Time-dependent Degradation Pattern of Cardiac Troponin T in Cases of Death by Burn

S Kumar[1], W Ali[1], US Singh[1], AK Verma[2], S Bhattacharya[3], A Kumar[1], R Singh[2], R Rupani[2]

ABSTRACT

Objective: *Death due to burn occurs frequently. This study investigated time-dependent alterations in cardiac troponin T (CTnT) associated with fatal burns.*
Methods: *Cardiac tissue samples were collected from 10 medico-legal autopsies after informed consent from the relatives and post-mortem degradation by incubation of the cardiac tissue was studied at room temperature for different time periods. The cases included in this study were the subjects of burns without any prior history of disease who died in the hospital and their exact time of death was known. An efficient extraction protocol to analyse the banding pattern of cTnT in post-mortem tissue was developed.*
Results: *The data show a distinct time-dependent profile corresponding to the degradation of cTnT by proteases found in cardiac muscle. Both post-mortem interval and cardiac tissue of burned corpse had a statistically significant effect where the greatest amount of protein breakdown was observed within the first 41.20 hours, after which intact protein slowly disappears. The average molecular weight of all fragments showed intact cTnT to be rapidly degraded into smaller fragments.*
Conclusion: *In cases of burns, such knowledge will assist in knowing if there were previous scars that might have mimicked a burn and also help to properly evaluate the real cause of death.*

Keywords: Burn, degradation, human, SDS-PAGE, troponin T

Patrón de Degradación del Tiempo de la Troponina T Cardíaca en Casos de Muerte por Quemadura

S Kumar[1], W Ali[1], US Singh[1], AK Verma[2], S Bhattacharya[3], A Kumar[1], R Singh[2], R Rupani[2]

RESUMEN

Objetivo: *La muerte debido a quemaduras ocurre con frecuencia. Este estudio investigó las alteraciones dependientes del tiempo en la troponina T cardíaca asociada con quemaduras mortales.*
Métodos: *Se obtuvieron muestras de tejido cardíaco de 10 autopsias forenses después del consentimiento de los parientes, y se estudió la degradación post-mortem por incubación del tejido cardíaco a temperatura ambiente durante períodos de tiempo diferentes. Los casos incluidos en este estudio fueron objeto de quemaduras, no tenían ninguna historia previa de enfermedad, murieron en el hospital, y se conocía la hora exacta de la muerte. Se desarrolló un protocolo de extracción eficiente para analizar el patrón de bandas de cTnT en el tejido post-mortem.*
Resultados: *Los datos muestran un claro perfil dependiente del tiempo, correspondiente a la degradación de la troponina cardiaca T (cTnT) por las proteasas que se encuentran en el músculo cardiaco. Tanto el intervalo post mortem (IPM) como el tejido cardíaco de los cadáveres quemados tuvieron un efecto estadísticamente significativo, en el que se observó la mayor cantidad de descomposición de proteína en las primeras 41.20 horas, tras lo cual la proteína intacta desapareció lentamente. El peso molecular promedio de todos los fragmentos mostró que el cTnT intacto se degrada rápidamente en fragmentos más pequeños.*
Conclusión: *En los casos de quemaduras, este conocimiento servirá para saber si hubo cicatrices anteriores que podrían haber tomado la apariencia de una quemadura, y también ayudará a evaluar adecuadamente la verdadera causa de la muerte.*

From: [1]Post Graduate Department of Pathology, [2]Department of Forensic Medicine and Toxicology and [3]Department of Physiology, King George's Medical University UP, Lucknow, India.

Correspondence: Dr S Kumar, Post Graduate Department of Pathology, King George's Medical University UP, Lucknow, India. E-mail: sachilkgmc@gmail.com

DOI: 10.7727/wimj.2014.232

Palabras claves: Quemadura, degradación, humano, SDS-PAGE, troponina T

West Indian Med J 2015; 64 (3): 227

INTRODUCTION

Death due to burn occurs frequently. 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 occurs in low- and middle-income countries. Burns are extremely common and are a major public health problem in a developing country such as India (1, 2). The approach described here is based on time-dependent alterations in cardiac troponin T (cTnT) associated with fatal burns. In cases of burns, such knowledge will assist in knowing if there were previous scars that might have mimicked a burn and also help to properly evaluate the real cause of 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. Cardiac troponin T is a 37 KD protein that is (10–30%) dissimilar from skeletal troponin T isoforms (3). Proteases such as calpains, cathespins and serine proteases have shown an active role in degradation of cTnT (4, 5). It has been shown that the calcium-activated cysteine proteases such as μ-calpain (calpain 1) and caspase-3 are capable of degrading cTnT (and cTnI) *in vitro* (4). The degradation of cTnT in necrotic tissue and serum has been investigated with respect to immunodetection in clinical assays (6). In this study, the aim was to investigate time-dependent alterations in cardiac troponin T associated with fatal burns.

SUBJECTS AND METHODS

Cardiac tissue samples were collected from 10 medico-legal autopsies from the Department of Forensic Medicine and Toxicology, King George's Medical University (KGMU), Lucknow, India, after informed consent from the relatives, and post-mortem degradation by incubation of the cardiac tissue was studied at room temperature for different time periods (~7.30, 18.20, ... 88.40 hours). A prior approval was obtained from the KGMU 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 burns without any prior history of disease who died in the hospital and their exact time of death was known.

Tissue: 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 sodium hydroxide (NaOH) or 6 M hydrochloric acid (HCl) and 1 mL of the EZBlock™ protease inhibitor cocktail, EDTA-Free (K272-1 mL, BioVision). The samples were then centrifuged at 5000 g for five minutes. The resulting supernatant was aliquoted and stored at -80 ˚C until used. Protein content was quantified using the ELITech Clinical Systems with Biuret endpoint method.

Gel preparation: Mix all components of Running (Bottom) Gel in that order and promptly pipette into assembled gel plates evenly from side to side (dH2O, 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 minutes). Pour off butanol; blot 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 prevent air bubbles from persisting. Allow to dry (~5–30 minutes).

SDS-PAGE: The supernatant containing the protein of interest is diluted (1:1) with SDS-PAGE 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 three minutes and then separated by 12% SDS-PAGE electrophoresis at 65 V using a Bio-Rad mini-gel system.

Western blotting: The resolved protein is then transferred to PVDF membrane (IMMOBILON PO 0.45 um, 26.5 cm x 3.75 m, item no: IPVH00010, MILLIPORE) through a Western blot protocol at RT at 30 V for 120 minutes using a Bio-Rad wet electro transfer apparatus. The membrane was blocked for 60 minutes in TBS (Tris-base 2.42 g, NaCl 8.78 g, added ddH2O 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 wash buffer (TBS containing 3% non-fat dry milk) and incubated overnight at 4 ˚C. The membrane was washed three times – each for five minutes – in TBST buffer (TBS buffer containing Tween-20) 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 minutes at room temperature. The membrane was washed four times – each for five minutes – in TBST buffer and finally once in TBS for 10 minutes. 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.

Analysis: The data were analysed using SPSS version 16.0. Simple linear regression analysis was done. The p-value ≤ 0.0001 was considered significant.

RESULTS

In post-mortem samples, standardizing protein levels poses a problem since the standard markers (*eg* CTnT) that are used can also undergo digestion (proteolytic degradation). 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. 1).

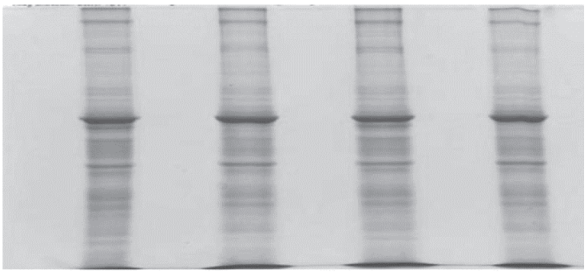

Fig. 1: SDS-PAGE gel demonstrating that each lane has an equal amount of total protein.

As exemplified by cTnT (Fig. 2), Western blotting of these isolated proteins results in clearly defined bands at suitable molecular weights that are amenable to scanning and quantification.

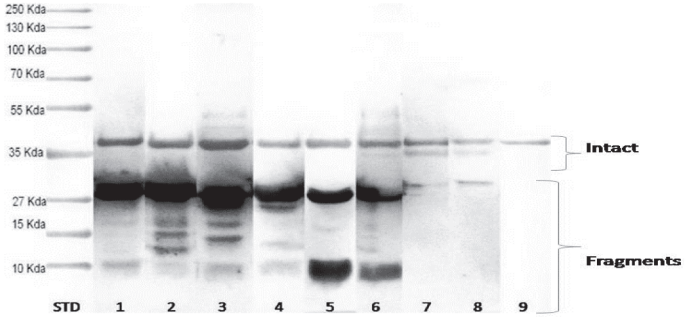

Fig. 2: Western blot of human cardiac troponin T (cTnT) post-mortem profile probed with anti-cTnT monoclonal antibody. Lane identification: (1) 7.30 hours, (2) 18.20 hours, (3) 30.30 hours, (4) 41.20 hours, (5) 41.40 hours, (6) 54.30 hours, (7) 65.20 hours, (8) 88.40 hours, (9) cTnT standard; STD: prestained protein ladder.

The data show a distinct time-dependent profile corresponding to the degradation of cTnT by proteases found in cardiac muscle. The Western blot was probed with anti-cTnT specific antibodies to visualize the cTnT fragments formed over time. As time post-mortem progresses, the intact cTnT band degrades into fragments that are easily detected by the monoclonal antibodies. The coloured bands of the Western blot were scanned for intensity and migration distance utilizing the Gel Doc Image Lab software. The digitization of the Western blot data shows a profile of cTnT proteolysis that takes place over time.

The disappearance of intact cTnT and the appearance of lower molecular weight bands are easily observed. There are several clusters of cTnT bands that show up at different times (Fig. 1). Western blot data clearly showed the intact protein at 42 kDa, three major (28 kDa, 30kDa, 10kDa) fragments, three additional minor fragments (12 kDa, 14kDa and 15 kDa) and formation of low molecular weight fragments. Within the first 7.30 hours, the original band of cTnT (42 kDa) decreased markedly into two major fragments (28 kDa, 30 kDa) and three minor fragments (10 kDa, 15 kDa and 27 kda). After, at 18.20 hours, Western blot data clearly showed the intact protein at ~42 kDa, two major (~28 kDa, 30 kDa) fragments and five additional minor fragments (~10 kDa, 12 kDa, 14 KDa, 15 kDa and 27 kDa.). At 30.30 hours, one minor fragment at 12 kDa slightly disappeared, as at 18.20 hours. Troponin T degradation, Western blot data probed for cTnT showed the intact protein, two major (~28 kDa, 30 k Da) and three minor (10 kDa, 12 kDa, 27 kDa) fragments detected at 41.20 hours and one additional major fragments (10 kDa) at 41.40 hours. At 54.30 hours, there was formation of one additional fragment at 37 kDa and the 10 kDa major fragment lost its intensity as compared to 41.40 hours. Within 65.20 hours, Western blot data clearly showed the intact protein at ~42 kDa, one major (~30 kDa) fragment and two additional minor fragments (~28 kDa and 37 kDa). But at 88.40 hours, blot shows only intact protein of very less intensity and two minor fragments (30 kDa and 37 kDa).

Overall, both post-mortem interval (PMI) and cardiac tissue of burned corpses had a statistically significant effect where the greatest amount of protein breakdown was observed within the first 41.20 hours, after which intact protein slowly disappeared. The average molecular weight of all fragments showed intact cTnT to be rapidly degraded into smaller fragments (Fig. 3).

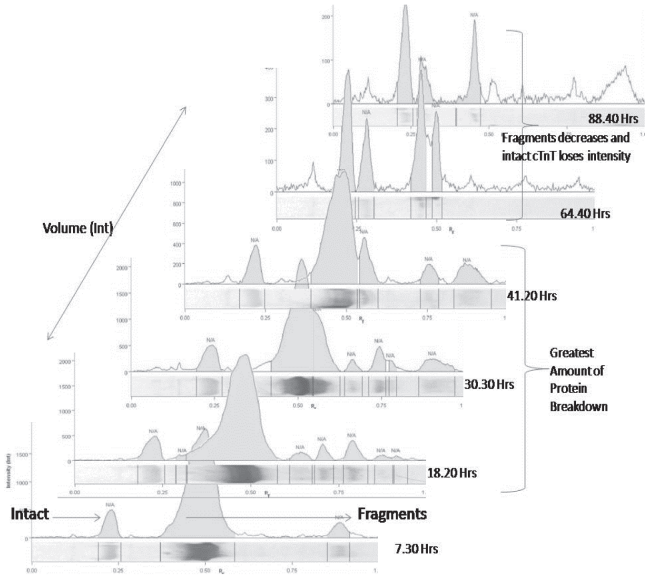

Fig. 3: Western blot showing decrease in volume (Int) of intact cardiac troponin T and formation of lower molecular weight fragments with time.

DISCUSSION

Depending on the electrophoretic conditions and which molecular markers are used, the molecular mass of intact cTnT may seem to vary *eg* 42 kDa in the present study *vs* 39 kDa in

a previously published study (7). The disappearance of intact cTnT and the appearance of lower molecular weight bands are easily observed. Wu *et al* showed the presence of immunoreactive cTnT fragments in the circulation of acute myocardial infarction (AMI) patients (7). Somewhat later, in a study by Labugger *et al* on troponin I and T degradation in patients with AMI, Western blot data clearly showed the intact protein at ~42 kDa, one major (~26 kDa) fragment and two additional minor fragments [~32 kDa] (8). In addition to their presence in serum, cTnT fragments have also been detected in healthy human heart tissue homogenates using the antibodies of the third generation cTnT immunoassay (9, 10). These data all support the hypothesis that cTnT is present in the circulation in degraded form. Both PMI and cardiac tissue of burned corpses had a statistically significant effect where the greatest amount of protein breakdown was observed within the first 41.20 hours, after which intact protein slowly disappeared. Sabucedo and Furton (11) examined the breakdown pattern of cardiac troponin I in human post-mortem samples and found that it could be a very useful marker for estimating PMIs of 1–5 days. Xiao and Chen (12) examined the breakdown patterns of actin and tubulin in the liver tissue extracts of rats and found that actin was completely degraded by 10 days post-mortem while β tubulin could not be detected after four days postmortem.

However, it should be noted that a limitation of the current study was that it represented a small study size. The mechanisms involved in the release of cTnT in case of deaths due to burn have not been determined.

CONCLUSION

Time since death plays a very important role in the degradation of cTnT, where the greatest amount of breakdown was observed in the first hours of death. Such knowledge will help to properly evaluate the cause of death in burn cases.

ACKNOWLEDGEMENT

Special thanks to the Department of Forensic Medicine and Toxicology, KGMU, Lucknow, India, for providing the post-mortem human cardiac tissues requested for this study.

REFERENCES

1. Kumar S, Ali W, Verma AK, Pandey A, Rathore S. Epidemiology and mortality of burns in the Lucknow Region, India: a 5-year study. Burns 2013; **39:** 1599–605.
2. Kumar S, Verma AK, Ali W, Singh US, Krishna A. Intentional and non-intentional burns related deaths: a comparative study of socio-demographic profile. Burns 2015; **41:** 265–70.
3. Mair J. Cardiac troponin I and troponin T: are enzymes still relevant as cardiac markers? Clin Chem Acta 1997; **257:** 99–115.
4. Di Lisa F, De Tullio R, Salamino F, Barbato R, Melloni E, Siliprandi N et al. Specific degradation of troponin T and I by mu-calpain and its modulation by substrate phosphorylation. Biochem J 1995; **308:** 57–61.
5. Noda T, Isogai K, Hayashi H, Katunuma N. Susceptibilities of various myofibrillar proteins to cathespin B and morphological alteration of isolated myofibrils by this enzyme. J Biochem 1981; **90:** 371–9.
6 Bodor G. Cardiac troponin-I: a highly specific biochemical marker for myocardial infarction. J Clin Immunoass 1994; **17:** 40–4.
7. Wu AH, Feng YJ, Moore R, Apple FS, McPherson PH, Buechler KF et al. Characterization of cardiac troponin subunit release into serum after acute myocardial infarction and comparison of assays for troponin T and I. American Association for Clinical Chemistry Subcommittee on cTnI Standardization. Clin Chem 1998; **44:** 1198–208.
8. Labugger R, Organ L, Collier C, Atar D, Van Eyk JE. Extensive troponin I and T modification detected in serum from patients with acute myocardial infarction. Circulation 2000; **102:** 1221–6.
9. Ricchiuti V, Voss EM, Ney A, Odland M, Anderson PA, Apple FS. Cardiac troponin T isoforms expressed in renal disease skeletal muscle will not cause false-positive results by the second generation cardiac troponin T assay by Boehringer Mannheim. Clin Chem 1998; **44:** 1919–24.
10. Davis GK, Labugger R, Van Eyk JE, Apple FS. Cardiac troponin T is not detected in western blots of diseased renal tissue. Clin Chem 2001; **47:** 782–3.
11. Sabucedo AJ, Furton KG. Estimation of postmortem interval using the protein marker cardiac troponin I. Forensic Sci Int 2003; **134:** 11–6.
12. Xiao JH, Chen YC. A study on the postmortem relationship between the degradation of protein and the postmortem interval. Fa Yi Xue Za Zhi 2005; **21:** 110–2.