Meat Science 93 (2013) 378–386

Contents lists available at SciVerse ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

Relationships between structural characteristics of bovine intramuscular connective tissue assessed by image analysis and collagen and proteoglycan content

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ARTICLE INFO

Article history:
Received 3 May 2012
Received in revised form 12 September 2012
Accepted 19 September 2012

Keywords:
Perimysium
Endomysium
Beef breed
Muscle
Collagen
Proteoglycans

ABSTRACT

Three muscles (*Longissimus thoracis*, *Semimembranosus*, *Biceps femoris*) of 40 young bulls from 3 breeds were used to quantify structural characteristics of bovine connective tissue by image analysis, with both macroscopic and microscopic approaches. Collagen and proteoglycan content was also investigated. Perimysium occupied a greater area (8 *vs* 6%), and was wider (42 *vs* 2 μm) and shorter per unit area (1.9 *vs* 30 mm mm^{-2}) than endomysium. Perimysium and endomysium from *Longissimus* were thinner, less ramified than in *Biceps*. *Longissimus* showed less total collagen and cross-linking and more proteoglycans ($P<0.0001$) than *Biceps* muscle. Blond d'Aquitaine perimysium occupied less area, was more ramified and muscles contained less collagen, cross-linking and more proteoglycans than Angus ($P<0.001$). Limousin was intermediate. High proteoglycan content in muscle containing less total collagen suggested a complementarity between these molecules. They might influence mechanical properties of intramuscular connective tissue. This was especially true given that proteoglycans and total collagen were negatively and positively linked with structural parameters, respectively.
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1. Introduction

Connective tissue in skeletal muscle is divided into endomysium, surrounding each muscle fibre individually, perimysium surrounding groups of muscle fibres and epimysium surrounding the muscle as a whole. Intramuscular connective tissue (IMCT) (endomysium and perimysium) is a three-dimensional network whose morphology and composition varies from one type (species, gender, age) of animal to another and from one muscle to another according to muscle position and function, while having a complex link with meat tenderness (Purslow, 2005).

The first structural descriptions of IMCT were done manually and subjectively, by projection of stained muscle sections using an overhead projector (for large sections and muscle fibre bundle studies) or by microscopic observation (small sections and study of microscopic structures) (Dumont, 1982; Dumont & Schmitt, 1973; Schmitt, Degas, Perot, Langlois, & Dumont, 1979; Schmitt & Dumont, 1969). More recently, image analysis associated with histological methods have been developed. Except Sifre-Maunier, Taylor, Berge, Culioli, and Bonny (2006) and Del Moral, O'Valle, Masseroli, and Del Moral (2007), authors have rarely described their method accurately. Image analysis has been used to study the structural characteristics of perimysium thickness (An et al., 2010; Brooks & Savell, 2004; Das et al., 2010; Fang, Nishimura, & Takahashi, 1999; Lachowicz, Zochowska, & Sobczak, 2004; Nishimura, Fang, Wakamatsu, & Takahashi, 2009; Sifre-Maunier et al., 2006; Torrescano et al., 2001) or muscle fibre bundles, but few authors have been interested in endomysium (An et al., 2010; Lachowicz et al., 2004; Torrescano et al., 2001). This may be explained by the fact that the major part of the IMCT is perimysium (Light & Champion, 1984; Light, Champion, Voyle, & Bailey, 1985). Nevertheless, a negative correlation has been found between endomysium thickness and meat tenderness in breast muscle (*Pectoralis superficialis* and *Pectoralis profundus*) of chicken (An et al., 2010) and a positive correlation has been found in pork (*Biceps femoris*, *Semimembranosus*, *Quadriceps femoris*, *Longissimus dorsi*, all muscles combined) (Lachowicz et al., 2004). Therefore, the study of perimysium in conjunction with endomysium would seem important. We have developed an image analysis method and we have worked at two scales, macroscopic (magnification ×5) and microscopic (magnification ×100).

IMCT is mainly composed of fibrillar collagens embedded in a matrix of proteoglycans (PGs). These two classes of molecules form a continuous network inside perimysium but also between perimysium, endomysium and muscle fibres (Voermans et al., 2008). Fibrillar collagens make up the structure of IMCT and are linked between each other by different chemical cross-links. The main cross-links in muscle are the pyridinolines (hydroxylysylpyridinoline and dehydroxylysylpyridinoline). They are trivalent and thermo-stable. PGs play an important role in the stabilization of the intramuscular collagen network (Nishiumi, Fukuda, & Nishimura, 1997), are sensitive to physiological changes (growth, age) (Nakano & Scott, 1996; Rooney & Kumar, 1993) and are quickly degraded by proteases during post-mortem storage (Nishimura, Hattori, & Takahashi, 1996). The degradation of PGs during post-mortem ageing would expose collagen to collagenases and facilitate its degradation

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0309-1740/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.meatsci.2012.09.020

(Nishimura, Hattori, & Takahashi, 1996). Thus, PGs could influence the mechanical properties of IMCT and tenderness. With regard to beef meat tenderness, proteoglycans have been little considered while collagen and its cross-links have been extensively studied and compared between muscles (Rhee, Wheeler, Shackelford, & Koohmaraie, 2004; Torrescano, Sanchez-Escalante, Gimenez, Roncales, & Beltran, 2003), but much less data exist between breeds. Nevertheless, it has been shown that muscularity affects the connective tissue structural parameters and its composition in collagen (Dumont, 1982; Listrat et al., 2001). In addition, late-maturing cattle breeds (as Belgian blue, Limousin and Blond d'Aquitaine) grow more slowly and deposit more muscles and less fat, compared to early-maturing cattle breeds (as Angus and Japanese Black cattle) which tend to have muscles richer in collagen with a higher proportion of insoluble collagen (Campo et al., 2000; Christensen et al., 2011; Jurie et al., 2011; Blanco et al., in press; Monson, Sanudo, & Sierra, 2004).

The aim of the present study was to i) quantify structural characteristics of bovine IMCT (perimysium and endomysium) by image analysis, ii) show whether there was a relationship between these parameters and the biochemical composition of IMCT (collagen and PGs), and iii) determine which parameters best discriminate muscles and breeds.

2. Materials and methods

This study was carried out in compliance with the French recommendations and those of the Animal Care and Use Committee of the National Institute for Agricultural Research (INRA, Institut National de la Recherche Agronomique) of Clermont-Ferrand/Theix, France for the use of experimental animals including animal welfare.

2.1. Beef production and muscle sampling

The experiment was performed on 40 young entire males of Aberdeen Angus (AA) (n = 12), Limousin (LI) (n = 14) and Blond d'Aquitaine (BA) (n = 14) pure breeds. Animals (12 month-old) were assigned for a 100 day finishing period. The animals were housed in straw pens, individually fed and weighed every 2 weeks. Diets consisted of concentrate (75%) and straw (25%). Animals were slaughtered at the same age (around 17 months) and final live weight (around 665 kg) (Table 1) in order to avoid weight and age effects on IMCT characteristics. They were slaughtered at the experimental slaughter-house of the INRA Research centre. For the other parameters (average daily gain, muscle and fat carcass characteristics), AA was significantly different to LI and BA, which were similar. AA had 17% higher daily gain, 34% higher fatty tissue weight and 19% lower carcass muscle weight than LI and BA.

Three muscles were taken for each animal: *Longissimus thoracis* (LT), *Semimembranosus* (SM) and *Biceps femoris* (BF). For each muscle and each analysis, samples were taken at the same location. Muscle samples were removed from the 9th rib for LT and from the centre of the muscle for SM and BF.

For histological and PG measurements, muscle samples were taken 15 min after exsanguination. For histology, muscle samples of 1.5 × 1.5 × 1 cm were taken and for PG measurement, muscle samples (60–80 g) were cut up into small pieces. The two sets of samples were stored at −80 °C until preparation for analysis. For histology, samples were initially frozen in isopentane chilled in liquid nitrogen.

The carcasses were chilled in a cold room (+2 °C) and muscle samples were taken at 24 h post-mortem for collagen and cross-link measurements. Muscle samples (about 150 g) were cut into pieces of 1 cm cross-section, sealed under vacuum in plastic bags and stored at −20 °C until preparation for analysis.

2.2. Histology associated with image analysis

Muscle blocks (1.5 x 1.5 x 1 cm) were fixed with Tissue-Tek® OCT™ (Sakura) in a cryostat (HM 500 M, Microm, France) maintained at −25 °C, and cross-sections of 10 μm thickness were prepared. For each animal, the three muscle sections were placed on the same slide. A total of 40 slides were stained. For each staining bath, the three breeds were mixed to limit staining differences between two staining baths. The staining was carried out according the Picro-Sirius red method previously described by Flint et al. (Flint & Pickering, 1984) with slight modifications. Briefly, after acetone fixation (60 min), cross-sections were fixed 10 min in Picro-Formalin solution, rinsed in 90% ethanol (1 min), then in distilled water (10 min) and immersed in Sirius red staining solution (60 min) before washing in 0.01 M hydrochloric acid (5 min). After rinsing in distilled water (1 min), cross-sections were dehydrated successively in 95% ethanol and then twice in 100% ethanol. Stained sections were cleared in Safesolvant (Labonord) and mounted with Safemount (Labonord). IMCT (perimysium and endomysium) was stained red and muscle fibres yellow (dark grey and light grey, respectively, in Fig. 1). The aim was to create a contrast between IMCT and muscle fibres in order to develop an image analysis method to study perimysium, endomysium and muscle fibre morphology, on the same section, in an automatic and objective manner.

2.2.1. Image acquisitions

2.2.1.1. Perimysium study — macroscopy. Each stained muscle section (1.5 × 1.5 cm) was scanned entirely in transmission mode using an EPSON Expression 10000XL PRO A3 scanner (resolution of 2400 dpi, corresponding to a spatial resolution of 10.58 μm/pixel).

2.2.1.2. Endomysium study — microscopy. Stained sections were visualized in bright field mode with an Olympus BX51 microscope (Olympus, Tokyo, Japan) using a 10x objective (NA = 0.3). Five images were acquired randomly from the stained sections of 1.5 × 1.5 cm, used for perimysium study, with a DP-72 colour camera and Cell-D image

Table 1
Finishing and slaughtering traits (mean, standard deviation [SD], Minimum, Maximum) of experimental animals.

	Aberdeen Angus (n = 12)				Limousin (n = 14)				Blond d'Aquitaine (n = 14)				*P*-value
	Mean	SD	Minimum	Maximum	Mean	SD	Minimum	Maximum	Mean	SD	Minimum	Maximum	
Age at slaughter (mo)	16.8	0.7	16.0	18.5	16.8	0.7	15.9	18.0	16.5	0.5	15.7	17.3	0.46
Final live weight (kg)	689	50	607	784	659	32	580	718	645	53	543	744	0.06
Hot carcass weight (kg)	385 [b]	33	327	434	417 [a]	23	364	442	423 [a]	41	356	500	0.01
Average daily gain (g d^{-1})	1,697[a]	214	1,429	2,161	1,347[b]	164	1,029	1,609	1,460[b]	366	811	1,876	0.01
Carcass fatty tissues weight (kg)	73.7[a]	14.5	51.4	93.1	51.5[b]	4.9	44.5	61.1	45.0[b]	8.1	28.3	57.1	<0.0001
Carcass fat (%)	19.1[a]	3.0	14.8	22.8	12.4[b]	1.0	11.0	14.1	10.6[c]	1.5	8.0	13.4	<0.0001
Muscles carcass weight (kg)	256[b]	22.2	223.0	304.5	311[a]	18	270	332	322[a]	31	279	387	<0.0001
Muscle/Bone ratio	4.64[b]	0.18	4.25	4.92	5.75[a]	0.20	5.46	6.13	5.73[a]	0.16	5.46	5.98	<0.0001

[a–b]Within a row, means without a common superscript letter differ (P<0.05). Means with no superscript letters are not significantly different.

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