The influence of fixing condition on myoglobin stainability of striated muscle as a tool for forensic diagnosis

Chie Kobayashi [a], Masanobu Miura [a, b], Yukie Yamasaki [a], Kaori Taniguchi [a], Satoru Miyaishi [a]

[a] *Department of Legal Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan.*
[b] *Department of Legal medicine, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan.*

Corresponding author
Masanobu Miura
Department of Legal medicine, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan.
TEL: +81-86-462-1111
E-mail: masanobu@med.kawasaki-m.ac.jp

Highlights

· Stainability of myoglobin staining was examined with various fixing conditions.
· ELISA verified myoglobin diffusion form muscle into formalin during fixation.
· Fixing condition of formalin suitable for myoglobin staining was revealed.

Keywords
Immunohistochemical Stainability
Myoglobin
Fixing condition
Formalin fixation

1. Introduction

Immunostaining is a method for visualizing a specific protein on a thin section of sample. It is useful for elucidating protein localization in the examined tissue, with many authors having reported its applicability to forensic autopsy diagnosis [1-19]. Stainability of the target protein depends on the preservation of its immunogenicity in tissue, which is typically performed by use of 10% neutral-buffered formalin. Existing studies have reported the investigation of appropriate conditions of fixation for immunostaining using this reagent. It was reported that the type of fixation solution, fixation time, and pH affected immunohistochemical stainability [20], while it was said that there was no optimal common fixatives for all antigens [21]. Some authors observed that staining intensity began to reduce after different duration of fixation among antigens [22, 23]. These researchers suggest that the appropriate condition of fixation for immunostaining differs between targeted antigens [20-23].

It was noted that different stainability is observed in the case of short fixation of tissue in formalin, because the remaining unfixed part is fixed in ethanol during the embedding process [22, 24]. On the other hand, it is known that prolonged fixation in this reagent reduces stainability, because of masking epitope resulting from a change in tertiary structure [24]. According to such previous reports [22, 24], immunostaining using tissue fixed in inappropriate conditions may result in unreliable forensic diagnosis. For example, negative staining due to masking epitope could be misidentified as loss of protein.

Myoglobin (Mb) has been well known as the target protein in forensic diagnosis for myocardial ischemia/infarction using immunostaining [1, 2, 4-6, 8, 9, 12]. In the beginning of 1980s, Ishiyama et al. [1] reported that myocardial ischemia could be diagnosed using Mb immunostainig, applying the experimental results using dogs [25]. Though they emphasized its applicability to the sample fixed for such extended period of time as 3 years, no description about the concentration of the used formalin could be found. In many reports as well as mentioned one, the usefulness of Mb staining for the diagnosis of myocardial ischemia/infarction was referred to without describing fixing duration and formalin concentration [2, 4-6, 8, 9], while such conditions were to affect stainability [20, 22-24]. In the reports on the usefulness of Mb staining for skeletal muscle injury, fixing condition was not also described [3, 7, 10]. Concerning the amount of fixative as fixing condition, it was not described in most previous reports [1-10]. In addition to fixing condition, we want to emphasize to analyze the deletion of Mb from striated muscle due to its exudation into fixative during fixation as a novel viewpoint for the evaluating stainability of this protein. Myoglobin has been known as a very water-soluble protein.

In the present study, we experimentally fixed striated muscle using three different amounts of 10% neutral-buffered formalin for various durations and investigated their effects on stainability of Mb. We also examined the possible exudation of Mb from striated muscle into this fixative during fixation biochemically. Stainability of Mb in autopsy cases was additionally interpreted considering the results of above-mentioned approaches.

2. Materials and Methods

2.1 Striated muscle

Sheep (lamb) heart was sliced with thickness of 1 cm, then 21 pieces of myocardial tissue containing both endocardium and epicardium of approximately 1 g were excised from the left ventricle of the obtained slices. Total 105 pieces of myocardial tissue were prepared using 5 hearts and all pieces were weighed before supplying them experiment.

Skeletal muscle was obtained from human resources, in which 5 specimens without injury/disease (2 of iliopsoas and 3 of quadriceps) were collected from autopsy cases within 4 days after death, 4 males and 1 female, from 24 to 89 years old (average 57 years).

For the investigation of the actual situation of stainability of autopsy material, iliopsoas muscle was collected from 10 cases within 4 months of postmortem interval. The cases included 5 males and 5 females, of ages between 19 to 97 years old (average 56 years). Samples were embedded in paraffin after 5 to 10 days fixation in 10% neutral-buffered formalin (pH 7.0). Paraffin-embedded myocardium samples of the left ventricle of which duration of fixation was known (1 week, 1 month and 4 years) were also collected from 3 cases with one missing of 4 year-fixation. All samples were from males aged 48, 82 or 19 years old, collected within 3 days of postmortem interval. No injury and disease of skeletal and heart muscle was documented in autopsy records.

2.2. Reagents

In immunostaining, mouse anti-human Mb monoclonal antibody (MG-1, Sigma-Aldrich, U.S.A.) was selected as the primary antibody. Its cross-reactivity sheep Mb was confirmed by Basic Local Alignment Search Tool [26]. ImmPRESS REAGENT anti-Mouse IgG (Vector Laboratories, Inc., Burlingame, CA) was used for the horse radish peroxidase (HRP) conjugated secondary antibody. Color development was obtained using diaminobenzidine (DAB, Nacalai Tesque, Inc., Japan). Counterstaining was performed using Mayer's hematoxylin. Enzyme immunoassay was conducted using anti-human Mb (MG-1) as primary antibody, EnVision+ HRP, anti-mouse (Agilent Technologies, Inc. Santa Clara, CA, (Dako)) as secondary antibody and 2,2'-azino-di [3-ethyl-benzthiazoline sulfonate (6) (ABTS, LGC Clinical Diagnostics, Inc, UK, (SeraCare)) as enzyme substrate.

2.3. Criteria for staining intensity and definition of the stainability of examined muscle tissue

Myoglobin-stained 105 sections made in the process described in Section 2.4. were microscopically observed, and 10 arbitrary fields in the magnification of 400x were saved from all sections as images in TIFF format. All images were analyzed by IHC profiler [27] plugged in Image J (1.53u) [28]. By the analysis of an image using IHC profiler, we obtained percentage contribution of high positive, positive, low positive and negative as degrees of stainability. We gave the coefficients of 2, 1.5, 1 and 0 to high positive, positive, low positive and negative, respectively. Percentage

contribution of each degree was multiplied by corresponding coefficient. The summed-up value of all multiplied ones was defined as the total positive (TP) of the field, and total 1050 TP values were collected from 105 sections. The range between maximum and minimum of all TP values were equally divided into 9, and 11 grades were set by adding the grades of ‘under minimum’ and ‘over maximum’ for the determination of the staining intensity of tissue (ST), which was defined as the grade corresponding to the mean TP value of 10 fields.

2.4. Experimental analysis of the relation between condition of fixation and myoglobin stainability of striated muscle

Sheep myocardial tissue was fixed in room temperature using 10% neutral-buffered formalin (pH 6.9) under 21 different conditions based on combinations of the following: three ratios of volume of formalin (mL) to weight of myocardium (g) (RFM) of 1, 4 or 9, seven different durations of fixation (DF) of 0.5, 3 or 6 hours, and 1, 2, 5 or 7 days. For all 21 conditions five pieces of myocardial tissue were used. After fixation, they were rinsed with running water for 30 minutes, cut vertically to endo-/epicardium with thickness of 0.5 cm, and embedded in paraffin putting the inner side to the surface of thin slice. ST of each sample was determined after the immunostaining for Mb using a 2.5 μm thick section, and the relationship between condition of fixation and stainability was investigated.

Heat-induced antigen retrieval for Mb staining was conducted at 10 minutes in 10 mM citrate buffer (pH 6.0) at 90°C. Reaction time was 60 minutes for the primary antibody, 30 minutes for secondary antibody and 5 minutes for color development.

2.5 Investigation of relationship between pH of formalin and myoglobin stainability

The pH of formalin used in the experiment mentioned in Section 2.4 was determined at the end of fixation. The influence of formalin pH on ST was investigated by taking the conditions of fixation into consideration.

2.6. Detection of myoglobin diffused into formalin from skeletal muscle during fixation by ELISA

Five samples of skeletal muscle collected from autopsy were cut into smaller sections of approximately 1 g and fixed in formalin at the RFM of 9 for 0.5 or 3 hours and 1, 2, 5, or 7 days. After fixation, a piece of nitrocellulose membrane of 0.2 cm^2 (pore size 0.22 micrometers, Bio-Rad Laboratories, Inc. Hercules, CA.) was immersed overnight in 40 μL of the formalin used for fixation at refrigerated temperature for trapping Mb diffused into formalin. After rinsing off the formalin, the membrane was reacted with 40 μL primary antibody solution adjusted in concentration of 7 μg/ml for 60 minutes, then with 40 μL of secondary antibody solution for 30 minutes, and 150 μL of ABTS solution for 15 minutes in this order. After stopping chemical reaction by adding 100 μL of SDS, Mb diffused into formalin was detected by measuring the absorbance of 200 μL reacted solution using microtiter plate with a wavelength of 405 nm. Detection of Mb was performed 3 times for each sample.

2.7. Evaluation of stainability of myoglobin of striated muscle in routine autopsy examination

The pH of the formalin fixing iliopsoas muscle was measured at the end of fixation. Sections with thickness of 2.5 μm were prepared from all samples of iliopsoas muscle as well as from the sample of myocardium in three cases. These sections were subjected to Mb staining with the same condition as described in 2.4. and their ST values were obtained. Actual situation of Mb stainability in routine examination of autopsy cases was evaluated using ST values in comparison with experimental study.

Ethical approval

This study was approved by the institutional ethics committee at the authors' University (approval number 2202-041).

3. Results

3.1 Determination of staining intensity based on myoglobin staining results of sheep myocardial tissue and comparative study by fixation conditions.

As in the section 2.4 of Materials and Methods, TP in all 1050 fields ranged from a maximum of 105 to a minimum of 19, based on ST classification from 0 to 10. The results of ST for all 105 sheep myocardial tissues are shown in Fig. 1.

In sheep myocardial tissue, Mb ST initially increased in all RFMs, with RFM1 showing an ST of 4.8 ± 0.75 at DF 0.5 hours, which peaked at 6.0 ± 0 at DF 6 hours. RFMs 4 and 9 exhibited peak ST at DF 3 hours, with ST increasing from 4.6 ± 1.0 to 6.4 ± 0.49 and from 4.8 ± 0.75 to 5.8 ± 0.40, respectively. Subsequently, ST decreased over time in all RFMs, reaching its minimum at DF 7 days, with values of 3.2 ± 0.75 for RFM1 and 2.4 ± 1.0 and 2.6 ± 0.80 for RFMs 4 and 9, respectively. The highest ST among all staining conditions occurred in RFM4 at DF 3 hours. Furthermore, from DF 1 day onwards, ST was consistently highest in RFM1.

Statistical analytics on ST between the DF with the highest ST in each RFM and other DFs was conducted by Mann-Whitney test. It was found that in RFM1, ST significantly decreased at DF 0.5 hours, 1, 2, 5, and 7 days compared to DF 6 hours ($p < 0.05$). In RFM4 and RFM9, the highest ST was observed at DF 3 hours. Conversely, in RFM4, ST significantly decreased at DF 0.5 hours, 2, 5, and 7 days, and in RFM9, ST values at DF longer than 1 day were significantly low compared with the highest ST value at DF 6 hours ($p < 0.05$). In other words, in all RFMs, ST significantly decreased from the time point of maximum ST to DF 2 days onwards.

The pH of formalin fixed in sheep myocardial tissue was pH 6.9 before tissue fixation, but in RFM1 it became 6.6 at DF 0.5 hours, and rapidly decreased until DF 1 day. Ultimately, at DF 7 days, the pH decreased to 5.5. A similar trend of pH decrease was observed in RFM4 and 9, with rapid decline until DF 1 day, albeit to a lesser extent compared to RFM1. At DF 7 days, the pH remained at 6.3 and 6.7 for RFM4 and 9, respectively, when compared to RFM1 (Fig. 2).

3.2 Detection of myoglobin from formalin-fixed skeletal muscle via ELISA

The results of the ELISA performed in the section 2.6 of Materials and Methods using formalin for skeletal muscles from five locations are shown in Fig. 3. At DF 0.5 hours, high absorbance values around 2 were obtained for all muscles, confirming exudation of Mb into formalin. Subsequently, at DF 3 hours, sufficient absorbance values exceeding 1 were obtained for all skeletal muscles. However, by DF 1 day, except for one instance, absorbance values became very low (0.2-0.4), and by DF 2 days, absorbance values were 0.5 or below for all samples. The absorbance remained low thereafter, with values around 0.1 for all samples by DF 7 days.

3.3 Myoglobin staining properties in formalin-fixed striated muscle for autopsy examination

The case summaries and the results of ST for each case after Mb staining of the iliopsoas muscle tissue are presented in Table. 1. In all cases, ST ranged from 6 to 9, showing variability among cases. The highest ST was observed in a case of acute heart failure with a postmortem interval of about 2.5 days, while the lowest was observed in one case each of drowning and burning, with postmortem intervals of 1-2 months and 3-3.5 days, respectively. As to the cytoplasmic staining two types were observed: all muscle fibers were homogenously and equally stained (Even) and both stained and non-stained fibers were mixed (Uneven) (Fig. 4). Eight of ten cases were classified in type of Even, while type of Uneven, appeared in remaining two cases, which had ST values of 8. Among the cases with ST value of 8, three cases were type of Even.

On the 4th day of fixation, the pH of formalin for fixing autopsy samples was 6.6 at the maximum and 5.3 at the minimum. A graph depicting the average pH up to the 4th day of fixation, categorized by the degree of decomposition of the cases. Regardless of the degree of decomposition, there was a trend of pH decrease from the fixation day to the 1st day, with a more pronounced decrease observed as the degree of decomposition increased. However, no significant relationship was found between ST and pH or the degree of decomposition or postmortem interval (Fig.5a, b, c).

The ST of myocardial tissue from three autopsy cases fixed in formalin for 7 days showed excellent staining with a score of 9 in all cases. However, when fixed for one month, the ST was 7 for Case 1 and Case 2, and 5 for Case 3, indicating a decrease in ST in all cases. ST of samples fixed for 4 years was 0 (Fig. 6).

4. Discussion

When Mb staining was performed using 1 g of sheep myocardium after changing the amount of fixed formalin and the fixation time, the highest ST among all fixation conditions was observed in RFM4 at DF 3 hours. Regardless of the RFM, there was a consistent trend of an initial increase in ST followed by a subsequent decrease, with significant decreases in ST observed in all RFMs from DF 2 days onwards compared to the maximum ST value. The reason for insufficient

staining intensity in the early stages of fixation may be attributed to the fact that formalin fixation progresses at a rate of 1 mm per hour [29-31]. Therefore, it is presumed that inadequate cross-linking formation occurred due to the fixation time being too short to allow for the necessary cross-linking. Regarding the samples in the early stages of fixation, it is speculated that they were washed with water in a state with few cross-links, causing water-soluble Mb to exudate from the tissue and resulting in reduced staining intensity. On the other hand, even when it took DF 2 days or more, ST decreased regardless of the amount of formalin used. This could be attributed to antigen masking by formaldehyde. J.D. Webster et al. reported strong Mb staining up to the 7th week of fixation using dog muscle tissue [32]. However, our study revealed that prolonged fixation is not desirable in this case. Additionally, while we confirmed cross-reactivity in our experiments, using anti-human antibodies on sheep tissue may have resulted in overall lower staining intensity compared to the expected staining.

By changing the RFM, differences were observed in the fixation time at which ST reached its maximum, with DF 6 hours being the peak time for RFM1 and DF 3 hours for RFM4 and 9. When measuring the pH of formalin used to fix sheep myocardium, a gradual decrease in formalin pH over time was observed in all RFMs. Particularly in RFM1, there was a rapid decrease in formalin pH until DF 1 day, ultimately dropping to 5.5 by DF 7 days. On the other hand, in the case of RFM9, even at DF 7 days, the pH remained at 6.7, indicating that a larger amount of formalin leads to better pH maintenance. It is known that amino acids are detected in tissues after death [33], and that amino acids become acidic by reacting with formaldehyde [34]. When the amount of formalin is small, the buffering capacity may not be sufficient, leading to acidic pH of formalin. Conversely, when there is a large amount of formalin, the buffering capacity of formalin is sufficient, and significant decreases in formalin pH may not be observed. However, while the differences in pH due to RFM did not have a significant impact on the magnitude of ST values, differences were observed in the time to reach the peak ST value. In RFM1, where the pH decreased the most, the fixation time to reach the peak ST was longer compared to RFM4 and 9. It is known that fixation conditions can change due to pH, and under acidic conditions, the number of tissue cross-links by formalin decreases. Therefore, it was inferred that the decrease in pH resulted in a longer time to reach the peak staining intensity. In a study by Sato et al, it was reported that Ki-67 immunostaining had a higher positivity rate under acidic conditions [35]. It is believed that the optimal pH for preserving samples for immunostaining varies depending on the characteristics of the target protein. However, in our study, no clear correlation was found between pH and staining intensity for Mb staining. It was evident that regardless of whether the pH was maintained or decreased, staining intensity decreased as DF increased. However, since the decrease in formalin pH is known to affect phenomena such as formalin pigment precipitation [29], it is considered necessary to adjust the amount of formalin according to the intended use of the tissue.

In the investigation regarding the depletion of Mb in skeletal muscle fixation with formalin, factors other than formalin fixation conditions were considered, particularly the possibility of Mb

exudation during fixation. Water-soluble proteins in unfixed conditions have the potential to exudate into formalin. In addition to proteins, past reports have indicated that drugs such as phenobarbital and butalbital can be detected in organs fixed in neutral-buffered formalin [36]. There have also been reports of mercury leaking from organs into formalin [37]. There are no similar reports regarding Mb, but given that Mb, a water-soluble protein, has been reported to leak into the bloodstream early after death [38, 39], it seemed plausible that leakage into the liquid phase could occur in formalin as well. Therefore, we attempted to detect Mb using ELISA from formalin containing muscle tissue.

In the ELISA assay, Mb was detected in the early stages of formalin fixation, confirming the exudation of Mb from the skeletal muscle cross-section into the formalin. However, it was hardly detected in formalin after one day of fixation. It is believed that skeletal muscle fragmented into 1 cm^3, as used in this study, would be sufficiently fixed throughout the tissue after one day of fixation. As a result, the exudation of Mb likely decreased, contributing to this outcome. Additionally, the reason why the initially exudated Mb was no longer detected was not because the Mb in the formalin had disappeared, but rather because the Mb in the exudated solution had bound to formaldehyde, leading to antigenic inactivation and making it undetectable. The experimental method used in this study suggested that Mb exudate from skeletal muscle even during formalin fixation. This phenomenon was also considered to be one of the reasons for the decrease in staining intensity observed in the experiments using sheep myocardium mentioned earlier, as fixation time increased.

In our facility's medicolegal autopsies, we examined whether the experimental results obtained from sheep myocardium, as mentioned earlier, were reflected in the Mb staining of 10 human skeletal muscle cases and 3 human cardiac muscle cases routinely preserved and stained for Mb. The 10 skeletal muscle cases used for practical purposes varied in formalin fixation time, ranging from 5 to 10 days, as they were utilized for routine purposes. While the precise measurement of the amount of formalin used was not conducted, it is customary in our facility to fix tissues with approximately the same volume of formalin as the amount of tissue to be preserved. The Mb staining intensity of the skeletal muscles preserved under the aforementioned conditions was evaluated using the ST values defined in the sheep myocardium experiment, resulting in a range of 6-9, with significant variation observed among cases. The obtained staining intensity was generally higher than that observed in the sheep myocardium experiment, which was attributed to differences in the reactivity of the antibodies to the tissues. We investigated the correlation between staining intensity in these 10 cases and factors such as formalin pH, degree of decomposition, and postmortem interval. The average formalin pH on the 4th day of fixation in the 10 cases was 6.2, indicating an acidic trend, with a significant decrease in pH observed particularly in severely decomposed bodies. Human decomposition is known to start approximately 4 minutes after death [33], and it is presumed that amino acids released from muscle proteins by bacteria decomposition may affect pH. Furthermore, considering the reported growth of *Mycobacterium species* from lungs

fixed in formalin for 80 days [40], it is possible that changes due to decomposition may continue even after formalin penetration, particularly leading to pH decrease in decomposed bodies. However, in the Mb staining of muscle tissue samples used in this study, no correlation was found between decomposition, pH decrease, and staining intensity, nor was there any correlation with postmortem interval. In human skeletal muscles stored for 5-10 days, although the reasons remain unclear, the results of Mb staining showed variability among cases, and the cause of this variability was also unknown. Therefore, it seems prudent to exercise caution when using tissues stored for more than 2 days for diagnosis.

In addition, uneven cytoplasmic staining was observed in 2 out of 10 cases (Fig. 4), where staining was sparse and did not correlate with staining intensity. Similar phenomena have been reported in Mb staining of human cardiac muscle tissues [13], suggesting it may be a common occurrence in striated muscles. The cause of this phenomenon may be attributed to incomplete formalin fixation, resulting in a mixture of formalin and ethanol fixation during the staining process, which can affect antigenicity and staining properties [22, 24, 41]. While some reports have mentioned that only the periphery of the tissue is fixed, resulting in stronger staining in the center or periphery, the conditions under which this occurs remain unclear [24]. In our study, the reason for the observed differential staining patterns was not clearly understood. The variation in staining intensity within the cytoplasm of muscle fibers, especially in muscles where no lesions or damages were identified and the fact that the cause of this is unknown raise significant concerns about the reliability of the assay. If Mb depletion from the cytoplasm occurs either partially or entirely, it may lead to misdiagnosis as indicating some form of pathology or injury. While our study was not aimed at elucidating the underlying cause, there is a pressing need to uncover the actual mechanisms involved. Moreover, in several instances, Mb was strongly stained in the extracellular spaces such as the interstitium or within blood vessels rather than within the cytoplasm. This observation supports the findings of our experimental investigation, which confirmed the exudation of Mb into formalin and the early postmortem leakage of Mb into the bloodstream [38, 39]. When performing Mb staining on autopsy specimens, it is essential to consider the possibility that some Mb may have exudated out of the cytoplasm even before the tissue is embedded in paraffin.

Similarly, in three cases of human cardiac muscle where routine Mb staining was performed and preserved during autopsy, all showed high ST values of 9 at the 7th day of fixation. However, like the findings in human skeletal muscle, even though the ST values were high, examination of the tissues revealed strong staining outside the cytoplasm rather than inside, leading to elevated ST values. Additionally, sparse staining patterns were observed in some areas. Therefore, it was considered difficult to immediately rely on the staining images obtained after 7 days of fixation for diagnosis.

In all three cases of human cardiac muscle, the staining intensity decreased after 1 month of fixation compared to 7 days of fixation. Additionally, when additional Mb staining was performed on samples that had been fixed in formalin for 4 years in Cases 2 and 3, all of them showed an ST

value of 0, indicating no Mb staining at all (Fig. 6). The reason for this phenomenon was presumed to be similar to the reasons mentioned earlier, namely, changes in the antigenicity of Mb due to over-fixation. In forensic medicine, examinations using specimens stored for a long time are often conducted during re-evaluations. However, it is crucial to exercise caution in interpreting immunostaining results of formalin-fixed tissues.

5. Conclusion

According to our animal experiments, it has been demonstrated that the most suitable formalin fixation condition for Mb immunostaining is RFM4 with DF of 3 hours to 1day. Similar staining intensity was also recognized across RFM1 to RFM9 with the same fixation duration. Irrespective of the RFMs, staining intensity decreased with fixation durations exceeding 2 days. Fixation time was deemed the most influential factor affecting the properties of Mb staining in skeletal muscles. In the study using autopsy material with unknown fixation condition, there were some of samples showing distinctly different stainability with unknown cause comparing with other samples. Therefore, it is preferable to fix the muscle tissue in the following condition to obtain stable stainability for forensic autopsy diagnosis: the ratio of volume of formalin to weight of muscle is 4:1 and duration of fixation for 3 hours to 1 day. Extended duration of fixation like 4 years should be avoided for analysis.

[1] I. Ishiyama, M. Kamiya, M. Rose, E. Komuro, A. Takatsu, Fulminant deletion of myoglobin from myocardial fibres in a state of acute cardiac failure inducing sudden cardiac arrest, Lancet 2(8313) (1982) 1468-1469.

[2] S. Leadbeatter, H.M. Wawman, B. Jasani, Immunocytochemical diagnosis of early myocardial ischaemic/hypoxic damage, Forensic Sci. Int. 40(2) (1989) 171-180.

[3] G. Fechner, R. Hauser, M.A. Sepulchre, B. Brinkmann, Immunohistochemical investigations to demonstrate vital direct traumatic damage of skeletal muscle, Int. J. Legal Med. 104(4) (1991) 215-219.

[4] B. Brinkmann, M.A. Sepulchre, G. Fechner, The application of selected histochemical and immunohistochemical markers and procedures to the diagnosis of early myocardial damage, Int. J. Legal Med. 106(3) (1993) 135-141.

[5] C. Ortmann, H. Pfeiffer, B. Brinkmann, A comparative study on the immunohistochemical detection of early myocardial damage, Int. J. Legal Med. 113(4) (2000) 215-220.

[6] Z. Xiaohong, C. Xiaorui, H. Jun, Q. Qisheng, The contrast of immunohistochemical studies of myocardial fibrinogen and myoglobin in early myocardial ischemia in rats, Leg. Med. (Tokyo) 4(1) (2002) 47-51.

[7] A. Fieguth, D. Franz, R. Lessig, W.J. Kleemann, Fatal trauma to the neck: immunohistochemical study of local injuries, Forensic Sci. Int. 135(3) (2003) 218-225.

[8] C.P. Campobasso, A.S. Dell'Erba, A. Addante, F. Zotti, A. Marzullo, M.F. Colonna, Sudden cardiac death and myocardial ischemia indicators: a comparative study of four immunohistochemical markers, Am. J. Forensic Med. Pathol. 29(2) (2008) 154-161.

[9] H.A.A. Amin, A.M.Y. El-Hennawy, G.A.A. Nakhla, S.A.-H. Tabak, H.H. Hassan, Immuno-histochemistry in the detection of early myocardial infarction (a post-mortem study), Egyptian Journal of Forensic Sciences 1(1) (2011) 5-12.

[10] N. Kenji, Y. Ihama, M. Fukasawa, T. Nagai, C. Fuke, T. Miyazaki, Morphologic investigation of injury caused by locally applied negative pressure in a rat model, Leg. Med. (Tokyo) 14(1) (2012) 21-26.

[11] T. Sugimura, E.L. Wang, M. Kashiwagi, K. Hara, A. Matsusue, B. Waters, S. Kubo, Renal immunohistochemical investigation for the differentiation of the cause of multiple trauma fatalities, Leg. Med. (Tokyo) 14(1) (2012) 1-5.

[12] S. Sabatasso, P. Mangin, T. Fracasso, M. Moretti, M. Docquier, V. Djonov, Early markers for myocardial ischemia and sudden cardiac death, Int. J. Legal Med. 130(5) (2016) 1265-1280.

[13] S. Sabatasso, M. Moretti, P. Mangin, T. Fracasso, Early markers of myocardial ischemia: from the experimental model to forensic pathology cases of sudden cardiac death, Int. J. Legal Med. 132(1) (2018) 197-203.

[14] S. Bohnert, A. Seiffert, S. Trella, M. Bohnert, L. Distel, B. Ondruschka, C.M. Monoranu, TMEM119 as a specific marker of microglia reaction in traumatic brain injury in postmortem examination, Int. J. Legal Med. 134(6) (2020) 2167-2176.

[15] C. Mondello, E. Ventura Spagnolo, G. Bartoloni, A. Alibrandi, L. Cardia, D. Sapienza, P. Gualniera , A. Asmundo, Dystrophin and metalloproteinase 9 in myocardial ischemia: A post-mortem immunohisto chemical study, Leg. Med. (Tokyo) 53 (2021) 101948.
[16] T. Kondo, M. Takahashi, G. Yamasaki, M. Sugimoto, A. Kuse, M. Morichika, K. Nakagawa, M. Sa kurada, M. Asano, Y. Ueno, Immunohistochemical analysis of thrombomodulin expression in myocardial tissue from autopsy cases of ischemic heart disease, Leg. Med. (Tokyo) 51 (2021) 101897.
[17] T. Kondo, M. Takahashi, G. Yamasaki, M. Sugimoto, A. Kuse, M. Morichika, K. Nakagawa, M. Sa kurada, M. Asano, Y. Ueno, Immunohistochemical analysis of CD31 expression in myocardial tissues fr om autopsies of patients with ischemic heart disease, Leg. Med. (Tokyo) 59 (2022) 102127.
[18] T. Kondo, M. Takahashi, G. Yamasaki, M. Sugimoto, A. Kuse, M. Morichika, K. Nakagawa, M. Sa kurada, M. Asano, Y. Ueno, Immunohistochemical analysis of vimentin expression in myocardial tissue from autopsy cases of ischemic heart disease, Leg. Med. (Tokyo) 54 (2022) 102003.
[19] Y. Shang, Y. Wang, Y. Guo, L. Ren, X. Zhang, S. Wang, C. Zhang, J. Cai, Analysis of the risk of traumatic brain injury and evaluation neurogranin and myelin basic protein as potential biomarkers of traumatic brain injury in postmortem examination, Forensic Sci. Med. Pathol. 18(3) (2022) 288-298.
[20] J.H. Williams, B.L. Mepham, D.H. Wright, Tissue preparation for immunocytochemistry, J. Clin. Pat hol. 50(5) (1997) 422-428.
[21] M.M. Arnold, S. Srivastava, J. Fredenburgh, C.R. Stockard, R.B. Myers, W.E. Grizzle, Effects of fi xation and tissue processing on immunohistochemical demonstration of specific antigens, Biotech. Histo chem. 71(5) (1996) 224-230.
[22] R. Wasielewski, M. Mengel, M. Nolte, M. Werner, Influence of Fixation, Antibody Clones, and Sign al Amplification on Steroid Receptor Analysis, The Breast Journal 4(1) (1998) 33-40.
[23] S. Sunni, J.C. Geer, S.P. Kent, Staining in normal and ischemic human myocardium. A study of myoglobin, IgG, glycogen, and diastase-PAS, Arch. Pathol. Lab. Med. 108(8) (1984) 649-653.
[24] M. Werner, A. Chott, A. Fabiano, H. Battifora, Effect of formalin tissue fixation and processing on immunohistochemistry, Am. J. Surg. Pathol. 24(7) (2000) 1016-1019.
[25] S.P. Kent, Diffusion of myoglobin in the diagnosis of early myocardial ischemia, Lab. Invest. 46(3) (1982) 265-270.
[26] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215(3) (1990) 403-410.
[27] F. Varghese, A.B. Bukhari, R. Malhotra, A. De, IHC Profiler: an open source plugin for the quantit ative evaluation and automated scoring of immunohistochemistry images of human tissue samples, PLo S One 9(5) (2014) e96801.
[28] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis, Nat Methods 9(7) (2012) 671-675.
[29] I. Eltoum, J. Fredenburgh, R.B. Myers, W.E. Grizzle, Introduction to the theory and practice of fixa tion of tissues, J Histotechnol 24(3) (2001) 173-190.

[30] M.E. Hammond, D.F. Hayes, M. Dowsett, D.C. Allred, K.L. Hagerty, S. Badve, P.L. Fitzgibbons, G. Francis, N.S. Goldstein, M. Hayes, D.G. Hicks, S. Lester, R. Love, P.B. Mangu, L. McShane, K. Miller, C.K. Osborne, S. Paik, J. Perlmutter, A. Rhodes, H. Sasano, J.N. Schwartz, F.C. Sweep, S. Taube, E.E. Torlakovic, P. Valenstein, G. Viale, D. Visscher, T. Wheeler, R.B. Williams, J.L. Wittliff, A.C. Wolff, O. American Society of Clinical, P. College of American, American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (unabridged version), Arch. Pathol. Lab. Med. 134(7) (2010) e48-72.

[31] K.G. Helander, Kinetic studies of formaldehyde binding in tissue, Biotech. Histochem. 69(3) (1994) 177-179.

[32] J.D. Webster, M.A. Miller, D. Dusold, J. Ramos-Vara, Effects of prolonged formalin fixation on diagnostic immunohistochemistry in domestic animals, J. Histochem. Cytochem. 57(8) (2009) 753-761.

[33] A.A. Vass, S.A. Barshick, G. Sega, J. Caton, J.T. Skeen, J.C. Love, J.A. Synstelien, Decomposition chemistry of human remains: a new methodology for determining the postmortem interval, J. Forensic Sci. 47(3) (2002) 542-553.

[34] R. Cammack, T. Attwood, P. Campbell, H. Parish, A. Smith, F. Vella, J. Stirling, formol titration, Oxford Dictionary of Biochemistry and Molecular Biology, Oxford University Press, Oxford, 2006, p. 252.

[35] M. Sato, M. Kojima, A.K. Nagatsuma, Y. Nakamura, N. Saito, A. Ochiai, Optimal fixation for total preanalytic phase evaluation in pathology laboratories: a comprehensive study including immunohistochemistry, DNA, and mRNA assays, Pathol. Int. 64(5) (2014) 209-216.

[36] M. Cingolani, M. Cippitelli, R. Froldi, G. Tassoni, D. Mirtella, Stability of barbiturates in fixed tissues and formalin solutions, J. Anal. Toxicol. 29(3) (2005) 205-208.

[37] T. Miyama, T. Suzuki, Release of Mercury from Organs and Breakage of Carbon-Mercury Bond in Tissues Due to Formalin Fixation, Sangyo Igaku 13(6) (1971) 554-555.

[38] K. Puschel, U. Lockemann, J. Bartel, Postmortem investigation of serum myoglobin levels with special reference to electrical fatalities, Forensic Sci. Int. 72(3) (1995) 171-177.

[39] S. Miyaishi, An enzyme immunoassay for human myoglobin and its application to forensic medicine, Nihon Hoigaku Zasshi 45(1) (1991) 6-25.

[40] K.F. Gerston, L. Blumberg, V.A. Tshabalala, J. Murray, Viability of mycobacteria in formalin-fixed lungs, Hum. Pathol. 35(5) (2004) 571-575.

[41] H. Yaziji, T. Barry, Diagnostic Immunohistochemistry: what can go wrong?, Adv. Anat. Pathol. 13(5) (2006) 238-246.

Table. 1. Myoglobin stainability in the iliopsoas obtained of 10 autopsy cases with each summary.

Case No.	Age (year)	Sex	Causes of death	PMI	Degree of putrefaction*	Staining intensity	Cytoplasmic staining	Formalin pH (Fixed 4 days)
1	97	F	Heart failure	2.5 d	Zero	9	Even	6.40
2	34	M	Burning	2.5-3 d	Zero	8	Even	6.40
3	56	M	Hypothermia	2-3 w	Early	8	Uneven	6.60
4	19	F	Interrupted aorta	1-1.5 d	Zero	8	Even	6.30
5	53	M	Drowning	3-4 w	Middle	7	Even	6.10
6	58	F	Drowning	3.5-4 d	Zero	8	Even	6.43
7	55	M	Gastrointestinal hemorrhage	3-4 d	Zero	7	Even	6.23
8	20	F	Drowning	1-2 m	Delay	6	Even	6.13
9	90	F	Burning	3-3.5 d	Early	6	Even	6.50
10	81	M	Coronary artery disease	3-4 m	Delay	8	Uneven	5.33

M: males, F: females

PMI: Postmortem interval, d: days, w: weeks, m: month

*: Degree of putrefaction was evaluated using the extent of external color change. Zero: none, Early: some parts, Middle: about half of body, Delay: whole body.

Figure legends

Fig. 1. There was no clear difference in myoglobin stainability depending on their amount of formalin, and the stainability increase initial and then decrease gradually. The ratios of three samples are 1:1 (a), 1:4 (b) and 1:9 (c).

Fig. 2. The pH of formalin in which the sheep hearts were fixed decreased with smaller amount of formalin. Circle is formalin volume of 1mL. Triangle is formalin volume of 4 mL. Rhombus is formalin volume of 9mL.

Fig.3. Myoglobin was detected from the formalin fixing muscle samples immediately after fixation by ELISA. The absorbance rapidly reduced from 0.5 hours to 1day, low absorbance remained from 2 days for all skeletal muscles.

Fig. 4. Myoglobin was evenly stained in muscle cells in many autopsy cases (a). However, myoglobin sometimes exuded to interstitium from the cytoplasm and muscle cells unevenly stained immunohistochemically, even if these skeletal muscles were intact (b).

Fig. 5. No correlations between staining intensity of myoglobin and degree of putrefaction (a), postmortem interval (b) and pH of formalin (c) and for 10 autopsy cases of myoglobin-stained iliopsoas were observed.

Fig. 6. Myoglobin stainability of muscles fixed in formalin for a long duration obviously decreased in autopsy samples. The muscles fixed by a: 7 days, b: 1 month, c: 4 years. Fixed in formalin for 7 days showed staining with a score of 9. However, when fixed for one-month, staining intensity of tissues was 7. The myocardial tissues fixed in formalin for 4 years showed staining intensity of tissues of 0.

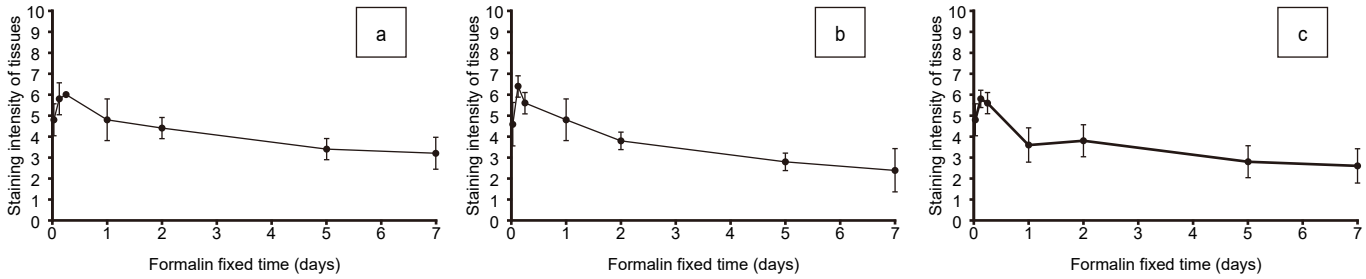
a
b
c
Staining intensity of tissues
Formalin fixed time (days)
0
1
2
3
4
5
6
7
8
9
10

Fig. 1. .

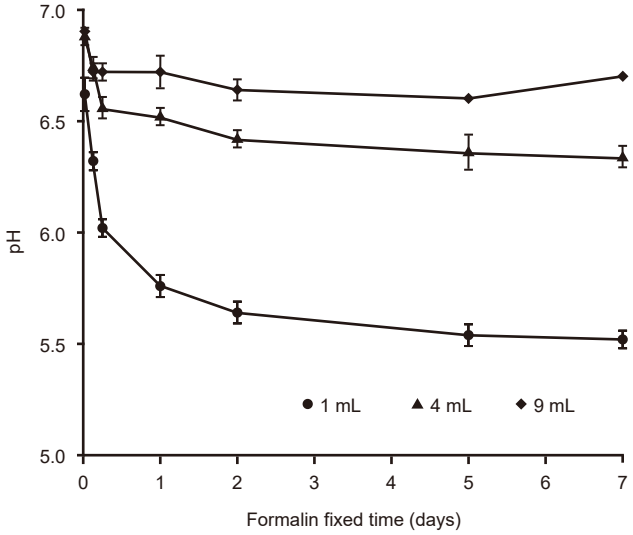
7.0
6.5
6.0
5.5
5.0
pH
1 mL
4 mL
9 mL
0
1
2
3
4
5
6
7
Formalin fixed time (days)

Fig. 2.

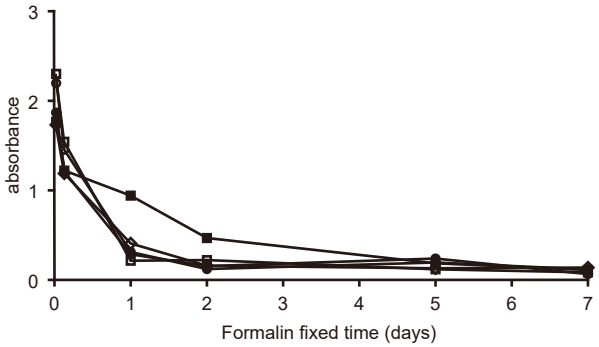

Fig.3.

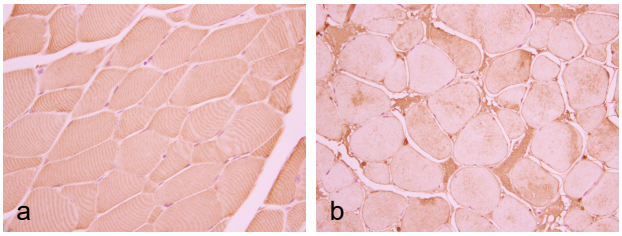

Fig. 4.

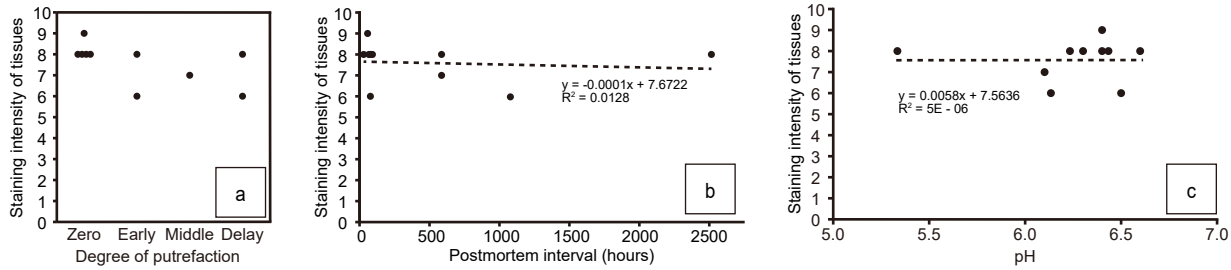
Staining intensity of tissues
Zero
Early
Middle
Delay
Degree of putrefaction
a
y = -0.0001x + 7.6722
R² = 0.0128
Postmortem interval (hours)
b
y = 0.0058x + 7.5636
R² = 5E - 06
pH
c

Fig. 5.

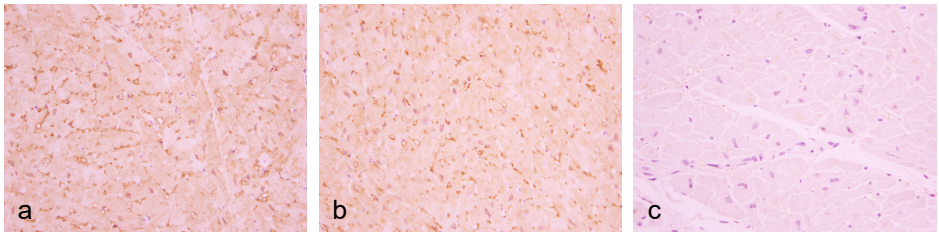

Fig. 6.