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Evaluation of *post mortem* stability of porcine skeletal muscle RNA

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ABSTRACT

The objective of this study was to evaluate the effect of *post mortem* times on the quality of porcine skeletal muscle total RNA in order to consider the possibility to use *post mortem* material for gene expression analysis. Samples of *Musculus semimembranosus* were collected at 20 min, 2 h, 6 h, 24 h and 48 h *post mortem* from the left legs of four commercial heavy pigs. Total RNA was analysed by agarose gel electrophoresis stained with ethidium bromide and by microfluidic capillary electrophoresis on an Agilent 2100 Bioanalyzer instrument obtaining 28S:18S rRNA peak ratios and RIN values. The average RIN values of the analysed samples were 7.45 ± 0.13, 7.43 ± 0.15, 7.45 ± 0.10, 7.33 ± 0.15 and 3.95 ± 0.58 for the same *post mortem* times, respectively, indicating that RNA degradation was present at 48 h *post mortem*. In a similar experiment, carried out by other authors on beef cattle muscle total RNA extracted at different *post mortem* times, RNA was stable up to 8 days after death as indicated by intact 28S and 18S rRNA bands. Thus, differences among species or other environmental factors might affect the level of RNA degradation. In the porcine *post mortem* samples, qualitative assessment of *GAPDH* transcripts by PCR amplification of different cDNA fragments indicated that *post mortem* stages did not affect the possibility of analysing this housekeeping gene. Thus, *post mortem* porcine skeletal muscle can be an useful tissue to obtain gene expression based information.

1. Introduction

Post mortem conversion of muscle to meat involves several biochemical modifications and processes that begin with a metabolic shift from an aerobic to an anaerobic state followed by transformation of glycogen into lactic acid and consequent pH reduction, activation of different proteinase systems with degradation of muscle proteins and activity of other enzymatic mechanisms with effects and/or production of specific metabolites (Lawrie, 1985). New approaches and technologies have been changing the way in which these mechanisms and their final results and products are investigated. The primary emphasis has been towards the use of proteomics to analyse the muscle *post mortem* processes and to identify biomarkers associated with meat quality traits and technological processing (Bendixen, 2005; Hollung, Veiset, Jia, Færgested, & Hildrum, 2007; Lametsch & Bendixen, 2001; van de Wiel & Zhang, 2007). However, despite the increasing number of studies on structural and protein *post mortem* modifications and degradation, few studies have been produced on the fate and dynamics of total RNA and specific mRNAs in meat tissues (Bahar et al., 2007). mRNA contains gene expression information that can be useful in analysing animal health and identifying animal specific traits, tissue signatures and action of dietary nutrients, drugs and contaminants with potential impacts in understanding meat quality processes and developing gene expression biomarkers for livestock production and product authentication (Bahar et al., 2007; Byrne et al., 2005; Eggen & Hocquette, 2004; Kaput & Rodriguez, 2004; McDaneld, Hancock, & Moody, 2004; Qu, Rothschild, & Stahl, 2007; Reecy, Spurlock, & Stahl, 2006; Wimmers et al., 2007; Zhang et al., 2007).

Compared to DNA, mRNA is more unstable and has a much shorter half-life. This characteristic is indispensable for regulation of gene expression in the cells that is determined by a balance between the rate of synthesis and decay. Degradation of RNA involves different cell machineries and pathways. In particular, eukaryotic mRNA degradation depends mainly by processes of poly(A)-tail shortening (3′ → 5′ degradation) and 5′-cap removing (decapping; 5′ → 3′ degradation) operated by ubiquitous ribonuclease (RNases) systems (Garneau, Wilusz, & Wilusz, 2007; Meyer, Temme, & Wahle, 2004). The specific way in which *post mortem* RNA degradation occurs has not been the subject of deep investigation but it can be assumed to happen following the same mechanisms in apoptotic cells (Del Prete et al., 2002; Samali, Gilje, Døskeland, Cotter, & Houge, 1997). The result is that, after death, RNA is progressively RNAse degraded, making it difficult to preserve in tissues for long periods without deep freezing and/or inhibition of RNases. Monitoring total RNA quality is an essential pre-requisite in transcriptome studies as the quality of the results largely depends on the

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quality of the extracted RNA (Auer et al., 2003; Copois et al., 2007; Thompson, Pine, Rosenzweig, Turpaz, & Retief, 2007). To this aim, the extent of *post mortem* total RNA and mRNA degradation in animal models and humans has been the subject of studies addressing the question of gene expression in *post mortem* tissues for pathology and forensic investigations (Bauer, Gramlich, Polzin, & Patzelt, 2003; Catts et al., 2005; Heinrich, Matt, Lutz-Bonengel, & Schmidt, 2007; Humphreys-Beher, King, Bunnel, & Brody, 1986; Jewell et al., 2002; Johnson, Morgan, & Finch, 1986; Kuliwaba, Fazzalari, & Findlay, 2005; Marchuk, Sciore, Reno, Frank, & Hart, 1998; Sanoudou et al., 2004). Long-term stability of total RNA or the integrity of specific transcripts up to 48–96 h *post mortem* has been shown in human, rat and rabbit brain (Bauer et al., 2003; Chevyreva, Faull, Green, & Nicholson, 2008; Cummings, Strum, Yoon, Szymanski, & Hulette, 2001; Ervin et al., 2007; Heinrich et al., 2007; Inoue, Kimura, & Tuji, 2002; Schramm et al., 1999; Stan et al., 2006; Yasojima, McGeer, & McGeer, 2001), perinatal and postnatal rabbit and human lung (De Paepe et al., 2002; Jewell et al., 2002; Marchuk et al., 1998), human bone (Kuliwaba et al., 2005), rat and human heart (Heinrich et al., 2007; Inoue et al., 2002), porcine retinal (Malik, Chen, & Olsen, 2003), rabbit connective (Marchuk et al., 1998) and bovine reproductive and adipose tissues (Bahar et al., 2007; Fitzpatrick et al., 2002). Organs such as pancreas, spleen, liver and kidney exhibit more rapid RNA fragmentation because of their high content in RNases, even if contradictory results have been reported for human, rat, rabbit and cattle *post mortem* tissues (Bahar et al., 2007; Finger, Mercer, Cotton, & Danks, 1987; Heinrich et al., 2007; Humphreys-Beher et al., 1986; Inoue et al., 2002; Marchuk et al., 1998; Thompson et al., 2007). Potential sources of variability in total RNA or specific transcripts stability are due to pre and *post mortem* factors, like agonal state, time from death to sampling, tissue handling and pH, that have been shown to affect, mainly in brain, the level of tissue RNA degradation and gene expression after death (Barton, Pearson, Najlerahim, & Harrison, 1993; Chevyreva et al., 2008; Harrison et al., 1995; Kingsbury et al., 1995; Li et al., 2004; Mexal et al., 2006).

Integrity of skeletal muscle RNA extracted at different *post mortem* times has been analysed in few species. Sanoudou et al. (2004) reported that autopsy specimens of human skeletal muscles collected up to 46 h *post mortem* was not degraded and still useful for gene expression investigation. In beef cattle a time-course study of RNA integrity was conducted on skeletal muscles at refrigeration temperature indicating that, despite evident total RNA degradation after 8 days *post mortem*, specific mRNAs were detectable up to 22 days after death (Bahar et al., 2007). RNA stability of porcine skeletal muscle during time *post mortem* has not been investigated yet.

The objective of this study was to assess the quality of total RNA extracted from porcine *semimembranosus* muscle at different times *post mortem* up to 48 h from death, following the abattoir processing conditions of the fresh thighs destined for Parma ham production, in order to evaluate if this material can be used in gene expression analysis experiments.

2. Materials and methods

2.1. Animals

The pigs chosen for this study were four commercial heavy hybrids of female sex, raised for the production of Parma ham in the same commercial farm. Animals were slaughtered at about 160 kg live weight in the same commercial abattoir within 1 min of each other. The pigs were stunned by CO_2 (concentration 87%) using a dip lift system (Butina, Denmark) and bled in a lying position.

2.2. Sample collection

Semimembranosus muscle from the left legs was sampled (3–5 g), using a sterile surgical knife, at 20 min after the bleeding of the animals following the normal operations of the abattoir. Twenty min *post mortem* was the time at which carcass dissection procedures separated the upper portion of the hind quarters making *Semimembranosus* muscles accessible. Collection of samples from the same muscle was carried out at the same position of the previous sampling at 2, 6, 24 and 48 h *post mortem* (cutting away the cut surface of the previous sampling) following the cold-chain at the abattoir until 4 °C. At 2 h *post mortem* the hams are moved to cold rooms in which their internal temperature reached approximately 4 °C in about other 4 h (6 h *post mortem* that was chosen as a sampling time point). The other two time points (24 and 48 h) were chosen in order to monitor for longer periods total RNA degradation. After sampling, tissues were snap frozen in liquid nitrogen and then stored at −80 °C till RNA extraction. The pH_1 (at 20 min *post mortem*), $pH_{120\ min}$ (at 2 h *post mortem*) and pH_u (at 24 h *post mortem*) were determined on the same sampled muscle using a Crison pH-meter equipped with an Ingold Xerolite electrode (Mettler Toledo, Udorf, Switzerland).

2.3. RNA extraction

Total RNA was extracted with RNeasy Midi kit (Qiagen, Hamburg, Germany) following the protocol for animal tissue. Briefly, after having scraped away the cutting surface, about 100 mg of muscle was homogenized with an Ultra-Turrax T10 basic instrument (Ika Werke gMBh & Co., Staufen, Germany) in a guanidine-isothiocyanate and β-mercaptoethanol containing lysis buffer. Then, the homogenate tissue was digested with proteinase K for 20 min at 55 °C. Debris was pelleted by centrifugation and the supernatant was eluted in column after addition of ethanol 100%. Then, several washes with kit buffers were carried out and RNA was finally eluted from the column membrane with RNase-free water.

2.4. RNA quality evaluation

The concentration and purity of the extracted RNA (1 μl) was determined by reading the absorbance spectra (A) on a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) from 220 to 750 nm and taking values at 230, 260 and 280 nm. Then, the A_{260}:A_{280} and A_{260}:A_{230} ratios were calculated.

A first check of total RNA quality was carried out by gel electrophoresis of 1 μg of muscle RNA on 1% agarose gel in 1× TBE buffer. RNA was visualized with ethidium bromide excited on a UV apparatus (UVP Transilluminator, Upland, CA, USA). Gel images were captured with a Kodak EDAS 290 system (Eastman Kodak Company, Rochester, NY, USA). Furthermore, total RNA was evaluated by microfluidic capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) using the RNA 6000 Nano LabChip® kits. All chips were prepared and loaded according to the manufacturer's instructions with 250 ng of RNA isolated from the different *post mortem* times. The results were displayed as electropherograms and gel-like images. RNA quality was evaluated using the RNA integrity number (RIN) calculated with the Agilent 2100 expert software (RIN beta release; Schroeder et al., 2006). The RIN algorithm allows calculation of RNA integrity using a trained artificial neural network based on the determination of most informative features that can be extracted from the shape of the curve in the electrophoretic profiles. The RIN ranges from 1 to 10, with 1 being the most degraded profile and 10 the most intact (Schroeder et al., 2006). In addition, the 28S:18S ribo-