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Original communication

# The effect of elapsed time on cardiac troponin-T (cTnT) degradation and its relation to postmortem interval in cases of electrocution

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ABSTRACT

*Background:* The estimation of postmortem interval (PMI) is of paramount importance for the police in their investigation when arriving at the scene of a questionable death. The aim of present study is to evaluate the effect of elapsed time on cardiac Troponin-T degradation and its association with PMI in cases of death due to electrocution.

*Methods:* Cardiac tissue samples were collected from medico-legal autopsies, after informed consent from the relatives. The cases included were the subjects of electrocution without any prior history of disease who died in the hospital and their exact time of death was known. The analysis involves extraction of the protein at room temperature for different time periods (~5, 26, 50, 84, 132, 157, 180, 205 and 230 Hrs), separation by SDS-PAGE and visualization by Western blot using cTnT specific monoclonal antibodies.

*Results:* The results specify a characteristic banding pattern amongst human cadavers ($n = 5$), a pseudo-linear relationship between percent cTnT degraded and the time since death ($R^2 = 0.87$, $p = 0.0001$) was observed. The area of the bands within a lane was quantified by scanning and digitizing the image using Gel Doc (Universal Hood II).

*Conclusions:* 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 electrocution.



## 1. Introduction

The estimation of postmortem interval (PMI) is of paramount importance for the police in their investigation when arriving at the scene of a questionable death. A precise determination of a victim's time of death makes it possible to focus the investigation not only on a narrow time frame, but often also on a narrower range of suspects. The aim of this study was to investigate the degradation or proteolysis of a cardiac protein (Troponin-T) in cases of death due to electrocution as a marker of PMI. Electrocution occurs frequently, with a high percentage of incidents resulting in death.[1,2] 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.

Cardiac Troponin T is a 37 KD protein that is (10–30) % dissimilar from skeletal Troponin T isoforms.[3] 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.[4,5] Different groups have developed monoclonal

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antibodies specific for cTnT by exploiting residues that are unique to the cardiac isoform.[6,7] Proteases such as calpains, cathespins and serine proteases have showed active role in degradation of cTnT.[8,9] It has been shown that the calcium activated cysteine proteases such as μ-calpain (calpain1) and caspase-3 are capable of degrading cTnT (and cTnI) in vitro.[8] 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.[10] Furthermore, addition of inhibitors of μ-calpain showed a decrease in degradation of cTnT.[10] Proteolysis of proteins in necrotic tissue is well documented following ischemia.[11] Two major cTnT fragments found in serum were reported to be a complex between TnT-TIC (TC) in the presence of calcium.[12] The degradation of cardiac Troponin T in necrotic tissue and serum has been investigated with respect to immunodetection in clinical assays.[13,14]

## 2. Material and methods

Cardiac tissue samples were collected from 5 medico-legal autopsies, (department of Forensic Medicine and Toxicology), King George's Medical University (K.G.M.U.), Lucknow India, after informed consent from the relatives and studied post-mortem degradation by incubation of the cardiac tissue at room temperature for different time periods (~5, 26, 50, 84, 132, 157, 180, 205 and 230 Hrs). A prior approval was obtained from the K.G.M.U. ethics committee vide letter no-865/R-Cell-12. Ref. code: 55 E C M.II A/P20 to conduct this research. The cases included in this study were the subjects of electrocution without any prior history of disease who died in the hospital and their exact time of death was known. The experimental design for this work is summarized in Fig. 1.

### 2.1. Tissue homogenization

Tissue homogenization was done by taking 1 g of cardiac tissue sample with 4 ml extraction buffer consisting of 25 mM acetic acid/acetate in 6 M urea, pH 4.6, using 6 M NaoH or 6 M HCL and one ml of the EZBlock™ protease inhibitor cocktail, EDTA-Free (K272-1 ML, BioVision). The samples were then centrifuged at 5000 g for 5 min. The resulting supernatant was aliquoted and stored at −80 °C until used. Protein content was quantified using the ELITech clinical systems with Biuret end point method

### 2.2. Gel preparation

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 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 dH2O, blots dry (Whatman paper). Mix all components of Stacking (Top) Gel (dH2O, 0.5M 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 allow drying (~5–30 min).

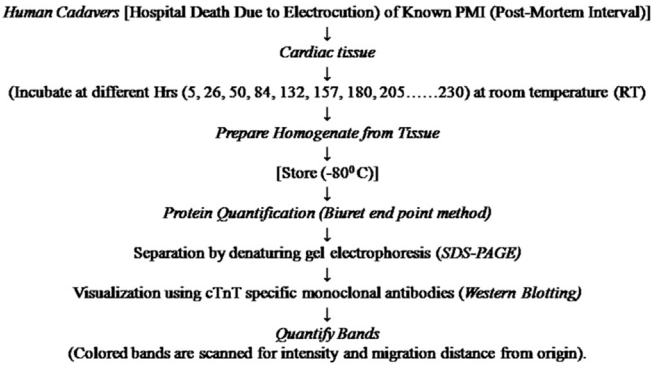


**Fig. 1.** A flow chart of the experimental design used.

### 2.3. SDS-PAGE

The supernatant containing the protein of interest is diluted (1:1) with Laemmli sample buffer (2% SDS, 0.0625 M Tris–HCL (pH 6.8), 5% 2-β-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue). Samples are then boiled at 100 °C for 3 min and separated by 12% SDS-PAGE electrophoresis at 60 V using a Bio-Rad mini-gel system

### 2.4. Western blotting

The resolved protein is then transferred to PVDF membrane (IMMOBILON PO .45um, 26.5 cm × 3.75 m, item no: IPVH00010, MILLIPORE) through a western blot protocol at 30 V for 120 min using a Bio-Rad wet electro transfer apparatus. The membrane was blocked for 60 min in TBS buffer (Tris-base 2.42 g, NaCl 8.78 g, added $ddH_2O$ to 1L, 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 TBS buffer containing 3% non-fat dry milk and incubated overnight at 4 °C. The membrane was washed three times-each 5 min in TBST buffer and then secondary antibody (goat anti-mouse IgG-AP: sc-2008) was added at a 1:5000 dilution in TBS buffer containing 3% non-fat dry milk and incubated for 60 min at RT. The membrane was washed four times-each 5 min in TBST buffer and finally once in TBS for 5 min. The membrane was developed with colorimetric precipitating substrate (sc-358798) specific for alkaline phosphatase enzyme (NBT/BCIP). The coloured bands are scanned for intensity and migration distance from origin

### 2.5. 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

## 3. Results

In postmortem samples, standardizing protein levels causes' problem since the standard markers (e.g., cTnT) that are used can also undergo digestion. We have found that our approach of loading fixed amounts of cell extracts based upon predetermined protein concentrations generates protein profiles in SDS-PAGE gels that show equivalent protein loading [Fig. 2 (a)]. As exemplified by cTnT [Fig. 2 (b)], Western blotting of these isolated proteins results in clearly defined bands at suitable molecular weights that are amenable to scanning and quantification. Human hearts (n = 5) were used to optimize the extraction, separation and visualization of cTnT degradation in heart tissue.

The human heart model was incubated at room temperature (20 ± 2 °C) for different time periods (~5, 26, 50, 84, 132, 157, 180, 205 and 230 Hrs). The data shows a distinct temporal profile corresponding to the degradation of cTnT by proteases found in cardiac muscle. The Western blot was probed with anti-cTnT specific