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Original Research Article

Characterization of autofluorescence in normal and necrosed muscles in chickens

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ABSTRACT

The histopathological diagnosis of muscle necrosis and hyalinosis frequently poses considerable difficulty and has a contradictory diagnosis. The present study described the morphologic features of nine clinically affected chicken pectoral muscles and one normal muscle using fluorescence microscopy on formalin fixed-paraffin embedded tissues. Histopathological examination of samples (normal and necrosed) was routinely done using stained sections with heamatoxylin and eosin. Sections examined by fluorescent microscopy showed significant or intense autoflouresncence in necrosed muscles. The subsequent image/color analysis of the fluorescent images was carried out to characterize the color intensity of autofluorescence emitted from chickens' muscles and to compare autoflourescence with the normal ones. In necrosed muscles, samples exhibited a marked increase in fluorescence intensity. Normally stained section with non-specific autoflourescent revealed 99.48% for normal specimens compared to 82.93% for necrosed ones, and that of specific autoflourscent revealed 0.62% for normal specimens compared to 17.08% for necrosed ones. The technique allows imaging of chickens muscle samples, facilitating the determination of the degree of necrosis throughout the muscle using statistical analysis, particularly in those related to comparative pathology, and avoiding the disadvantages of routine histopathological examination.

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1. Introduction

Autofluorescence is the natural emission of light by biological structures such as lysosomes and mitochondria when they have absorbed light (Monici, 2005). The most commonly observed autofluorescencing molecules (endogenous fluorophores) are pyridinic (NADPH) and flavin coenzymes;the extracellular matrix could also contribute to autofluorescence as a result of the intrinsic properties of collagen and elastin (Monici,2005). Generally, proteins containing an increased amount of some amino acids as tryptophan, tyrosine and phenylalanine show some degree of autofluorescence (Menter, 2006). In a few cases, autofluorescence may actually illuminate the structures of interest, or act as a useful diagnostic indicator (Monici, 2005). For example, cellular autofluorescence could be used as cytotoxicity indicator without using fluorescent markers (Fritzsche and Mandenius, 2010). Changes occurring in the cell and tissue state during physiological and/or pathological processes result in modifications of the amount and distribution of endogenous fluorophores and chemical–physical properties of their microenvironment. In addition, the analytical techniques based on autofluorescence monitoring can be utilized in order to obtain information about morphological and physiological state of cells and tissues (Monici, 2005).

In HE-stained sections, significant fluorescence was not only expressed in various tissues without exogenous markers; epithelial, connective tissue and or parenchymatous organs but also the biological agents (fungi, bacteria or parasites) (Graham, 1983; Elston, 2001; Monici, 2005; Menter, 2006, Bhatta et al., 2006; Daugschies et al., 2006; Deeb et al., 2010).

The aim of the present study was to promote using of autofluorsecence as a useful diagnostic indicator for the healthy status of pectoral chicken muscles.

2. Materials and methods

Pectoral muscles samples were collected from ten broiler chicken aged 15 to 35 days (one control and nine clinically affected which were avian infectious bronchitis rt-PCR +ve cases). These samples were fixed in formalin 10% for 48hrs. After that they were dehydrated, cleared, embedded in paraffin wax, blocked and underwent microtomy (4-5 µm) and stained routinely with heamatoxylin and eosin (HE) (Bancroft and Gamble, 2012). Stained sectioned were examined by Carlzeiss, Inc. fluorescent microscope with ARC LAMP and power supply 50W HBO AC and connected to digital camera. Quantitative analysis using image analysis to verify that degenerated or necrosed sections exhibit increased autofluorescence intensity compared to the normal one (Bankhead, 2014).

3. Results

Gross examination

Macroscopically, pectoral muscle showed no alterations except paleness in some cases.

Histopathological examination

Normal pectoral muscle fibers appeared normal. The sarcoplasm had ovoid or elongated nuclei located near the sarcolemma. Some nuclei were also located centrally within the muscle fibers. The myofibrils are striated and consisted of numerous myofilaments. Numerous muscle fibers grouped in fasicicles. Each fasicicle is surrounded by perimysium of loose connective tissue (Fig. 1A).

Affected pectoral muscles were characterized by coagulative necrosis and hyalinosis with fragmentation of some muscle fibers. The necrosed muscles fibers revealed condensation of their nuclei with structurless, homogenous and esinophilic cytoplasm (Fig. 2A).

Fluorescent microscopy

The control sample exhibited a faint dull green fluorescence (Fig. 1B), while the necrosed one showed stronger and significant bright green autofluorescence. Meanwhile, enhanced autofluorescence was significantly observed in muscle fibers showing severe hyalinosis (Fig. 2B). Quite differences between the color intensity of the nine degenerated pectoral muscle samples were observed.

Quantitative analysis

Necrosed sections exhibited marked increase in autofluorescence intensity in which percent of non-specific autoflourescent 99.48 for normal sectioned stained muscle compared to 82.925 for degenerated one and percent of specific autoflourscent 0.62 for normal compared to 17.075 for degenerated one (Fig. 3).

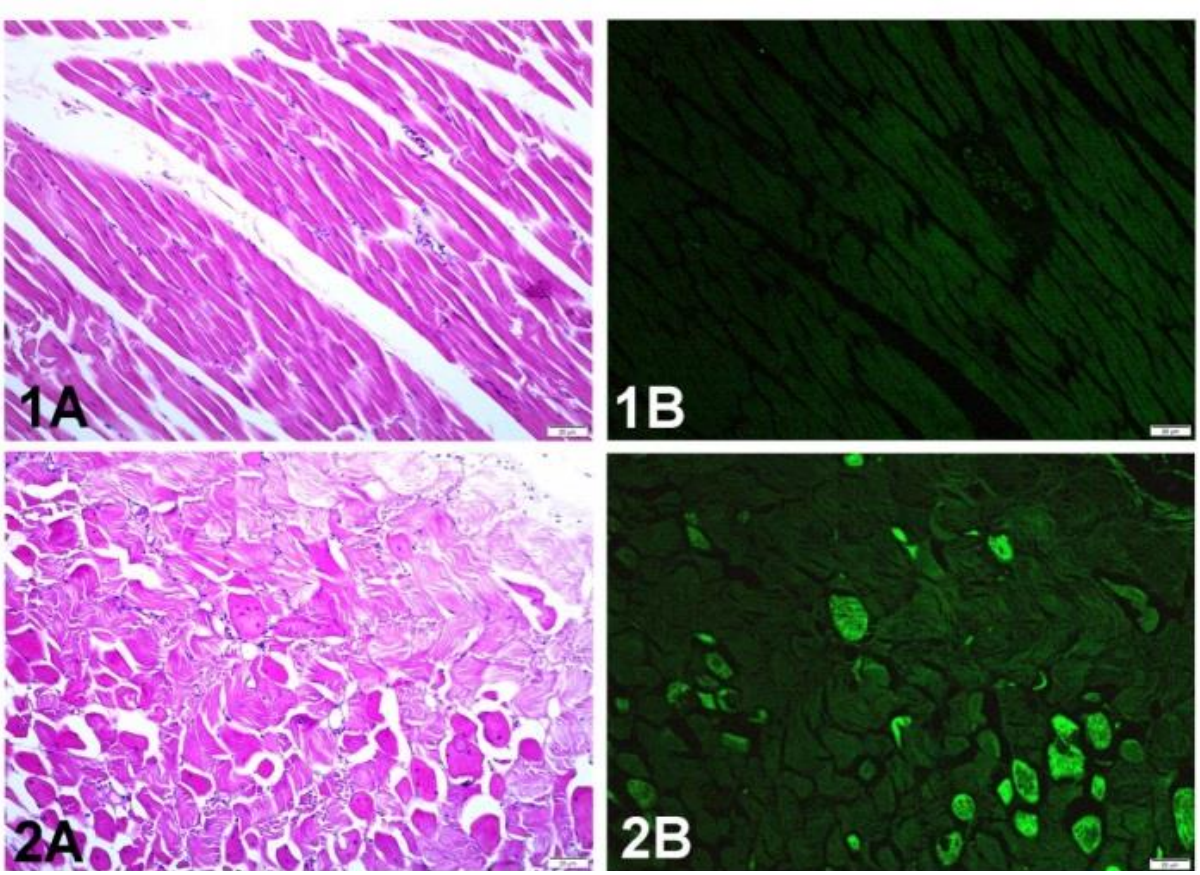

Fig.1. A) Light microscopic image of normalpectoral muscle of broiler chicken showing striation and peripheral nuclei of muscle fibers (HE; X200). **1B)** Fluorescent microscopic image of normal pectoral muscle of broiler chicken showing faint olive green autofluorescent of muscle fibers (HE; X200).

Fig.2. A) Light microscopic image of pectoral muscle of broiler chicken showing degeneration and hyalinosis of muscle fibers (HE; X200). **B)** Fluorescent microscopic image of pectoral muscle of broiler chicken showing bright green autofluorescent of necrosed muscle fibers (HE; X200).

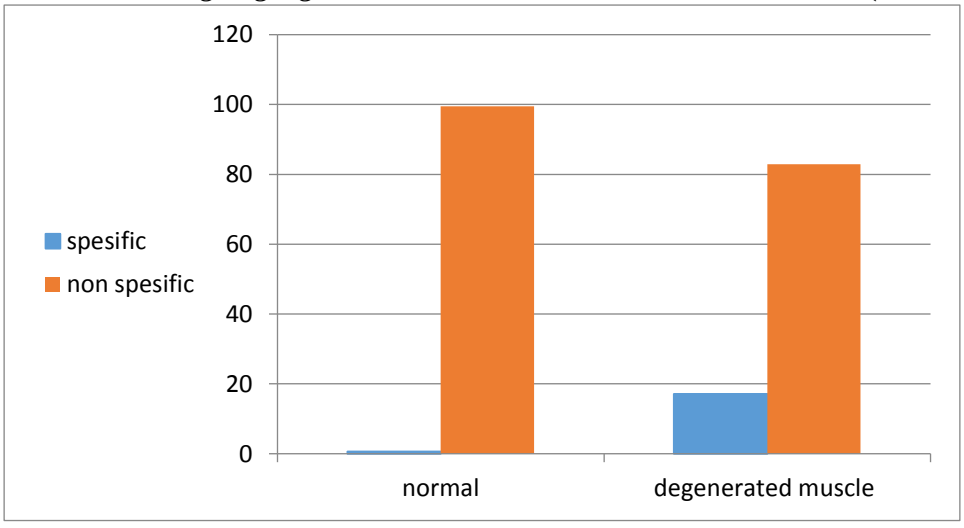

Fig.3. Percentage of specific and non-specific auto-fluorescent of normal and necrosed muscles.

4. Discussion

Using of autofluorescence could be very helpful in obtaining information about morphological and pathological states of tissues and cells especially if it is based on analytical morphological techniques. In H&E stained sections, significant fluorescence was not only expressed in various tissues; epithelial, connective tissue and or parenchymatous organs but also the biological agents (fungi, bacteria or parasites (Elston, 2001; Deeb et al., 2010). Normally stratum corneum expressed strong fluorescence more than the other epidermal layers (Elston, 2001). This technique was more helpful for identifying fungal infection in the skin and parenchymal organs (Graham, 1983; Elston, 2001). Several factors may influence the fluorescence pattern of fungi as the wavelength of light, fixation method and mounting media (Graf, 1996).

The dominant fluorophores (NADH and FAD) are the sources of the fluorescence and they are increased with the higher metabolic rate of tissues. For example, fluorescence intensity was most prominent in cases of dysplastic epithelial tissue relative to the normal samples (Chance, 1989; Drezek et al., 2001). This leads to a decreased concentration in the oxidized electron carrier FAD and increased concentration in the reduced electron carrier NADH.

In skeletal muscle, several distinct fiber types are found; oxidative and glycogenic fibers. Oxidative fibers are distinguished from glycolytic fibers by their high level of NADH dehydrogenase activity (Allen et al., 2001). The autofluorescence is associated with the fibers containing the highest levels of NADH dehydrogenase activity; this indicates that the autofluorescent fibers are oxidative. Alkaline or acidic pH may influence autofluorescene illumination; alkaline pH increase autofluorescnce relative to acid pH (Jackson et al., 2004) and this come with our result in which the more prominent autofluorescence intensity indicate the more tissue necrosis.

Autofluorescence emitted from structural proteins, particularly collagen and elastin, which can be considered the most importantfluorophores in the extracellular matrix (Fujimoto, 1977; Blomfield et al., 1969), so, there was faint autoflorescent emitted from muscles in which collagen and elastin act as the main component of myofibers protein. Several emissions have been observed for collagen and elastin within which the molecular origin has not been completely explored (Richards and Sevick, 1996). The fluorescence emission of these proteins is mainly due to the cross-links (Fujimoto, 1977; Deyl et al., 1980). Changes of collagen and elastin contents in tissues have been found in several pathological conditions (Campo et al., 1992). Thus, monitoring of autofluorescence emitted by muscle structural protein have diagnostic value and indication for healthy state of muscles, as our result showed that intensity of flourscene was high in necrosed muscles with special brightness to those muscle fibers showed severe hyalinosis relative to normal muscle fibers.

The biochemical properties of tissues may be changed significantly *in vitro* and *in vivo*. The ratio of NAD/ NADH may also be changed, as well as blood content and oxidation state. These changes can influence UV-visible autofluorescence (Bigio et al., 1980). Consequently, fluorescence patterns of tissues in vitro differ from those in vivo. Moreover, in vitro the fluorescence from NADH appears to decay exponentially with time, while the fluorescence from collagen and FAD remains relatively constant (Schomacker, 1992). And this may explore our result in relation to collagen and oxidative state from normal to degenerated and necrosed muscles.

The anaerobic component of energy metabolism increases while the aerobic component decreases (Warburg, 1956). The decreased efficiency of the aerobic component increase the NADH (Obi-Tabot, 1993) of tissue, from normal to abnormal state, is accompanied with a decrease in the absolute fluorescence related to collagen, and an increase in the relative contribution of NADH. Thus, differentiation of the various tissues state is determined by the extent of each of these factors (Ramanujam, 1994), therefore, our results could differentiate between normal and necrosed muscle. Such finding came in accordance with that given by Salinas and Sotelo (1986) in which bright yellow auto fluorescence was observed in the necrotic tubules stained with HE. Finally, this method identifies strongly autofluorescence intensity in necrosed muscle fibers than normal ones and also facilitates determining the healthy state and quality of muscles.

5. Conclusion

Autofluorescene provides a reliable determination of the degree of necrosis in muscles, and can overcome the disadvantages of the routine histopathology.

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