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Research Article

SENSITIVE, RAPID AND COST EFFECTIVE APPROACH FOR THE ESTIMATION OF TIME ELAPSED AFTER DEATH USING HUMAN CARDIAC TROPONIN I

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A B S T R A C T

Estimation of postmortem interval is critical in forensic investigations related to homicides, suicides and accidents. The aim of the present investigation was to develop a simple and cost effective method to estimate the time since death based on the degradation of a protein, cardiac Troponin I (cTnI), which is a part of a troponin complex responsible for calcium dependent muscle contraction. A simple and efficient extraction protocol to analyze the banding pattern of cTnI in postmortem tissue was developed. The analysis involves extraction of the protein and separation by SDS-PAGE. Human cardiac tissue was used to develop and optimize the protocol of banding profile. The results show a characteristic banding pattern of cTnI of human cadaver's heart tissue. The degradation band pattern of standards of human cardiac tissues of known time since death are compared with the degradation band pattern of the unknown samples in a simple comparative analysis to predict the actual time since death. The human heart degradation-banding pattern of tissue cTnI is useful in the determination of time since death up to 5 days. Overall, this technique offers advantages such a wide postmortem interval, measurable degradation pattern, cost effectiveness and is sensitive enough to be used to estimate the postmortem interval.

INTRODUCTION

In the cases of accidents, suicides and homicides, estimation of time elapsed after death(TEAD) is very important to catch the accused or to eliminate the innocent people, also sometimes to check whether the witness is saying truth or not.Time elapsed after death is considered as a corroborative evidence in medico-legal cases like criminal and civil cases. After death the body goes through decomposition due to environmental conditions like temperature, humidity, microbial population etc. Time elapsed after death depends on many factors like temperature, humidity, sunlight and many more (Y. Poloz, 2009). Due to this multivariate dependency time elapsed after death estimation is always difficult to be very precise. In early era rectal and outer ear temperature were used for approximation of time elapsed after death but it is not more than an approximation (C. Henssge, 1994). The changes in nerve conduction pattern show comparatively more accuracy in early postmortem interval (I. Elmasa, 2001). Postmortem staining measuring using photometric changes for estimation of time elapsed with more power (4) and immunohistochemical and bio-chemical markers postmortem activity of lactate and malate dehydrogenase gave step up to the older methods (T Gos, 1993).

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Proteolysis of muscle with time elapsed after death in bovine muscle and autolysis of muscle fibrils after death gave an idea of repose of rigor mortis (D, Goll). Since inner organs are comparatively less affected than external, electrophoresis of internal organ reduced the error band of TEAD (J. Mittmeyer. 1979). During literature survey we foundthat after death biochemical changes in the biomolecules and electrolytes like protein fraction, glucose, urea, creatinine, iron, calcium, potassium, sodium and chloride in the body used for estimation of time elapsed after death have made significant advancement(C. Henssge. 1994), (F. Gallois-Montbrun, 1988), (F. Wehner. 1999), (P. Neis, 1999), (H.J. Mittmeyer, 1980), (H.J. Mittmeyer 1979). Internal organ like heart is not directly exposed to external factors and is well protected for a considerable time and hence will have lot of significance in TEAD study.

Troponin is a complex ofregulatory proteins (Troponin C, Troponin T and Troponin I) that is integral to muscle contraction in skeletal muscle and cardiac muscle that is useful as a diagnostic marker or therapeutic target for various heart disorders. Cardiac troponin I (cTnI) is used for detection of myocardial infraction, cardiac specific troponin I is used from 1978 onward (G. Bodor, 1994). Troponin I consists of 181-211 amino acid residues, and the cardiac isoform is larger due to

the presence of an additional approximately 30-membered N-terminal peptide. The troponin I isoforms are coded by three different genes. It is known that the gene of human cardiac troponin I is located on the 19th chromosome and consists of eight exons. Both the cardiac and the slow skeletal isoforms of troponin I are expressed in the heart of the human fetus. After birth the expression of the slow skeletal isoform is blocked, whereas the expression of the cardiac isoform is enhanced (J. Wilkinson, 1979). After death cell lysis occurs and proteases degrade the proteins present in cells, the rate of degradation can be used for the determination of TEAD.

Our research group has worked on the estimation of the time elapsed after death using different body fluids and the biological markers like albumin (A. Parmar,2015) in CSF, amino acid tryptophan (Ansari *et al*, 2017), cysteine (Ansari *et al*, 2016) and protein (N, Ansari 2016) from vitreous humor using nanoparticles for TEAD,. The encouraging results during the above studies prompted us to focus on internal organ like heart to correlate the biochemical changes with TEAD.

A. Sabucedo (2003) and co-workershave reportedstudies on cardiac troponin I for determination time elapsed after death using bovine cardiac troponin I. They extracted bovine troponin I using troponin I antibody coated magnetic particles and proteases inhibitor cocktail to preserve troponinI from the effect of proteases. They gave semi quantitative approach for TEAD. However sincecardiac troponin I of bovine is having different molecular structure and molecular weight as compared to human cardiac troponin I (K. Mittamann 1990), the degradation rate of cTnI of bovine cardiac tissue will be different from human cardiac tissue and so will not be advisable to use as reference standard for human cTnI. Also the cause of death of human heart donor is not mentioned in their study. The studies reported (S Kumar 2016) shows that the cause of death also make the difference in the degradation rate. Apart from that this method is very costly because of the use of cardiac troponin I coated magnetic particles and proteases cocktail in the mentioned study. In the present investigation we worked on human cardiac tissue to get results as real as possible, we also tried to develop the extraction method for cTnI from human cardiac tissue without the use of magnetic particles. Also the whole process was carried out under 4 ^{0}Cto eliminate the use of protease cocktail, because all proteases get deactivated below4 ^{0}C (Worthington Biochemical Corporation).

MATERIALS AND METHOD

All chemicals used were of MB gradepurchased from Sigma Aldrich, Ranbaxy or Bangalore Genei.

Sample collection

All samples were collected from post mortem room of B.J. Medical College Civil Hospital Ahmedabad. With ethical permission cardiac tissue samples were collected using sterilized surgical gloves used for the prevention of infection during collection of tissue. The samples collected were from natural death, accidental death and a case of burn death so as to avoid the cause of death interfering with the degradation pattern. Approximately 20 to25g of heart tissue (from right ventricle) were collected using medical sterilized surgical blade because right ventricle have more muscles and does not have fat tissue on it.

Tissues were transported using thermo cool box containing coolant bag, cardiac tissues were collected from 23 different cadavers out of which 20 cardiac tissue samples were used for the preparation of standard band pattern and 3cardiac tissue samples were used as test samples. The test cardiac tissue samples collected were at 12, 36 and 108th hours after the time of death. All the three samples were preserved in cryofuse tube in liquid nitrogen (temp. -196^{o}C) in a cryocan to prevent more degradation of cTnI in tissue samples.

Preservation of sample

The 20 cardiac tissues samples were left at room temperature for degradation. Six consecutive samples (2 g) were collected at 12, 24, 48, 72, 96 and120th hour from the time of death. In order to maintain the degradation, weighed samples (200 mg) were preserved in cryofuse tube of 1cm diameter and 2 cm length in liquid nitrogen (temp. -196^{o}C) in a cryocan.

Isolation of troponin I from human cardiac muscle

Preparation of extraction buffer and elution buffer

Extraction buffer was prepared using 10 mM disodium hydrogen phosphate, 100 mM Tris, 200 mM sodium chloride and 0.1% sodium azide. Elution buffer was prepared by using100 mM glycine, 6 M urea and 250 mM sodium chloride.

Extraction and elution of protein (cTnI) From Human cardiac muscle

For the extraction of troponin I, 200 mg of tissue sample was homogenized in mortar-pestle with 25ml of liquid nitrogen. Liquid nitrogen was used to prevent incomplete maceration. The homogenized tissue was transferred in to sterilized 2 ml eppendorf tube and 1 ml ice chilled extraction buffer was added to inhibit the protease enzyme activity. This mixture was centrifuged at 6000 rpm for 10 min at 4^{0}C in refrigerated centrifuge "Sigma Labrzentrifuge 2 k15" which is used for maintaining the temperature below 4 ^{0}C.The activity of protease enzyme is inhibited below 4 ^{0}C so it cannot degrade the protein (Troponin I). Supernatant was decanted into another autoclaved 2 ml eppendorf tube, for elution of cTnI. 1 ml of elution buffer was added in to the same tube and centrifuged at 6000 rpm for 10 min at 4 ^{0}C. Supernatant was transferred in to another autoclaved eppendorf tube and this contains cTnI.

SDS PAGE Electrophoresis of extracted protein, cTnI

We used discontinuous Tris glycine buffer (pH 8.3) systems for SDS-Polyacrylamide gel electrophoresis to be carried out in vertical gels. Vertical Mini Gel Electrophoresis systems are used for separation of proteins. We prepared sample in the 2 µl of 1x SDS gel-loading buffer and heated them to 100^{o}C for 3 minto denature the proteins. For visualization and identification higher molecular weight protein marker of Bangalore Genei was used.

RESULTS

From the postmortem room of B.J. Medical College, Civil hospital, Ahmedabad, total 23 heart tissue samples were collected and 20 samples were used for preparation of standard visualization using six samples from each cadaver collected at the interval of 12, 24, 48, 72, 96 and 120th h after death. Human cardiac troponin I wasextracted and eluted with the newly developed method and SDS PAGE was performed for their separation. 25 µl of each of the sample in predefined

order was introduced into the bottom of wells with the help of micropipette. Gel was run at voltage of 100 volts until the bromophenol blue reaches the bottom of gel. After the running, gel was stained using Coomassie Brilliant Blue for overnight and destained using 30% glacial acetic acid in methanol. From SDS PAGE theband profiles areobtained. To get the visual change and to record data, the gel documentation system Syngene Genius was used. As postmortem interval prolongs, the intact cTnI band degrades to lower molecular weight or polypeptide chains or amino acids (Figure 1) confirming the presence of cTnI in the extracted sample.

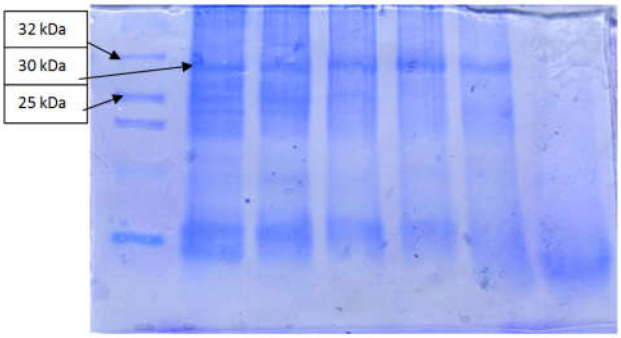

Figure 1 Identification of human CTnI (30kDa) band in between 32kDa and 25kDa with the use of standard molecular protein markerof Bangalore Genei

Standard human banding profile

Standard human banding profile was prepared using six samplesat the time interval of 12, 24, 48, 72, 96 and 120th h after death **(Fig.2).**Banding profile of all 120 samples were obtained, Fig 2 shows a representative case. In all the cases degradation pattern remained almost the same.

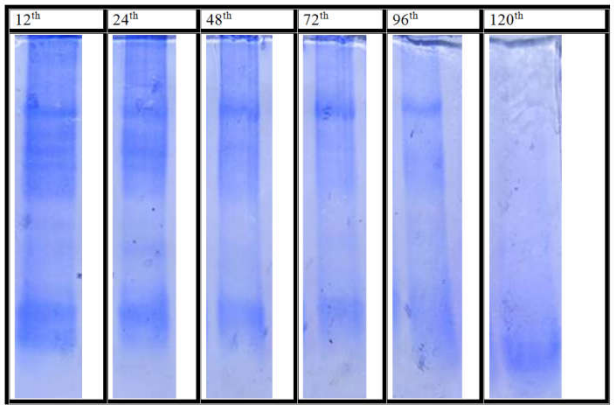

Figure 2 Standard human cTnI banding profile (time in hours)

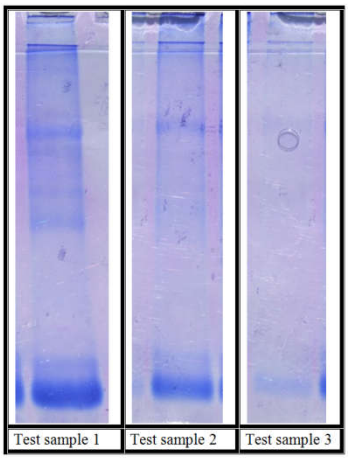

Figure 3 Human heart banding profile of sample 1, 2 and 3 of 12, 36 and 108 h of TEAD respectively.

The banding profile of test samples were compared with the standard banding profile (Figure 3) to get the postmortem interval with respect to the test sample. The results obtained were in concordance with the known postmortem interval of the test samples.

DISCUSSION

In the present study we developed a simple, rapid and sensitive methodand successfully extracted the Human Cardiac Troponin I(Fig 1) showing the single band of 30kDa between 25kDa and 32kDa in SDS PAGE. 23 heart tissue samples were gathered and 20 samples were utilized for preparation of standard banding profile, six examples from every cardiac tissue gathered at the interim of 12, 24, 48, 72, 96 and 120th h after death. Banding profile of all 120 samples were obtained using SDS PAGE, in all cases degradation pattern remained the same, Fig 2 shows a representative case. In the visual comparison of data generated by the qualitative approach as given in (Fig 3), Test sample 1 taken after 12th hour of time elapsed after death shows visual resemblance with the band patterns of standard band profile of 12th h. Test sample 2 taken after 36th hour of TEAD shows visual resemblance with the band patterns of standard band profile between 24th - 48th h. Test sample 3 taken after 108th hour of TEAD shows visual resemblance with the band patterns of standard band profile between 96th h to 120th h. The result confirms the reliability of method developed for time elapsed after death evaluation.

The method reported earlier (A. Sabucedo 2003) extracted bovine troponin I utilizing human cTnI antibody coated magnetic particlewhich is meant for human cTnI. Antibody meant for human cTnT poorly binds with bovine cTnT(S. Monte 2007) and hence the use of bovine cTn I as standard may lead to erroneous results in any investigation. Furthersince cardiac troponin I of bovine is having different concentration of different amino acid and molecular weight as compared to human cardiac troponin I this will effect on the degradation rate of cTnI of bovine cardiac tissue and will be different from human cardiac tissue and so is not advisable to use as reference standard for comparison with human cTnI. Also human cTnI antibody coated magnetic particles will decrease the cost effectiveness. Our newly developedmethod gives the same result of extraction and banding profile which is relatively fast and less costly but with the same level of accuracy. In our extraction method we also removed use of proteases inhibitor cocktail to preserve troponin I from the effect of proteases with the help of working under 4^{0}C.. The main advantage of present study is that it can resolve prolonged TEAD up to 5 days.This technique is precisely measuring the molecular changes after death and hence only very less amount of tissue is required compared to traditional method of thanatochemistry. Also in the case of burn dead bodies where there are no external signs to determine the time since death, it is the only technique which can be fruitful.

CONCLUSION

The method developed is reliable, accurate, cheaper and faster than the previously reported method using cTnI for the estimation of time elapse after death. The sample requirement of heart tissue is very less and it is suitablefor all the cases especially for the case of burn injuries.

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