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The effect of elapsed time on the cardiac Troponin-T (cTnT) proteolysis in case of death due to burn: A study to evaluate the potential forensic use of cTnT to determine the postmortem interval

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ABSTRACT

Background: After the death of an organism, intracellular enzymes cause protein to degrade into smaller fragments as the time passes, if these fragments can be isolated and visualized, and if the fragmentation is proved to be measurable and quantifiable, it can be a good sign of the post-mortem interval (PMI). The aim of this study is to evaluate the effect of PMI on Troponin-T protein degradation in cardiac tissues of cadavers through quantitative analysis of Troponin-T degradation by easily electrophoretic method and its association with PMI in case of deaths due to burn.

Methods: An efficient extraction protocol to analyze the banding pattern of cTnT in post-mortem tissue was developed. The analysis involves extraction of the protein (at different PMI (~7.30, 18.20, 30.30, 41.20, 41.40, 54.30.....88.40 h), from the heart muscles of cadavers (burn cases), separation by denaturing gel electrophoresis (SDS-PAGE) and visualization by Western blot using cTnT specific monoclonal antibodies.

Results: The results specify a characteristic banding pattern amongst human cadavers ($n = 9$), a pseudo-linear relationship between percent cTnT degraded and the time since death ($r = 0.87$, $p = 0.0001$). The area of the bands within a lane was quantified by scanning and digitizing the image using commonly available scanners.

Conclusions: The present research used a simple, easy, applicable and highly informative electrophoresis method that make it an ideal for the busy forensic laboratory. The post-mortem Troponin-T fragmentation observed in this study reveals a sequential, time-dependent process with the potential for use as a predictor of PMI in cases of burning. This result shows a potential for use as a future applied method of evaluating time since death.

1. Introduction

A more reliable time of death marker would be a great benefit in homicides, suicides and unintentional deaths as well as other modes of death, where time of death might impact the course of a criminal investigation. According to the World Health Organization, it is estimated that each year over 3,000,000 people die from fire related burns. The vast majority (over 95%) of fire-related burns occur in low and middle-income countries [1]. Burns are extremely common and are a major public health problem in a developing country like India [2].

The approach described here is based on analyzing the degradation or proteolysis of a cardiac protein in cases of deaths due to burn (cadavers) as a marker of time since death. Isolation of cTnT from heart tissue was selected because of its abundance in a highly protected internal organ and this protein is an excellent substrate for proteases. The proteolytic degradation of cTnT is the result of its primary amino acid sequence, which is rich in sites that serve as a substrate for protease.

Troponin T (TnT) binds to tropomyosin (Tm) to anchor the troponin complex in the thin filament and it thus serves as a vital link in the Ca^{2+} regulation of striated muscle contraction.

The enzyme linked immunoassay was developed for cTnT and showed its potential role towards cardiac myocytes as an AMI marker in 1989 and 1991 by Katus and Gerhardt respectively [3]. Cardiac Troponin T is a 37 KD protein that is (10–30)% dissimilar from skeletal Troponin T isoforms [4]. Different groups have developed monoclonal antibodies specific for cTnT by exploiting residues that are unique to the cardiac isoform [5,6]. Proteases such as calpains, cathespins and serine proteases have showed active role in degradation of cTnT [7,8]. It has been shown that the calcium activated cysteine proteases such

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as μ-calpain (calpain1) and caspase-3 are capable of degrading cTnT (and cTnI) in vitro [7]. Studies in the rat and mouse have shown that μ-calpain activation results in a 27 kDa fragment after ex-vivo ischemia and reperfusion of the isolated heart [9]. Furthermore, addition of inhibitors of μ-calpain showed a decrease in degradation of cTnT [9]. Proteolysis of proteins in necrotic tissue is well documented following ischemia [10]. Two major cTnT fragments found in serum were reported to be a complex between TnT-TIC (TC) in the presence of calcium [11]. The degradation of cardiac Troponin T in necrotic tissue and serum has been investigated with respect to immunodetection in clinical assays [12,13]. The aim of the present study is to evaluate the effect of elapsed time on cardiac Troponin-T degradation and its association with PMI in case of deaths due to burn by easily electrophoretic method.

2. Material and methods

The experimental design for this work is summarized in Fig. 1.

2.1. Tissue homogenization

Cardiac tissue samples (known PMI) were obtained after written informed consent from the next of kin for use of this sample in research and studied post-mortem degradation in a "human heart model incubated at room temperature (RT) for several days". 1 g of cardiac tissue sample is then homogenized with 4 ml extraction buffer consisting of 25 mM acetic acid/acetate in 6 M of urea, pH 4.6, using 6 M of NaOH or 6 M of HCL and 1 ml of the EZBlock™ protease Inhibitor Cocktail, EDTA-Free (K272-1ML, BioVision). The samples are then centrifuged at 5000 *g* for 5 min. The resulting supernatant was aliquoted and stored at −80 °C until used.

2.2. Protein quantification

An aliquot from each tissue sample was thawed and the protein content was quantified using the ELITech Clinical Systems with Biuret End point method which is based on the principle, proteins form a colored complex in the presence of copper salt in alkaline solution.

2.3. Gel preparation (12% SDS-PAGE GEL)

Mix all components of Running (Bottom) Gel in that order and promptly pipette into assembled gel plates evenly from side to side (dH_2O, 1.5 M of Tris (pH 8.8), 10% SDS, acrylamide:bisacrylamide ratio of 29:1, 10% APS, TEMED). Add a small layer of water-saturated butanol in order to produce a clean, straight top of the running gel; allow Running Gel to dry (~5–30 min). Pour off butanol; wash once with dH_2O, blots dry (Whatman paper). Mix all components of Stacking (Top) Gel (dH_2O, 0.5 M of Tris (pH 6.8), 10% SDS, acrylamide:bisacrylamide ratio of 29:1, 10% APS, TEMED) in that order and promptly pipette into the assembled gel plates on top of the Running Gel, evenly from side to side. Fill plates with stacking buffer so that it will overflow upon addition of the comb. Insert the comb, and prevent air bubbles from persisting. Allow to dry (~5–30 min).

2.4. SDS-PAGE

The supernatant containing the protein of interest is diluted (1:1) with SDS-PAGE Laemmli sample buffer (2% SDS, 0.0625 M of Tris–HCL (pH 6.8), 5% 2-β-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue) and pH adjusted with 6 M sodium hydroxide. Samples are then boiled at 100 °C for 3 min and then separated by 12% SDS-PAGE electrophoresis at 65 V with an acrylamide: bisacrylamide ratio of 29:1 using a Bio-Rad mini-gel system.

2.5. Western blotting

The resolved protein is then transferred to PVDF membrane (IMMOBILON PO .45 μm, 26.5 cm × 3.75 m, item no: IPVH00010, MILLIPORE) through a Western blot protocol at room temperature at 30 V for 120 min using a Bio-Rad wet electro transfer apparatus. The membrane was blocked for 60 min in TBS (Tris-base 2.42 g, NaCl 8.78 g, added ddH_2O to 1 l, pH 7) containing 5% non-fat dry milk. The primary monoclonal anti-cTnT antibody (Troponin T-C (2G3): sc-33721) was added at a 1:800 dilution in wash buffer (TBS containing 3% non-fat dry milk) and incubated overnight at 4 °C. The membrane was washed three times-each 5 min in wash buffer and the secondary antibody (goat anti-mouse IgG-AP: sc-2008) was then added at a 1:5000 dilution in wash buffer (TBS containing 3% non-fat dry milk) and incubated for 60 min at room temperature. The membrane was washed four times-each 5 min in wash buffer and finally once in TBS for 10 min. The membrane was developed with colorimetric precipitating substrate (sc-358798) *specific* for alkaline phosphatase enzyme (NBT/BCIP). The antibody used is cardiac TnT specific Troponin T-C (2G3) is a mouse monoclonal antibody raised against cardiac Troponin T of human origin, with epitope mapping to amino acids 94–180. The colored bands are scanned for intensity and migration distance from origin. This protocol was approved by the King George's Medical University ethics committee wide letter no-865/R-Cell-12. Ref. code: 55 E.C.M.II A/P20.

2.6. Analysis

The data was analyzed by using SPSS 16.0 version. The simple linear regression analysis was done. The p-value < 0.0001 was considered significant.

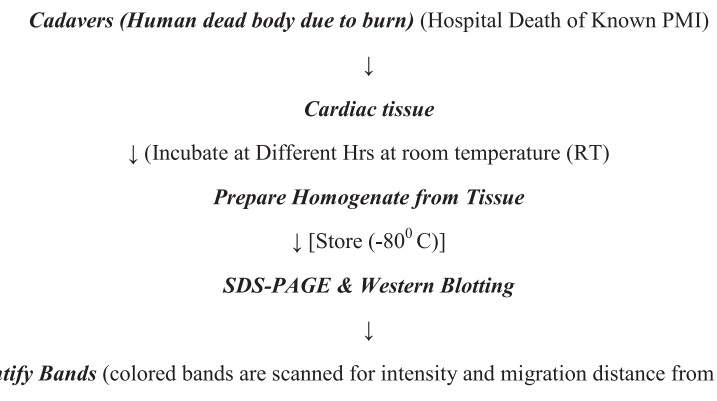

Fig. 1. A flow chart of the experimental design used in this study.