

IMMUNOHISTOCHEMICAL STUDY ON THE DEPLETION OF DESMIN, ACTIN AND MYOGLOBIN IN AUTOLYZED AND PUTREFIED NORMAL HUMAN MYOCARDIA

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Abstract: *Background.* The depletion of several cardiac cellular proteins have been studied for postmortem diagnosis of early myocardial ischemia in the field of forensic medicine. The diagnostic value would be markedly limited if its immunohistochemical demonstration were affected by autolysis and putrefaction.

Objectives. The aim of this study was to investigate the impact of autolysis and putrefaction on the immunohistochemical demonstration of desmin, actin and myoglobin for the postmortem diagnosis of early myocardial ischemia.

Methods. The tissue blocks taken from the left ventricles of normal autopsy hearts without coronary artery diseases were stored at 4°C in the refrigerator for different periods. The depletion areas of desmin, actin and myoglobin were studied with immunohistochemistry, image analysis technique and statistics system.

Results. The normal myocardia kept at 4°C for 1 to 2 days showed homogenous brown reactions for desmin, actin and myoglobin. Depletion of desmin, actin and myoglobin became evident when normal myocardia were kept at 4°C over 3 days postmortem, and the average depletion areas increased with the lapse of postmortem interval.

Conclusion. The results of the present study indicate that depletion of desmin, actin and myoglobin could be caused not only by myocardial ischemia, but also by the postmortem changes related to autolysis and putrefaction, so these three cardiac cellular proteins are only suitable for fresh corpses(1 to 2 days postmortem) to detect early myocardial ischemia in forensic practice.

Key words: Early myocardial ischemia, desmin, actin, myoglobin, immunohistochemistry, postmortem change.

INTRODUCTION

In past years, the depletion of several cardiac cellular proteins have been studied for post-mortem diagnosis of early myocardial ischemia in the field of forensic medicine. The extensively studied markers include myoglobin of cytoplasmatic proteins, desmin of cytoskeletal proteins, and actin of contractile proteins [1-9]. Up to now, almost all reports dealt with the analysis of these antigens in non-putrefied materials, but the autolytic and putrefied materials have to be investigated in forensic practice. The diagnostic value would be markedly limited if its immunohistochemical demonstration were affected by autolysis and putrefaction. Therefore, investigations concerning the post-mortem stability of these antigens are necessary.

In the present study, immunohistochemical staining of desmin, actin and myoglobin were analyzed in experimentally induced autolysis and putrefaction in human autopsy hearts, to evaluate their significance for the post-mortem diagnosis of early myocardial ischemia.

MATERIALS AND METHODS

Study design and subjects

The experiments were approved by the institutional ethic committee. For the experimental group, the eight tissue blocks of the left ventricles were taken from each of five normal human autopsy hearts without coronary atherosclerosis. All the cases died immediately of acute cranio-cerebral injuries without resuscitation, autopsied at 6-8 hours post-mortem,

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aged from 17 to 35 years old, two males and three females. The heart tissue blocks were stored at 4°C in the refrigerator, and sampled for processing at intervals over a period of 1 day, 2 days, 3 days, 4 days, 7 days, 14 days, 21 days, 28 days, separately.

Five cases of acute myocardial infarction who died suddenly without resuscitation were used as controls. There were four males and one female, aged from 49 to 81 years old, autopsied 9-48 hours post-mortem, with macroscopic and microscopic evidence of acute myocardial infarction, as well as moderate or severe coronary stenosis (greater than 50%). Other causes of death were excluded.

All samples were fixed in 10% formaldehyde for 1 to 2 days, embedded in paraffin, sectioned serially at 4-5µm, stained for H&E and immunohistochemistry, respectively.

Immunohistochemical staining

The streptavidin-biotin-peroxidase method was applied for immunohistochemical staining, using the primary antibodies against desmin (monoclonal mouse anti-human desmin antibody, D33, ready to use, Maxim Co.), muscle actin (monoclonal mouse anti-human muscle actin antibody, HHF35, ready to use, Maxim Co.), myoglobin (rabbit anti-human myoglobin polyclonal antibody, ready to use, Maxim Co.). Streptavidin-biotin-peroxidase Kit and liquid DAB (ready to use) were also from Maxim Co. Phosphate buffer saline (PBS) took the place of primary antibodies as the negative controls. Sections were counter-stained with Mayer's hematoxylin.

Image analysis and statistics

The depletion areas of desmin, actin and myoglobin were measured quantitatively by Interactive Building Analysis System. The grey level range is 0-256, every slide was examined by light microscopy. After the fields of depletion of desmin, actin and myoglobin were found at 100-fold magnification, the microscopy was adjusted to 200-fold magnification, then five microscopic fields were selected at random on every section.

All the measuring data were presented as mean \pm standard deviation ($\bar{x}\pm s$) and put into Microsoft Excel. Due to heterogeneity of variance between groups, the mean and standard deviation ($\bar{x}\pm s$) of each group was calculated after the value of depletion area of each section was converted into a natural logarithm, then subjected to a one-way ANOVA followed by the Student-Newman-Keuls^a (SNK) post hoc test by Statistics Package for

Social Science for Windows. Significant differences were identified between groups. $P<0.05$ was regarded as indicating significant differences between groups.

RESULTS

H&E staining

For the experimental group, the cardiomyocytes of normal heart tissues stored at 4°C in the refrigerator for 1 day were stained clearly, with distinct cross-striation and oval, dense nuclei. Mild or severe autolytic changes appeared after 2 to 3 days, cross-striation became blurred or effaced, some nuclei also disappeared, muscle fibers were disorganized, with fragmentation and segmentation of the myofibrils. After 14 days, the muscle fibers were widely spaced with mostly invisible nuclei, fungal mycelia, bacterial clusters, and some gas bubbles were present. After 21 days, the myocardial tissues were almost totally blurred, stained homogeneously pale red, and more gas bubbles could be observed.

For the control group, the changes of acute myocardial infarction could be found in all 5 cases of definite myocardial infarction, such as hyper-eosinophilia, disappearance of nuclei, effaced cross-striation, infiltration of granulocytes.

Immunohistochemical staining

For the experimental group, cardiomyocytes of normal heart tissues stored at 4°C in the refrigerator within 2 days showed homogenous brown reactions for the three antibodies, no evident depletion was found. Depletion of the three markers could be observed in the cardiomyocytes of normal heart tissues stored at 4°C in the refrigerator after 3 days, and the depletion areas increased with the lapse of post-mortem interval (Figs 1-3).

For the control group, all cases showed variable degrees of depletion of desmin, actin and myoglobin. Some sections showed no brown staining, some sections showed clear loss of staining with some positivity (brown colour) remaining (Figs 4-6).

Image analysis and statistics

The average depletion areas of desmin, actin and myoglobin are presented in Table 1. After natural logarithm conversion of the value of depletion area of each section, the mean and standard deviation ($\bar{x}\pm s$) of each group and the statistical analysis results are presented in Table 2.

From Table 1 and ANOVA/SNK in Table 2,

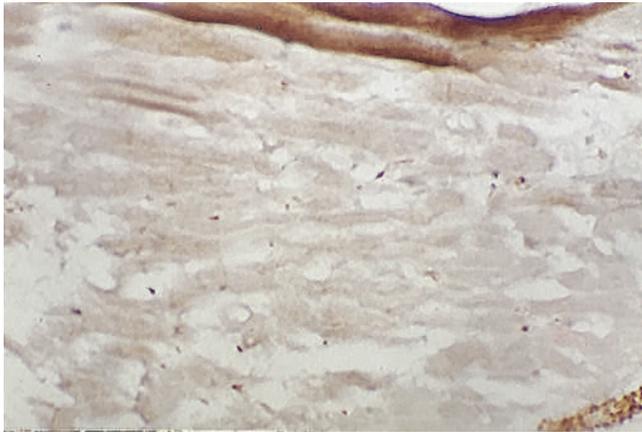


Figure 1. Depletion of desmin in the normal myocardium stored at 4°C for 5 days. ×200.

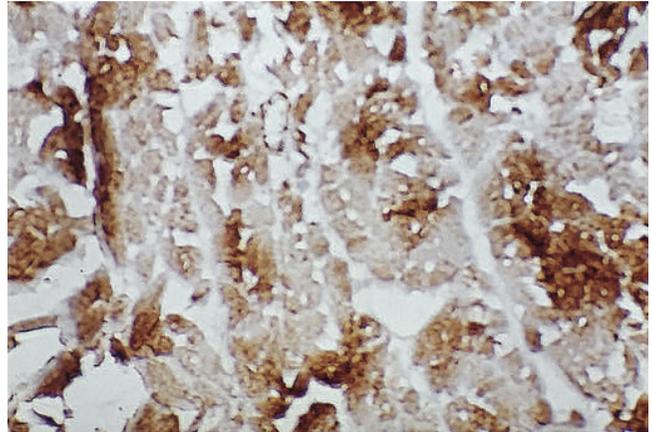


Figure 2. Depletion of actin in the normal myocardium stored at 4°C for 5 days. ×200.

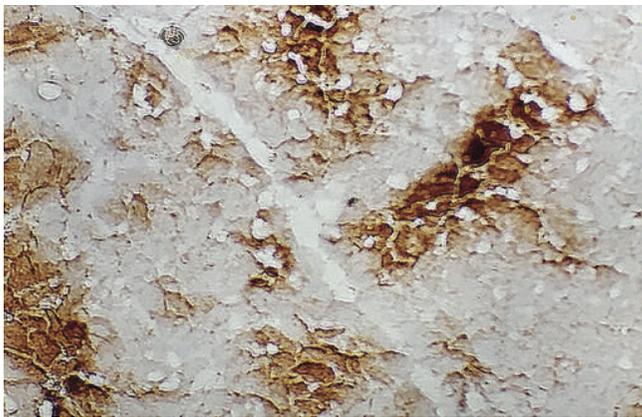


Figure 3. Depletion of myoglobin in the normal myocardium stored at 4°C for 5 days. ×200.



Figure 4. Depletion of desmin in the infarcted myocardium. ×200.

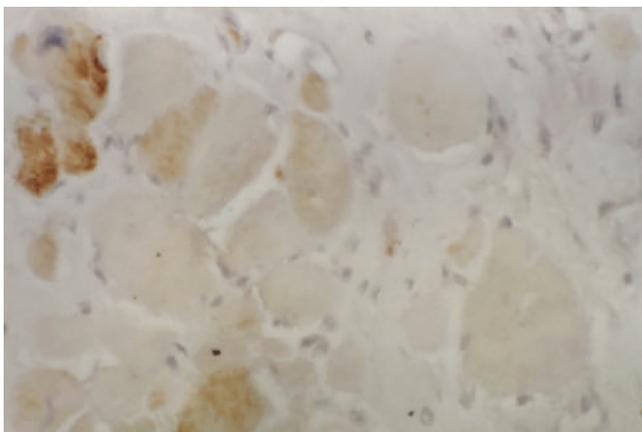


Figure 5. Depletion of actin in the infarcted myocardium. ×400.

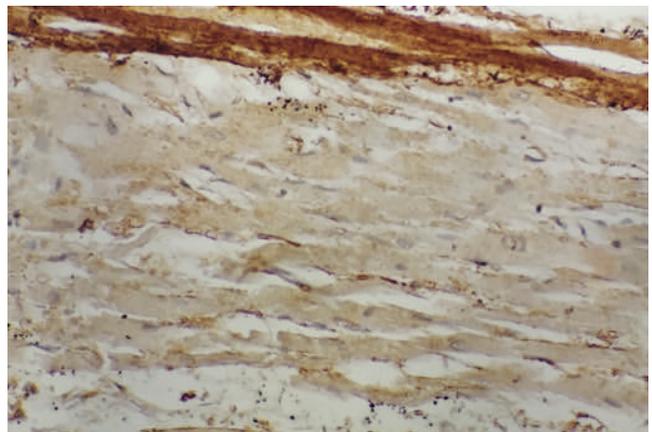


Figure 6. Depletion of myoglobin in the infarcted myocardium. ×200.

$F_{\text{desmin}} = 882.671$, $P_{\text{desmin}} < 0.01$, $F_{\text{actin}} = 882.671$, $P_{\text{actin}} < 0.01$, $F_{\text{myoglobin}} = 907.564$, $P_{\text{myoglobin}} < 0.01$, denoting that for each marker, the average depletion areas are significantly different between these nine groups, and gradually increased with the prolongation of storage period. It is worth noting that the average depletion areas of desmin, actin and myoglobin in the normal human autopsy

hearts stored at 4°C in the refrigerator more than 3 days were significantly different from those within 2 days.

DISCUSSION

The post-mortem diagnosis of early myocardial ischemia is still a puzzling problem in forensic practice.

Table 1. The average depletion areas of desmin, actin and myoglobin in the myocardia stored at 4°C for different time after death(x±s, n=5)

Group	desmin(μm^2)	actin(μm^2)	myoglobin (μm^2)
1 days	2637.99±152.43	2624.93±205.79	2523.56±225.54
2 days	2673.19±122.31	3185.96±213.88	3002.51±341.31
3 days	3366.28 ± 246.33	9098.24±574.15	8288.53±370.21
4 days	4864.19 ±126.79	26485.79±1910.85	18413.64±1195.60
7 days	33715.06±1904.90	30889.87±1367.11	34530.41±2333.41
14 days	43070.38±1502.92	32250.19±2252.00	43272.85±2117.06
21 days	46027.37±2172.05	34876.79±1802.53	48003.58±2344.48
28 days	48899.66±2264.73	46050.22±2643.78	55208.10±2411.12
MI*	55064.70±15085.35	45016.43±8456.42	49748.62±9610.58

Notes: MI - Myocardial infarction.

Table 2. The average depletion areas of desmin, actin and myoglobin in the myocardia stored at 4°C for different time after death after natural logarithm conversion(x±s, n=5)

Group	desmin		actin		myoglobin	
	Area(μm^2)	Comparison	Area(μm^2)	Comparison	Area(μm^2)	Comparison
1 days	7.88±0.06	A*	7.87 ±0.08	A*	7.83± 0.09	A*
2 days	7.89±0.05	A*	8.06 ±0.07	A*	8.00±0.11	A*
3 days	8.12±0.07	B*	9.11±0.06	B*	9.02±0.04	B*
4 days	8.49±0.03	C*	10.18±0.07	C*	9.82±0.06	C*
7 days	10.42±0.06	D*	10.34±0.04	D*	10.45± 0.07	D*
14 days	10.67±0.03	E*	10.38±0.07	D*	10.67±0.05	E*
21 days	10.74±0.05	EF*	10.46±0.05	D*	10.78± 0.05	EF*
28 days	10.80±0.05	EF*	10.87±0.06	E*	10.92±0.04	F*
MI*	10.89±0.28	F*	10.70±0.20	E*	10.80± 0.19	EF*
ANOVA	F =882.671, P<0.01		F =882.671, P<0.01		F =907.564, P<0.01	

Notes: A,B,C,D,E and F in column of Comparison - Area means with the same letter are not significantly different, while those with different letters are significantly different from each other ($\alpha=0.05$). MI - Myocardial infarction.

Since it is not always possible to perform an autopsy soon after the death, whether one marker can be used in forensic practice depends on the post-mortem alteration of immunohistochemical expression because of autolysis and putrefaction. So far, not many studies have investigated the immunohistochemical markers with respect to alterations by autolysis and putrefaction [10]. In a study by Thomsen and Held, C5b-9(m) was demonstrated immunohistochemically in necrotic myocardium due to infarction up to the 11th day of experimentally induced autolysis and putrefaction [11]. Ortmann *et al.* found that positive results of C5b-9(m) could be detected in necrotic myocardia even after long periods (over the 8-week) of artificial and natural putrefaction [3]. In another study by Ortmann *et al.*, fibronectin could be demonstrated immunohistochemically in two cases with a post-mortem interval range of 3 to 4 days [2]. Several reports showed that immunohistochemical expression of myoglobin could be used for detection of myocardial infarction within 2 to 3 days after death [1, 10, 12]. Toupalík and Bouska also reported that after a longer post-mortem interval, extensive artificial losses of myoglobin were observed [13]. However,

in the previous researches, the sample size is small (1 to 2 cases on average), and no control group (deaths because of non-cardiac causes) was considered [10], so it is not easy to distinguish pathological depletion (true positive) from protein alteration due to autolysis and putrefaction, which also manifests itself in depletion (false positive).

In the present study, the impact of autolysis and putrefaction on the post-mortem alteration of expression of desmin, actin and myoglobin was investigated. In order to differentiate the depletion caused by protein alteration because of autolysis and putrefaction from pathological depletion, the normal heart without coronary atherosclerosis were chosen for study, and the myocardial tissues were stored at 4°C in the refrigerator for different periods to experimentally induce autolysis and putrefaction. Our results showed that under the same circumstances, the post-mortem interval had significant influence on the depletion of desmin, actin and myoglobin. When the normal myocardial tissues were stored at 4°C in the refrigerator within 1 to 2 days, the immunohistochemical staining of desmin, actin and myoglobin showed brown homogenous reaction without obvious depletion. When

the period of storage exceeded 3 days, the depletion of desmin, actin and myoglobin became obvious, and the average depletion areas increased gradually with the prolongation of storage period. The statistical analysis showed that the average depletion areas of desmin, actin and myoglobin stored at 4°C in the refrigerator more than 3 days were significantly different from those within 2 days. It is suggested that these three markers are resistant to autolysis for 2 days at 4°C, which is in accordance with immunohistochemical expression of myoglobin reported by Brinkmann *et al.* [1]. After 3 days, it will be difficult to differentiate the immunohistochemical depletion caused by myocardial infarction from that due to autolysis and putrefaction. Under this circumstance, the distribution pattern of the depletion should be taken into account. The regional depletion caused by ischemia is usually with the presence of the pathological changes of the regional coronary arteries, which differs from the diffuse depletion caused by autolysis and putrefaction.

The mechanism that autolysis and putrefaction may affect the immunohistochemical expression of these markers lies on that, autolysis may cause breakdown of the myocardial tissues, with degeneration of the protein structures resulting from activation of the intracellular enzymes promoted by acidosis [10]. While putrefaction may cause destruction of cells and organs by invasion and spreading of bacterial flora and fungal clusters. Western blot analysis of post-mortem protein degradation showed that in the process of post-mortem muscle protein degradation, loss of native protein bands or appearance of degradation products occur due to autolysis [14]. Desmin in human and Sprague Dawley rats muscle samples showed complete degradation of native band with a molecular weight of approximately 50 kDa as well as appearance of degradation products of approximately 41 kDa, 38 kDa, 35 kDa and 32 kDa with increasing PMI [15-17]. Obviously, the processes of autolysis and putrefaction depend on various factors, both intrinsic to the corpse (type of death, physical constitution, age, and sex) and environmental (temperature, humidity, clothing, and ventilation) [10, 17]. The reason why autolysis and putrefaction were experimentally induced at 4°C in the refrigerator in the present study is that the corpses are usually stored at 4°C in the refrigerators before performing autopsies in several countries around the world. Therefore, the results of this study will be applicable in forensic practice in those countries. However, considering that normally forensic bodies are usually discovered either at home/indoors or outdoors (with highly variable

temperatures), other temperature conditions including the room temperature should be investigated in the future studies.

In the present study, in order to avoid the superimposed effect of depletion caused by autolysis and putrefaction on pathological depletion due to myocardial infarction, the post-mortem changes of the three markers in the infarcted myocardia were not studied. Further immunohistochemical studies should be carried out with particular regard to the influence of autolysis and putrefaction on the ischemic myocardia in different states of the corpses.

In conclusion, according to the results of the present study, depletion of desmin, actin and myoglobin became evident when normal myocardia were kept at 4°C over 3 days post-mortem, and the average depletion areas increased with the lapse of post-mortem interval. The findings of this study suggested that the depletion of desmin, actin and myoglobin could be caused not only by myocardial ischemia, but also by the post-mortem changes related to autolysis and putrefaction, so these three cardiac cellular proteins are only suitable for fresh corpses (1 to 2 days post-mortem) to detect early myocardial ischemia in forensic practice.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Brinkmann B, Sepulchre MA, Fechner G. The application of selected histochemical and immunohistochemical markers and procedures to the diagnosis of early myocardial damage. *Int J Legal Med.* 1993;106(3):135-141.
2. Ortman C, Pfeiffer H, Brinkmann B. A comparative study on the immunohistochemical detection of early myocardial damage. *Int J Legal Med.* 2000;113(4) :215-220.
3. Ortman C, Pfeiffer H, Brinkmann B. Demonstration of myocardial necrosis in the presence of advanced putrefaction. *Int J Legal Med.* 2000;114 (1-2):50-55.
4. Mondello C, Cardia L, Ventura-Spagnolo E. Immunohistochemical detection of early myocardial infarction: a systematic review. *Int J Legal Med.* 2017;131(2):411-421.
5. Aljakna A, Fracasso T, Sabatasso S. Molecular tissue changes in early myocardial ischemia: from pathophysiology to the identification of new diagnostic markers. *Int J Legal Med.* 2018;132(2):425-438.
6. Sabatasso S, Moretti M, Mangin P, Fracasso T. Early markers of myocardial ischemia: from the experimental model to forensic pathology cases of sudden cardiac death. *Int J Legal Med.* 2018;132(1):197-203.
7. Hu BJ, Zhu JZ. Sequence and time course of depletion of cardiac cellular proteins and accumulation of plasma proteins in rat early ischemic myocardium. *Legal Med. (Tokyo)* 2019;38:36-44. doi: 10.1016/j.legalmed.2019.04.001.

8. Sabatasso S, Mangin P, Fracasso T, Moretti M, Docquier M, Djonov V. Early markers for myocardial ischemia and sudden cardiac death. *Int J Legal Med.* 2016;130(6):1265-1280.
9. Campobasso CP, Dell'Erba AS, Addante A, Zotti F, Marzullo A, Colonna MF. Sudden cardiac death and myocardial ischemia indicators: a comparative study of four immunohistochemical markers. *Am J Forensic Med Pathol.* 2008;29(2):154-161.
10. Barranco R, Ventura F. Immunohistochemistry in the detection of early myocardial infarction: systematic review and analysis of limitations because of autolysis and putrefaction. *Appl Immunohistochem Mol Morphol.* 2020;28(2):95-102.
11. Thomsen H, Held H. Susceptibility of C5b-9(m) to postmortem changes. *Int J Legal Med.* 1994;106(6):291-293.
12. Amin HAA, El-Hennawy AMY, Nakhla GAA, Tabak SA-H, Hassan HH. Immunohistochemistry in the detection of early myocardial infarction - A postmortem study. *Egyptian J Forensic Sci.* 2011;1(1):5-12.
13. Toupalik P, Bouska I. The effect of autolysis on the interpretation of results of immunohistochemical detection of myoglobin and fibrinogen in the myocardium. *Soud Lek.* 1999; 44(4):44-46.
14. Ehrenfellner B, Zissler A, Steinbacher P, Monticelli FC, Pittner S. Are animal models predictive for human postmortem muscle protein degradation? *Int J Legal Med.* 2017;131(6):1615-1621.
15. Choi KM, Zissler A, Kim E, Ehrenfellner B, Cho E, Lee S, Steinbacher P, Yun KN, Shin JH, Kim JY, Pittner S. Postmortem proteomics to discover biomarkers for forensic PMI estimation. *Int J Legal Med.* 2019; 133(3):899-908.
16. Zissler A, Ehrenfellner B, Foditsch EE, Monticelli FC, Pittner S. Does altered protein metabolism interfere with postmortem degradation analysis for PMI estimation? *Int J Legal Med.* 2018;132(5):1349-1356.
17. Pittner S, Ehrenfellner B, Monticelli FC, Zissler A, Sanger AM, Stoiber W, Steinbacher P. Postmortem muscle protein degradation in humans as a tool for PMI delimitation. *Int J Legal Med.* 2016;130(6):1547-1555.