Original Article

นิพนธ์ต้นฉบับ

Study of immunohistochemical staining method with fibronectin antibody for detection of early myocardial infarction in comparison to triphenyl tetrazolium chloride (TTC) staining

การศึกษาการย้อมอิมมูโนฮิสโตเคมีด้วยไฟโบรเนคตินแอนติบอดี้เปรียบเทียบกับไตรฟีนิลเตตระโซเลียมคลอไรด์ในการตรวจพบกล้ามเนื้อหัวใจตายเฉียบพลัน

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Abstract

Early acute myocardial infarction within a few hours after onset of ischemia usually cannot be detected on naked eye examination and by routine hematoxylin and eosin staining under light microscopic study, it is usually visible at six hours after onset of ischemia. Triphenyl tetrazolium (TTC) chloride staining is reported to be able to detect gross ischemic change of the heart within two hours after ischemia. Immunohistochemical staining with fibronectin was reported to be able to detect ischemic change after coronary ligation in pigs within 30 minutes.

The objective of this study was to compare different methods of detecting early myocardial infarction. Hearts from 41 autopsy cases that were Troponin T- positive with coronary artery occlusion for 70% and over were collected at Forensic Pathology Division, Department of Forensic Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University. The hearts were cut transversely across both ventricles at 1 cm thick. for TTC staining. Positive and negative result of TTC staining areas were selected for paraffin embedding, then sectioned at 3 microns for hematoxylin and eosin staining and immmunohistochemical staining with fibronectin.The results showed that the percentage of cell damage in areas with fibronectin stain, compared to hematoxylin and eosin stained areas was significantly different ($p<.01$) and fibronectin had a sensitivity of 100% and a specificity of 50%. When comparing between fibronectin and TTC, for which TTC groups were divided into positive and negative result groups, the amount of cell damage determined by fibronectin staining showed no significant difference. From this study, fibronectin detected myocardial injury in cases that were Troponin T

positive and TTC negative with statistical significance (p<.05). Therefore, fibronectin can detect myocardial infarct earlier than TTC.

Keywords: Fibronectin, TTC, Myocardial infarction

บทคัดย่อ

กล้ามเนื้อหัวใจตายเฉียบพลันที่เกิดขึ้นโดยฉับพลันไม่สามารถตรวจพบได้โดยตาเปล่า การตรวจพบโดยการย้อมฮีมาท็อกซิลินและอิโอซินสามารถพบได้หกชั่วโมงหลังจากกล้ามเนื้อหัวใจขาดเลือด จากการศึกษาการย้อมทีทีซีในชิ้นเนื้อหัวใจพบว่าสามารถพบกล้ามเนื้อหัวใจตายได้ในสองชั่วโมงหลังจากกล้ามเนื้อหัวใจขาดเลือด การย้อมโดยวิธีอิมมูโนฮีสโตเคมีด้วยไฟโบรเนคตินสามารถตรวจพบกล้ามเนื้อหัวใจตายในหมูได้ภายในสามสิบนาที วัตถุประสงค์ของการศึกษานี้จึงต้องการเปรียบเทียบวิธีการตรวจพบกล้ามเนื้อหัวใจตายเฉียบพลัน โดยศึกษาในหัวใจจำนวน41รายที่มีหลอดเลือดโคโรนารีตีบเกิน70%และมีโทรโปนินผลเป็นบวกซึ่งได้จากการผ่าศพที่สาขานิติพยาธิวิทยา ภาควิชานิติเวชศาสตร์ คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล โดยนำหัวใจมาหั่นตามขวางให้มีความหนาหนึ่งเซนติเมตรเพื่อนำไปย้อมทีทีซี เมื่อได้ผลการย้อมทีทีซีทั้งพื้นที่ที่ผลบวกและผลลบก็จะถูกเลือกเพื่อนำไปผ่านกระบวนการจนได้เป็นพาราฟินบล็อคและตัดชิ้นเนื้อให้มีขนาดสามไมครอนเพื่อนำไปย้อมฮีมาท็อกซิลินและอิโอซินและการย้อมโดยวิธีอิมมูโนฮิสโตเคมีด้วยไฟโบรเนคติน

จากผลการศึกษาพบว่าการเปรียบเทียบจำนวนเซลล์ของกล้ามเนื้อหัวใจตายระหว่างการย้อมฮีมาท็อกซิลินและอิโอซินและการย้อมโดยวิธีอิมมูโนฮิสโตเคมีด้วยไฟโบรเนคตินมีความแตกต่างอย่างมีนัยสำคัญ (p<.01) และไฟรโบรเนคตินมีค่าความไว 100% ค่าความจำเพาะ 50% เมื่อเปรียบเทียบจำนวนเซลล์ของกล้ามเนื้อหัวใจตายโดยวิธีอิมมูโนฮิสโตเคมีด้วยไฟโบรเนคตินระหว่างทีทีซีสองกลุ่มผลบวกและผลลบ พบว่าจำนวนเซลล์ของกล้ามเนื้อหัวใจตายไม่แตกต่างกัน และไฟโปรเนคตินสามารถตรวจพบกล้ามเนื้อหัวใจตายในรายที่ทีทีซีตรวจไม่พบกล้ามเนื้อหัวใจตายโดยมีค่าความแตกต่างอย่างมีนัยสำคัญทางสถิติ (p<.05) ดังนั้นจึงสรุปได้ว่าไฟโบรเนคตินสามารถตรวจพบกล้ามเนื้อหัวใจตายได้ก่อนการตรวจพบด้วยทีทีซี

คำสำคัญ: ไฟโปรเนคติน, ทีทีซี, กล้ามเนื้อหัวใจตาย

Introduction

Myocardial Infarction (MI) is by far the most important form of IHD and alone is the leading cause of death in the industrialized nations. About 1.5 million individuals in the United States suffer an acute MI annually and approximately one third of them die. At least 250,000 people a year died of a heart attack before they reached the hospital.[1] Bureau of Non Communicable Disease in Thailand reported that during the year 2008 people died about 2.12 million of ischemic heart disease.[2] However, early recognition of acute myocardial infarcts by pathologists can be difficult, particularly when death has occurred within few hours after onset of symptoms. Previous study showed that myocardial infarction

occurred occasionally as dark mottling areas in 4 to 12 hours after major coronary occlusion seen by naked eye. However, myocardial infarcts less than 6 hours old are usually not apparent on gross examination. Variable waviness of fiber border can be seen under microscopic examination in a minimum period of ½ -4 hours after ischemic attacking with hematoxylin and eosin (H&E) staining but usually none. [1] In those cases where sudden deaths occurred in the very early stage of infarction, the myocardial lesion could not be easily detected. Many morphological, histochemical, enzyme histochemical, electron microscopic, or immunohistochemical methods have been introduced for postmortem detection of acute myocardial infarction, but many have been shown to be non-specific, unreliable or difficult to use in forensic practice. [3,9,10]

Regarding to immunohistochemical technique by fibronectin (FN) comparing to enzyme histochemical technique by Triphenyl tetrazolium chloride (TTC). Enzyme histochemical technique is based on the fact that membranes of ischemic myocardial cell loses their integrity and releases enzyme into blood. [4] This enzyme histochemical stain imparts a brick-red color to intact, noninfarcted myocardium where the dehydrogenase enzymes are preserved. Because dehydrogenase depleted in the area of ischemic necrosis (they leak out through the damaged cell membranes), an infarcted area is revealed as an unstained pale zone (while old scarred infarcts appear white and glistening).[1] TTC is diagnosing the infarcts of around 2 hours of age.[1] According to the immunohistochemical method by using FN antibody, FN mediates a wide variety of cellular interactions with the extracellular matrix (ECM) and plays important roles in cell adhesion, migration, growth and differentiation.[5,6] FN have binding domain for collagens, fibrin, heparin sulfate, and many types of cells, including platelets. One domain has homology to tissue plasminogen activator, moreover, FN was localized in the interstitial space between myocytes, and beneath arterial, venous, and capillary endothelium. Previous study in rat hearts found at 4 hours after coronary ligation, FN was localized in a patchy fashion in the cytoplasm and interstitial space of some of the myocytes in the area supplied by the ligated vessel. At 48 hours, the intensity of staining for FN was maximal in and between the necrotic myocytes. Similar patterns of localization were observed at 3 and 7 days after coronary ligation, but progressive decreases in the intensity of staining.[7] In pig hearts, after 30 minutes and sometimes 60 minutes after coronary occlusion, individual myocytes and groups of myocytes were observed to be stained for FN.[11] Troponin is released from injured cardiac myocytes about 3 hours after ischemic injury and the elevation may persist for up to several weeks.1 Moreover, Troponin T is relatively stable the first 3 days after death.[8] So Troponin-T positive cases were selected for TTC method and immunohistochemical staining.

In this study, selected hearts were obtained from autopsy cases at Forensic Pathology Division, Forensic Medicine Department, Faculty of Medicine Siriraj Hospital, Mahidol University. All hearts were collected from cases of sudden unexpected death revealing troponin t positive results and coronary atherosclerosis with lumen occlusion of 70% and over. Hearts were transversely sliced and stained with TTC method and selected tissue for H&E staining and for immunohistochemical staining with FN antibody. The objective of this study is to find effective method for detection of early myocardial infarction, by comparing the amount of cell damage in early MI detected by FN and H&E and also to compare sensitivity and specificity between TTC and FN.

Materials and methods

Materials

41 hearts were collected from autopsy cases at Forensic Pathology Division, Forensic Medicine Department, Faculty of Medicine Siriraj Hospital, Mahidol University. All cases were sudden unexpected death revealing coronary atherosclerosis with luminal occlusion for 70% and over, and all were Troponin T positive by Troponin T test.

Methods

1. Troponin – T Test

Femoral blood drawn from each selected autopsy case, then put blood in EDTA tube, drop 150 µl of blood into Troponin T kit and read for the result in 20 minutes. No line represents error in the sample or Troponin T kit, one line represents internal control and negative result, two lines represent positive result. (Figure1)

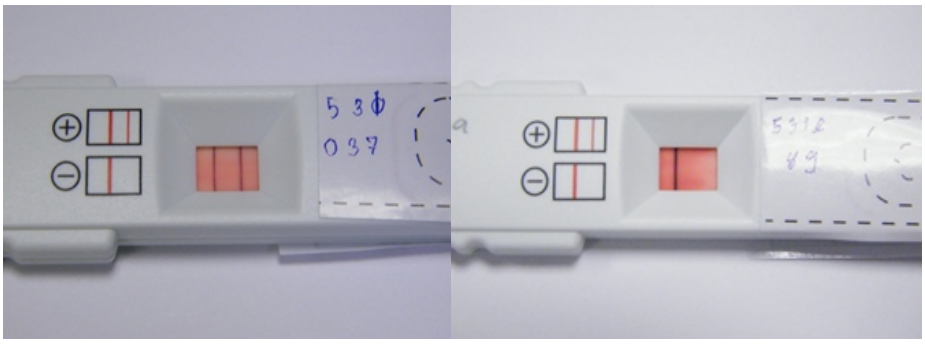

Figure1 Troponin T kit, one line represents the internal control and negative result, two lines represent positive result.

2. TTC staining method

The 41 selected hearts were cut transversely across both ventricles, 1 cm. in thickness. Wash the heart slices rapidly in tap water to remove excess blood, taking care not to macerate the tissue.

Then place heart slices with 1 % TTC in sodium phosphate buffer pH 8.5. TTC solution was prepared avoiding light because TTC is very sensitive to light. Therefore, TTC solution was wrapped by foil every time. Incubated heart slices in TTC solution in water bath temperature 37 - 40 oC for 30 – 40 minutes (turn the heart slice once at 15 - 20 minutes). Take pictures from digital camera. Normal hearts stain brick- red color (TTC negative for MI) and myocardial infarction area revealed unstain or pale color (TTC positive for MI)

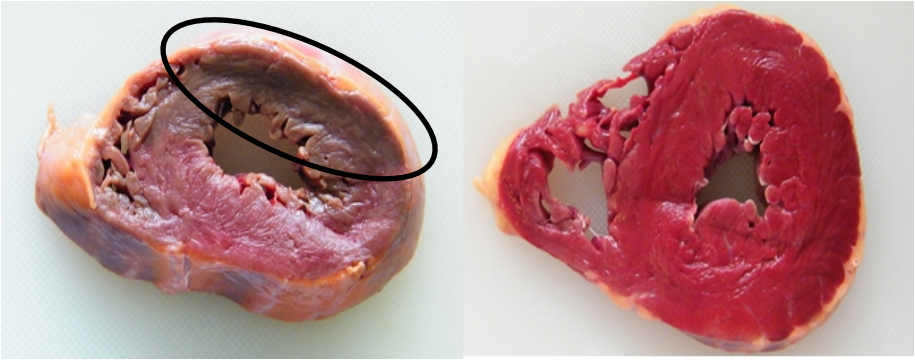

Figure2 Left: heart slice reveals pale unstained area of MI = TTC: positive result for MI,
Right: entire slice stain red = TTC negative result for MI

3. Preparation of tissue for paraffin sections

Cut tissue of heart from selected areas for appropriate size and fixed in 10% formalin overnight. After overnight fixation, select area for sectioning and process tissue in tissue processer and embedded in paraffin block to be sectioned for H&E staining and immunohistochemical staining.

3.1 H&E staining

Paraffin block were sectioned at 3 microns by microtome and tissue floated in warm water bath and placed on glass slides. The slides were left to dry, then placed slides in hot air oven over night at 60°C for better tissue attached and paraffin bake. Then stain the slide with H&E staining method.

Microscopic findings composed of edema, wavy fibers, contraction bands, early coagulation necrosis, hemorrhage, the earliest finding was hypereosinophilia of myocytes (Figure3). Percentages of amount of cell damage in MI for H&E staining were grading in table1.

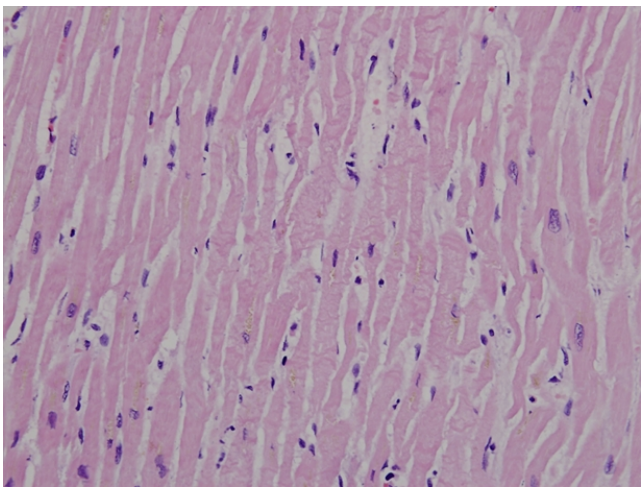

Figure3 Photography showing wavy myocardial fibers with contraction band necrosis (H&E×100).

Grading	Percentages of amount of cell damage in MI
0	None
1	<25%
2	25-75%
3	>75%

Table1 Grading of percentages of amount of cell damage detected by H&E staining method

3.2. Immunohistochemical staining with FN antibody

Paraffin blocks were sectioned at 3 microns by microtome and the ribbon of tissue was placed on superfrost plus microscope slides, let the slides dried, then placed the slides in hot air oven overnight at 60^{o}C for better tissue attached and paraffin bake. Then proceed with immunohistochemical staining with FN antibody

All stained slides were examined under light microscope. FN positive areas were stained dark brown which could be detected in cytoplasm, interstitial space of myocytes, the capillary endothelium and necrotic myocytes in myocardial infarction (Figure4). Percentages of amount of cell damage in MI shown by FN staining were similarly grading as shown in table1.

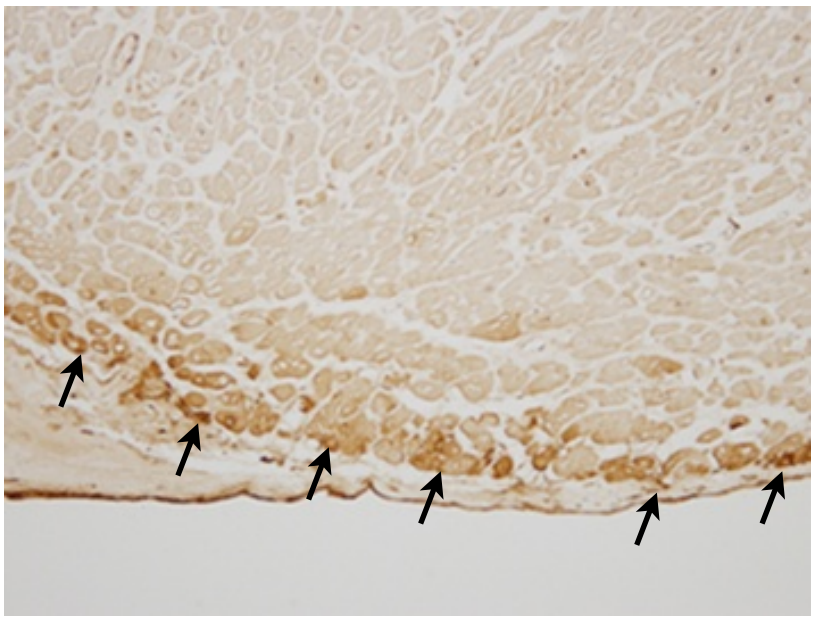

Figure4 Photograph of FN antibody staining and FN positive area stained dark brown on myocytes and interstitial tissue (×40).

Statistic method

Data statistics were analyzed using SPSS program.

1. Pair sample T- test to compare for mean of percentages of amount of cell damage in MI between FN antibody and H&E staining in all cases, TTC negative and TTC positive results.

2. Independent-Samples T test to compare for mean of percentages of amount of cell damage study by FN antibody staining and H&E staining between TTC positive result and TTC negative result.

3. Comparing sensitivity and specitivity between TTC method and FN antibody staining.

Results

The results of MI in 41 cases of heart slices detected by TTC method showed positive staining for MI in 25 cases and negative staining for MI in 16 cases. Microscopic appearance by immunohistochemical staining with FN antibody showed grade1 of cell damage in 6 cases, grade2 of cell damage in 15 cases and grade3 of cell damage in 20 cases . H&E staining showed grade1 of cell damage in 12 cases, grade2 of cell damage in 20 cases and grade3 of cell damage in 9 cases.

1. Comparison for mean of percentages of amount of cell damage between FN antibody and H&E staining.

The mean of percentage of amount of cell damage were 60.12 ± 26.445% (S.D.) in FN antibody staining and 48.78 ± 26.594% in H&E staining. The statistic of mean in percentage of cell

damage of myocardial infarction used pair sample T- test showed significant difference between FN antibody and H&E staining ($p<.01$).

2. Correlations

The correlations coefficient revealed 0.768 relationship were strongly positive and significant between FN antibody and H&E staining ($p<.01$) (Figure5).

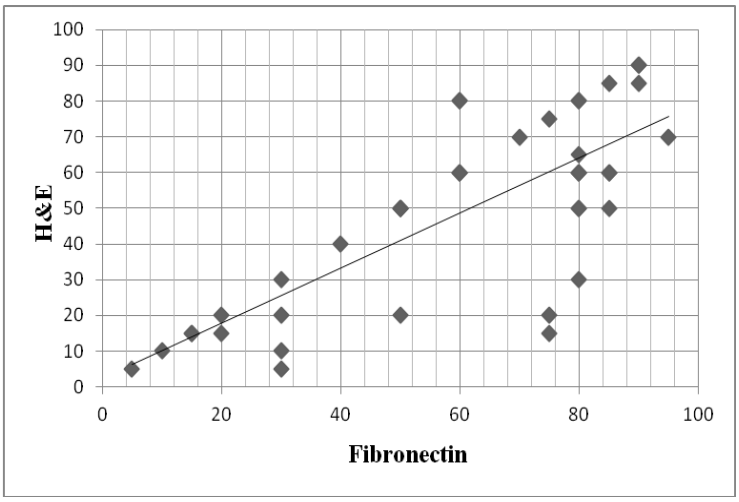

Figure5 Correlations for percentages of amount of cell damage between FN and H&E staining.

3. Comparison for mean of percentages of amount of cell damage study by FN antibody staining between TTC positive result and TTC negative result.

The mean of percentage of amount of cell damage staining for FN finding were 49.69 ± 30.467% (S.D.) in TTC positive result and 66.80 ± 21.597% in TTC negative result. The mean of two groups were not significantly different ($p>.05$)

4. Comparison for mean of percentage of amount of cell damage study by H&E staining between TTC positive result and TTC negative result.

The mean of percentage of amount of cell damage were 37.19 ± 25.558% (S.D.) in TTC positive result and 56.20 ± 24.970% in TTC negative result. There were a significant difference between TTC negative result and TTC positive result ($p<.05$). Moreover, TTC positive result had significantly higher percentage of amount of cell damage detected by FN antibody than those in TTC negative result.

5. Comparison for mean of percentage of amount of cell damage between FN antibody and H&E staining in TTC positive result.

The mean of percentage of amount of cell injury were 66.60 ± 22.066% (S.D.) in FN antibody staining and 56.20± 24.970% in H&E staining. The statistic for mean of percentage of amount of cell

damage in TTC positive result used pair sample T- test showed significant difference between FN antibody and H&E staining ($p<.01$) (Figure6).

6. Comparison for mean of percentage of amount of cell damage between FN antibody and H&E staining in TTC negative result.

The mean of percentage of amount of cell damage were 49.69 ± 30.467% (S.D.) in FN antibody staining and 37.19± 25.558% in H&E staining. The statistic of mean of amount of percentage of cell damage staining in TTC negative result used pair sample T- test showed significant difference between FN antibody staining and H&E staining ($p<.05$)(Figure6).

7. Sensitivity and Specificity

Fibronectin antibody can detect myocardial infarctions with sensitivity of 100% and 50% specificity.

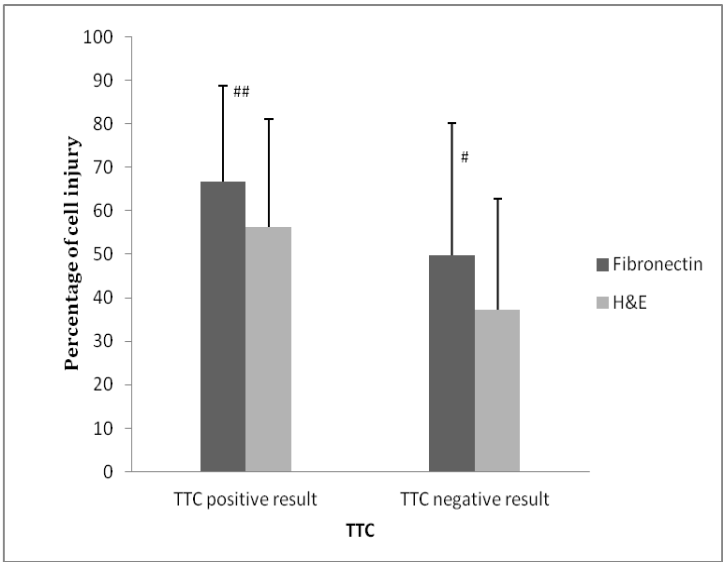

Figure6 The mean of percentage of amount of cell damage between FN and H&E staining in TTC positive and TTC negative results.
different significantly ($p< 0.05$) ##different significantly ($p< 0.01$)

Disscussion

Myocardial infarction due to atherosclerotic ischemic heart disease is probably the commonest disease diagnosed in majority of sudden death cases subjected to clinical and medicolegal autopies. This is so because “Heart Attack” as a cause of sudden death has been readily accepted not only amongst non-medical personnels like police officials, coroners and judges, but also amongst medical profession. The scientific reason for frequent resortance to this diagnosis during autopsies is that, an indirect diagnosis of myocardial infarction is accepted in many such instances. That is, the diagnosis is not based on actual visualization of the presence of infarcted myocardial tissue by any means but

the presence of significant narrowing of one or more major coronary vessels is considered as sufficient evidence for coming to a conclusion. This criterion is applied in cases where the patient did not survive for sufficient time for either gross or microscopic findings of infarct to appear after sustaining fatal ischemic attack. It is known that microscopic and gross findings can be detected in the infarcted tissue only if the infarct is at least six or twelve hours old respectively.[7]

Many morphological, histochemical, enzyme histochemical, electron microscopic or immunohistochemical methods have been introduced for postmortem detection of acute myocardial infarction.[3]

This study compared different method to detect for early myocardial infarction, by comparing for mean of percentage of amount of cell damage detected by FN antibody staining and morphological changes seen by H&E. The results revealed percentage of damaged cells showing significant difference by FN antibody staining compared to H&E staining ($p<.01$). In TTC positive and TTC negative result groups , the mean of percentage of amount of cell damage showed significant difference between FN antibody staining and H&E staining ($p<.01$) and ($p<.05$). Therefore, FN antibody staining showed better detection for damaged cells than H&E staining. Previous study showed that variable wavy fibers seen as early as 1-3 hours and when present, can occur early in a minimum period of ½ -4 hours but usually none1 with microscopic examination. FN is a very reliable marker for irreversible injured myocytes, it usually was detected in the blood vessel walls and in the interstitium between myocytes, but the cytoplasm of myocytes showed no staining.[3] At 4 hours after coronary ligation FN was localized in a patchy fashion in the cytoplasm and interstitial space of some of the myocytes in the area supplied by the ligated vessel.[7] In pig hearts, after 30 minutes and sometimes 60 minutes of coronary occlusion, individual myocytes and groups of myocytes were observed to be stained by FN.[4]

Ischemic myocardium can be reliably delineated and quantitated as early as 2-3 hours after the occlusion of a coronary artery by TTC staining method.[1] This study showed that comparison for mean of percentage of amount of cell damage with FN antibody between TTC positive result group and TTC negative result group showed no significant difference ($p>.05$). Moreover, comparison for mean of percentage of amount of cell damage with H&E staining between TTC positive result group and TTC negative result group showed significant difference ($p<.05$). In TTC positive result group, the comparison for mean of percentage of amount of cell damage between FN antibody staining and H&E staining, the results showed significant difference ($p<.01$). In TTC negative result group, the comparison for mean of percentage of amount of cell damage between FN antibody staining and H&E

staining, the results showed significant difference ($p<.05$). Therefore, fibronectin antibody staining method was better in detecting early MI than TTC staining method. However, TTC is often possible in highlighting for area of MI, thus, helping in guiding for sampling the heart tissue for examining by routine H&E staining for confirming of MI, and this certainly is better than routine sampling with no highlighted areas by TTC staining.

Previous study showed that TTC method can detect MI with sensitivity of 77.4% and specificity 92.6%.[5] In this study the results showed that FN antibody staining can detect MI with sensitivity of 100% and specificity only 50%. FN antibody staining showed better sensitivity than TTC method. However, TTC method showed more specificity than FN antibody because of progressive decreases in the intensity of FN antibody staining after 7 days of coronary ligation.[7] This study found over 7 days of MI in 5 of 41 cases for which no FN staining in infarcted areas while TTC staining showed positive results in all these 5 cases.

Therefore, FN is better than TTC staining method in detecting early MI but it has less specificity than TTC staining method when study in late MI of over 7 days.

However, FN antibody staining method is more expensive and time consuming when compare to TTC staining method which is less expensive and less time consuming and can detect early MI in close proximity with FN antibody staining method with more specificity than FN antibody staining method.

Moreover, TTC method should be used in the detection of acute MI because TTC method has more specificity than FN antibody.

However, selection of the method depends on storage of bodies. When the bodies were stored at 4°C, it would take several hours for the internal organs to actually cool down to 4°C, thus giving room for some autolysis and loss of dehydrogenase enzyme activity. It has also been shown that in the human heart, a rapid loss of enzymes occurs in the first 12 hours after death.[5] FN was demonstrated in ischemic myocytes at all postmortem intervals in bodies refrigerated and autopsied between 8 hours and 4 days after death.[3]

Conclusion

The study revealed statistic significant difference of percentage of cell damage between FN antibody staining and H&E staining, TTC staining method can be used to highlight the area of infarction and help in selecting samples of heart tissue for H&E staining and FN antibody staining. Moreover, FN antibody can detect MI with sensitivity of 100% better than TTC staining method which

had sensitivity of 77.4% but TTC staining method had specificity of 92.6% which was higher than 50% specificity of FN antibody staining. In addition, selection of the method depends on storage of dead bodies. The bodies that kept in storage temperature of 4 °C for 12 hours after death had been revealed loss of TTC staining.[5] However, the bodies stored in refrigeration between 8 hours and 4 days. FN was still able to detect MI in those storage bodies.[3]

In conclusion, FN antibody staining is more sensitive but less specific than TTC staining method. Therefore, TTC staining is still better method for detecting early MI.

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