Postmortem changes in muscle fibres autofluorescence

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Abstract – After slaughter, the muscle cells undergo biochemical and physicochemical changes which may affect their autofluorescence characteristics. The *postmortem* metabolism kinetic of rat EDL and soleus muscles was assessed by glycogen depletion determination while autofluorescent response of different muscle fiber types was investigated by Deep UV synchrotron microspectroscopy at slaughter and and 24 hours *postmortem*. Following a 275 nm excitation, emission fluorescence spectra showed discrimination depending on *postmortem* time (T0 versus T24h) on both muscles at 346 and 302 nm and to a lesser extent at 408 and 325 nm. Taken individually, all fiber types are discriminated but with variable accuracy, the type IIA showing better separation of T0 comparing to T24h than other fiber types.

These results highlight the relevance of using the autofluorescent response of muscle cells to rapidly assess their state of *postmortem* changes.

I. INTRODUCTION

After slaughter of farm animals, muscle undergoes significant metabolic, physical, structural and biochemical changes that determine the qualities of meat and meat products (Bendall, 1973). After bleeding, muscle maintains their homeostasis by degrading their glycogen stock through anaerobic glycolysis. The lactate, which accumulates in cells, is accompanied by a drop in pH that stabilizes when the energy reserves are depleted. Then, additional biochemical changes and significant ultrastructural alterations are observed and are related to the improvement of meat quality, especially meat tenderness. The speed of these *postmortem* changes (also called meat maturation) is dependent on the species, and mostly on the metabolic and contractile type of considered muscles (Ouali, 1990). Degradation of myofibrillar structure is faster in the white fibers than in red ones.

Characterization of *postmortem* changes, related to meat quality, can be assessed by mechanical measurements, biochemical and / or ultrastructural analysis, but these methods are usually destructive and time consuming. Fluorescence spectroscopy methods allow characterizing slight differences in muscle composition (Dufour *et al.*, 2003). This highly sensitive method allows detecting fluorophores naturally present in muscle and whose properties are very sensitive to changes in their environment. Collagen, tryptophan, tyrosine or NADH are among the most abundant autofluorescent molecules in muscle (Dufour *et al.* 2003). It has been shown that collagen and tryptophan autofluorescence signals allow the discrimination of different muscles and the meat storage time (Frencia *et al.* 2003).

More recently, the combination of a microscope to a UV fluorescence spectrometer coupled to synchrotron radiation has been used to characterize animal tissues at ultrastructural level (Jamme *et al.*, 2013).

Our objective was to characterize the *postmortem* metabolism and structural changes of two rat muscles very different on their contractile and metabolic types and to explore the effect of a 24h maturation time on the muscle fibers autofluorescence response, taking into account their metabolic and contractile types.

II. MATERIALS AND METHODS

1. Animals and samples

Twenty four male Wistar rats, aged 5 months were sacrificed by decapitation following an isoflurane gas anesthesia. Immediately after slaughter, the lower limbs were carving and

dissected under sterile conditions. Extensor Digitorum Longus (EDL) and Soleus muscles from right and left posterior legs were extracted from tendon to tendon. Muscles from three rats were immediately cryofixed at the end of dissection (10 minutes after decapitation). For the 21 other rats, muscles were suspended at 20 °C in a sterile moist chamber to prevent drying of the muscle and contamination by microorganisms. Lead ballast (1.5g) was attached at the caudal tendon of each muscle to preserve the anatomical muscle length.
After respectively 30min, 1h, 2h, 4h, 8h, 12h and 24h *postmortem*, muscles of three rats were removed from their moist chambers and prepared for biochemical and imaging analysis.

2. Glycogen determination

Glycogen content was determined on each right EDL and Soleus rat muscles at each *postmortem* time by enzymatic procedures according to Bergmeyer (1974), Results were expressed in micromoles per gram of fresh tissue.

3. Histology

Parts of left EDL and Soleus muscles from 0 (T0) and 24h (T24) *postmortem* time, were cryofixed by immersion in -160°C isopentane cooled with liquid nitrogen (-196°C). Serial cross-sections of entire muscles (10 µm thick) were realized using a cryostat (Microm, HM 560) and collected on glass slides for histological stains and on quartz coverslips for DUV microspectroscopy fluorescence analyses.
Fiber types were identified by highlighting the different myosin heavy chains isoforms (MyHC) thank's to specific mouse monoclonal antibodies BA-D5, SC-71, BFF3 (AGRO-BIO France). The different primary MyHC antibodies were revelated by an Alexa Fluor 488 labelled goat anti-mouse IgG secondary antibody (A 11001, Invitrogen). The myofiber response to the different MyHC antibodies allowed to identify fiber types I, IIA, IIB and hybrid IIX-IIB. IIX fiber types corresponded to the remaining unmarked cells. Controls were performed without primary antibody to validate the results.
Observations and images acquisitions were performed using a light microscope (Olympus BX 61) coupled to a high resolution digital camera (Olympus DP 71) and the Cell F software.

4. UV fluorescence microspectrocopy

From 2 rat Soleus and EDL muscles (0 and 24h post-mortem), a unstained serial section was deposited on quartz coverslips, and thank's to fiber types identification by immunohistofluorescence on other serial sections, 10 cells of each type (I, IIA, IIX, IIX-IIB, IIB) were spotted for fluorescence spectra acquisition by Deep UV microspectroscopy which was performed on the DISCO beamline of the SOLEIL synchrotron radiation facility (Saint-Aubin, France). Deep UV monochromatized light was used at 275 nm excitation wavelength to excite tissue sections, the emission spectra were acquired from 290 to 540 nm. Each excited pixel was recorded by the fluorescence spectrum arising. On each spotted cell, 20 acquisitions were made in the intracellular space (10 in the central and 10 on periphery of the cell).
Autofluorescence spectra were spike and noise filtered using an in-house program written in MATLAB version 7.3 (The MathWorks, Natick, MA) and images of each individual fluorescent component were produced. The Unscrambler software (v9.8, Camo Software AS, Norway) was used to perform a baseline adjustment to zero, to apply unit vector normalization and to analyze the processed spectra in principal component analysis (PCA). Loading plots derived from the first (PC1) and second principal components (PC2) were used to reveal and identify characteristics fluorescence peaks.

III. RESULTS AND DISCUSSION

1. *Postmortem* metabolism evolution.

The initial concentration of glycogen (around 23µmoles / g**,** Figure 1) is lower than data from Calder & Geddes (1990) and Manabe *et al.* (2013) who observed 30-40 µmol/ g of fresh rat muscle. This could be due to the depletion of glycogen during the 10 min dissection (Calder *et al.* 1990). The initial glycogen content is lower than in muscle of farm animals (about 60-70 µmol / g in average) and residual glycogen reserves remained relatively high compared to farm animals whose meat glycogen reserves fall to less than 10 micromoles / g.

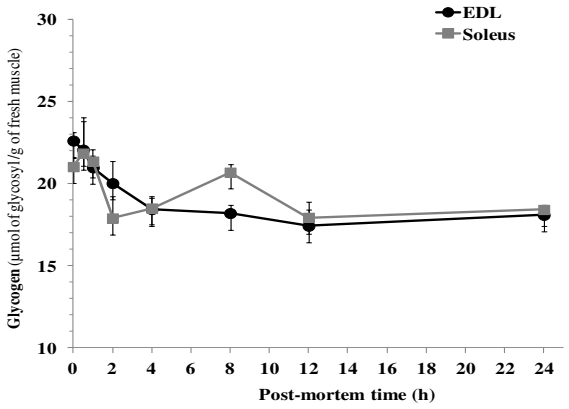

Figure 1: *Postmortem* evolution of glycogen content in EDL and Soleus muscles

2. Fluorescence response of muscle fibers according to *postmortem* time

The results of Principal component analysis (PCA) are presented in Figures 2-4.

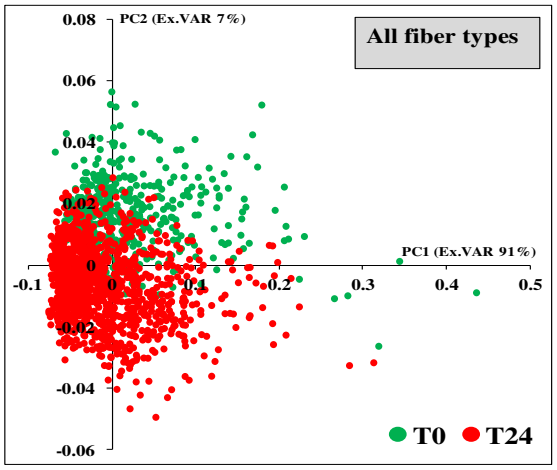

Figure 2: PCA score plots of fluorescence response of fresch (T0) and 24 hour *postmortem* (T24) EDL muscle

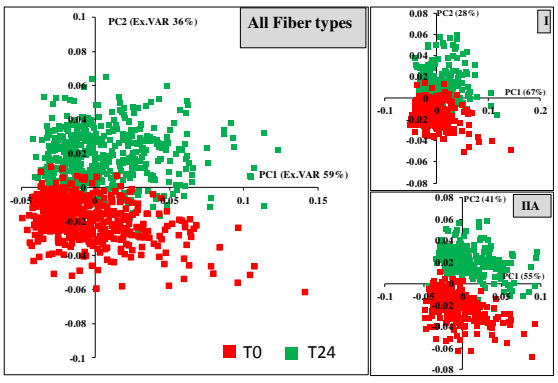

Figure 3: PCA score plots of fluorescence response of fresch (T0) and 24 hour *postmortem* (T24) soleus muscle.

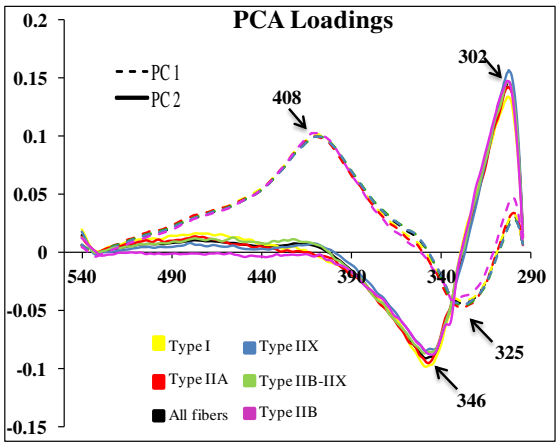

Figure 4: PCA loadings of fluorescence response of fresh (T0) and 24 hour *postmortem* (T24) EDL and soleus muscle.

PCA score plots and loadings indicate a separation of the two *postmortem* times (T0 and T24) essentially in the PC2 on the fluorescence intensities at 302 and 346 nm, whatever the considered muscle. In general, 302 nm and 408 nm fluorescence intensities were higher and 346 nm and 325 nm fluorescence intensities lower for T0 than T24 samples. Separation on the EDL PCA is less clear than in the Soleus, mainly because the EDL is composed of four types of fiber while the Soleus is composed of only I and IIA fibers.

Some cell types allow a better separation of *postmortem* times like type IIA fibers which show excellent discrimination of post mortem times on both the EDL (not shown) and Soleus muscles. Discriminatory wavelengths of PC1 and PC2 are exactly the same for the 2 muscles (EDL and Soleus) and all types of fibers: 325 nm and 408 nm for PC1, 302 and 346 for PC2. So it seems that only few fluorophores are concerned in *postmortem* time discrimination.

The emission fluorescence at 302 nm is assigned to tyrosine (Jamme et al. 2013). Emission fluorescence at 326, and 346 could be assigned to tryptophan but with no certainty. The fluorescence peak at 408 nm could be attributed to NADH and / or pyridoxine since on living cells these compounds are suspected to fluoresce in this wavelength range (Jamme *et al.* 2013). From animal slaughtering, the muscle undergoes structural, biochemical and physicochemical changes. The pH drop is accompanied by a glycogen decrease, lactate accumulation in cells and muscle contraction. Quickly, the plasma membranes are perforated and gradually ionic

gradients plasma membrane, nucleus and organelles (mitochondria, lysosome sarcoplasmic reticulum …) collapse leading to the ion concentration equilibrium in the cells. Muscle proteins are degraded into peptides under the action of endogenous proteolytic enzymes (Ouali, 1990). These phenomena substantially modify the intracellular composition with consequent effects on its pH, ionic strength and osmotic pressure. It is unlikely that the amino acid components of the peptides derived from protein hydrolysis are degraded.

Therefore, T0 and T24 differences in fluorescence intensity of amino acids are probably related to changes in their environment (Lakowicz, 2006).

The level of discrimination seems dependent on both muscle (EDL or Soleus) and fiber type. Compared to type I fibers, the better *postmortem* time separation on IIA fibers suggested more marked environmental changes.

IV. CONCLUSION

The muscle physicochemical and biochemical changes, subsequent to its extended anoxia, lead to a variation of the fluorescence characteristics of endogenous molecules emitting at 302 and 346 nm and to a lesser extent at 408 and 325 nm. The analysis of the fluorescence spectra allows the discrimination of 24 hours *postmortem* muscles from 10 minutes *postmortem* ones. The quality of discrimination depends on the fiber type; the most discriminated being the IIA fiber type suggesting a more efficient discrimination of *postmortem* time on muscle with high type IIA fiber proportion.

Our results demonstrate the benefit of exploiting the variation of autofluorescence muscle cells to rapidly characterize the effect of prolonged anoxia. These observations bring into perspective the possibility of using the DUV spectroscopy to characterize the degree of maturation a meat.

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