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Author(s)	Kakimoto, Yu; Tsuruyama, Tatsuaki; Miyao, Masashi; Abiru, Hitoshi; Sumiyoshi, Shinji; Kotani, Hirokazu; Haga, Hironori; Tamaki, Keiji
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The effectiveness and limitations of triphenyl tetrazolium chloride to detect acute

myocardial infarction at forensic autopsy

Yu Kakimoto[1], Tatsuaki Tsuruyama [1], Masashi Miyao[1], Hitoshi Abiru[1], Shinji Sumiyoshi[2],

Hirokazu Kotani[1], Hironori Haga[2], Keiji Tamaki[1]*

1 Department of Forensic Medicine and Molecular Pathology, Kyoto University Graduate

 School of Medicine, Yoshida-Konoe-cho, Sakyo-ku, Kyoto, 606-8501, Japan

2 Department of Diagnostic Pathology, Kyoto University Hospital, 54 Shogoin-Kawahara-cho,

 Sakyo-ku, Kyoto, 606-8507, Japan

Corresponding author. Tel.: +81 75 753 4474; Fax: +81 75 761 9591.

E-mail address: ktamaki@fp.med.kyoto-u.ac.jp

Abstract

Triphenyl tetrazolium chloride (TTC) is one of the most conventional stains to detect infarcted area of the heart in animal experiments. However, its availability and limitations have not been thoroughly discussed in the forensic field. Here, authors stained human hearts with TTC soon after the harvest. Photographs of the samples were analyzed using image analysis software, which evaluated the occupying ratio of the stained area on the surface of each slice. The results showed that the stainability of TTC declines with the length of the postmortem interval (PMI). Specimens reacted well to TTC within 1.5 days after death, and then decreased the stainability logarithmically with PMI ($y = - 0.294 \ln (x) + 1.0441$; $x =$ PMI, $y =$ TTC-stained area / total myocardial area, $R^2 = 0.5673$). Samples with old myocardial infarction produced clear TTC contrast; normal tissue is vivid red and fibrotic myocardium is white discoloration. In AMI cases where death occurred within 9 hours after the attack, however, the detection of infarcted area was very difficult even when PMI was less than 1.5 days. In summary, the TTC method may be useful within 1.5 days after death, but short suffering period before death disturbs its staining efficiency.

Keywords

INTRODUCTION

The most common cause of sudden, unexpected, natural deaths in adults is arteriosclerotic cardiovascular disease, which accounts for as many as 34-55% of all such deaths (1-3). Although most clinicians tend to label these deaths as acute myocardial infarction (AMI) by their episodes and symptoms, some patients do not show histopathologically ischemic changes at the very acute phase. In fact, most AMI cases have chronic stenosis with calcification in coronary arteries; however, more than 50% of the fatal thrombi occur at sites with < 75% cross-sectional stenosis by plaque (4). The infarcted area was difficult to identify according to the extent of coronary arteriosclerosis, as severe stenosis is not always prerequisite for AMI. Besides, about 20-40% of AMI failed to show direct evidence of thrombi (5-7). Microscopic changes, including waviness of fibers or coagulation necrosis, appear in 30 minutes after infarction, while macroscopic changes require more than 4 to 12 hours to be detected as a reddish-blue or yellow-tan area of discoloration (8, 9). If the infarcted area is reperfused more than 6 hours after the onset of chest pain, extensive hemorrhage is observed in that region (10). However, sudden death within a few hours after the infarct without reperfusion shows little histological changes. Therefore, the postmortem diagnosis of AMI can be difficult, particularly when death has occurred soon after coronary occlusion.

Triphenyltetrazolium chloride (TTC) has been used to visually detect the infarcted area of postmortem organs (11-16). TTC is reduced into triphenyltetrazolium formazan (TTF), which imparts a brick-red color to intact myocardium, where dehydrogenase activity is preserved. On the other hand, infarcted myocardia, in which the enzymes are inactivated or lacking, appear as an unstained pale zone. Accordingly, TTC staining clearly shows the division between the normal and the ischemic area. It has been possible to highlight the infarcted area by TTC if

more than 3 hours have passed since the artery occlusion before death in dog experiments (11-13). Actually, this method has also been used in human pathological autopsies, and the infarct was recognized in patients who died 8 hours after the onset of the clinical symptoms (14, 15). With a postmortem interval (PMI) shorter than 18 hours, the diagnostic sensitivity and specificity are up to 77.4% and 92.6% respectively, in pathological cases (16). Although the diagnostic sensitivity is not prominent, the specificity is higher than that of the commercial kits detecting troponin T or heart-type fatty acid-binding protein (H-FABP) (17). TTC staining, however, has been seldom used at forensic autopsies. It is not realistic to apply methods confirmed in animal or pathological cases with short PMI to forensic samples under worse conditions. Here, we examined the availability of the TTC method to diagnose AMI in forensic samples of patients who died in the very acute phase and revealed the limitations of the method for old samples, i.e., how long after death this staining is applicable, by using image analysis software. This is the first attempt to evaluate the effectiveness and limitations of TTC staining in the forensic field.

MATERIALS AND METHODS

Subjects

The heart samples were obtained from forensic autopsies performed at Kyoto University Graduate School of Medicine between November 2010 and May 2011. This project was approved by the Ethics Committee of Medicine at Kyoto University, and the studies were conducted according to the principles of Helsinki's Declaration. The samples were selected and classified into three groups: acute myocardial infarction (AMI), old myocardial infarction

(OMI), and non-myocardial infarction (NMI).

AMI was diagnosed with the following criteria: (ⅰ) sudden death within 12 hours from the onset of symptoms; (ⅱ) fresh thrombi, plaque erosion, or plaque rupture; (ⅲ) coronary atherosclosis (> 75% occlusion); (ⅳ) microscopic findings, including waviness of fibers, coagulation necrosis, myocyte hypereosinosis, contraction band, or neutrophilic infiltrate; and (ⅴ) H-FABP in the blood ≥ 6.2 ng/ml.

OMI was diagnosed on the basis of the following criteria: (ⅰ) visually white fibrosis scarring in myocardia; (ⅱ) coronary atherosclosis (> 50% occlusion); (ⅲ) microscopic findings, including increased collagen deposition, fatty metaplasia, or decreased cellularity; and (ⅳ) H-FABP in the blood < 6.2 ng/ml.

NMI was diagnosed after exclusion of AMI and OMI and in the following cases: (ⅰ) OMI complicated by AMI; (ⅱ) other heart diseases, such as cardiomyopathy, myocarditis, and congenital heart abnormality; (ⅲ) abnormal temperature circumstances, such as death by burns, heatstroke, and cold; and (ⅳ) age < 20.

A total of 27 cases were collected (17 males and 10 females, 53.5 ± 19 years, PMI = 2.8 ± 2.3 days: Table 1). Four cases were AMI (all males, 53.5 ± 14 years, PMI = 1.4 ± 0.2 days), Three cases were OMI (all males, 72.3 ± 13.8 years, PMI = 2.2 ± 0.8 days), and 20 cases were NMI (10 males and 10 females, 50.7 ± 19 years, PMI = 3.1 ± 2.6 days). These results are expressed as the means ± SD.

Tissue Preparation

In each case, two approximately 1-cm-thick transverse sections of the heart, located 1~2 cm under the atrioventricular sulcus, were taken out for TTC staining. The blood on the surface of the tissue was washed off to exclude hemoglobin mimicking the TTF color (18). After

macro-staining, the heart sections were fixed with 10% formaldehyde for more than 2 weeks and embedded in paraffin. The blocks were sectioned to 4 μm thickness. Samples for microscopy were taken from the left ventricular posterior, lateral, and anterior walls, the septum, and the right ventricle wall of the paraffin blocks, including the main perfusion area of the main coronary arteries.

Histochemical Staining

One of the sections from each heart was wrapped with gauze and incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, US) at 37℃ for 1 hour. Another section was incubated in phosphate-buffered saline (PBS) as a negative control for the same period. To avoid oxidative reaction with the air, each section was sealed with the buffer in a zipped freezer bag after carefully removing the air from the bag. During the incubation, we gently shook the bags a few times in a hot bath to obtain uniform staining. Microscopic staining was performed with Azan as well as hematoxylin and eosin (HE). H-FABP in the blood was measured using a commercial kit (Rapicheck; DS Pharm Biomedical, JP), which is effective within 6 hours, especially within 3 hours, after AMI (17).

Planimetric Measurements

Photographs were taken using a single-lens reflex digital camera (EOS kiss x3; Canon, JP) with a polarizing filter (CPL; Marumi, JP) to minimize reflecting light, which would present white spots similar to the infracted area on the picture. Planimetry was performed with Adobe Photoshop (version 6.0; Adobe, CA). On the surface of each slice, the total myocardial area (MA), visually fibrosis area without TTC staining (FA), visually non-fibrotic area without TTC (NFA), TTC-stained area (TA), and non-TTC-stained area (NTA) were classified by the color

gamut. Then, their pixels were counted and converted from pixels squared to millimeters squared as previously reported (19, 20). We calculated the occupying ratios of each area to the total myocardial area (FA/MA, TA/MA, and NTA/MA).

In NMI cases, we plotted the postmortem day and occupying ratio (TA/MA) to the scatter diagram and approximated the graph to a logarithmic function by Excel (Microsoft Office 2010; Microsoft Inc., US).

RESULTS

AMI

All AMI samples in this study were examined within 41 hours postmortem. They were completely stained vivid red with TTC (TA/MA = 1.0, Fig.1a) despite the presence of thrombus with more than 90% occlusion in the left ascending artery of Case 2 and in the left circumflex artery of Case 4 (Fig. 1b), plaque erosion with more than 75% occlusion in the right coronary artery of Case 1, and plaque rapture with more than 90% occlusion in the left ascending artery of Case 3. Microscopic findings also showed coronary sclerosis with fresh thrombus (Fig. 2a,b) and myocardial infarction in the acute phase (Fig. 2c).

OMI

OMI hearts were stained within 71 hours after cardiac arrest. In each case, the occupying ratio of the visually fibrosis area before staining (FA/MA) and that of the non-TTC-stained area (NTA/MA) were examined (Fig. 3). Cases 5 and 7 showed a smaller white area after staining (FA/MA > NTA/MA), and Case 6 showed the opposite (FA/MA < NTA/MA). The color contrast

on the TTC-stained slice (TA versus NTA) was much clearer than that on the control slice (NFA versus FA) in every case. In NTA and its peripheral area, dense collagenous scars and fatty changes were observed.

NMI

In NMI cases, samples within 36 hours after death were completely stained with TTC (TA/MA = 1.0), while some hearts harvested more than 40 hours after death were insufficiently stained (TA/MA < 1.0, Fig. 4). In the regression analysis of all NMI cases, the ability of staining decreased with the PMI, as shown below (Fig. 5): *y = - 0.294 In (x) + 1.0441; y = TA/MA, x = PMI > 1.2 days, R^2 = 0.5673* (NMI cases, n=20).

DISCUSSION

Regarding the use of the TTC method to detect AMI, forensic samples, compared with animal models or human pathological cases, present two main problems related to time. First, the time from the critical attack to death is much shorter. Most forensic victims have no chance to call an ambulance or die before arriving at the hospital. Second, PMI is much longer. The bodies of individuals, who do not die at a hospital and do not have a witness, may undergo a degree of damage and decomposition depending on the postmortem environment. Therefore, it is difficult to directly apply the methods established for animal experiments or pathological autopsies to forensic samples. In this first forensic trial, the results show that the first problem, i.e., the time between the critical insult and death, plays a more significant role in the postmortem detection of AMI than the second one, i.e., the time between death and autopsy. The cases, where death

had occurred within 9 hours after the attack and no macroscopic histological change had yet appeared, did not show discoloration of TTC.

Some NMI cases whose PMI is more than 1.5 days have NTA, although no findings of AMI were observed in each area microscopically. According to the staining results, plenty of dehydrogenases are preserved in heart samples 1.5 days after death. In this observation, the PMI of AMI cases is relatively short ($\leq$ 1.7days), and the stainability of TTC to detect the infarcted area might be well preserved. In fact, the AMI samples were completely stained without NTA against their ischemic changes on microscopy. This contradiction is due to the brief period between the critical myocardial infarction and death rather than to the postmortem effects. The earliest time to detect AMI as a dehydrogenase defect in animal models is reportedly 3 hours after a heart attack (11-13), while human cases require a longer period of at least 8 hours (14, 15). In our examination, the diagnosis of AMI by TTC within 9 hours of coronary occlusion was difficult, which is consistent with previous pathological reports.

OMI area was macroscopically distinguished by TTC staining. The difference between FA/MA and NTA/MA seems to depend on PMI and the degree of fibrosis in marginal areas. The longer the time after death is, the more unclear the contrast between FA and NFA or TA and NTA becomes in the samples. The fibrosis parts are supposed to lack dehydrogenase, given that collagen fibers replace myocardial cells. It is difficult to conclude how much fibrosis could be detected by the TTC method from this small number of samples. Moreover, the fibrosis area is originally different in each slice, as the sections are serial and 1 cm in thickness.

The least stained case for PMI was case 19, the oldest patient in NMI. Its stainability was only 35.8% of the expected value based on the formula. Its cause of death, blood loss, does not seem to contribute its inferior result, reviewing other same cases (Case 8, Case 10, and Case 14). The second senior patient (Case 22) also showed less stainability, 86.4% of that expected. These

discolorations may reflect the ischemic condition caused by their high atherosclerosis or imply the fragility and less mitochondrial activity of an aging heart. As senescent myocardium is more vulnerable to stress or injury (21-23), the enzymes necessary for TTC might easily dissipate at the time of death. Considering the dysfunction of the mitochondrial respiratory chain (24-26), the activity of dehydrogenases or electron carriers may be diminished in elderly hearts.

In fact, even if a patient died within 30 minutes after the occlusion of the coronary artery without visible thrombosis, it is possible to diagnose AMI based on electron microscopic findings: sarcoplasmic edema, mitochondrial swelling, and loss of glycogen (8, 9). Immunochemical staining against myoglobin, α-actin, vinculin, and desmin also reveals the AMI area 1 hour after the onset (27). An *in situ* apoptosis (DNA fragmentation) assay shows the AMI region 2 hours after the event (28, 29). However, such microscopic examinations without macroscopic orientation require extensive sampling, and tissue processing is costly as well as time-consuming. Macroscopic detection of the focus is essential to determine the area to sample for microscopic examination. Whole staining, which can be easily performed at forensic autopsy, will hopefully increase the efficiency and accuracy of postmortem diagnosis.

Sudden coronary death is the result of very complex pathophysiology with many parameters. Not only atherosclerosis but also hypertension and hyperglycemia contribute to its deterioration (30-33). However, forensic examiners often lack the information about such clinical risk factors. Consequently, visible morphological findings are very important at autopsy. TTC is a very inexpensive substance (about 3 dollars/g), and the staining method is quite simple. The pigment of TTF stays only on the surface of the tissue and thus does not interfere with subsequent HE staining (14). Even though the number of applicable cases is limited in forensic scenes, TTC can be useful as a rapid screening technique when AMI is strongly suspected.

In this present study, we stained only one slice with TTC per heart in order to compare the

color tone with that of a slice incubated in PBS as a negative control. Although the stained slice covered the area perfused by main coronary arteries, the risk that we missed the infarcted myocardia outside the slice could not be definitively excluded. At practical autopsies, better efficiency is expected by staining serial slices of a whole heart.

To summarize, the TTC method is available in cases within 1.5 days after death, except for aged patients (≥ 80 years) and the deceased with abnormal conditions of high temperature. The stainability of TTC logarithmically decreases with the PMI after 1.5 days. Considering another limitation that TTC hardly detects the infarcted lesion in sudden death with ischemic duration less than 9 hours, this staining method by itself cannot be a final diagnosis tool of AMI. When the clinical history, blood test, or coronary occlusion of a fresh autopsy case suggests AMI with some agonal period, TTC may be helpful to identify the infarcted parts for subsequent microscopic examination.

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FIGURE LEGENDS

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FIGURE 1.   AMI heart (Case 4).

a: The upper sample is negative control just in PBS, and the lower one is stained with TTC. The TTC slice is light brick-red in color, while the control is brownish. No NTA is found on the TTC slice.

b : Severe sclerosis of LCX (after fixation).

FIGURE 2.   Histological findings of AMI (Case 4).

a: Coronary artery with massively thickened intima.

b: A higher magnification of the inset in a. Fresh thrombus accompanied with fibrin precipitation

c: Waviness of fibers indicating ischemic lesioin at the lateral wall.

Original magnification a ×20, b ×200, c ×100. Azan stain.

FIGURE 3.   OMI heart (Case 5).

The upper sample is the control, and the lower one is stained with TTC. FA is observed on the endocardial side (arrows). That area is revealed clearly after TTC staining (arrowheads). The scale is 5 mm.

FIGURE 4.   NMI hearts.

The upper samples are the control, and the lower ones are stained with TTC.

a: Fresh heart; PMI = 1.3 days (Case 9). The TTC slice is vivid red in color, while the control is dark.

b: Old heart; PMI = 5.5 days (Case 25). Some non-stained spots are found on the

TTC slice (arrows). The scale is 5 mm.

FIGURE 5.　　　Regression analysis of the stainability of NMI after death.

The X-axis is the PMI, and the Y-axis is TA/MA. The curve shows the approximation
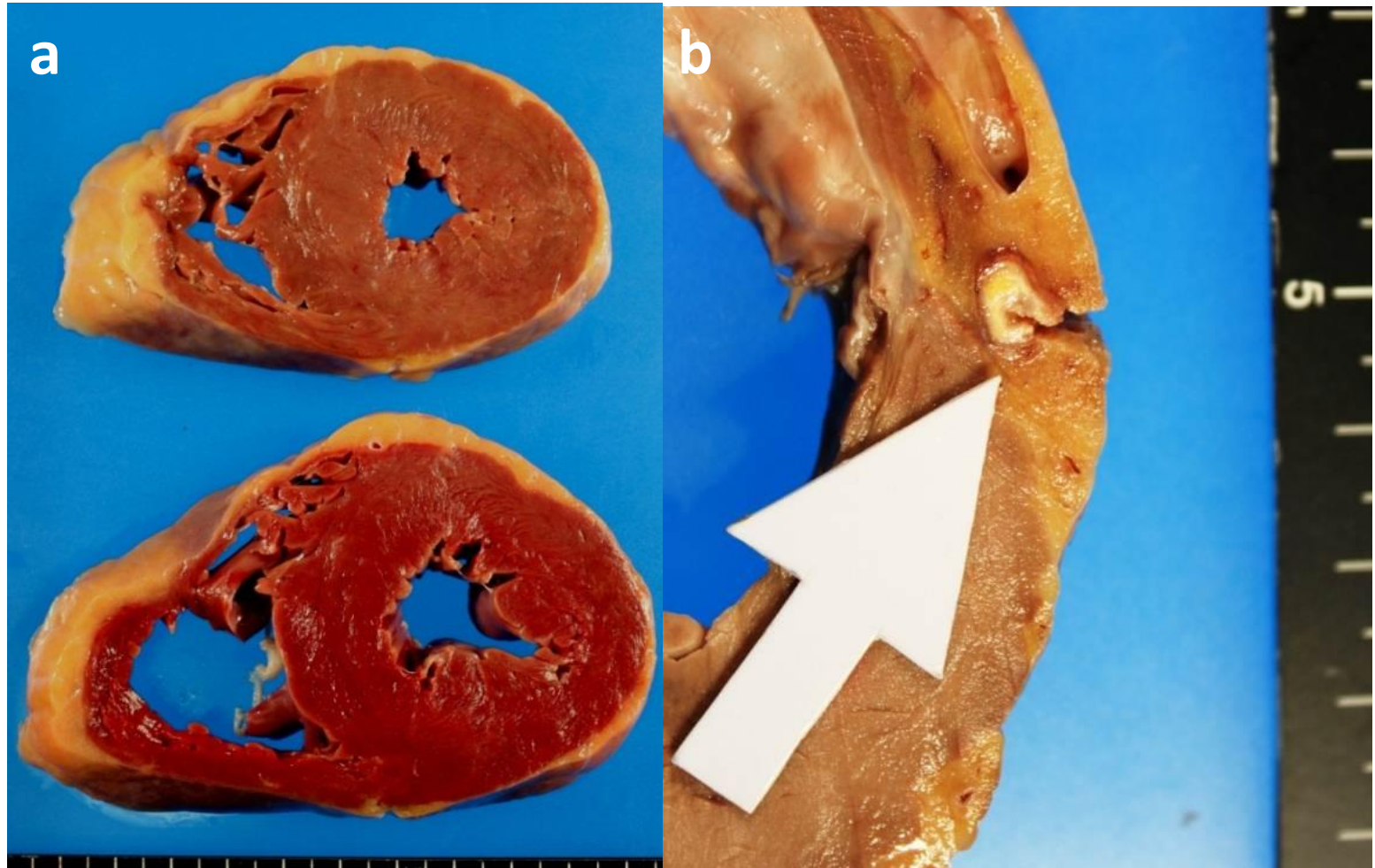
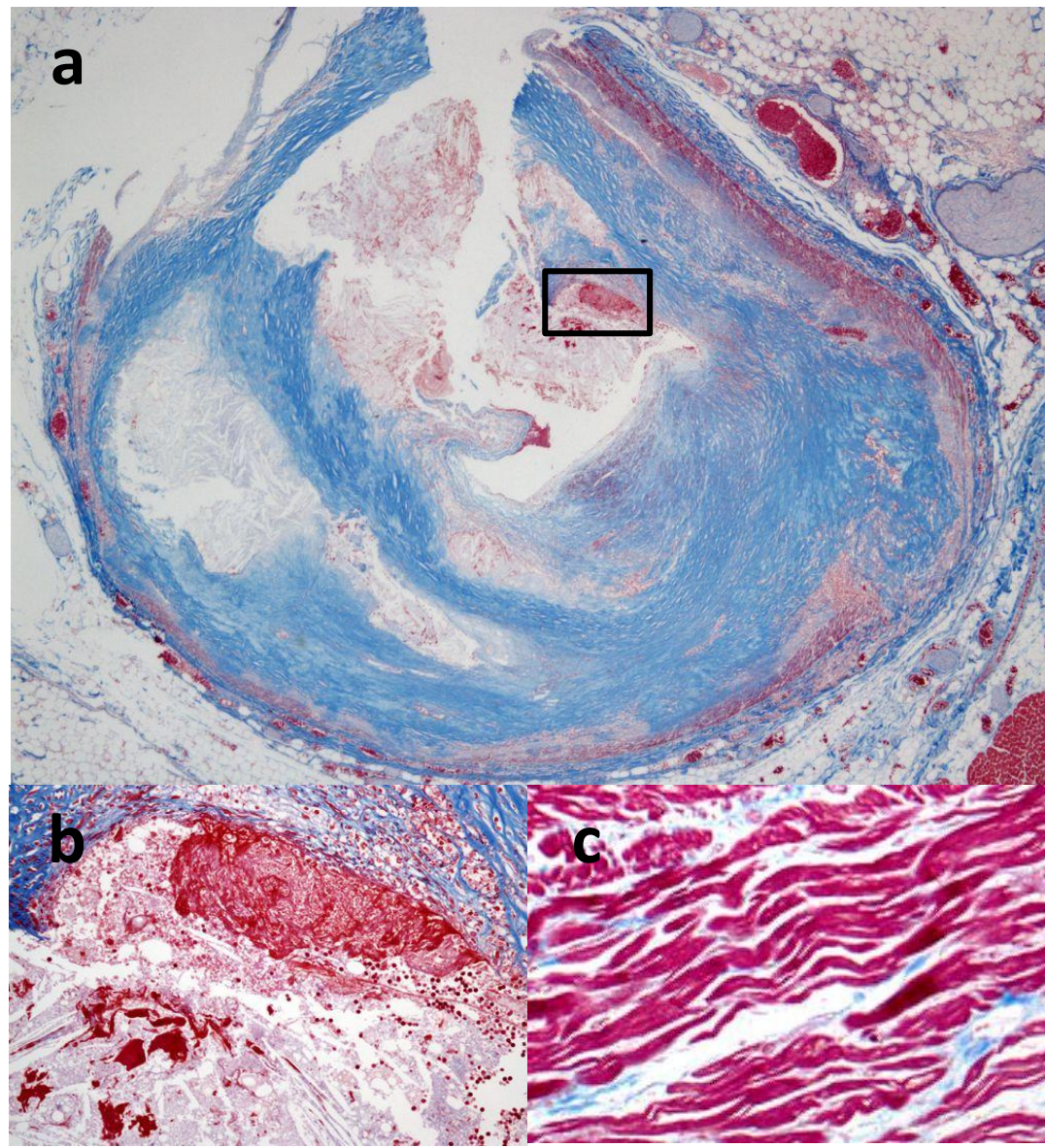formula: y = - 0.294 In (x) + 1.0441, x > 1.2, R^2 = 0.5673.
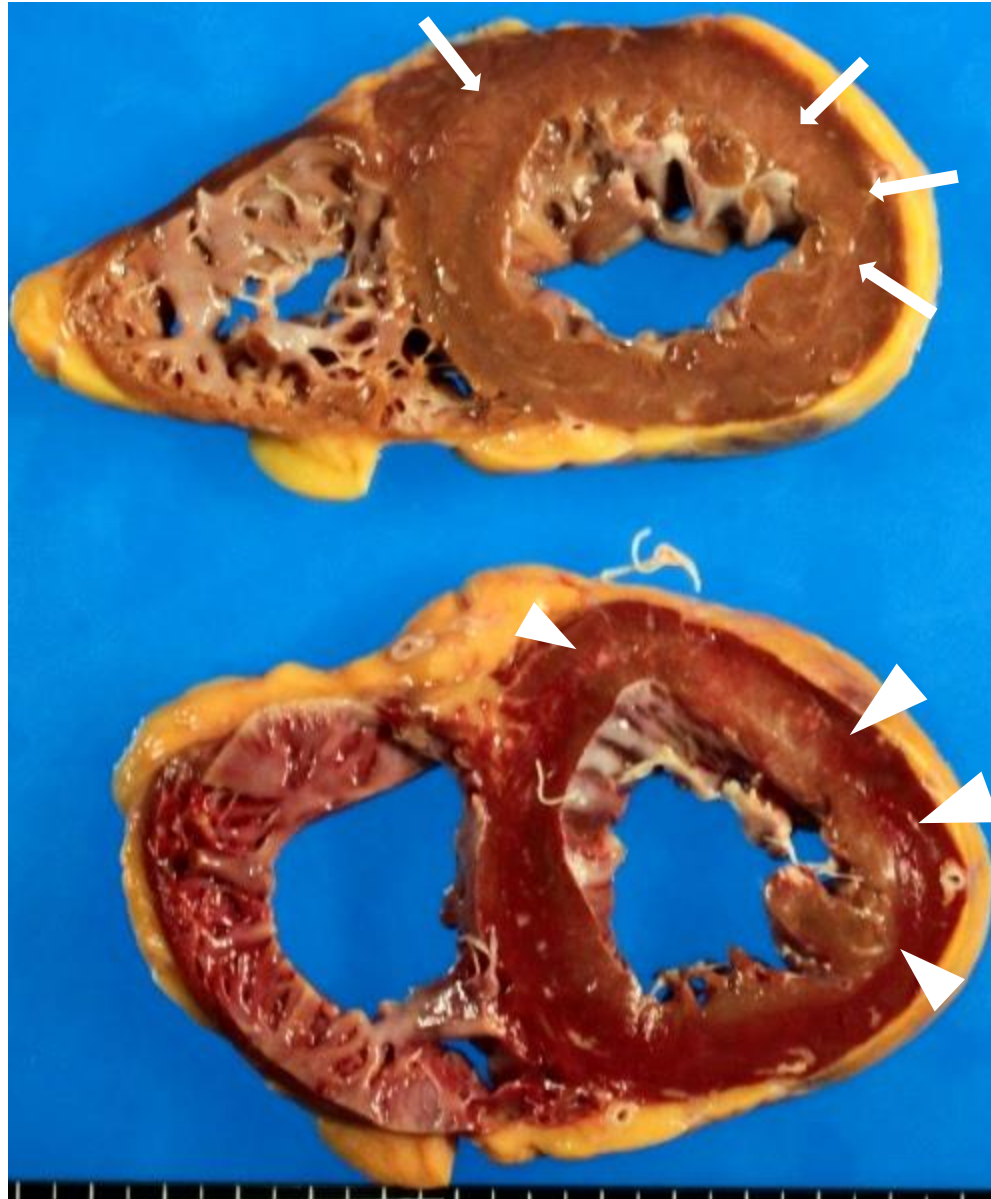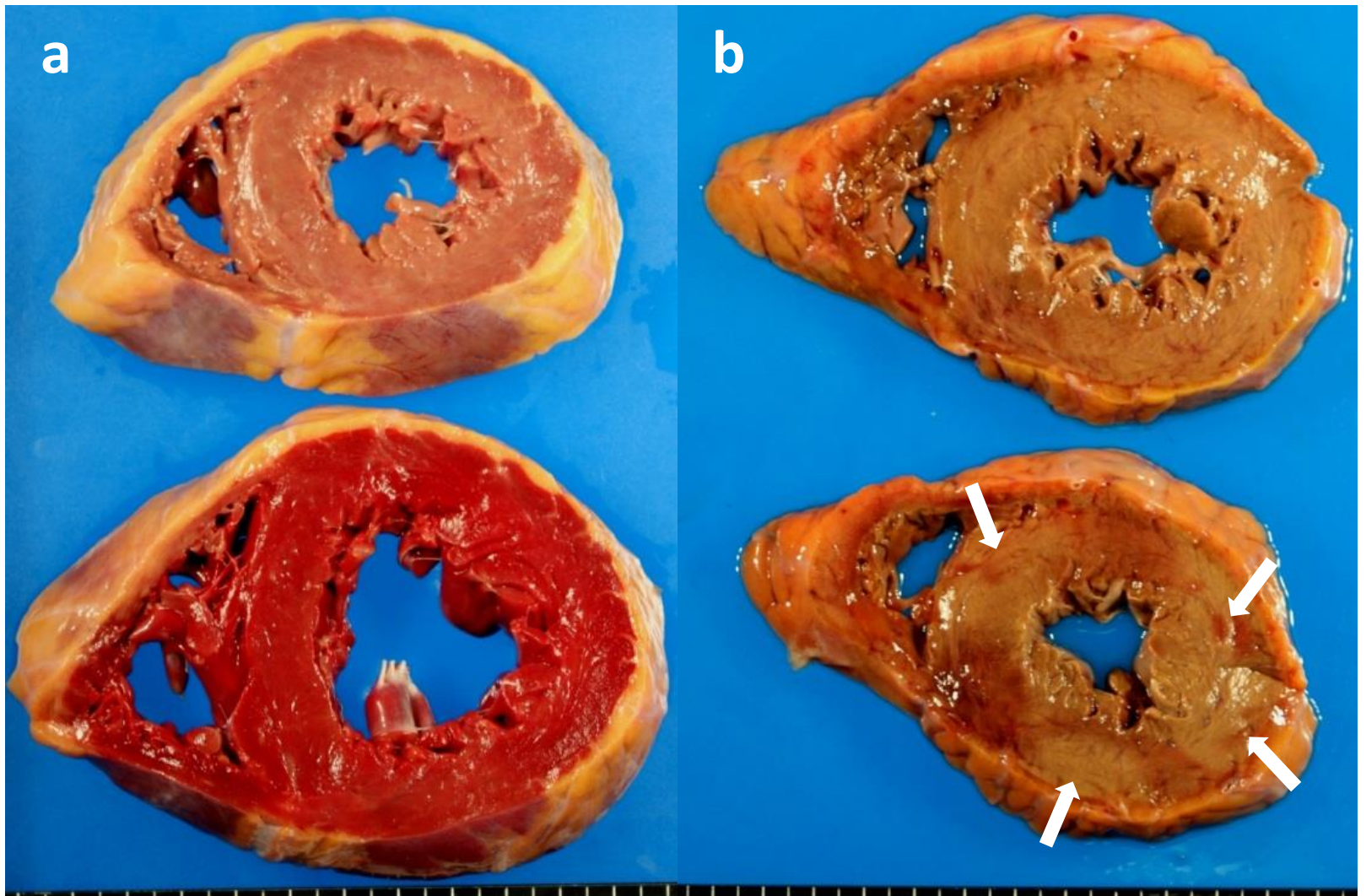
FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

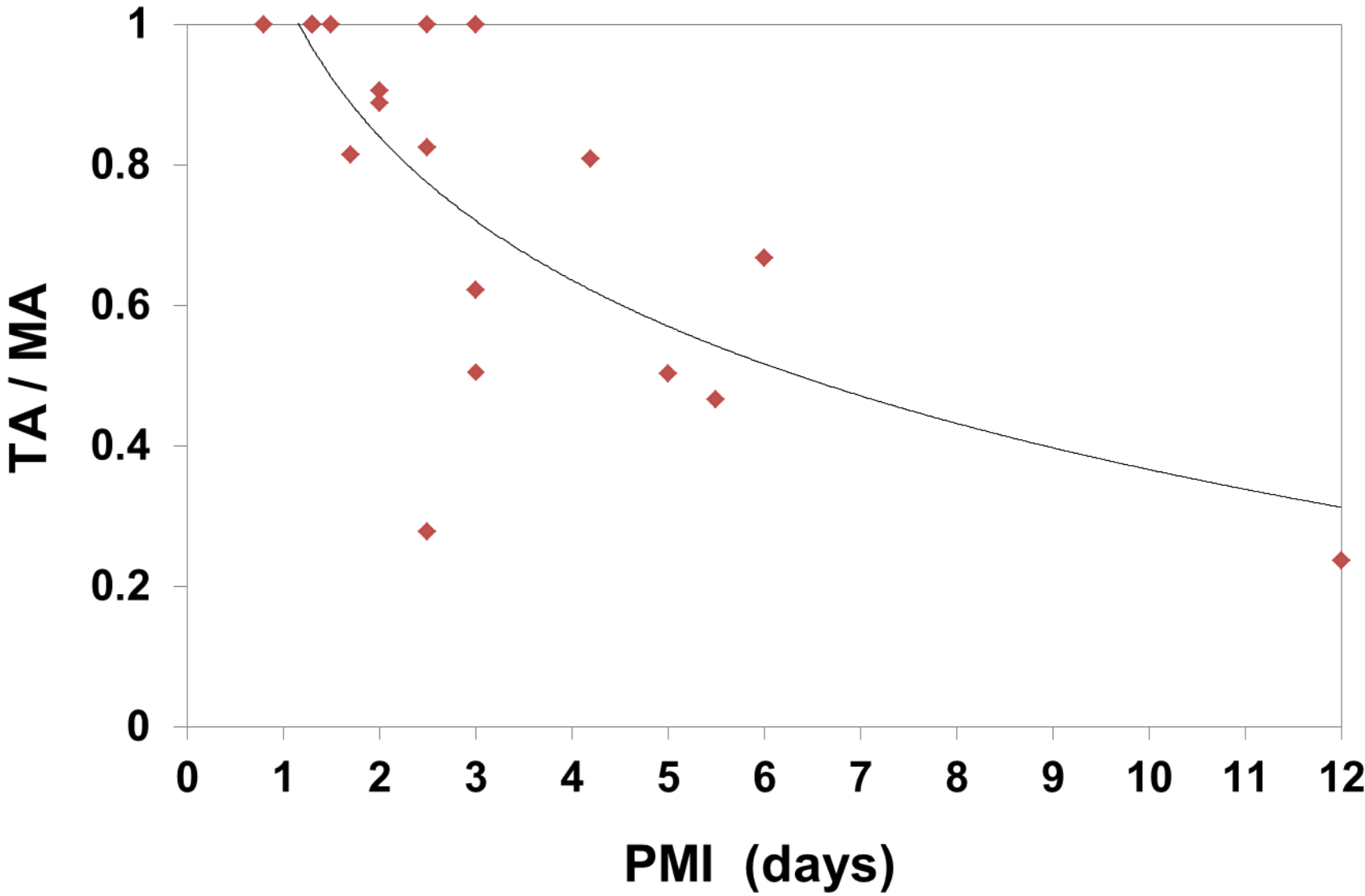**TABLE 1.** Details of Patient

Case	Sex	Age	Time from onset to death	PMI (day)	Cause of death	Sclerosis of coronary arteries	TA / MA	
1	M	72	< 9 hr	1.2	AMI	High, Plaque erosion RA	1	
2	M	40	< 7 hr	1.3	AMI	Severe, Thrombus in LAD	1	
3	M	48	< 2 hr	1.5	AMI	Severe, Plaque rapture in LAD	1	
4	M	54	< 20 min	1.7	AMI	Severe, Thrombus in LCX	1	
5	M	88	8 days	1.5	Brain contusion	Mild	0.546	(NTA/MA = 0.391)
6	M	62	A few days	2	Meningoencephalitis	Mild	0.482	(NTA/MA = 0.503)
7	M	67	Unknown	3	Acute respiratory failure	Severe	0.315	(NTA/MA = 0.136)
8	F	47	< 30 min	0.8	Blood loss	None	1	
9	M	39	9 hr	1.3	Brain contusion	Mild	1	
10	F	79	< 2 hr	1.3	Blood loss	Little	1	
11	M	41	5 days	1.3	Brain contusion	None	1	
12	F	69	< 1 hr	1.3	Subarachnoid hemorrhage	Little	1	
13	F	37	< 30 min	1.5	Asphyxia	None	1	
14	M	29	9 hr	1.7	Blood loss	None	0.814	
15	M	33	Unknown	2	Pulmonary edema	None	0.888	
16	M	61	1 hr	2	Asphyxia	Little	0.906	
17	F	36	< 10 min	2.5	Asphyxia	None	1	
18	M	52	< 10 min	2.5	Brain contusion	High	0.825	
19	M	86	Unknown	2.5	Blood loss	Mild	0.277	
20	M	73	< 30 min	3	Brain contusion	Little	1	
21	F	39	22 hr	3	Hypoxic encephalopathy	None	0.505	
22	F	80	Unknown	3	Pancreatic carcinoma	High	0.622	
23	M	36	< 10 min	4.2	Asphyxia	High	0.809	
24	F	44	Unknown	5	Cirrhosis	None	0.502	
25	F	70	Unknown	5.5	Cerebral hemorrhage	Mild	0.466	
26	F	41	Unknown	6	Pneumonia	None	0.667	
27	M	21	Unknown	12	Unknown	None	0.237	

Sclerosis of coronary arteries is categorized according to the most serious occlusion at cross section;

Severe > 90%,High > 75%, Mild > 50%,Little > 25%, None ≤ 25%.

RA: right coronary artery, LAD: left anterior descending coronary artery, LCX: left circumflex coronary artery.