during which the proliferation of the myofibrils and hypertrophy of the muscle occurs. Thus, our study showed an increase of the fiber surface (factor of 2 between 1 to 120 days) with age. This result is in agreement with Maltin et al., 1989 who found between the period from 0 to 76 days an increase (factor 3) of the surface type I. As to, the fast fibers type IIA, they were formed with bigger surfaces since 1 month, compared to other types fibers (I and IIA) and this result was also shown by Maltin et al., 1989. Our morphological analyses showed a large increase of the surface of type IIA fibers, with a factor 2 between 1 month (surface = 993 ± 218μm^2) and 4 months (surface = 2253 ± 441μm^2), which continued to increase until 12 months (surface = 2989 ± 1208μm^2). The same observation was obtained in the study of Maltin et al., 1989.

To conclude on the evolution of the fiber type surface with age, our study showed that the surface of the fiber type I, IIA and IIC was in the same range of values at 4 months, suggesting a transition of the morphological properties at this age.

To our knowledge, the longitudinal variation of the fiber type within the muscle was studied by few authors. Punkt et al., 1998 studied the evolution along the length of the muscle aged of 18 months and found a significant increase (30%) of type I from the proximal to the distal region. Our study also showed a significant increase of type I from the belly to the proximal and distal region but only for muscles aged of 1 month.

4.2.3 Evolution of the total number of fibers

The increase of forces between 1 and 4 months is not related to an increase of fiber number during this period. Thus, our study measured the total number of fibers (about 1900 fibers) in the entire belly section of the muscle, which remained constant between R1 and R4. This

result is in agreement with the study of Timson and Dudenhoeffer (1990) who concluded no evolution of the fibers number for rats aged of 1, 6 (about 2672 fibers) and 12 months. However, our study showed a significant increase of the total number of fibers at 12 months.
It should be noted that our total number of fibers was less that the findings of Timson and Dudenhoeffer (1990). This may be due to different counting techniques.

4.3 Mechanical properties of muscles and fibers between 4 and 12 months

The dynamic and static tensions developed by the skinned fibers and muscles at 12 months were statistically significantly lower than the forces developed by those of 4 months.

At the fiber level, the decrease in the resting tension (about 50% with regard to 4 months) of the skinned fiber aged of 12 months could indicate the beginning of a myofibril loss process reported by Ansved and Edström (1991) for moderately old rats (21-25 months). More precisely these authors studied the effects of age (5-6 months and 21-25 months) on fiber structure and ultrastructure in fast and slow twitch rat muscles. In fact, their observations based on light microscopy showed a loss of myofilament in the central region of the old rat fiber with a slight increase in the size of intermyofibrillar spaces. According to these authors, these modifications are similar to the process of denervation which implies a loss of myofibril at the periphery of the fibers. However, an atrophy of the myofibrils with an increase of the inter myofibrilar space could be also an explanation of the decrease in resistance to stretch.
At the muscle level, the decrease of the resting tension (about 50% with regard to 4 months) of the muscles aged of 12 months may be due to their sedentary life, which acts as well on the collagen quality as well as on the collagen content, which decreases with age.
This decrease of the resting tension could also be due to the same phenomena of myofibril loss occurring at the microscopic level, which will be amplified at the macroscopic level.

Conclusion

The originality of this study was to compare the passive mechanical properties of skeletal tissue at the macroscopic and microscopic level. This work allows to conclude a parallel behaviour between the muscle and the fiber from the soleus (rat).

Thus, a similar significant increase of the dynamic and static forces between 1 and 4 months was obtained. These increases in forces were related to the muscle growth (increase of the fiber surface) and to the increase of the collagen content. After 4 months, the resting tension decreases significantly for both levels due to the intrinsic structure of the fiber (myofilament, intermyofibrillar spaces) and muscle (collagen content), which will have different effects on the passive mechanical properties.

ACKNOWLEDGEMENTS

We thank Dr. Ringleb, Ph.D, from the Orthopedic Biomechanics Laboratory from Mayo Clinic College of Medicine, for critical reading of this paper.

5. REFERENCES

Alnaqeed, M.A., Zaid, N.S., Goldspink, G., 1984. Connective tissue changes and physical properties of developing and ageing skeletal muscle. J. Anat. 139, 677-689.

Anderson, J., Zhenlin, L., Goubel, F., 2001. Passive stiffness is increased in soleus muscle of desmin knockout mouse. Muscle & Nerve 24, 1090-1092.

Anderson, J., Joumaa, V., Stevens, L., Neagoe, C., Li, Z., Mounier, Y., Linke, W.A., Goubel, F., 2002. Passive stiffness changes in soleus muscles from desmin knockout mice are not due to titin modifications. Pflügers Archiv 444, 771-776.

Ansved, T. and Edström, L., 1991. Effects of age on fiber structure, ultrastructure and expression of desmin and spectrin in fast- and slow-twich rat muscles. J. Anat. 174, 61-79.

Bartoo, M.L., Linke, W.A., Pollack, G.H., 1997. Basis of passive tension and stiffness in isolated rabbit myofibrils. Am. J. Physiol. 273, 266-276.

Baldwin, K.M., 1984. Muscle development: Neonatal to adult. Exerc. Sport. Sci. Rev. 12, 1-9.

Bensamoun, S., 2003. Détermination des propriétés mécaniques et morphologiques du tissu musculo squelettique. PhD.thesis, University of technology of Compiègne, Press University

Brooke, M.H. and Kaiser, K.K., 1970. Muscle fiber types: how many and what kind? Arch. Neurol. 23, 369-379.

Colomo, F., Piroddi, N., Poggesi, C., Kronnie, G., Tesi, C. 1997. Active and passive forces of isolated myofibrils from cardiac and fast skeletal muscle of the frog. J Physiol 500: 535-548.

Ducomps, C., Mauriège, P., Darche, B., Combes, S., Lebas, F., Doutreloux, J.P., 2003. Effects of jump training on passive mechanical stress and stiffness in rabbit skeletal muscle: role of collagen. Acta. Physiol. Scand. 178, 215-224.

Goldspink, G., 1970. The proliferation of myofibrils during muscle fibre growth.
J. Cell. Sci. 6, 593-603.

Horowitz, R., Kempner, E.S., Bisher M.E., Podolsky, R.J., 1986. A physiological role for titin and nebulin in skeletal muscle. Nature 323, 160-164.

Joumaa, V., Anderson, J., Stevens, L., Mounier, Y., Goubel, F. 2002. Resting tensions in slow and fast soleus muscle fibers. XXVII Congrès de la Société de la Biomécanique, Valenciennes p.17.

Kovanen, V., Suominen, H., Heikkinen, E., 1984a. Collagen in slow twitch and fast twitch muscle fibres in different types of rat skeletal muscle. Eur. J. App. Physiol. 52, 235-242.

Kovanen, V., Suominen, H., Heikkinen, E., 1984b. Mechanical properties of fast and slow skeletal muscle with special reference to collagen and endurance training.
J. Biomechanics 17, 725-735.

Kovanen, V. and Suominen, H., 1987. Effects of age and life-time physical training on fibre composition of slow and fast skeletal muscle in rats. Pflügers Archiv 408, 543-551.

Kovanen, V. and Suominen, H., 1989. Age and training-related changes in the collagen metabolism of rat skeletal muscle. Eur. J. Appl. Physiol 58, 765-771.

Maltin, C.A., Delday, M.I., Baillie, A.G.S., Grubb, D.A., Garlick, P.J., 1989. Fiber-type composition of nine rat muscles. I. Changes during the first year of life.
Am. J. Physiol. 257, E823-E827.

Mutungi, G. and Ranatunga, K.W., 1996. The viscous, viscoelastic and elastic characteristics of resting fast and slow mammalian (rat) muscle fibers. J. Physiol. 496, 827-836.

Neagoe, C., Kulke, M., Del Monte, F., Gwathmey, J.K., De Tombe, P.P., Hajjar, R.J., Linke, W.A., 2002. Titin isoform switch in ischemic human heart disease.
Circulation 106, 1333-1341.

Punkt, K., Mehlhorn, H., Hilbig, H., 1998. Region and age dependent variation of muscle fibre properties. Acta. Histochem. 100, 37-58.

Ranatunga, K.W., 1982. Temperature-dependance of shortening velocity and rate of isometric tension development in rat skeletal muscle. J. Physiol. 329, 465-483.

Salviati, G., Ceoldo, S., Fachechi-Cassano, G., Betto, R., 1990. Morphological and functional characterization of the endosarcomeric elastic filament. Am. J. Physiol. 259, 144-149.

Szymanowicz, G., Laurain, G., 1981. An automatic method of 4-hydroxyproline determination convenient for large series. Anal Biochem. 113, 58-61.

Timson, B.F., Dudenhoeffer, G.A., 1990. Skeletal muscle fibre number in the rat from youth to adulthood. J. Anat. 173, 33-36.

Toursel, T., Stevens, L., Granzier, H., Mounier, Y. 2002 Passive tension of rat skeletal soleus muscle fibers: effect of unloading conditions. J Appl Physiol 92:1465-1472.

Wang, K., McCarter, R., Wright, J., Beverly, J., Ramirez-Mitchell, R. 1993. Viscoelasticity of the sarcomere matrix of skeletal muscles. Biophys J 64:1161-1177.

Woessner, J.F., 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch. Biochem. Biophys. 93, 440-447.

Figure(s)

Figure 1: Experimental set up of isolated soleus muscle (R1, R4, R12).

Figure 2: Schematic representation of a passive test applied on isolated soleus fiber. Recording of the resting tension during a relaxation test is illustrated in the oscilloscope box. (A: peak value and B: relaxed value)

Figure 3a: Experimental representation of the developed forces for isolated soleus R4 fiber (A) and muscle (B) during a relaxation test (Fd : dynamic force Fs : static force, T: time).

Figure 3b: Experimental representation of the developed forces for isolated soleus R12 fiber (A) and muscle (B) during a ramp stretch (ΔL: stretch, Fs : static force, T : temps).

Figure 3c: Experimental representation of the developed forces for isolated soleus R4 fiber (A) and muscle (B) during a stretch release cycle (Fd : dynamic force, Fs : static force, T: time).

Figure 4: Subdivision of the soleus muscle into 5 regions (1…5) in the distal, belly and proximal areas.

Figure 5: Representation of a belly section (A) with the three different type of fibers (B) and the detection of the slow fibers (C)

Figure 6: Evolution of the surface fibers in function of fiber type (I, IIc and IIa) and age.

Figure 7: Range of slow fibers diameters values for R1, R4 and R12 (** : $P < 0,001$).

Figure 8: Repartition of the different fibers type in the belly region and along the longitudinal axis (* : $P < 0,05$).

Figure 9: Evolution of collagen content (weight dry) with age (** : $P < 0,001$).

Table 1. Minimum, maximum, median and average values of the diameters.
Average values (with SD) of the forces and tensions developed by the soleus fibers after the three different mechanical tests (R1: fiber of 1 month, R4: fiber of 4 months, R12: fiber of 12 months).

Table 2. Average values (with SD) of the muscle weight, length and surface.
Average values (with SD) of the forces and tensions developed by the soleus muscles after the three different mechanical tests (R1: muscle of 1 month, R4: muscle of 4 months, R12: muscle of 12 months).

Table 3. Statistical analysis of the fibers mechanical parameters between the three populations of rats named R1 (rat of 1 month), R4 (rat of 4 months) and R12 (rat of 12 months) (* $P < 0.05$, ** $P < 0.001$).

Table 4. Statistical analysis of the muscles mechanical parameters between the three populations of rats named R1 (rat of 1 month), R4 (rat of 4 months) and R12 (rat of 12 months) (* $P < 0.05$, ** $P < 0.001$).

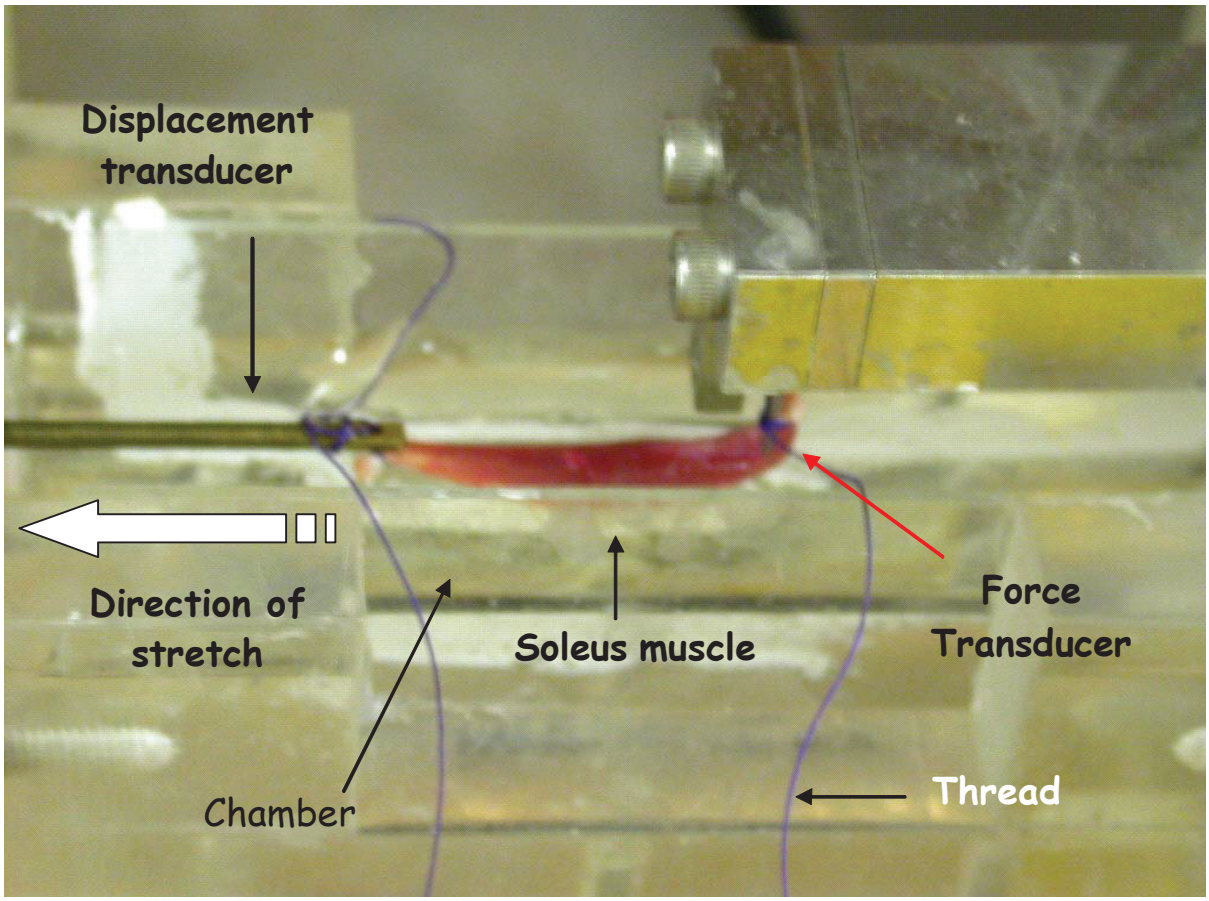

Figure 1: Experimental set up of isolated soleus muscle (R1, R4, R12).

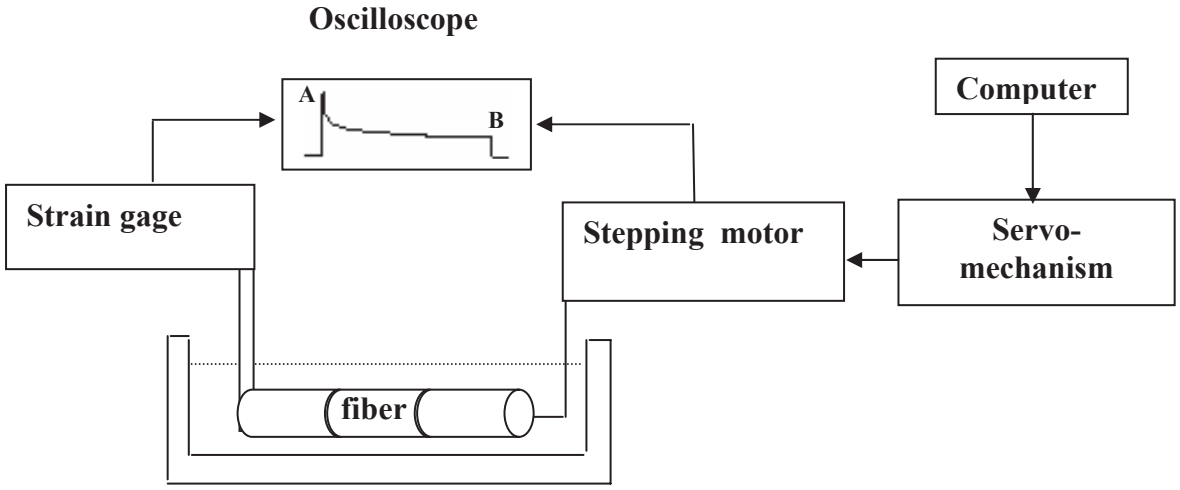

Figure 2: Schematic representation of a passive test applied on isolated soleus fiber. Recording of the resting tension during a relaxation test is illustrated in the oscilloscope box. (A: peak value and B: relaxed value)

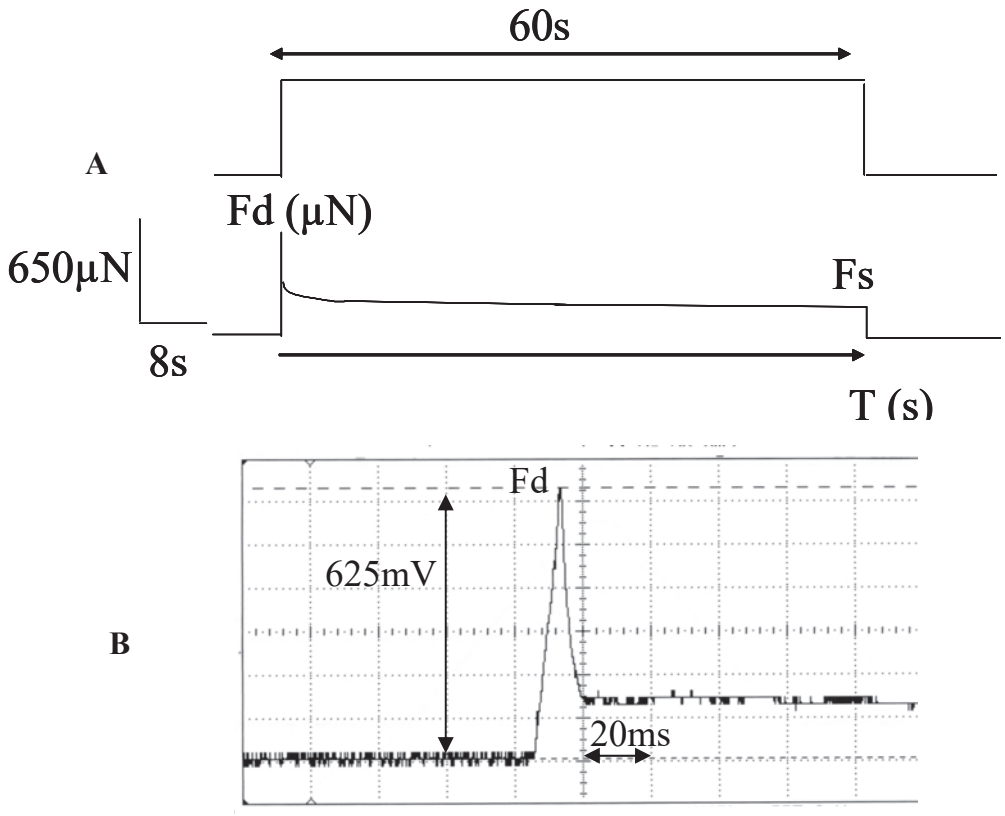

Figure 3a: Experimental representation of the developed forces for isolated soleus R4 fiber (A) and muscle (B) during a relaxation test (Fd : dynamic force Fs : static force, T: time).

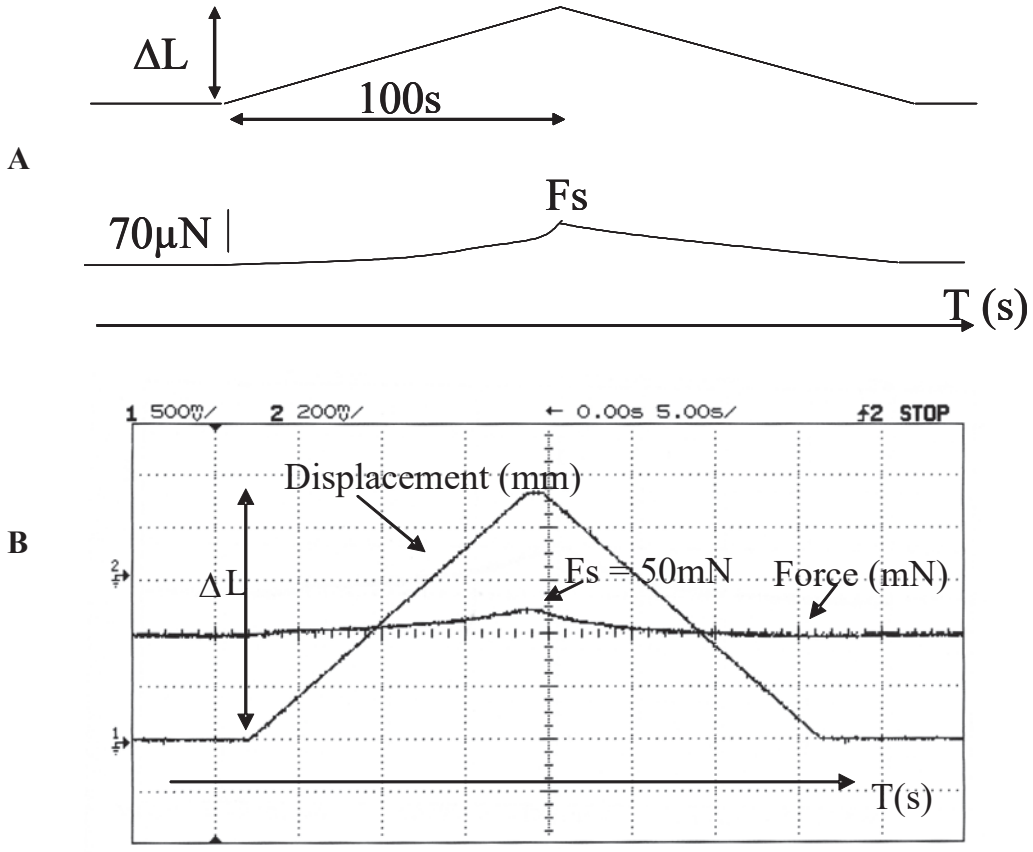

Figure 3b: Experimental representation of the developed forces for isolated soleus R12 fiber (A) and muscle (B) during a ramp stretch (ΔL: stretch, Fs : static force, T : temps).

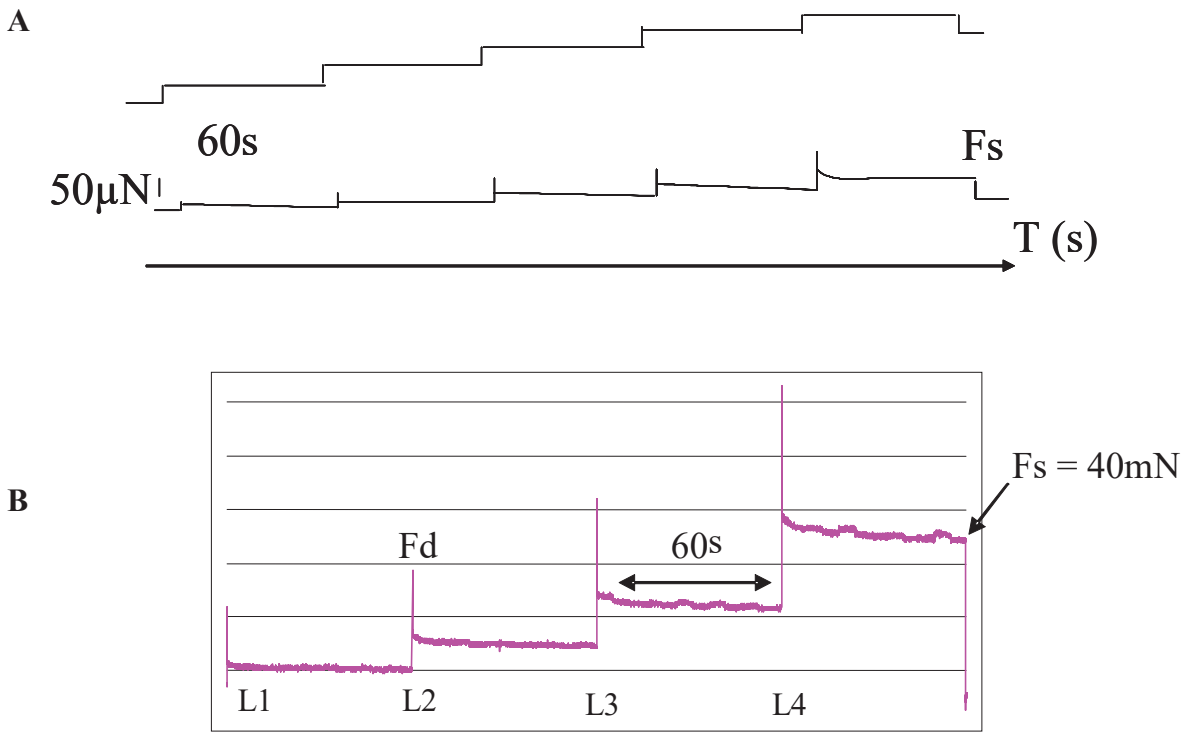

Figure 3c: Experimental representation of the developed forces for isolated soleus R4 fiber (A) and muscle (B) during a stretch release cycle (Fd : dynamic force, Fs : static force, T: time).

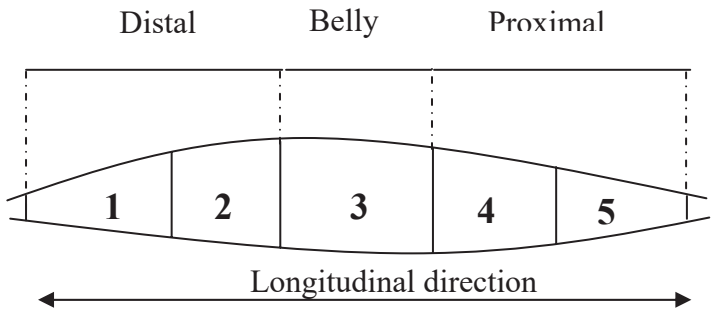

Figure 4: Subdivision of the soleus muscle into 5 regions (1…5) in the distal, belly and proximal areas.

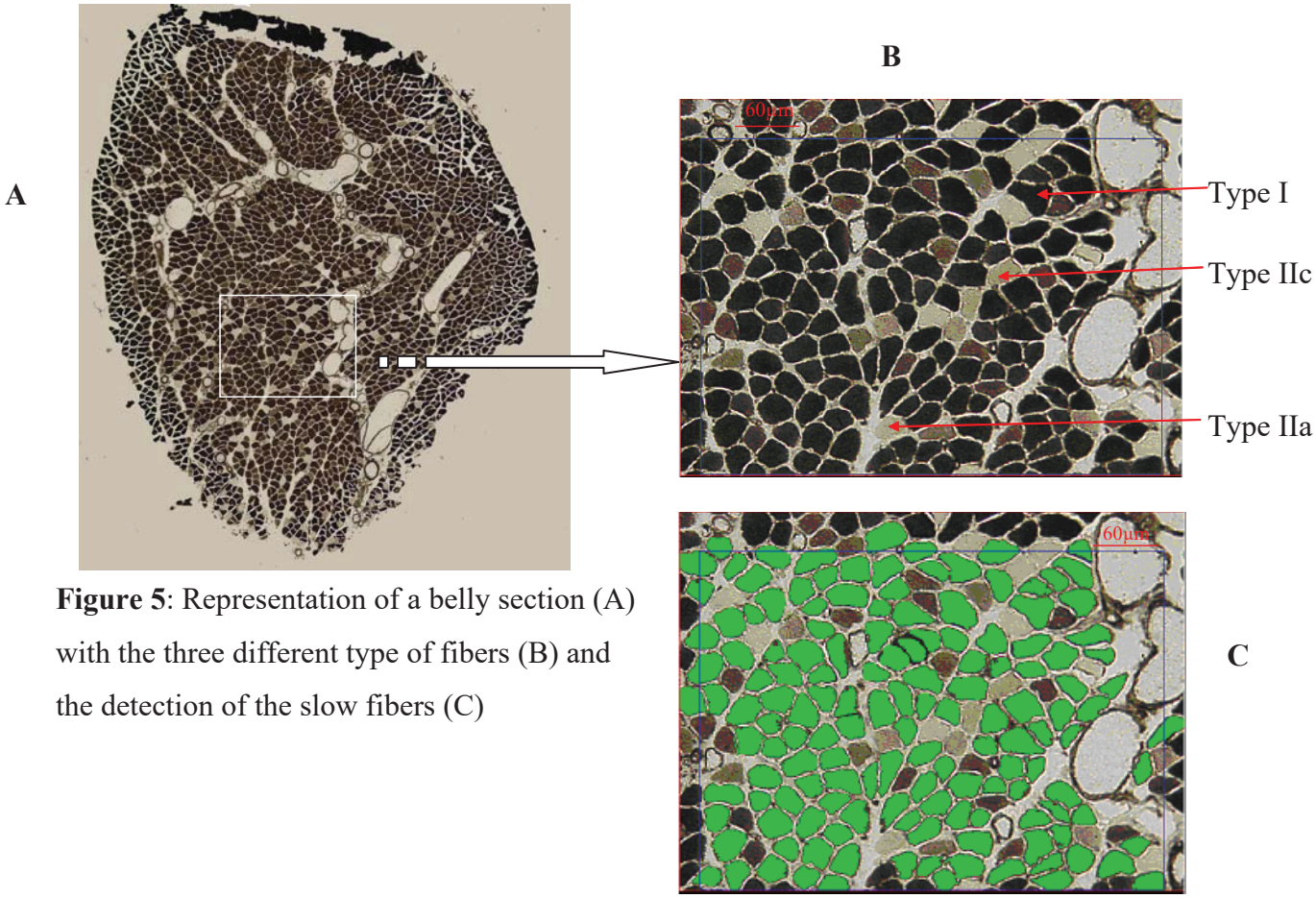

Figure 5: Representation of a belly section (A) with the three different type of fibers (B) and the detection of the slow fibers (C)

		R1 n = 19	R4 n = 18	R12 n = 24
Mechanical tests	Diameter (μm)	15 – 50 25 29 ± 11	38 - 98 50 55 ± 19	38 - 138 99 87 ± 29
Relaxation test	Fd (μN)	80 ± 50	400 ± 300	300 ± 200
	σd (KN/m^2)	138 ± 66	124 ± 32	61± 42
	Fs (μN)	20 ± 20	80 ± 40	100 ± 100
	σs (KN/m^2)	33 ± 24	35 ± 15	22 ± 22
Ramp stretch	Fs (μN)	30 ± 20	200 ± 100	100 ± 70
	σs (KN/m^2)	53 ± 43	51 ± 27	25 ± 22
	E (KN/m^2)	106 ± 86	103 ± 53	49 ± 44
Stretch Release cycle	Fs (μN)	20 ± 20	100 ± 70	90 ± 60
	σs (KN/m^2)	41 ± 41	39 ± 19	18 ± 22

Table 1. Minimum, maximum, median and average values of the diameters. Average values (with SD) of the forces and tensions developed by the soleus fibers after the three different mechanical tests (R1: fiber of 1 month, R4: fiber of 4 months, R12: fiber of 12 months).

		R1 n = 16	R4 n = 16	R12 n = 10
Mechanical tests	Muscle weight (mg) Muscle length (mm) Section (mm^2)	73,7 ± 5,2 17 ± 1 6,11 ± 0,42	201,6 ± 23,9 24 ± 1 11,99 ± 1,22	271,7 ± 23,6 30 ± 1 13,2 ± 1,21
Relaxation test	Fd (mN)	104 ± 12	296 ± 66	191 ± 21
	σd (kN/m^2)	17,34 ± 2,67	24,88 ± 4,75	14,79 ± 2,32
	Fs (mN)	26 ± 4	40 ± 11	21 ± 3
	σs (kN/m^2)	5,51 ± 1,48	3,25 ± 0,69	1,63 ± 0,18
Ramp stretch	Fs (mN)	30 ± 11	52 ± 12	27 ± 4
	σs (kN/m^2)	5,33 ± 2,15	4,36 ± 0,93	2,04 ± 0,32
	E (kN/m^2)	31,04 ± 11,09	21,82 ± 4,64	10,21 ± 1,61
Stretch Release cycle	Fs (mN)	27 ± 7	41 ± 12	22 ± 4
	σs (kN/m^2)	5,46 ± 1,97	3,40 ± 0,76	1,65 ± 0,25

Table 2. Average values (with SD) of the muscle weight, length and surface. Average values (with SD) of the forces and tensions developed by the soleus muscles after the three different mechanical tests (R1: muscle of 1 month, R4: muscle of 4 months, R12: muscle of 12 months).

		Kruskall-Wallis test
Relaxation test	Fd	R1≠R4**, R1≠R12**, R4 =R12
	σd	R1=R4, R1≠R12**, R4≠ R12**
	Fs	R1≠R4**, R1≠R12**, R4 =R12
	σs	R1=R4, R1≠R12*, R4≠ R12**
Ramp stretch	Fs	R1≠R4**, R1≠R12**, R4 =R12
	σs	R1=R4, R1≠R12*, R4≠ R12**
	E	R1=R4, R1≠R12*, R4≠ R12**
Stretch release cycle	Fs	R1≠R4**, R1≠R12**, R4 =R12
	σs	R1=R4, R1≠R12*, R4≠ R12**

Table 3. Statistical analysis of the fibers mechanical parameters between the three populations of rats named R1 (rat of 1 month), R4 (rat of four month) and R12 (rat of 12 month) (* $P < 0.05$, ** $P < 0.001$).

		Test ANOVA – Kruskall wallis		
Relaxation test	Fd	R1≠R4**	R1≠R12**	R4 ≠R12**
	σd	R1≠R4**	R1=R12	R4 ≠R12**
	Fs	R1≠R4**	R1≠R12**	R4 ≠R12**
	σs	R1=R4	R1≠R12**	R4 ≠R12**
Ramp stretch	Fs	R1≠R4**	R1=R12	R4 ≠R12**
	σs	R1=R4	R1≠R12**	R4 ≠R12**
	E	R1=R4	R1≠R12**	R4 ≠R12**
Stretch release cycle	Fs	R1≠R4**	R1≠R12*	R4 ≠R12**
	σs	R1=R4	R1≠R12**	R4 ≠R12**

Table 4. Statistical analysis of the muscles mechanical parameters between the three populations of rats named R1 (rat of 1 month), R4 (rat of four month) and R12 (rat of 12 month) (* $P < 0.05$, ** $P < 0.001$).

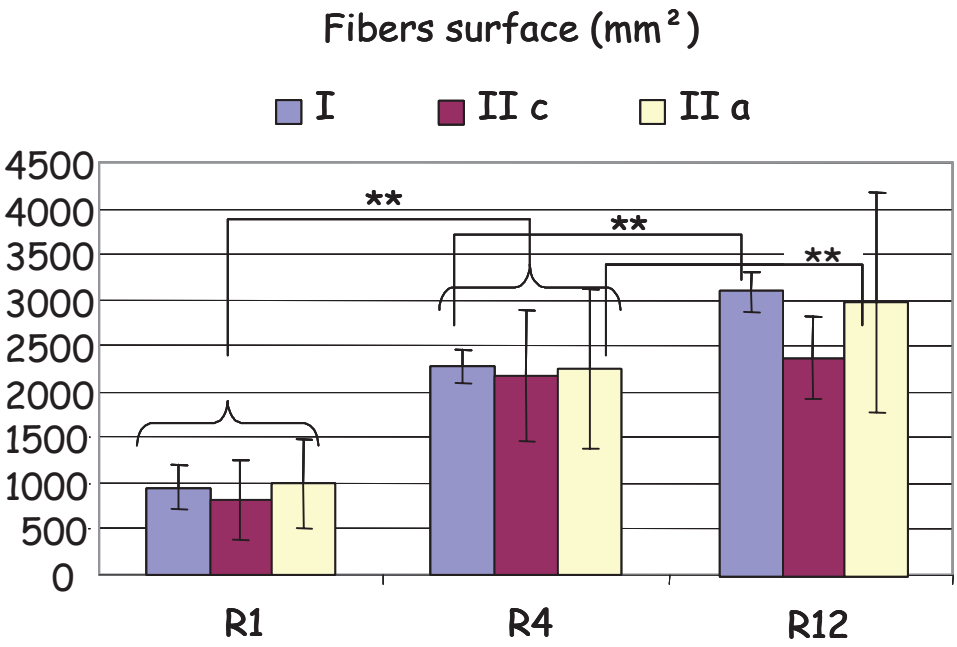

Figure 6: Evolution of the surface fibers in function of fiber type (I, IIc and IIa) and age.

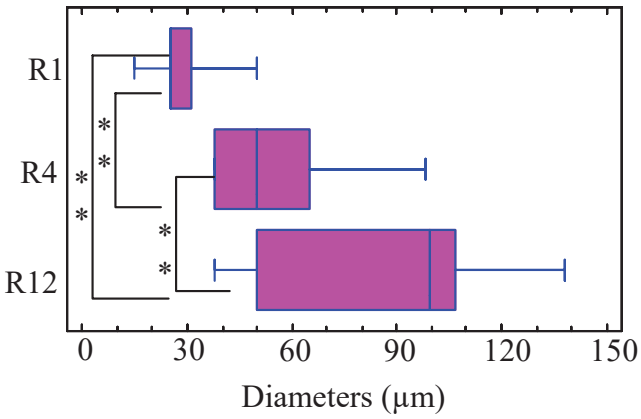

Figure 7: Range of slow fibers diameters values for R1, R4 and R12 (** : P < 0,001).

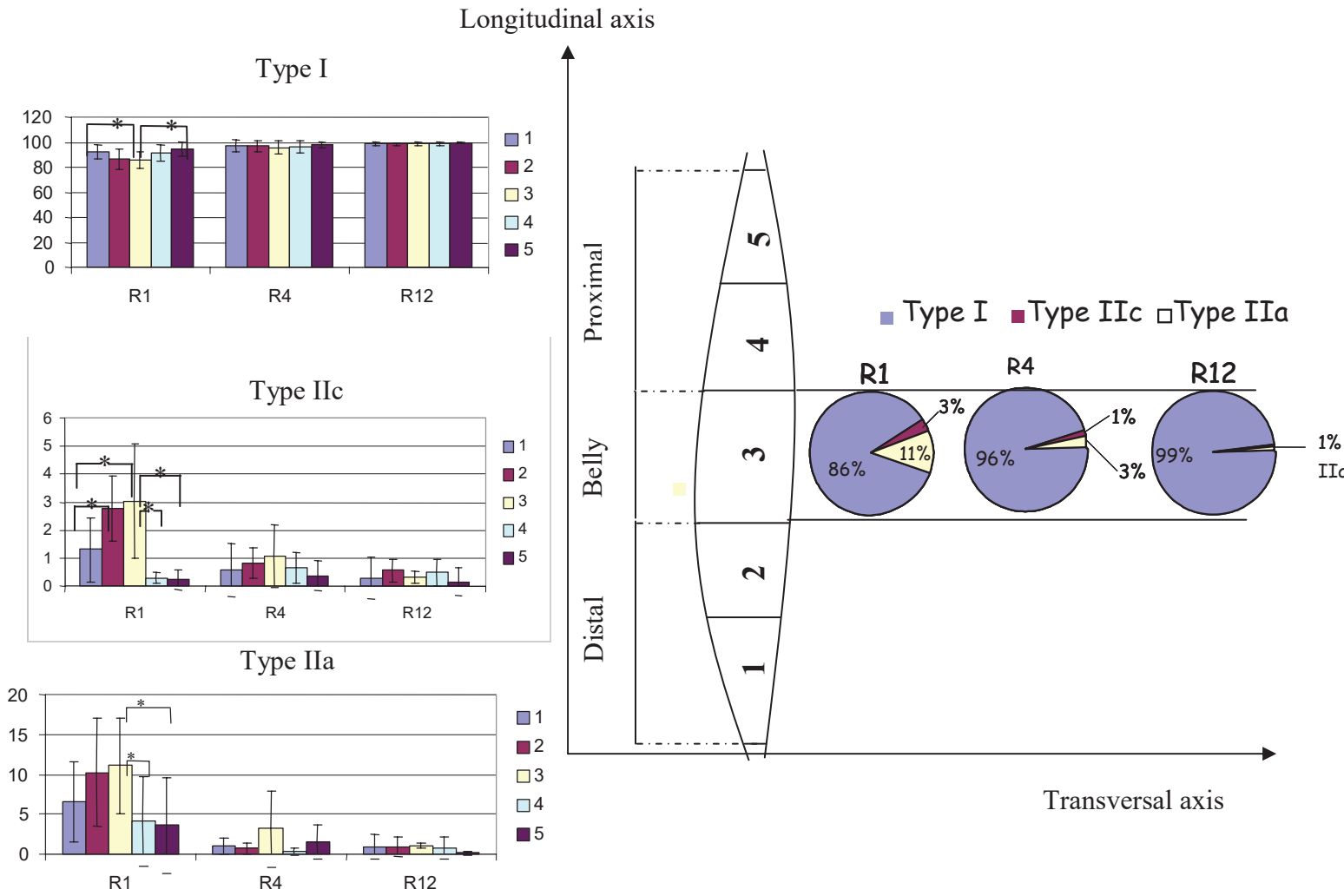

Figure 8: Repartition of the different fibers type in the belly region and along the longitudinal axis (* : P < 0,05).

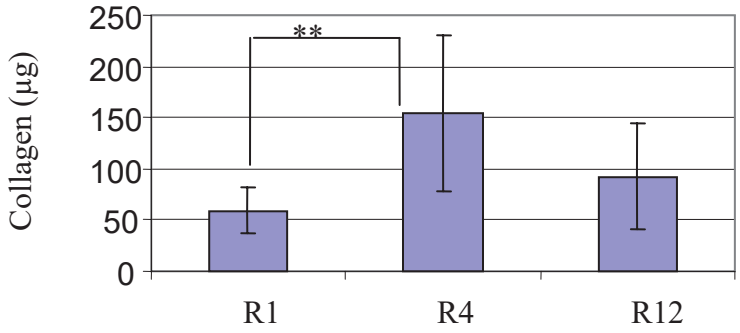

Figure 9: Evolution of collagen content (weight dry) with age (** : P < 0,001).