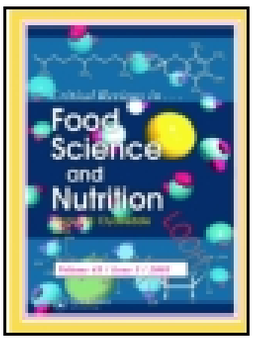

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BIOMARKERS: NON DESTRUCTIVE METHOD FOR PREDICTING MEAT TENDERIZATION

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Abstract:

Meat tenderness is the primary and most important quality attribute for the consumers worldwide. Tenderness is the process of breakdown of collagen tissue in meat to make it palatable. The earlier methods of tenderness evaluation like taste panels and shear force methods are destructive, time consuming and ill suited as they requires removing a piece of steak from the carcass for performing the test. Therefore, a non-destructive method for predicting the tenderness would be more desirable. The development of a meat quality grading and guarantee system through muscle profiling research can help to meet this demand. Biomarkers have the ability to identify if an exposure has occurred. Biomarkers of the meat quality are of prime importance for

meat industry, which has ability to satisfy consumers' expectations. The biomarkers so far identified have been then sorted and grouped according to their common biological functions. All of them refer to a series of biological pathways including glycolytic and oxidative energy production, cell detoxification, protease inhibition and production of Heat Shock Proteins. On this basis, a detailed analysis of these metabolic pathways helps in identifying tenderization of meat having some domains of interest. It was, therefore, stressed forward that biomarkers can be used to determine meat tenderness. This review article summarizes the uses of several biomarkers for predicting the meat tenderness.

Keywords:

carcass, meat, tenderness, non-destructive method, quality.

INTRODUCTION

Meat refers to the animal tissue or muscles of warm blooded animals, which were suitable for use as food. Although meat is composed of numerous types of tissues, but the major component of meat is muscle. Meat is rich in most of the nutrients required by the body. It is rich in proteins, contains all essential amino acids. It is also rich in minerals and vitamins. Phosphorus, copper and iron are present in significant amount in meat. Thiamine, riboflavin and niacin occur in good amount in all the meats. Proteins are the most important functional components of muscle foods, where they confer many of the desirable physicochemical and sensory attributes (Thu, 2006). Muscle proteins comprise 15–22% of the total muscle weight and can be divided into three major groups on the basis of solubility characteristics: sarcoplasmic proteins (water-soluble), myofibrillar proteins (salt-soluble) and stromal proteins (insoluble).

Muscle tissue is composed of long cylindrical cells termed myofibres that are surrounded by collagen fibres in the extra cellular space (Fig. 1). Muscle cells are packed with smaller cylindrical organelles called myofibrils that occupy over 80% of the cell volume. There may be as many as 1000 of these 1-2 μm diameter myofibrils in a cross section of a muscle fibre. Observation of these organelles in a phase contrast microscope reveals alternating light and dark bands (Kemp et al., 2010). Electron microscopy shows that the bands arise because of the presence of two major filaments: thick filaments in the A-band and thin filaments in the I-band. A dense line bisects the I-band perpendicular to the myofibril's long axis and is termed the Z-line. An M-line is located in the middle of the A-band. The filaments are composed of proteins, with myosin being the major constituent of the thick filaments while actin, tropomyosin and

troponin make up most of the thin filaments. Costameres link myofibrils to the sarcolemma, and N2-lines have been reported to be areas where titin and nebulin filaments, form a cytoskeletal network linking thick and thin filaments, respectively, to the Z-disk (Craig and Pardo, 1983).

MEAT TENDERIZATION

Tenderization is a variable process depending on a number of biological factors i.e. age, sex-type, muscle type and species. This is further affected by the rate of glycolysis, rate of pH decline, osmolarity of muscle cells, temperature, and genetic factors inherent to the animal, amongst others. Tenderness of meat originates in the biochemical and structural properties of skeletal muscle fibres (myofibrils, intermediate filaments, intramuscular connective tissue, the endomysium and the perimysium, which are composed of collagen fibrils and fibres). The connective tissue content and its properties are responsible for approximately 20% of variation in meat tenderness and the weakening myofibril structure primarily control meat texture. The mechanisms underlying myofibrillar fragmentation or disintegration of the myofibril structure are, the result of proteolytic action of various enzyme systems from which the calpain proteolytic system seems to play a major role. The final tenderness of meat depends on the degree of alteration of the muscle structural and associated proteins. Specific myofibrillar, myofibril cytoskeleton and costamere proteins, such as titin, desmin and vinculin respectively, are subjected to cleavage, with some cleavage of the major myofibrillar proteins such as actin, myosin (Hopkins and Taylor, 2002). The protease must be endogenous to skeletal muscle cells and it must be able to mimic post-mortem changes in myofibrils in vitro under optimum conditions and finally it must have access to myofibrils in tissue to be involved in post-mortem proteolysis and meat tenderisation (Koohmaraie, 1988). Tenderness is the most desirable quality

in meat. Tenderization is a process to break down collagens tissues in meat to make it more palatable for consumption. Tenderness can therefore be defined as:

1) Ease of penetration by teeth,

2) Ease with which meat breaks into fragments,

3) The amount of residue remaining after chewing (Jeremiah et al., 2003).

The amount of distribution of connective tissues and the size of both muscle fibres and bundles of fibres determine the tenderness of meat. The activities of calpains and their inhibitors, calpastatins have been especially associated with the tenderization process.

There are a number of ways to tenderize meat:

- Mechanical tenderization, such as pounding, cutting, grinding, piercing use of ultrasonic vibrations.
- The tenderization that occurs through cooking, such as braising.
- Tenderizing in the form of naturally occurring enzymes, which are added to food before cooking, like papain, bromelin and ficin.
- Marinating the meat with vinegar, wine, lemon juice, buttermilk or yogurt.
- Brining the meat in a salt solution (brine).
- Dry aging of meat at 0 to 2 °C (32 to 36 °F).

IMPORTANCE OF MEAT TENDERNESS:

The meat industry at present is faced with a major problem, which has been indicated in numerous international consumer surveys namely the inconsistency in meat tenderness. A significant portion of commercially available meat is regarded as unacceptable, due to toughness. Meat tenderness is therefore considered as one of the most important meat qualities attributes.

Meat quality is a complex trait influenced by genetic components, by the handling of animals during production, transportation, and slaughter, and by the handling of the meat during the slaughter process. Immediately after slaughter, there is in the muscle tissues as in all other organs a shift of biochemical processes in response to the halt of the respiratory system and the circulation of blood (Bongioni et al., 2004). In muscle tissues, the morphological and ultra structural changes as well as the changes in the energy metabolism that occur in the post mortem period and extent to which the post mortem metabolic processes run greatly influence important traits such as tenderness, water-holding capacity and sensory properties of meat. With regard to the tenderness of meat, it is well-known that the post mortem storage of meat greatly influences tenderness, and it is believed that degradation and denaturation of proteins during post mortem aging is responsible for the tenderization of meat (Lawrie, 1998).

Among the different qualities of meat, Meat tenderness is one of the most important palatability characteristics and will always be the first quality attribute sought by the consumer. It is a very complex and multi factorial sensorial meat quality trait, which depends partly on muscle characteristics. Meat tenderness is characterised by a high and uncontrolled variability which depends, at least in part, on differences in muscle characteristics. In meat, tenderness has long been recognised as the key determinant of eating quality, with evidence demonstrating that consumers accept to pay more for guaranteed tenderness (Guillemin et al., 2012). Tenderness is an important criterion in consumer's meat appreciation, and so a determining factor in purchase. Meat represents an important economic sector in some countries. However, uncontrolled tenderness variability is the number one reason for consumer's dissatisfaction. This tenderness variability is due to the muscle nature, which is a complex biological structure, consisting of

fibers, adipocytes and connective tissue with different properties, each partially responsible for the tenderness variability (Guillemin et al., 2012). Besides genetic factors, meat tenderization is influenced by the nature of feeding, age of the animal, degree of stress prior to slaughter, carcass chilling, ageing time and cooking method. Final tenderness is determined by the rate and extent of post mortem proteolysis of key myofibrillar proteins in the muscle. The calpain system (calpain-I, calpain-II and calpastatin) is the principal contributor to post-mortem proteolysis which is closely related to meat tenderness. Among the factors affecting the tenderness, post rigor calpastatin activity has the largest effect (~40%) on aged meat muscle (Zor et al., 2009). Although meat tenderness is highly dependent on pre- and post-slaughter factors, physiological factors and measurable genotype differences (genetic factors) make a contribution to the total variation in tenderness. However, reliable eating quality guarantee systems are lacking, it is essential to gain an understanding of the contribution made by these factors (genetic and physiological), in order to develop parameters for them to aid in the assessment of meat tenderness.

EARLIER TECHNIQUES TO PREDICT MEAT TENDERNESS:

Currently, there is no available method on the market which can measure meat tenderness in a fast, accurate and objective way. Subjective measurements of this characteristic are made by structured taste panels. Objective measurements have been made traditionally by texture analysis methods. The measurement is usually made either by using taste panels which are expensive, time consuming and subjective, or by Warner–Bratzler Shear Force method, which is rather simple but destructive and is mostly evaluates the resistance of the meat during cutting without

providing direct meat tenderness determination (Platter et al., 2003). Tenderness was evaluated by following methods:

(i) Sensory panellists

Although instrumental measurements of tenderness can provide valuable information on the effectiveness of tenderization interventions, having a trained sensory panel is very important to assess how consumers would perceive the product in question. Trained panellists who rated meat samples on non structured line scales marked at the extremities 'low' and 'high' and subsequently scored as the distance in units of 1, from 1 to 10. Sensory analysis is generally considered as the reference method to evaluate eating quality. For the sensory evaluation 14 day aged samples at 4^{o}C, were grilled at 55–60^{o}C and then tasted by sensory panellists (Oury et al., 2009; Allais et al., 2011).

(ii) Warner–Bratzler shear force method (WB)

This method was developed in the 1930s and is frequently carried out using a texture analysis instrument. The texture analyzer is equipped with a Warner-Bratzler fixture to measure the force required to shear through a uniform piece of meat. The Warner-Bratzler consists of a rigid frame supporting a shear bar. Interchangeable blades fit into the frame. For meat testing, a triangular slotted blade is used. Sample steaks are cooked, cooled and then 6 core samples (12.7 mm diameter) taken, parallel to the longitudinal orientation of the muscle fibers. The force required to shear each sample using the slotted blade is measured by recording the resistance of the meat to shearing as a function of time in force-deformation plot. The maximum shear force is the highest peak of this curve. This device provides a measure of force required to shear through a uniform piece of meat. (Ross and Chris, 2008; Oury et al., 2009)

(iii) Penetrometry (Puncture Tests):

This test is based on penetrating the sample with a probe. Penetrometers represent the oldest, and the longest-used, group of texture-measuring instruments. In principle, they measure the forces required to achieve a partial or total penetration of a probe into the tested material. The greater the requisite force, or the smaller the penetration depth, the more resistant the material is. Penetrometers can be particularly useful in testing the consistency of fats and gels (Saláková, 2012). The method can be used in two ways:

•Measure the force required to penetrate to a certain depth, at a constant probe speed

•Measure the puncture depth attained within a set time or otherwise with constant force acting on the probe.

NEED OF BIOMARKERS IN MEAT TENDERNESS:

The above said methods of texture evaluation are destructive, time consuming and ill suited. Since this method requires removing a piece of steak from the carcass for performing the test, a non-destructive method for predicting the tenderness would be more desirable (Koohmaraie and Geesink, 2006). The development of a meat quality grading and guarantee system through muscle profiling research can help to meet this demand (Chriki et al., 2013). The variability of meat tenderness depends, at least partly, on differences in muscle characteristics. However, the association between eating quality traits (i.e tenderness) and muscle characteristics varies according to the breed (Chriki et al., 2013). Different research programs have revealed several genes or proteins which could be good markers of beef tenderness. In order to validate the relation of these markers with beef tenderness on a large population of bovines, it is

necessary to have a large-scale and trusty technique which can access different quantities of proteins related to tenderness (Guillemin et al., 2009).

BIOMARKERS

A biomarker, or biological marker, generally refers to a measured characteristic which may be used as an indicator of some biological state or condition. The term occasionally also refers to a substance whose presence indicates the existence of living organisms. Biomarkers are often evaluated and measured to examine normal biological processes pathogenic processes, or pharmacologic responses to a therapeutic intervention. Biomarkers are used in many scientific fields (Hulka, 1990). They are very useful in the world of exposure assessment when dealing with direct measurement methods. Use of biomarkers in exposure studies is also referred to as bio-monitoring. They are chemicals, metabolites, susceptibility characteristics, or changes in the body that relate to the exposure of an organism to a chemical (Richard, 2004).

Biomarkers have the ability to identify if an exposure has occurred. When dealing with exposure assessment, there are three types of biomarkers that can be useful, biomarkers of susceptibility, biomarkers of exposure, and biomarkers of effect. Biomarkers must be evaluated in terms of their ability to predict and quantify exposure and dose (Naylor, 2003). There are certain properties that are desirable when linking a biomarker with an exposure. An ideal biomarker should be safe and easy to measure. They also include high specificity (one exposure to one biomarker), linear relationship across time, strong correlation with a health effect, inexpensive study, and consistency that the same exposure will produce the same concentration of the biomarker every time (Kumar and Saran, 2009).

BIOMARKERS IN MEAT TENDERNESS

For the meat sector, it is of major interest to be able to predict for consumers tenderness of the meat of live animals, carcass or cuts, as variability in beef tenderness causes dissatisfaction of consumers. For several years, various scientific programs aimed to identify genomic biomarkers for tenderness (Picard et al., 2012). Transcriptomic and proteomic analysis on bovine muscles with low or high tenderness assessed by sensory analysis and/or mechanical measurements allowed to produce a list of several protein tenderness biomarkers (Guillemin et al., 2011). Functional genomics programs were developed to explain this tenderness variability and to identify tenderness biomarkers, at DNA (Hocquette et al., 2007), RNA (Bernard et al., 2007) and protein levels (Picard et al., 2010). These potential biomarkers have been revealed by comparisons of animals groups which differ in the quality of the meat they produce. Larger analyses, on different muscles, animal types and breeds, are necessary to confirm the roles of these biomarkers in the determinism of tenderness (Guillemin et al., 2009). Some markers have been identified in different contexts of countries, breeds, muscles, etc, but these markers and their effects are not identical according to the contexts. Commercialized genetic tests based on utilization of SNP (Single Nucleotide Polymorphism) markers of genes encoding μ-calpain and calpastatine, can predict the muscle tenderness class (Page et al., 2004). The meat industry supports functional genomics programs to identify potential tenderness biomarkers (Guillemin et al., 2012).

Since decades, the mechanisms of meat tenderization have focused much interest from meat scientists. Despite these extensive efforts, these mechanisms are still unclear and this probably explains the fact that we have not yet identified a good marker of this quality (Kemp et

al., 2010). It has been now agreed that the meat tenderizing mechanisms are enzymatic in nature and involve several intracellular proteolytic systems and the first step of the conversion of muscle into meat is the onset of apoptosis, a finely regulated and complex energy dependent cell death process (Ouali et al., 2006). Quality markers would be a reflection of the different metabolic pathways contributing to the postmortem development of meat tenderness. Hence, a better understanding of these pathways and their interactions is a prerequisite for a successful identification of accurate biological/biochemical markers of this primary quality attribute of meat. In this respect, the modern proteomic technology has undoubtedly contributed to a better understanding of these processes (D'Alessandro and Zolla, 2013).

MAJOR EVENTS OCCURRING DURING ANIMAL BLEEDING

After animal bleeding, tissues come into an ischemic anoxic state which will affect all metabolic pathways and will lead to an adaptation of most, if not all, metabolic processes (Ouali et al., 2006; 2007). During this process, the cell starts for the development of contradictory tools for cell death or cell survival pathways (Fig. 2). When cell enters into the anoxia state, its main objective will be to provide the energy needed for increased metabolic activities by improving its capacities. This led to an increase in the enzymes associated to glycolytic and tricarboxylic acid (TCA) cycles, as observed by quantitative analysis of 2D gel spots. A major consequence of this intense metabolic activity will be the accumulation of diverse harmful by products including CO_2, HCO_3^-, NH_4 and lactic acid, which are normally transported to the liver where they can be recycled. Later in the context we will consider the energy aspects in the last part of this review as it could be a good source for finding new biomarkers of meat tenderness (Ouali et al., 2013).

The second step after animal bleeding will be the preservation of cell functions by an increase in the concentration of several Heat Shock Proteins (HSPs) including HSPs 70, 40, 27, 20, α β-crystallin, and probably others. The battle between cell death and cell survival will finally turn to the advantage of the cell death process with the well-known characteristic changes associated to this status, especially cell shrinkage, and phosphatidylserine externalization, together with mitochondria alteration (Becila et al., 2010; Ouali et al., 2007). A set of pro- and anti-apoptotic proteins will be released from mitochondria and their ratio will define the rate and extent of apoptosis development. For the same reason than for the energy aspects, mitochondria will be reconsidered later in this review. So it could be interesting to develop studies on mitochondria, in order to establish whether this could be a way to better explain tenderness variability of carcasses (Ouali et al., 2013). TCA: Tricarboxylic acids, HSP: Heat Shock Proteins, PS: Phosphatidylserine IAP: Inhibitors of Apoptosis Proteins. (Ouali et al., 2013)

PARTICULAR STRUCTURAL APOPTOTIC CHANGES IN POSTMORTEM MUSCLE

Examination of the thin cuts was done with the aim to identify structural changes characteristic of the programmed cell death or apoptosis by Prochazkova et al. (2003). In Fig. 3a, an ante-mortem sample showing that muscle cells in close proximity to each other and very few extracellular spaces, after few minutes of bleeding an important shrinkage of muscle cells fibers and an increase in the extracellular space was observed (Fig. 3b, small arrows). During the past decades, much of the research work was devoted to the post-mortem evolution of intra- and extracellular spaces in relation with intracellular water movements in the muscle (Offer and

Knight, 1988a; b). It was generally recognized that the main cause of these changes was the distribution between the two compartments of water, which accounts in weight for approximately 75% of muscle tissue. Acidification of muscle decreases protein electrical charge and induces an increase in their hydrophobicity, thereby reducing water retention. This is confirmed by the very high correlation observed between the increase in extracellular space and muscle pH (Guignot et al., 1993). There was an early increase in extracellular space, starting immediately after slaughter, whereas pH was still very close to neutrality. It has recently reported that cell death could provide a realistic explanation of the early volume change in the extracellular compartment in postmortem muscle (Becila et al., 2010). This finding constitutes the first evidence supporting the early increase in the extracellular space resulting from the expulse of intracellular water towards the extracellular compartment whereas the pH is still high. Hence, cell death would provide a more realistic explanation of the early volume change in the extracellular compartment in postmortem muscle.

In Fig. 3c, DNA fragmentation was detected in mononucleated cells located within the extracellular matrix when muscle sample excised 15 min after bleeding. Note the important accumulation in the close proximity of other cells which could correspond to resident macrophages, also called ED2 and ED3 macrophages, known to be unable to undertake degenerative cells phagocytosis. Interestingly, such cells can be observed above and below where they seemed to delineate empty cells or blood vessels (Becila et al., 2010).

The alignment of these suspected resident macrophages around the dying cells might contribute to the isolation of these cells from their neighbours as soon as they are engaged in the "suicide" program. Macrophages are attracted by the cells engaged in apoptosis through the

translocation of phosphatidylserine to the external leaflet of the membrane, the major signal of its suicide status (Martin et al., 1995). By contrast, no DNA fragmentation seemed to take place in muscle cells nucleus. Fig. 3d, also taken from a 15 min sample, emphasized the DNA fragmentation occurring in an apoptotic mononucleated cell and further confirmed the accumulation of resident macrophages in the close proximity of the dying cell and also, above, aligned close to the muscle cell membranes (Becila et al., 2010). Sometimes it was observed that the presence of muscle cell membrane invaginations suggesting that they are ingesting extracellular material or dying small cells by phagocytosis for further degradation within muscle cells supporting the occurrence of phagocytotic activities in postmortem muscle. From these findings, it must be therefore stressed that the first cells to die postmortem are mononucleated cells from the extracellular matrix, which might be degraded by muscle cells after ingestion by phagocytosis.

CASPASES AND APOPTOSIS

Apoptosis is the process of programmed cell death (PCD) that may occur in multicellular organisms. Biochemical events lead to characteristic cell changes (morphology) and death. These changes include bleeding, cell shrinkage, nuclear fragmentation, chromatin condensation chromosomal DNA fragmentation memberane bleeding and the formation of apoptotic bodies without including and inflammatory responses (Wyllie et al., 1980). Caspases are a family of cysteine proteases that play essential roles in apoptosis (programmed cell death), necrosis, and inflammation. Caspases are essential in cells for apoptosis or programmed cell death, in development and most other stages of adult life, and have been termed "executioner" proteins for their roles in the cell. Some caspases are also required in the immune

system for the maturation of lymphocytes (Ouali et al., 2006; Sentandreu et al., 2002). Failure of apoptosis is one of the main contributions to tumour development and autoimmune diseases; this coupled with the unwanted apoptosis that occurs with Ischemia or Alzheimer's disease, has stimulated interest in caspases as potential therapeutic targets since they were discovered in the mid-1990s.

POTENTIAL BIOMARKERS OF MEAT TENDERNESS IDENTIFIED UP TO DATE

For many decades, meat scientists are looking for accurate biological markers of meat tenderness that would make possible: (1) the classification of meat cuts soon after slaughter on the basis of their potential ultimate tenderness and (2) the optimization of the genetic selection of meat animals on the basis of this quality. These challenges have aroused a great interest in this research field by meat scientists.

A series of potential markers of meat tenderness have been screened according to the metabolic or biological process they are involved, are overviewed in this review. There are 8 subgroups identified for biomarkers involved in biological process, the three most important of them, being glycolytic and oxidative energy supplying pathways together with Heat Shock Proteins (HSPs).

MARKERS FROM THE GLYCOLYTIC ENERGY METABOLISM PATHWAY

Glycolysis is a two-phase process. The first phase consuming 2 ATP that corresponds to the conversion of glucose-1-phosphate generated from either free glucose or from glycogen by phosphorylase, to two triose phosphate. The second one ensures the conversion of 2 triose-

phosphates (from1 glucose) to pyruvate and then lactate, producing 2 ATP and one NADH, H^+ molecules. All members of this selected group are enzymes of the glycolytic pathway (Table 1).

From this first phase, two enzymes, out of five, were identified as potential markers of meat tenderness. These are the first and the last enzymes of this phase, i.e. Phosphoglucomutase (Bjarnadottir et al., 2010; Chaze et al., 2013) and Triosephosphate isomerise (Kim et al., 2008). In muscle cells, the predominant triose is dihydroxyacetone phosphate (DHAP), an important metabolite at a cross-road between several metabolic pathways (Fig. 4). DHAP can also enter the glycerol–phosphate shuttle (shuttle between cytosolic and mitochondrial glycerol-3-phosphate dehydrogenase), besides the normal continuity of the glycolytic pathway through isomerization to glyceraldehyde-3-P (second phase of glycolysis), which plays an important role in skeletal muscle (Kalapos, 1999).

This glycerol–phosphate shuttle is indeed used to rapidly regenerate NAD necessary for glycolysis and hence, speed up this pathway. Increased levels of DHAP can also activate the methylglyoxal synthase and DHAP then enters the glyoxal pathway which produces methylglyoxal, a toxic component for the cell (Thornalley, 2008). This toxic metabolite can further follow a two-step conversion to lactate, a chain of reactions catalyzed by both Lactoylglutathione lyase and Hydroxyacylglutathione hydrolase or by Methylglyoxal reductase and aldehyde dehydrogenase.

Opposed to the first phase, a majority of the enzymes 5 out of 6, involved in the second phase of glycolytic energy metabolism pathway were identified as good markers of meat tenderness. These were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Colell et al., 2009), phosphoglycerate kinase (Jia et al., 2006), enolase or phosphopyruvate hydratase (Choi et

al., 2010; Laville et al., 2009), pyruvate kinase (Laville et al., 2009; Polati et al., 2012) and lactate dehydrogenase (Laville et al., 2009; Polati et al., 2012). A most interesting feature of second phase glycolysis is the multifunctional role of GAPDH which is present in different compartments of the cell and contribute to diverse adverse cellular functions. It thus has a pro-apoptotic or a pro-survival function in cell (Tarze et al., 2007; Colell et al., 2007). Aldehyde dehydrogenase (Hollung et al., 2007; Jia et al., 2006), is another potential marker indetified increasing in postmortem muscle, that contributes to the glycolytic pathway through the direct conversion of glyceraldehyde to 2-phosphoglycerate. This enzyme is a member of the aldehyde dehydrogenase family, which is known to be implicated in multiple other processes including amino acids and fatty acid metabolism. Some members of this family of aldehyde oxidase protect against aldehydes generated by lipid peroxidation and overall aldehyde cytotoxicity and against both oxidative and osmotic stress (Brocker et al., 2010; Pappa et al., 2005).

MARKERS FROM THE OXIDATIVE ENERGY METABOLISM PATHWAY

Oxidative energy metabolism ends up within the mitochondrion matrix where ultimate degradation products of lipids, amino acids and polysaccharides are oxidized, providing substrates to the electron transport chain and to the ATP syntheses responsible for ATP production. In other words, this pathway uses all ultimate products provided by degradation of sugars, especially glucose (glycolysis), proteins (and more accurately amino acids coming from either the pool of free amino acids or from protein hydrolysis) and fatty acids generated from triglycerides (Ouali et al., 2013). Seven markers of tenderness coming from these energy supplying pathways have been so far identified (Table 2). 3-Hydroxyisobutyrate dehydrogenase (Jia et al., 2006; Jia et al., 2007; Lokanath et al., 2005) is an enzyme involved in the degradation

of the branched amino acid valine to succinyl-CoA, a member of the tricarboxylic acid cycle (TCA) cycle. β-Hydroxyacyl CoA-dehydrogenase (HADH) (Hamill et al., 2012; Polati et al., 2012) is a member of the β-oxidation of lipids, also known as the Lynen cycle (or Lynen helix) which produces acetyl-CoA, a metabolite entering the TCA cycle. Cytochrome c (Ding et al., 2002) is a member of the electron transport chain located outside the inner membrane of mitochondria. The three other markers identified from this pathway, i.e. Succinate dehydrogenase, Succinyl Co-A synthase and Isocitrate dehydrogenase, are all members of the TCA cycle (Jia et al., 2006; Hollung et al., 2007; Kim et al., 2009; Zapata et al., 2009; Hamill et al., 2012).

From different studies, it was stressed forward that all cellular components including sugars, amino acids (from the free pool or from protein degradation) and lipids could concomitantly be used for energy production in postmortem muscle. Regarding mitochondrial enzymes, it might be wonder on how the enzyme's concentration increases postmortem. Whether this concentration change results from an up regulation of the proteins' expression or from a multiplication of the number of mitochondria by fission as suggested for apoptotic cells remains an open question. Supporting this hypothesis, several studies indeed showed that mitochondrial morphology changes during apoptosis, resulting in small, round and more numerous organelles (Youle and Karbowski, 2005; Suen et al., 2008).

MARKERS INVOLVED IN CELL DETOXIFICATION

In living cells, several waste metabolites are produced by cell metabolism. Some of them are normally eliminated directly or after blood transport to liver. The most important are: (1)

NH_4 generated during amino acid degradation for energy production, which is recycled, in vivo, through the urea cycle in the liver. (2) Methylglyoxal, by product of DHAP.

(3) Carbon dioxide (CO_2) released from the TCA cycle and during the conversion of glycerate to acetyl-CoA (4) Carbonic acid (H_2CO_3) formed from CO_2 conversion by carbonic anhydrase (5) Toxic aldehydes.

In postmortem muscle, some enzymes are able to metabolize more or less these metabolites. Carbonic anhydrase can catalyze the conversion of CO_2 to bicarbonate which could explain, the discontinuity observed in the pH profiles. Lactoylglutathione lyase, or Glyoxylase 1, is able to catalyze the first step of the conversion of methyglyoxal to lactate. Finally, besides its function in glycolysis (Jia et al., 2006), some members of the complex aldehyde dehydrogenase family (Vasiliou et al., 2012), could also protect cells against cytotoxic effects of various aldehydes accumulating in the cytosol (O'Brien et al., 2005). It is worth noting that these three enzymes have been identified as potential markers of tenderness (Table 3). Regarding ammonium ions (NH_4^+), these are normally recycled in liver (glucose–alanine cycle) where it enters the urea cycle. During postmortem, this transportation is no more available and thus ammonium ions will accumulate in muscle tissue. These ions can be then used in different ways (Adeva et al., 2012) like for

(1) alanine synthesis: pyruvate (limiting substrate) + NH_4 → alanine.

(2) For synthesizing glutamate from alpha-ketoglutarate NH_4 + alpha-ketoglutarate (limiting substrate) → glutamate.

(3) Used by glutamine synthetase to convert glutamate to glutamine

MARKERS FROM THE HEAT SHOCK PROTEIN FAMILY

A large set of HSPs have been associated with meat tenderness as summarized in Table 4. According to most studies, the increase in HSP levels results in meat toughening. However, it is difficult to globally analyze the contribution of HSPs to meat tenderization and each of them must be considered separately. The role of HSPs in post mortem muscle remains unclear and additional investigations on the underlying mechanisms will be needed (Arya et al., 2007; Lanneau et al., 2008).

Stress induces synthesis of protective proteins called Heat Shock Proteins (HSPs) which preserve cellular proteins against denaturation and possible loss of functions (Kultz, 2003). The many known Heat Shock Proteins are generally classified in subfamilies on the basis of their size (molecularweight in kDa): HSP 90, HSP 70, HSP 40, HSP 27, etc. Most of them play an important role as molecular chaperones during protein assembly (Haslbeck et al., 2005), protein folding and unfolding (Zietkiewicz et al., 2004) and in the refolding of damaged proteins (Marques et al., 2006). It was expected that HSPs, also called stress proteins, at the time of animal death will have a pro-survival function and, hence plays an anti-apoptotic role (Arrigo, 2005; Beere, 2005; Flower et al., 2005). Upon apoptotic stimuli, HSPs may therefore have diverse anti-apoptotic actions. They are involved in the formation of a complex with active caspases (initiators or effectors) thus hindering their function. Also helps in protection of target proteins (substrates) of effectors caspases, thus by preventing their degradation by these enzymes. They also help in the restoration of the initial and active structure of proteins having undergone structural damage following either the stress itself or the initiation of apoptosis.

ANNEXINS AS POTENTIAL MARKERS OF MEAT TENDERNESS

Annexins are members of large structurally-related and calcium sensitive protein family. Expressed in all eukaryotic cells, they participate in a variety of cellular processes including apoptosis and intracellular signaling. Annexins are a class of Ca_2^+ regulated proteins, characterized by the unique architecture of their Ca_2^+ binding sites, which enables them to peripherally localize onto negatively charged membrane surfaces in their Ca_2^+ bound conformation (Gerke and Moss, 2002; Monastyrskaya et al., 2009). They are multifunctional proteins, contributing to numerous cellular and physiological processes (Gerke et al., 2005; Kenis et al., 2010; Van Genderen et al., 2008):

- They provide a membrane scaffold, which is relevant to changes in the cell shape.
- They are involved in trafficking and organization of vesicles, exocytosis, endocytosis.
- They contribute to calcium ion channel formation.
- They can be transported to the extracellular space where their activity is linked to fibrinolysis, coagulation, inflammation and apoptosis.

Changes in the concentration of two annexin isoforms, Annexin A1 (also known as lipocortin-1) and Annexin A6, have been recently reported in postmortem muscle, a change probably related to apoptosis development and meat tenderization (Bjarnadottir et al., 2012; Zhao et al., 2010).

ANNEXIN A1

Annexin A1 has been implicated in the apoptotic process where they are rapidly translocated to the cell surface as an “eat me” message to promote the removal of cells that have undergone apoptosis. Similarly to phosphatidylserine, Annexin A1 is therefore a marker of apoptosis onset, but its exportation to the cell surface is dependent on caspase activation (Ouali et al., 2013). Caspase activation thus induces a recruitment of Annexin A1 from the cytosol, a

translocation to the outer plasma membrane leaflet where it colocalizes with phosphatidylserine and is also required for efficient clearance of apoptotic cells (Arur et al., 2003). The caspases are activated in postmortem muscle and suggests an over expression of these proteases in tender meat (Ouali et al., 2006).

ANNEXIN A6

Annexin A6 is involved in a large set of biological processes and promotes apoptosis (Cornely et al., 2011). In cells lacking Annexin A6, mitochondrial morphology is indeed abnormal, Ca_2^+ signaling and respiration are impaired and cells have increased resistance to Ca_2^+ mediated apoptosis. Mitochondrial fission is an early event during apoptosis, occurring before caspase activation. This process is mediated by binding of the fission GTPase Drp 1 (Dynamin-related protein 1) to the outer mitochondrial membrane leading to a preliminary release of small amounts of Cytochrome c (cyt c) (Chlystun et al., 2013; Suen et al., 2008). Released cyt c translocates to the endoplasmic reticulum where it selectively binds InsP3R (inositol (1,4,5) triosephosphate receptor) resulting in sustained cytosolic calcium increases. Mitochondrial fission can be inhibited by binding of Annexin A6 to Drp1, an inhibition relieved by high cytosolic calcium levels, which dissociates the Drp1-Annexin A6 complex and targets Annexin A6 to the plasma membrane (Boehning et al., 2005; Boehning et al., 2003). This event causes an amplification of Cytochrome c release and fission of mitochondria, generating a higher number of smaller organelles.

In postmortem muscle, Annexin A6 acts as a brake to apoptosis through inhibition of Drp 1 and subsequent intensification of Cytochrome c release occur soon after animal death (Fig 5). Cytochrome c released from Mitochondria binds to InsP3R resulting in sustained calcium efflux.

Bik, a BH3 member of B-cell lymphoma 2 family also mediates calcium release from ER. Increase in cytosolic calcium induces Drp1 recruitment to the mitochondria which initiates the fission process. Mitochondria take up calcium into the matrix via calcium channel as mitochondrial calcium uptake (MICU). The massive calcium influx into the matrix leads to mitochondrial fission, accelerates Cytochrome c release and amplify apoptosis.

Apoptosis regulation:

- Proteins (Prot) released from mitochondria comprise Cytochrome c, Apoptosis Inducing Factor (AIF), Endo G (endonuclease G), second mitochondria-derived activator of caspases encoded by the Diablo gene (Smac/Diablo) and Omi stress-regulated endopeptidase/high-temperature-requirement protein A2 (Omi/HtrA2).
- Cytochrome c binds to Apoptatic protease activating factor (Apaf1) to form the apoptosome, a complex activating procaspase 9.
- Endo G and AIF translocate to the nucleus where they contribute to chromatin condensation and DNA fragmentation.
- IAPs (Inhibitors of Apoptosis Proteins) are inhibitors of caspases 3, 9 and 7 and the interaction with their target enzymes can be reversed by SMAC/Diablo and Omi/HtrA2.
- SERPINA3 like are pseudo-irreversible inhibitors of initiator and effecter caspases and hence will be essential regulators of the caspase-dependent apoptotic process.

With regard to the present function of Annexin A6 in apoptosis, a lower abundance of this protein in tender meat would be expected as compared to tough meat, a proposal in good agreement with the conclusion of Bjarnadottir et al. (2012) who observed lower Annexin A6 levels in tender meat.

OTHER MARKERS OF TENDERNESS

GALECTINS 1

Galectin 1 (Bjarnadottir et al., 2012; Zapata et al., 2009), a member of the Galectin family and constitute an evolutionary conserved family of β-galactoside binding proteins that are ubiquitous in mammals and other vertebrate, invertebrates and fungi. Galectin 1 is believed to exhibit pro apoptotic activities whereas Galectin 3 is the only one considered to have an anti-apoptotic activity (Hernandez and Baum, 2002). The role of Galectin 1 in apoptosis is however highly controversial and contradictory findings are reported in the literature. It appears that a lower expression of Galectin 1 is associated with greater tenderness (Zapata et al., 2009; Bjarnadottir et al., 2012).

PEROXIREDOXIN 6

Peroxiredoxin-6 (Jia et al., 2009) is an antioxidant contributing to hydrogen peroxide degradation and exhibiting phospholipase activity. Peroxiredoxin 6 (Prdx6) was the sixth (and final) mammalian member of the Prdx family to be described and shares structural and functional properties with other members of this family but has important characteristics that make it unique among the Prdxs. The first one is structural in nature since it has only one conserved cysteine residue instead of two for other members. Second, Thioredoxin, the natural co-enzyme of most Prdxs, does not participate in the catalytic cycle as compared to the other members of this family. Third, Prdx6 is able to bind and reduce phospholipid hydroperoxides, an essential enzymatic activity in antioxidant defense. Finally, structural studies revealed that Prdx6 is a bi-functional enzyme with phospholipase A2 activity in addition to its peroxidase function (Fisher,

2011). Prdx6 functions in antioxidant defense mainly by hydrolysis of hydrogen peroxides and by facilitating repair of damaged cell membranes via reduction of peroxidized phospholipids (Manevich and Fisher, 2005). Jia et al. (2009) reported an over expression of Prdx6 in tender meat, a finding in contradiction with the antiapoptotic and/or cell survival activities of this protein. However, it is presently difficult to get conclusions about the functions of Prdx6 in postmortem muscle and thus more detailed investigations will be needed in order to clarify the exact nature of the relationship between Prdx6 increased activities and ultimate meat tenderness.

PROTEASES AND PROTEASE INHIBITORS

Since decades, meat tenderizing process is unanimously recognized to be enzymatic in nature and the most studied proteolytic systems were cathepsins, calpains, the 20S proteasome and, although more recently, members of the caspases family (Kemp and Parr, 2012; Ouali et al., 2006; Sentandreu et al., 2002). Proteolytic enzyme levels were indeed expected to be good predictors of meat tenderness. In this respect, the at-death enzyme/inhibitor ratio or the inhibitor concentrations alone are both the best predictors of meat tenderness reported up to date (Ouali and Talmant, 1990). Assays with calpastatin, the common specific inhibitor of calpains 1 and 2, have not been convincing and not reproducible. Cystatins, a group of cysteine protease inhibitors, have been identified as potential markers (Shackelford et al., 1991). It was thus stressed that at-death cystatin levels showed a much higher correlation with Warner Bratzler shear force at 7 days postmortem than calpastatin levels alone ($r = +0.62$ vs -0.23 for calpastatin), that confirming the unreliability of calpastatin as predictor of meat tenderness.

Serine protease inhibitors have been suggested to be a good marker of meat tenderness. Serine protease inhibitors revealed a complex protein family belonging to the serpin super

family. Serpins, an acronym of SERine Protease Inhibitors, were discovered in the beginning of the 80's. This super family comprises the largest family of protease inhibitors identified to date, now having over 3000 members in all the three kingdoms of life, the archaea, the bacteria, and the eukaryotes, as well as in some viruses. Serine protease inhibitor levels have been shown to be best predictors of meat tenderness among about thirty quantitative variables including calpains and calpastatin (Zamora et al., 2005). This unexpected feature was extremely surprising since no serine protease susceptible to contribute to myofibrillar softening was reported (Gagaoua et al., 2012; Ouali, 1990). Investigations then performed on bovine muscle aiming at identifying these intracellular serine protease inhibitors revealed that most of them belong to the serpin super family (an acronym of SERine Proteases INhibitors), the largest serine protease inhibitor subfamily. Serpins are pseudo irreversible inhibitors of serine proteases, cysteine proteases and possibly other proteases groups. Some of them exhibited no inhibitory activity and serves other functions (Olson and Gettins, 2011). As they bind tightly to caspases, they must be considered as efficient inhibitors of the caspase-dependent apoptotic process which seems to be the case in postmortem muscle.

STRUCTURAL MUSCLE PROTEINS

Meat tenderization process involves all intracellular proteases and probably also extracellular ones like plasmin, thrombin etc. It is therefore essential to emphasize here that the identification of the proteases suspected to be responsible for the degradation of a particular protein is of secondary importance. This is especially true as the degradation of a protein in vitro by a particular protease does not necessarily imply that it is the target protein in situ. Moreover, a given protein can be often degraded by several different proteases at least in vitro. Numerous

myofibrillar proteins are degraded in postmortem muscle and the first to be identified was probably troponin T, a regulator and non structural protein which does not contribute to meat texture, together with the suspected 30–32 kDa troponin proteolytic fragment which is in fact an electrophoretic band containing principally a proteolytic product of actin (Becila et al., 2010). One of the earliest structural changes resulting from hydrolysis of cytoskeletal structures is the rapid postmortem detachment of the basal lamina from the plasma membrane following the degradation of all transversal connection between muscle cells and the extracellular matrix (Nishimura et al., 1996; Taylor et al., 1997).

CONCLUSION

Biomarkers of the meat quality are of prime importance for meat industry which has to satisfy consumers' expectations. The tenderization of meat starts immediately after animal death with the onset of apoptosis followed by a cooperative action of endogenous proteolytic systems. It was thus emphasized that mitochondria, an important organelle in the production of energy from carbohydrates, lipids and proteins are a central element in the initiation and development of apoptosis. Apoptosis is generally believed to begin after caspase activation. Large set of proteins of interest as potential predictors of meat tenderness. Most of them are enzymes of the energy metabolic pathways and/or direct or indirect regulators of these pathways. Whatever the source of energy used by muscle cells, the limiting step will always be the mitochondrion where all end products of these pathways are finally oxidized to produce a maximum of ATP. Muscle cells were shown to express strong inhibitors of initiator and effector caspases belonging to the serpin family. As inhibitors are better predictors of meat tenderness than their target enzymes, such

investigations would help to clarify the role of the different endogenous proteolytic systems in postmortem proteolysis.

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Table 1: Markers from glycolytic pathway

Marker name	Localization	Functions
Phosphogluco-mutase	Cytoplasm	Glucose-1P <==> Glucose 6P
Triosephosphate isomerase	Cytoplasm	Dihydroxyacetone phosphate (DHAP) <===> glyceraldehyde 3-phosphate
Glyceraldehyde-3 phosphate dehydrogenase (GAPDH)	Cytoplasm, Nucleus	Glycéraldéhyde.3.phosphate <=> 1,3.diphosphoglycerase Cell death & cell survival
3-Phosphoglycerate kinase	Cytoplasm	3-Phosphoglycerate + ADP <==> 1,3-bisphosphoglycerate + ADP
Aldehyde dehydrogenase (ALDH)	Cytosol and organelles	Aldehyde oxidation to carboxylic acids (glycolysis, amino acids and lipids degradation)
Enolase3or phosphor pyruvate hydratase	Cytoplam & muscle specific	2-Phosphoglycerate <===> phosphoenolpyruvate
Pyruvate kinase	Cytoplasm	Phosphoenolpyruvate <===> pyruvate
Lactate dehydrogenase	Cytoplasm	Pyruvate <===> lactate

Table 2: Markers of meat tenderness belonging to oxidative energy metabolic pathway

Marker name	Localization	Functions
3-Hydroxyisobutyrate dehydrogenase (1)	Mitochondria	1 of the 9 steps of valine degrad. To succinyl-CoA
β-Hydroxyacyl CoA-dehydrogenase.	Matrix Mitochondria	Fatty acid degradation (Lynen helix)
Cytochrome c	Mitochondria	Electron transport chain external side of inner Mito mb. Apoptosis
Succinate dehydrogenase	Matrix Mitochondria	TCA cycle
Succinyl Co-A synthase	Matrix Mitochondria	TCA cycle
Isocitrate dehydrogenase.	Matrix Mitochondria	TCA cycle
ATP synthase	Inner Mitochondria	Mb ATP synthesis

Table 3: Markers of meat tenderness involved in cell detoxification

Marker name	**Localization**	**Functions**
Carbonic anhydrase	Cytosol, Mitochondria	Elimination CO_2; $H_2O + CO_2 \Rightarrow HCO_3 + H^+$
Lactoylglutat-hone lyase or Glyoxylase 1	Cytosol	Detoxification methylglyoxal => lactate
Aldehyde dehydrogenases (ALDHs)	Cytosol and all organelles	Protect cell from cytotoxic aldehydes

Table 4: Markers of meat tenderness from Heat Shock Proteins

Marker name	Localization	Functions
HSP 70	Cytoplasm	Slow down the process of cellular death. Protection of tissues against oxidative stress and fat deposition
DNAJA1 (HSP 40)	Nucleus/cytoplasm	DNAJA1 gene encodes HSP 40, a chaperone involved in protein import into mitochondria and a co-chaperone of HSP70
HSPB (HSP 27)	Cellsurface/ cytoplasm/ nucleus	Response to heat and stress. Regulation and stabilization of myofibrillar proteins, and protects actin filaments and desmin
α-Crystallin (CRYAB)	Nucleus	Protein homodimerization activity. Protection of structural proteins
HSP 60	Cell surface/cytoplasm	Prevent degradation & structure damage of proteins from apoptotic processes in muscle cells.

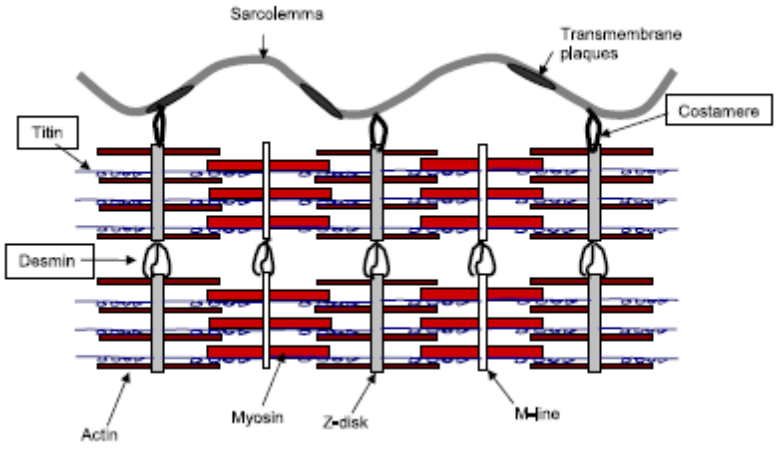

Fig 1. Schematic representation of muscle myofibrillar proteins with major components of the sarcromere. susceptible (Kemp et al., 2010).

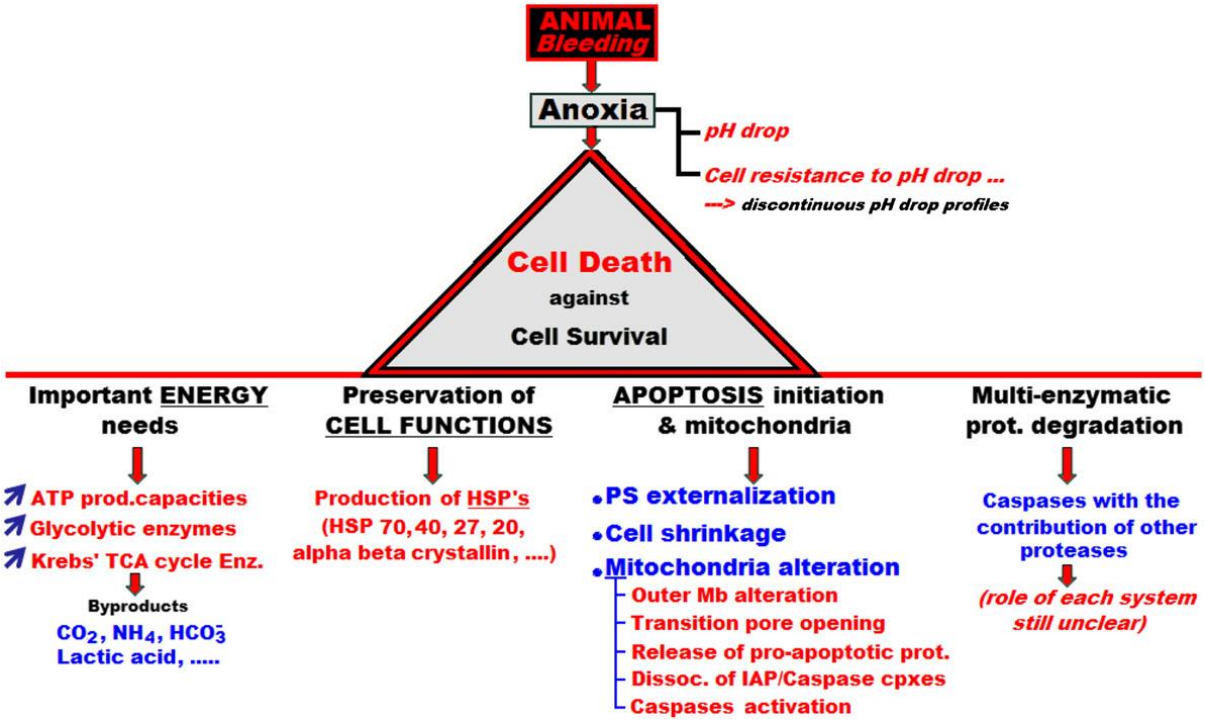

Fig.2. Major events occurring in postmortem muscle after cessation of blood flow.

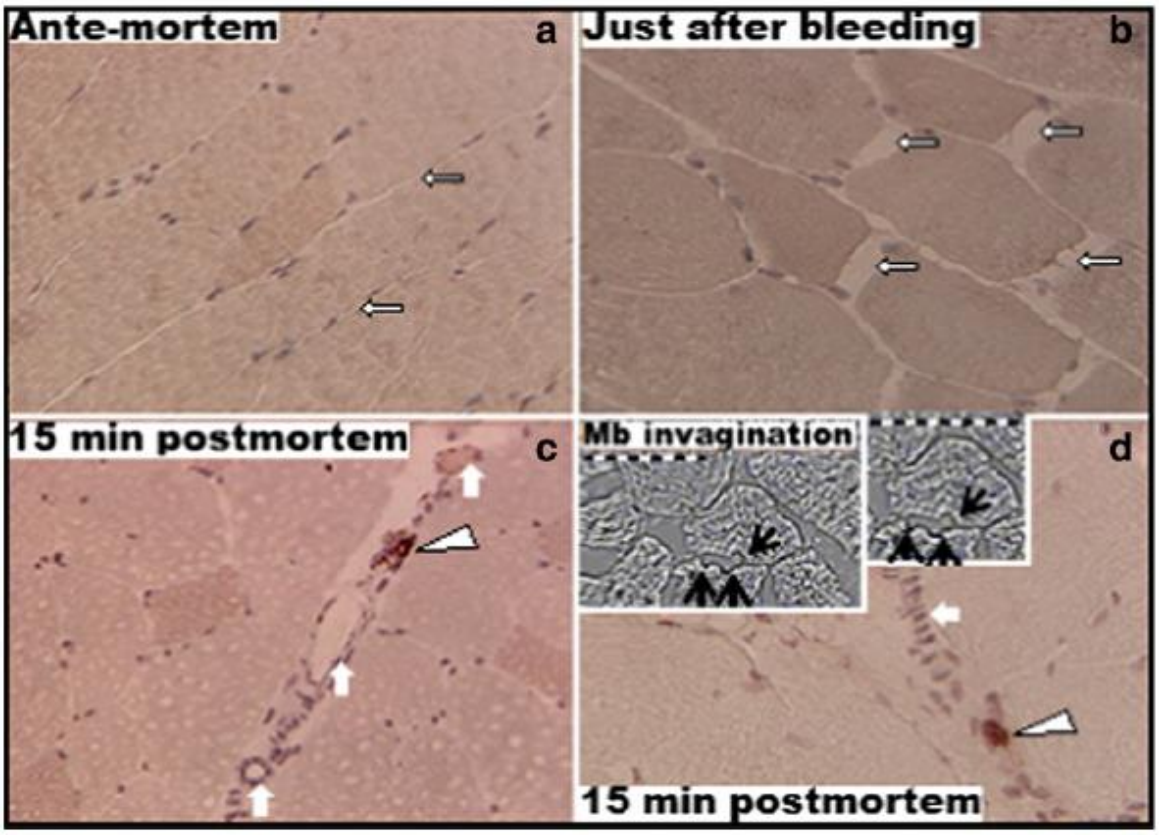

Fig. 3. Postmortem structural changes in transversal cuts of muscle fibers. (Ouali et al., 2013)

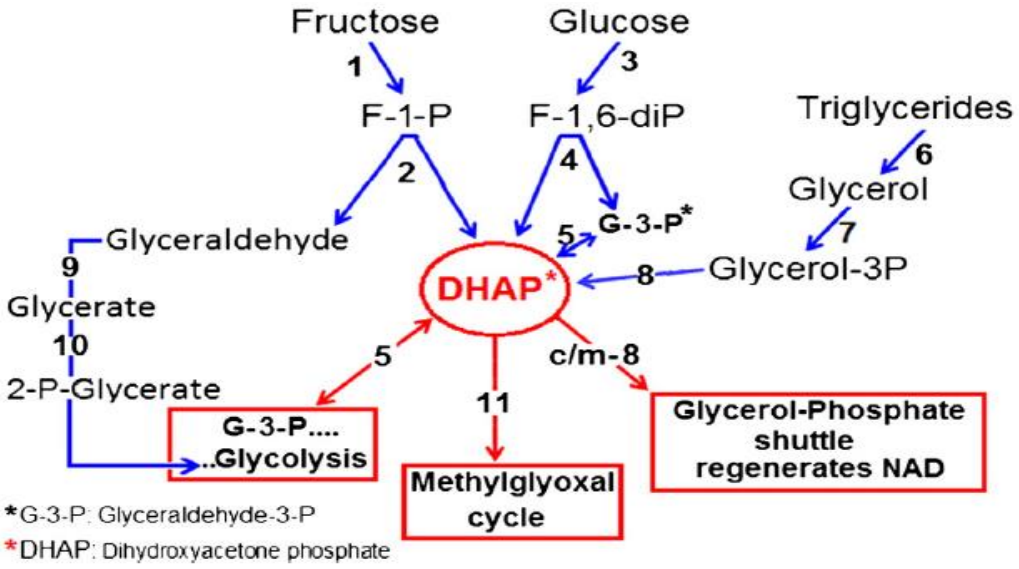

Fig. 4. Metabolic pathways generating or using dihydroxyacetone phosphate. Enzyme names: (1) Fructokinase (2) Fructose-1-P aldolase (3) Glycolysis (4) Aldolase (5) Triose phosphate isomerase (6) Lipases (7) Glycerol kinase (8) Glycerol-3-phosphate dehydrogenase (9) Aldehyde dehydrogenase (10) Glycerate kinase (11) Methylglyoxal synthase (Ouali et al., 2013)

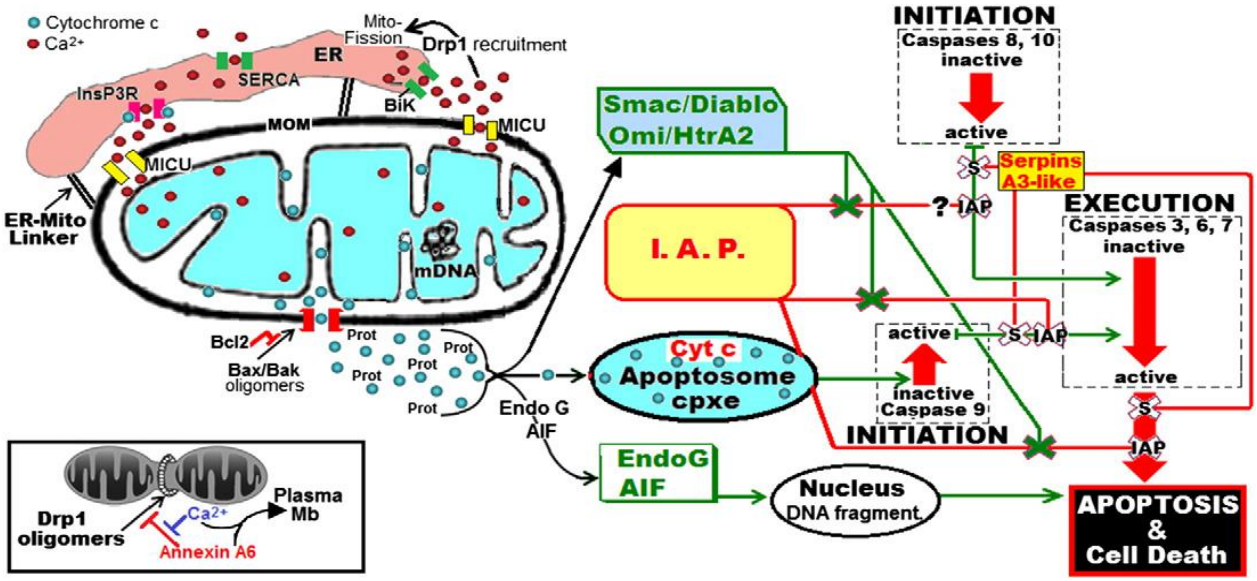

Fig 5. Calcium trafficking between endoplasmic reticulum (ER) and mitochondria which are close to each other. SERCA: sarco/endoplasmic reticulum Ca_2^+-ATPase (Ouali et al., 2013)