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Research Article

DEGRADATION OF CARDIAC TROPONIN T IN HUMAN SKELETAL MUSCLE: A MOLECULAR APPROACH IN POSTMORTEM INTERVAL ESTIMATION

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A B S T R A C T

Estimation of Post-mortem Interval (PMI) is an integral part of medico-legal investigation. In most unwitnessed death investigation, the knowledge of PMI is important as it gives a preliminary idea of the time of assault. The well-known methods for PMI estimation such as algor *mortis, rigor mortis, livor mortis*, putrefaction, forensic entomology, etc. are useful within 72 hours of death. The purpose of present study is to overcome this limitation by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting to analyse time course of degradation pattern of cardiac troponin T (cTnT) until postmortem. Our results showed cTnT degraded into two degraded products 1 and 2 (cTnT dp 1 and 2) in a certain and ordinary fashion. Logistic regression analysis showed no correlation between cTnT degradation and PMI. No correlation was found between confounding factors and cTnT degradation. To conclude, the degradation of cTnT can be used as a method of PMI estimation especially in the time interval for more than 24 hours.

INTRODUCTION

In criminal investigations, determination of the exact time when a person would have died has always been an important question. Various approaches have been taken for the delimitation of PMI, viz., physical changes, application of entomology, or biochemical changes. Physical changes have been the most favoured approach for the estimation of PMI. Some of the commonly used physical methods include *algor mortis*, *rigor mortis*, *livor mortis*, and putrefaction and supravital reactions. (1)

The biochemical approach has also been thoroughly worked upon for PMI estimation. Chemical composition of various body fluids changes after death. The most commonly used body fluids include blood (2), vitreous humor (3-6), and cerebrospinal fluid (CSF) (7).

Recently, a new biochemical approach using the post-mortem degradation of the skeletal muscle proteins has been proposed as a promising tool for PMI estimation. (8, 9) Animal study on Pigs done by Pittner *et al* (2015) (8) has used titin, nebulin, α-actinin, desmin, tropomyosin, cTnT and SERCA1 and measured using SDS-PAGE & western blotting for 10 days post-mortem. They showed protein degradation to be a 'very promising tool' for PMI estimation. Their results showed that titin & nebulin to be early degradation markers while desmin, cTnT & SERCA1 as late degradation markers. However, α-actinin & tropomyosin failed to show any degradation over the total study period of 10 days. Later, Pittner *et al* (2016) (9) came with another study to validate the results of their previous work on Humans. By far, this is the only piece of work done on humans to see the pattern of skeletal muscle protein degradation with time since death. They analyzed 40 forensic cases with well-known time since death & measured protein such as tropomyosin, desmin & cTnT using the SDS-PAGE & western blotting. Similar to their previous work, tropomyosin showed no degradation while both desmin & cTnT showed time-dependent degradation. It has also been noted that the degradation products of cTnT appeared earlier in humans than in pigs. One of the degradation products of cTnT (cTnT dp2) has also been claimed by them to be 'the most valuable decomposition marker in PMI analysis.' In 2017, Pittner *et al* (10) used the database obtained from their previous study and applied it practically upon a case of two dead bodies found in a lake. They discovered that when all the known methods of estimation of time since death failed, using muscle protein degradation as a novel approach helped in investigation and time since death estimation.

Since skeletal muscle is one of the largest organs and found in every part of the body, using skeletal muscle for PMI estimation could be of extreme importance in cases where other methods fail such as in the case of a mutilated body. (10) No such study could be found in Indian population. Therefore,

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the current study was conducted to correlate the PMI with degradation of skeletal muscle cTnT protein.

MATERIALS AND METHODS

A cross-sectional study was conducted at All India Institute of Medical Sciences Rishikesh for 1 year from December 2018 to December 2019. Samples were collected from all medico-legal cases undergoing post-mortem examination with a known time of death. We received 14 samples during the study. Approval from the Institutional Ethical Committee was taken. Case details were recorded as a measure of post-mortem interval. If there is a delay in the post-mortem examination and the body was kept in the cooling cabinet, then the duration for which the body was kept inside was also recorded. Physical methods used for time since death estimation was also recorded and both the methods were compared with each other. Evidences of any diseases like muscular dystrophy or any myopathy were excluded. Autopsies were carried out at the mortuary of AIIMS Rishikesh. It was conducted as per the standard protocol and procedure described in Current Methods of Autopsy Practices by J. Ludwig.

Skeletal muscle samples were obtained from *vastus lateralis*, approximately of 2x2x2 cm in all the cases. Samples were snap frozen in liquid nitrogen and stored at -80^{o}C. Frozen samples were homogenized by cryogenic grinding and sonication. Radioimmunoprecipitation assay (RIPA) buffer (SIGMA) with a protease inhibitor cocktail was used as a protein extraction buffer for further use. The homogenate was centrifuged at 1000 x g for 6 minutes. The supernatant was discarded and the precipitate was stored at -80^{o}C. Protein concentration was measured using the Pierce BCA assay kit.

SDS-PAGE

For detection of cTnT, 10% polyacrylamide gels was used as resolving gel and 5% polyacrylamide gels were used as stacking gel. 30µg of total protein was diluted in 15µl *aquabidest* and 5µl sample buffer [10% mercaptoethanol, 40%glycerine, 250mM Tris-HCl (pH 6.75), 0.04% bromophenol blue]. The sample was heated at 90^{o}C for 5 minutes and inserted into gel wells. At a constant voltage of 150 V, the electrophoresis was run until the dye front reached the bottom of the gel. The running buffer contained 195 mM glycine, 100 mM EDTA, 25 mM Tris, and 0.1% SDS. The proteins were transferred from gel to PVDF membrane at a constant current of 250 mA for 75 minutes in transfer buffer [192 mM glycine, 20% methanol, and 25 mM Tris].

Western Blotting

All bolts were blocked in 1 % dried milk in TBST for 1 hour. The primary antisera i.e. Mouse monoclonal anti-cTnT antibody was diluted in 1% dried milk in 1X TBST (CT3, DSHB, 1:250). The secondary antibody used was HRP-conjugated polyclonal goat anti-mouse antibodywas also diluted in 1% dried milk in 1X TBST (DAKO, 1:10,000). The membranes were washed extensively after each antibody incubation in 1X TBST. Then, the chromogenic substrate TMB was added. Once the colored band the visible, the reaction was stopped by adding stop solution. The blotting membrane was photographed using gel documentation system.

Statistical analysis

Logistic regression analysis was done for finding the relation between PMI and degradation of cTnT and also to find out the statistical influence of all the confounding factors i.e. duration of storage in cold cabinet, age, sex, and cause and manner of death.

RESULT

A total of 14 samples were received with exactly known time since death. Out of 14 samples, 2 were females and 12 were males. The mean age of the cases was 41.2±19.6 years. The causes of death were diverse. From a legal point of view, six were accidental, 3 natural, 1 homicidal, and 4 have an unclear manner of death. Post mortem interval was calculated with the help of hospital records. In 12 out of 14 cases, PMI was less than 25 hours and in the rest of 2 cases, it was 117 and 124 hours. Almost all the bodies were kept in cold storage for a variable period. The two cases with longer PMI, duration of cold storage were 72 hours and 115 hours, respectively. During the study, physical methods (i.e. *algor mortis, rigor mortis,* and *livor mortis*) were also simultaneously used for estimation of PMI.

Protein analysis

cTnT analysis revealed double bands between 30 and 40 kDa in all the cases. Only the two cases with PMI 117 and 124 hours showed two additional bands, representing degradation products of cTnT (cTnT dp1 and dp2) with a molecular weight between 20 and 30 kDa. Also, as time increases the intensity of the degradation product cTnT dp1 and dp2 increases. (Figure 1) Regression analysis showed no correlation between PMI and cTnT degradation (*P*value: 1).

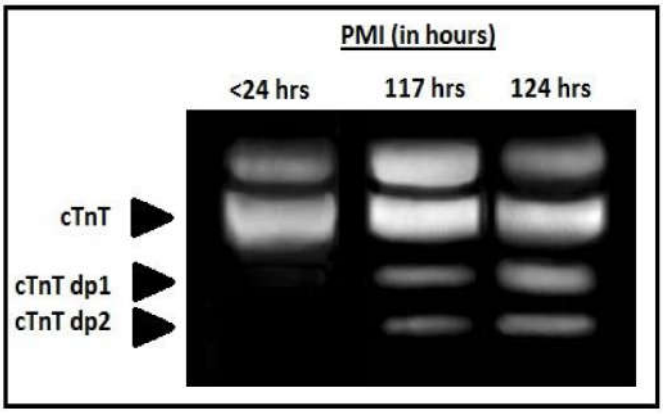

Figure 1 Post-mortem degradation pattern of cTnT at varying postmortem interval using the western blotting technique

Correlation between cTnT degradation and confounding factors

Logistic regression analysis showed no significant correlation between age, sex, cause and manner of death, and duration of storage of cold cabinet with cTnT degradation. (*P* value: 1)

DISCUSSION

In our study, cTnT showed time dependant degradation of proteins with two bands of degraded proteins in both the cases with higher PMI. Our results closely match with the results obtained by Pittner *et al*. (9) When we compared the two cases which showed the presence of degradation bands, the one with higher PMI showed bands with a higher intensity which means that even this degradation pattern is time dependant and increases with time.

While considering other influencing factors that might affect the results like age, sex, cause of death, and manner of death, they lacked any significant correlation with the results. Also, storage in a cold cabinet showed no significant effect on protein degradation.

In the study conducted by Pittner *et al* (9), various proteins were taken analysis out of which only cardiac troponin T (cTnT) showed results that can be useful for the estimation of time since death. In our study, we only chose cTnT for the validation of results and found similar results. But our study failed to show any significant statistical correlation which can be attributed to a lesser sample size. They also considered surrounding temperature as confounding and used ADD (adjusted degree days) instead of just time since death. In our scenario, we omitted the measurement of temperature because the surrounding temperature changes, very often due to seasonal, diurnal, and storage conditions which makes it difficult to monitor.

There are several limitations of the study. Firstly, it included a small number of sample size. Secondly, the major challenge faced was the timing of the collection of samples. As per the policies, post-mortem examinations were conducted in most of the cases within 24 hours. Only in the cases of unclaimed and unknown bodies, the post-mortem was done after 72 hours. So, finding cases with post-mortem interval between 24-72 hours became difficult and in our situation, we received no such cases. Also, in almost all such cases with unclaimed and unknown bodies, the exact time of death is not known.

CONCLUSION

We can conclude that the degradation of cTnT can be used as a method of PMI estimation especially in the time interval for more than 24 hours. Although, this method requires further research and investigation for the production of adequate data before it can put into practical application. Various other parameters such as sex, age groups, racial and regional variation, and also environmental condition, need to be assessed before using it as medico-legal evidence.

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Conflict of interest

There is no conflict of interest.

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