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Fiber type characterization of striated muscles related to micturition in female rabbits

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ABSTRACT

Pelvic and perineal striated muscles are relevant for reproduction and micturition in female mammals. Damage to these muscles is associated with pelvic organ prolapse and stress urinary incontinence. The fiber type composition of skeletal muscle influences the susceptibility for damage and/or regeneration. The aim of the present study was to determine the fiber type composition of a perineal muscle, the bulbospongiosus, and a pelvic muscle, the pubococcygeus. Both muscles were harvested from adult female rabbits (8–10 months old). NADH-TR (nicotinamide adenine dinucleotide tetrazolium reductase) histochemistry was undertaken to identify oxidative and glycolytic muscle fibers. Alkaline (pH 9.4) ATP-ase (actomyosin adenosine triphosphatase) histochemistry was used to classify type I, type IIb or type IIa/IId muscle fibers. Results showed that the content of glycolytic fibers in the bulbospongiosus muscle was higher than that of oxidative fibers. Meanwhile, the opposite was true for the pubococcygeus. In the bulbospongiosus muscle, the content of type IIb muscle fibers was higher than that of type I, but was similar to that of type IIa/IId. In contrast, the content of each fiber type was similar in the pubococcygeus muscle. The relative proportion of fibers in bulbospongiosus and pubococcygeus muscles is consistent with their function during voiding and storage phases of micturition.

Introduction

The pelvic and perineal skeletal muscles play important roles in the physiology of the lower urogenital tract (LUT) (Thor and de Groat, 2010). These muscles are involved in particular in reproductive and excretory functions (Martínez-Gómez et al., 2012). In micturition, the pelvic muscles participate in urine storage (DeLancey et al., 2008), whereas the perineal muscles participate in voiding of urine (Amarenco et al., 2002). Functional and structural alterations of these muscles are related to the onset of stress urinary incontinence and pelvic organ prolapse in women (Shafik, 2003; Ashton-Miller and DeLancey, 2007). Fiber type composition can offer valuable information with regard to better comprehension of the histopathological changes in pelvic and perineal muscles, whereas available information is mainly concerned with pelvic muscles (Gosling et al., 1981; Morley et al., 1996; Dimpfl et al., 1998; Jundt et al., 2005; Sumino et al., 2006; Yiou et al., 2009).

The fiber type composition of skeletal muscles related to the female LUT has been evaluated by histochemical approaches in squirrel monkeys (Pierce et al., 2007), dogs (Augsburger and Eggenberger, 2004), lambs (Rodríguez-Veiga et al., 2005) and rats (Buffini et al., 2010). The former two studies were conducted on pelvic muscles (*levator ani*), while the latter studies were done on the external urethral sphincter (EUS) and the external anal sphincter (EAS). Thus, it has been shown that the content of slow (type I) fibers is similar to that of fast muscle fibers (type IIb) in the *levator ani* (Augsburger and Eggenberger, 2004; Pierce et al., 2007). In contrast, fast fatigue-resistant fibers (type IIa/IId) are predominant in the EUS and EAS muscles (Rodríguez-Veiga et al., 2005; Buffini et al., 2010).

Abbreviations: ATPase, actomyosin, adenosine triphosphatase; Bsm, bulbospongiosus muscle; CSA„ cross sectional area; EAS„ external anal sphincter; EUS„ external urethral sphincter; F„ fast; G„ glycolytic; I„ intermediate; i.p.„ intraperitoneal; NADH-TR„ nicotinamide adenine dinucleotide tetrazolium reductase; NBT„ nitroblue tetrazolium; O„ oxidative; Pcm„ pubococcygeus muscle; S„ slow; v/v„ volume per volume; w/v„ weight per volume.

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The female rabbit is a suitable model to evaluate functional, metabolic, and histological characteristics of pelvic and perineal muscles (Martínez-Gómez et al., 1997, 2011; Fajardo et al., 2008; Corona-Quintanilla et al., 2009; Spettel et al., 2012; López-García et al., 2013). The perineal bulbospongiosus muscle develops larger twitch and tetanic tension force in response to electrical stimulation than the pelvic pubococcygeus muscle (Fajardo et al., 2008). These contractile responses were arbitrarily related to the apparent fiber type composition determined using the Sudan black staining technique. Thus, the bulbospongiosus muscle was reported to be mainly composed of fast fibers. In contrast, a similar fiber content of slow and intermediate fibers (type IIa/IId) was reported for the pubococcygeus muscle (Fajardo et al., 2008). However, the Sudan black technique stains the lipid content of fibers (Humason, 1979), which is not the most acceptable approach to identify fiber types in skeletal muscles according to their histoenzymatic properties. The specific fiber type composition is related to the contractile and metabolic properties of skeletal muscles (Zierath and Hawley, 2004). The aim of our study was to elucidate the fiber metabolism and fiber type composition of perineal and pelvic muscles. To accomplish this, NADH-TR (nicotinamide adenine dinucleotide tetrazolium reductase) and alkaline (pH 9.4) ATPase (actomyosin adenosine triphosphatase) histoenzymatic techniques were carried out to characterize the predominant metabolic activity and fiber type content in bulbospongiosus and pubococcygeus muscles of nulliparous virgin rabbits.

Materials and methods

Animals

Five young (8–10 months old) Chinchilla-breed female rabbits (*Oryctolagus cuniculus*) were housed in individual stainless-steel cages and kept at 20 ± 2 °C under artificial lighting conditions (L:D 16:8, lights on at 0600 h). They were provided daily with pellet food (Purina, México) and *ad libitum* access to water. The Ethics Committee from the Universidad Autónoma de Tlaxcala approved all experimental procedures. All animals used in this study were euthanized with an overdose of sodium pentobarbital (60 mg/kg, i.p., Pisa).

Tissue sampling and preparation

Striated muscles were dissected under deep anesthesia as described elsewhere (Martínez-Gómez et al., 1997). A midline incision was made from the abdomen to the perineal vagina, and abdominal muscles and adipose tissue were removed to harvest bulbospongiosus muscles (Fajardo et al., 2008). Pubococcygeus muscles were harvested after removal of the ischium and pubis; the left side muscles were used for histoenzymatic staining protocols. After dissection the animals were euthanized by an overdose of anesthetics. The bulbospongiosus and pubococcygeus muscles were immediately frozen in a mixture of isopentane and dry ice, and stored at −80 °C until assayed. These muscles were mounted on specimen holders in a cryoprotectant solution (Tissue-Tek®, Sakura Fine Tek, Torrance, CA, USA) and 8 μm-thick serial transverse sections were cut from the medial region of each muscle using a cryostat microtome at −20 °C (CM-1100, Leica Microsystems, Wetzlar, Germany). Sections were mounted on glass slides for staining.

Histoenzymatic analysis

The oxidative metabolism of the muscle fibers was determined in consecutive muscle sections as stained for NADH-TR (nicotinamide adenine dinucleotide tetrazolium reductase) activities as modified from Nachlas et al. (1958). Briefly, bulbospongiosus and pubococcygeus muscle sections were incubated 1 h at 37 °C in the NBT-NADH solution 1:1 (v/v) [1.2 mM nitro-blue tetrazolium (Sigma–Aldrich, St. Louis, MO, USA) diluted in 50 mM tris buffer (Biorad, Hercules, CA, USA), pH 7.6; 2.25 mM nicotinamide adenine dinucleotide (NADH; Sigma–Aldrich, USA) diluted in 50 mM tris buffer (BioRad, Hercules, CA, USA)]. Subsequently, tissue sections were washed three times with deionized water. The excess of NBT-NADH solution was removed by washing sections with increasing and decreasing acetone concentrations (30, 60 and 90%). Slides were washed several times with deionized water and covered with Cytoseal 60 mounting medium (Richard–Allan Scientific, Kalamazoo, MI, USA) and a coverslip.

In the subsequent serial tissue slide, the myofibrillar ATPase activity was visualized in alkaline conditions (pH 9.4) following the technique described by Guth and Samaha (1970) with slight modifications. Muscle sections were immersed in the pre-incubation solution [Tris base 10 mM (BioRad, USA) and 18 mM $CaCl_2$ (J.T. Baker, México), pH 9.4] for 15 min. Afterwards, sections were washed three times with deionized water and incubated 1 h at 37 °C in 1.5% (w/v) adenosine 5′-triphosphate (Sigma–Aldrich, St. Louis, MO, USA) in a pre-incubation solution at pH 9.4. Muscle sections were washed with 2% (w/v) $CaCl_2$ for 3 min, transferred to 2% (w/v) $CoCl_2$ (Sigma–Aldrich, UK) for 3 min, and then to 10% (v/v) ammonium sulfide (Sigma–Aldrich, St. Louis, MO, USA) for 3 min. Sections were washed with distilled water, dehydrated and mounted in Cytoseal 60 medium (Richard–Allan Scientific, USA) and a coverslip.

NADH-TR and alkaline ATPase (pH 9.4) stained muscle sections were observed by light microscopy using a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan). Photomicrographs were made at 200× magnification using an Olympus C-5060 digital camera (Olympus Corp., Tokyo, Japan) and utilized for reconstructing one image (JPEG file) of the whole muscle section using the PowerPoint program (Microsoft Office, 14.0 for Mac). Fibers in NADH-TR-stained muscle sections were classified according to the following criteria: dark fibers were identified as oxidative, while light fibers were considered to be glycolytic (Fig. 1A and B). By using the alkaline ATPase technique, light, gray and dark fibers were identified as slow (type I), fast (type IIb), and intermediate (type IIa/IId; Fig. 2A and B) (Soukup et al., 1979; Miyabara et al., 2005; Ruiz-Rosado et al., 2013). The total number of each fiber type (oxidative, glycolytic; fast, slow, or intermediate) was determined for reconstructed images of bulbospongiosus and pubococcygeus muscle sections using ImageJ version 1.45s for Mac program (NIH, Bethesda, MD, USA) and expressed as percentage of the entire number of fibers scored. For each muscle, muscle fiber size was evaluated measuring the cross sectional area (CSA), diameter and perimeter in 100 randomly selected fibers from the medial region of each type stained with NADH-TR and alkaline ATPase using the AxioVision Rel 4.3 program (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Values are the mean ± SE. Statistical level of significance was set at $P \leq 0.05$. Unpaired two-tail Student's *t* tests were done to determine significant differences in the percentage of oxidative and glycolytic fibers for each muscle. A Mann–Whitney *U* test was used to determine differences in morphometric variables between glycolytic and oxidative fibers. One-way ANOVA followed by Tukey's *post hoc* tests were done to determine significant differences between the content of slow (type I), fast (type IIb), and intermediate (type IIa/IId) fibers. Kruskal–Wallis tests followed by a Dunn's *post hoc* test were done to compare morphometric variables between slow (type I), fast (type IIb), and intermediate (type