A pilot study to analyse post-mortem interval by serial estimation of expression of autophagy and cardiac muscle specific genes

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DECLARATION

I hereby declare that thesis entitled **"A pilot study to analyse post-mortem interval by serial estimation of expression of autophagy and cardiac muscle specific genes"** embodies the original work carried out by the undersigned.

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CERTIFICATE

This is to certify that the thesis titled "**A pilot study to analyse post-mortem interval by serial estimation of expression of autophagy and cardiac muscle specific genes**" is the bonafide work of **Dr. Sahil Thakral** carried out under our guidance and supervision, in the Department of Forensic Medicine and Toxicology, All India Institute of Medical Sciences, Jodhpur.

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Acknowledgment (With the Blessings of Bajrang Bali)

"Every living entity, especially persons in the human race, must feel grateful for the benedictions offered by the grace of the supreme lord."

Shri Bhagwat Geeta

Nothing worth achieving has ever been achieved without the guidance of our mentors and the support of those around us. The timeless words of **Shri Bhagwat Geeta** could not be more undeniable as I stand on the verge of one of the most prestigious achievements of my life.

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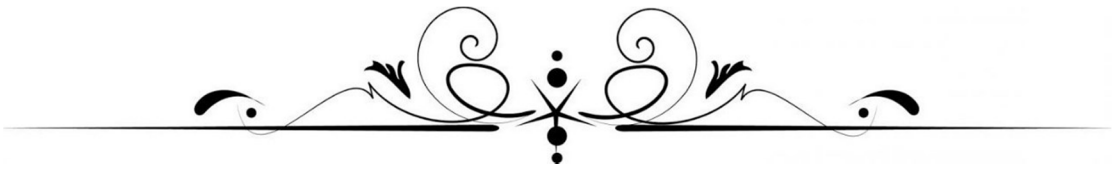
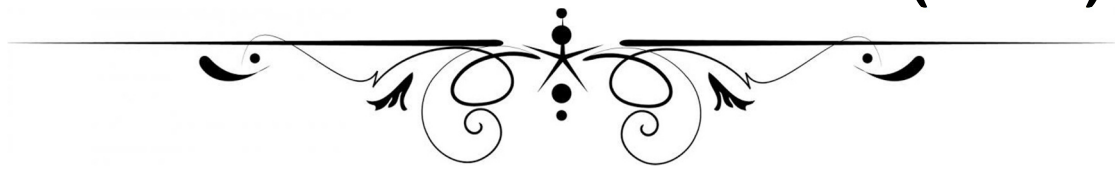


Dedicated To
My grandmother (Nani)
Late Smt. Shanti Grover (Maa)



Index

List of Abbreviations

ΔΔCt: delta delta cycle threshold

ΔCt: delta cycle threshold

AG: Assessed gene

ATP: Adenosine triphosphate

Bcl2: B cell lymphoma 2

cDNA: complementary deoxyribonucleic acid

Ct: cycle threshold

cTnI: Cardiac Troponin I

DAMP: Damage associated molecular pattern molecule

DEPC: Diethyl pyrocarbonate

DNA: Deoxyribonucleic acid

FCE: Fold change expressions

FDA: Food and Drug Administration

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

HG: Housekeeping gene

HMGB1: High-mobility group box 1

mRNA: messenger RNA

NT-proBNP: N-terminal pro-B-type natriuretic peptide

PCR: Polymerase chain reaction

PM: Postmortem

PMI: Post-mortem Interval

qRt-PCR: quantitative Real-time polymerase chain reaction

RCF: Relative Centrifugal Force

RM: Rigor Mortis

RNA: Ribonucleic acid

rRNA: ribosomal RNA

Rt-PCR: Real-time polymerase chain reaction

Tm◦: Melting temperature

Temp.: Temperature

tRNA: transfer RNA

TSD: Time Since Death

List of Tables

List of Figures

List of Flowcharts

List of Image

SUMMARY

Summary

Background- In Legal medicine, the determination of time since death is an important topic and one of the most difficult aspects. There are several methods used to estimate the post-mortem interval (PMI) like physicochemical, entomological, biochemical, metabolic, autolytic, and physical methods, which include post mortem staining, algor mortis, rigor mortis, and decomposition changes. These methods provide a wide range of PMI, as they are affected by different factors.

Objectives- The approach behind the present study is to calculate the accurate PMI and patterns by using mRNA degradation and gene expression of cardiac-specific genes (cTnI, NT-proBNP) and autophagy genes (HMGB1, Bcl2).

Methods- Sixteen cadaver heart tissues were analysed within a time frame of up to 24 hours from the time since death, at different time intervals at room temperature. Fold change gene expression was determined and the data were analysed using the value of average delta Ct (ΔCt) value of assessed gene and housekeeping gene (GAPDH). Livak method (ΔΔCt) was used to calculate the fold change expression at the different 7-time groups (0-6 hrs, 6-9 hrs, 9-12 hrs, 12-15 hrs,15-18 hrs,18-21 hrs, 21-24 hrs).

Results- The results obtained showed that FCE of cTnI is almost stable till 15 hours of PMI and then shows downregulation up to 24 hours PMI. The cTnI degraded about 15% in the first 6 hours of PMI and then degrade almost 13% to 17% every next 3 hours till 24 hours and also observed that continuous increase in the Ct values of cTnI up to 15-18 hours and then decreases up to 24 hours of PMI at room temperature. The FCE of NT-proBNP is stable till 12 hours PMI and then shows downregulation up to 24 hours PMI. The NT-proBNP degraded about 10 % in the first 6 hours after death and then degrade almost 13% to 22% in every next 3 hours till 21 hours of PMI, and 3% at 21 to 24 hours and also observed that the linear increase in the Ct values of NT-proBNP up to 12 to15 hours of PMI followed by a drop at 15 to18 hours of PMI, and continuously decreased up to 24 hours of PMI at room temperature. The FCE of HMGB1 is upregulated in the first 6-9 hours after death and then almost stable till 15-18 hours and then again shows upregulation up to 24 hours PMI. The HMGB1degraded about 32% in the first 6 hours after death and then degrade almost 12% to 21% in every next 3 hours till 15 hours of PMI, and 5% to 7% at in every next 3 hours till 24 hours and also observed that the linear decrease in the Ct values of HMGB1 up to 24 hours of PMI at room temperature. The Bcl2 shows no gene expression in the heart tissue after at any

point of time till 24 hours after death. Independent t-test was applied on the delta Ct values of NT-proBNP, cTnI, and HMGB1with GAPDH and the result was found to be significant (p-value <0.0001 respectively) at different times intervals.

Conclusion- The estimation of PMI by analysis of the FCE of cardiac-specific and autophagy genes is a new promising method in forensic medicine. The uniqueness of exploring such new methods lies in their accuracy and objectivity in estimating PMI and these genes may be a robust marker for estimation of time since death in medico-legal cases.


INTRODUCTION

Introduction

The precise estimation of time since death is climacteric in forensic medicine. If a forensic expert gets the answer to this crucial question, it provides a preliminary idea about the time of the incident, allowing us to narrow down the list of suspects.

Since time immemorial, post-mortem interval (PMI) has been tried to be estimated using various techniques. The earlier methods included physical, metabolic, autolytic, entomological, physicochemical, and biochemical methods. The various methods of estimating PMI include[1]:

- Physical methods- Changes in the eyes, Facial hair growth, Algor mortis, Post mortem staining.
- Physicochemical methods- Rigor mortis, Supravitality of skeletal muscle.
- Autolytic methods- Adipocere, Maceration, Marbling, Putrefaction.
- Biochemical methods- Enzymatic changes (Blood, CSF, Vitreous humor, pericardial fluid, synovial fluid).
- Entomological methods- Insect activity.

All these methods offer a broad range of time since death, as they are affected by various factors. The objectivity and reliability of the scientific methods provide a stronger value in comparison to eyewitnesses. Scientific methods when applied properly can be more reliable as compared to eyewitnesses, as the latter may have personal interests involved. However, these were never accurate and the quest for better methods led to the development of post mortem genetics focussed around mRNA degradation and gene expression. Various studies using different tissues as a source of mRNA have found a clear relationship between PMI and gene expression of various biomarkers. RNA stability has been described in the blood [2,3], human bone[4], retina[5], fetal and neonatal lung tissues[6].

Molecular biology is a new area where people are concentrating at present. The current study involves the estimation of various genes, cardiac Troponin I (cTnI), N-terminal pro–B-type natriuretic peptide (NT-proBNP), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), High mobility group box 1 protein (HMGB1), and B-cell lymphoma 2 (Bcl2) at various time intervals.

The N-terminal pro-B-type natriuretic peptide and cardiac troponin I are the leading cardiac biomarkers[7]. Numerous studies have been carried out to assess the level of cardiac biomarker proteins in different samples like antemortem and post-mortem serum levels, pericardial fluids, cerebrospinal fluids, and the non-human heart[8]. In 1995, the Food and Drug Administration (FDA) approved the first commercial cTnI enzyme immunoassay[9]. The 31 unique amino acid residues found at the N-terminus of cardiac protein differ from its skeletal isoform (sTnI)[10,11]. However, there are no studies to the best of our knowledge on human cadaveric heart tissue.

Some research has been done on autophagy proteins in this direction[12]. High-mobility group box 1 (HMGB1) has been labelled the best non-histone chromosomal protein which regulates autophagy in a localization-dependent manner with high electrophoretic mobility.[13,14] The HMGB, HMGN, and HMGA super-families are among them. HMGB1 is the most abundant and well-studied protein among these. It plays a key part in apoptotic cell death, as it is involved in a variety of routes and functions. It can be found both intracellularly and extracellularly, where it participates in many pathways. It also functions as a prototypic DAMP (damage associated molecular pattern molecule), eliciting immunological and inflammatory responses. Its involvement in autophagy, as well as autophagy-mediated disorders such as autoimmune diseases and cancer, has been thoroughly explored and documented[15]. The HMGB1 autophagy pathway associates nuclear release with cell death and apoptosis. Necrosis is induced by post-mortem changes, and necrotizing cells release HMGB1. In the serum of Wistar rats, HMGB1 has been reported to follow a serial increase in concentration with increasing time after death[12].

B cell lymphoma 2 (Bcl2) is the apoptosis-related gene. The process of programmed cell death or apoptosis is increased after death due to anoxia caused by the absence of blood flow in tissues[14,16]. Bcl2 has been reported in the liver of mice to follow a decrease in relative expression with increasing time after death[16,17].

In the present study, human heart cadaver tissue is used and the approach behind it is to assess the fold change expressions (FCE) of the cardiac muscle-specific genes and autophagy genes at different time intervals (0,6,12 hours) to calculate the time since death (TSD).

The aim of current study was to analyze the serial changes in gene expression with increasing time since death, by evaluating the fold change expressions of cardiac specific genes (cTnI, NT-proBNP) and autophagy gene (HMGB1, Bcl2).

AIMS & OBJECTIVES

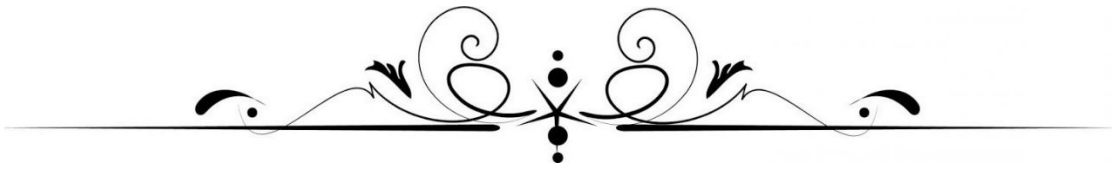
Aims and Objectives:

Aim:

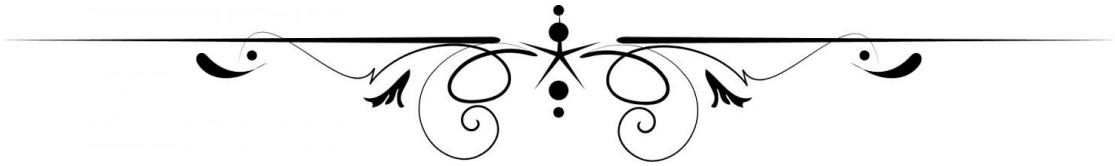
To study the role of expressions of various genes (NT-proBNP, cTnI, GAPDH, HMGB1, Bcl2) in estimating post-mortem interval.

Objectives:

1. To determine the PMI by serial estimation of autophagy genes (HMGB1, Bcl2) in cadaveric cardiac muscle at different time intervals.
2. To determine the PMI by serial estimation of cardiac tissue specific genes (NT-proBNP, cTnI) from cadaveric cardiac muscle at different time intervals.
3. To determine the patterns of autophagy genes (HMGB1, Bcl2) and genes expression (NT-proBNP, cTnI, GAPDH) from cadaveric cardiac muscle at different time intervals.



REVIEW OF LITERATURE

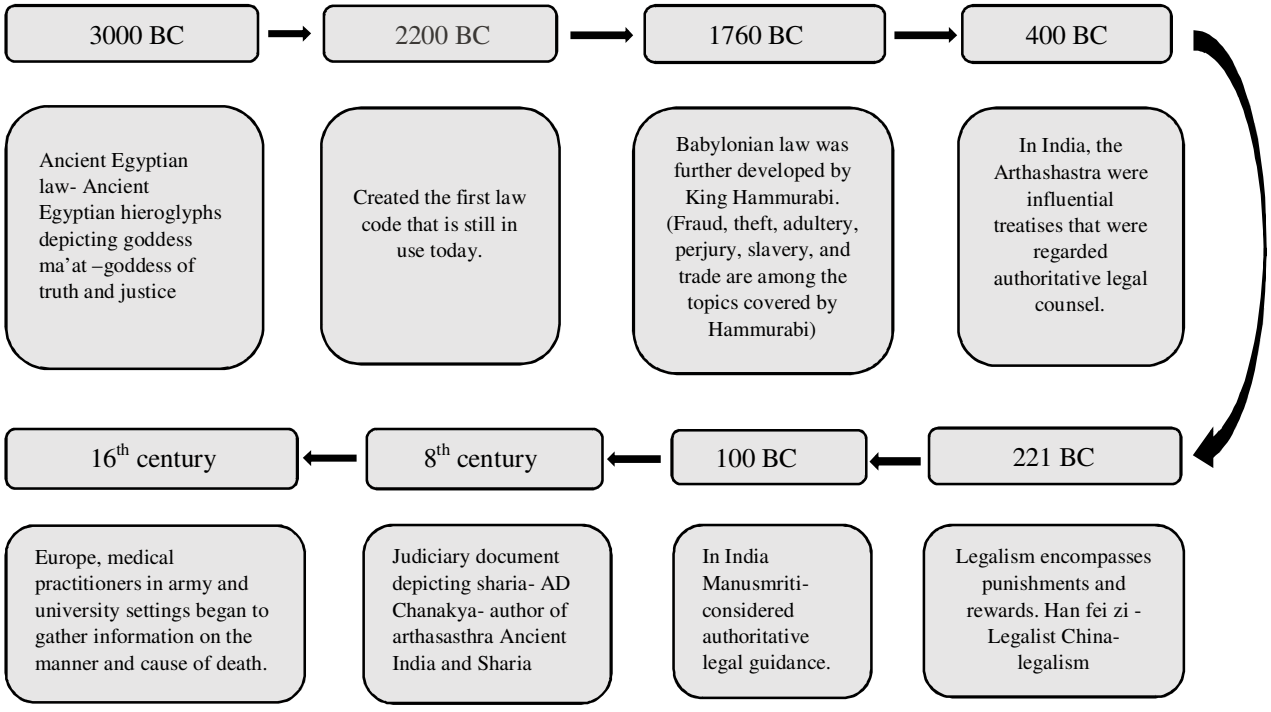


Review of literature

Forensic medicine and its origin

Forensic medicine is a discipline of medicine in which scientific principles and knowledge are used for legal (both civil and criminal) and justice issues [18]. Forensis means "forum" in Latin, and a forum was a gathering place in Rome where civil and judicial problems were debated. Fortunato Fedele, an Italian physician, authored the first treatise on forensic medicine in 1602. Paolo Zacchia is known as the "Father of Legal Medicine."[19]

History- The study of how and why the law has changed is known as legal history or law history. It is impossible to specify the single date as a point at which legal medicine appears as an identifiable, different scientific authority. However, we cannot say much about the origin until the mean of recording them has been achieved and the record we had, took us about 5000 years back[20]. The Egyptians were the first to practice the examination of internal organs of humans and discovered the application of autopsy 4000 years ago[21]. The timeline of the principal contributors in the history of forensic medicine is as below[20]-

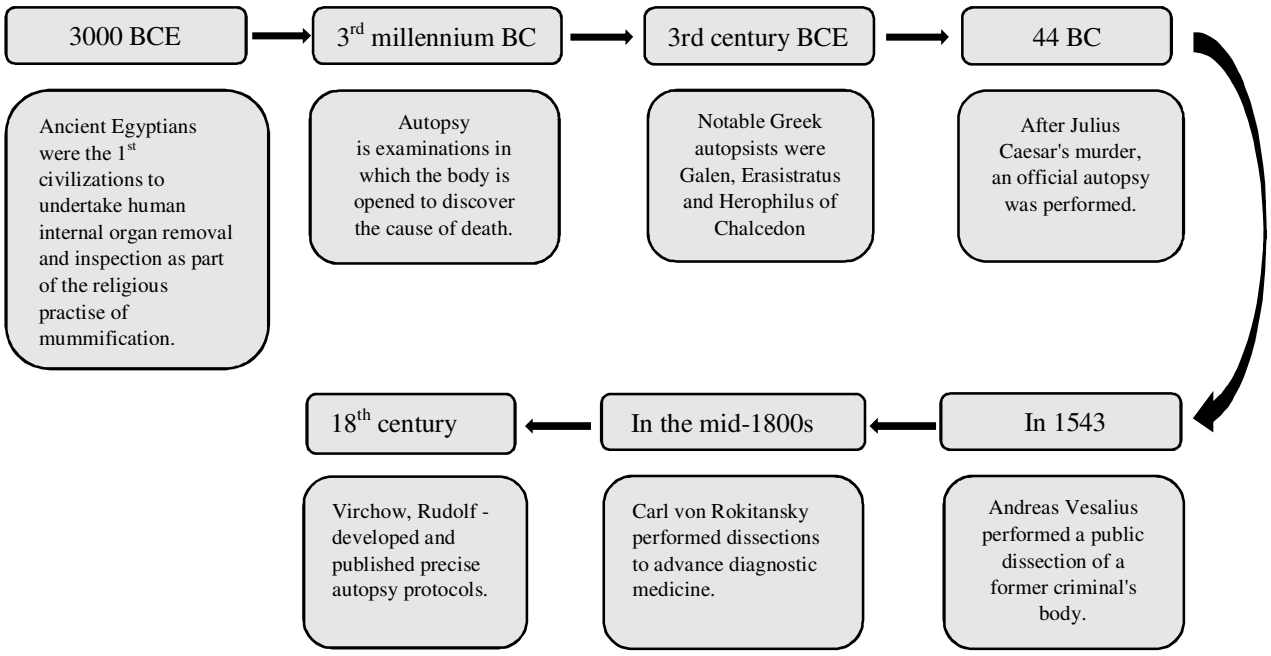


Flowchart 1: Timeline mentioning the principal contributors in the history of forensic medicine

Autopsy and its origin

The name "autopsy" comes from the Ancient Greek word autopsia, which means "to see for oneself"[22]. It is a surgical process that entails dissecting a body to establish the cause, method, and manner of death, as well as to assess any sickness or injury that may be present for research or educational purposes.

History- An autopsy's main goals are to determine the cause of death, the mechanism of death, and the manner of death. In the religious practice of mummification, the ancient Egyptians were one of the first cultures to remove and examine the internal organs of humans. The timeline mentions the principal contributors in the history of autopsy as below[21,22]-



Flowchart 2: Timeline mentioning the principal contributors in the history of Autopsy.

Death

Death is the end of all biological functions that keep a living organism alive[23]. Death is defined in section 46 IPC it states that the death of a human being unless the contrary appears from the context[23,24].

Changes that occur after death[23]:

Immediate changes (Somatic death)	Early changes (Cellular Death)	Late changes (Decomposition and decay)
❖ Insensibility and loss of voluntary power. ❖ Stoppage of Respiration ❖ Stoppage of Circulation.	❖ Skin discoloration and elasticity loss. ❖ Changes in the eye. ❖ Muscle flaccidity in its early stages. ❖ Algor Mortis ❖ Hypostasis (Suggilation, Cadaveric lividity PM lividity) ❖ Rigor Mortis	❖ Putrefaction ❖ Adiopocere formation ❖ Mummification

Time since death

The post-mortem interval, often known as the time since death, is the time between death and the moment of a body's examination[24,25].

Historical Background

During the 3rd and 4th centuries, the ancient Greeks and Egyptians developed methods for calculating the PMI. They believe that after death, deceased corpses grew cold and stiff in the following ways[25]:

Temperature of the body	Stiffness of body	Approximate TSD
Warm	Not stiff	Not more than 3 h dead
Warm	Stiff	Dead between 3 to 8 h
Cold	Stiff	Dead between 8 to 24 h
Cold	Not stiff	Dead more than 2 days.

Importance of PMI

- ❖ To determine the time of the crime.
- ❖ It offers the investigating officer a place to start their investigation.
- ❖ It enables them to deal with the information more effectively.
- ❖ It may allow some suspicions to be ruled out, and the hunt for the perpetrators can begin sooner.
- ❖ To verify or refute an alibi.
- ❖ To double-check a suspect's claims.

Estimation of PMI

There is no way to determine the exact time of death, thus only an approximate range may be given; there are significant biological variances in individual cases. Then local physical or environmental conditions at the crime scene, such as the existence of fires and household heating, open windows, air temperature, and so on, should be taken into account. The time range given is, at most, an informed prediction based on knowledge and experience, and it is prone to inaccuracy.

Different methods of estimation of PMI

Changes in the skin

As a result of blood draining from the skin's tiny blood veins. especially in fair people, the skin turns pale and ashy white. Within minutes of death, it loses its flexibility[25].

Changes in the eye

Blood clotting in the retinal vessels - occurs within 15 minutes and can last up to an hour. Corneal Clouding– Within 2 hours of death. If eyes are open, a Tache Noir developed in 3 to 4 hours. Intraocular tension halved at death, halved again in 30 minutes, and nil in 2 hours[25].

Algor mortis

It refers to the process of cooling the body after death. During the first 24 hours following death, the rate of drop in body temperature with time is used to determine the time since death[25–27]. Heat generation ceases after death, and body heat is lost to the environment by radiation, conduction, and convection. It's because the hypothalamus has lost its ability to regulate homeostasis. For measuring the temperature various body site have been used such as a nostril, axilla, ear, rectum, and abdominal skin surface. The most commonly used site is the rectum for measuring temperature[27–29]. In 1992 Nokes LD et al states the thumb rule that every hour there is a decrease of 1.5 degrees[30]. To estimate the TSD, numerous equations, algorithms, and charts have been created; Henssge published a 'nomogram approach' for determining the time of death from body temperature[1,28]. In 1976 and 1978 Brinkmann et al. and in 1984, Henssge et al. measured rectal temperatures, and nomograms for brain temperatures were used to produce PMI estimation utilizing algor mortis[28,31]. Algor mortis is one of the most accurate parameters in the early post-mortem phase for determining PMI. However, post-mortem cooling is not applicable everywhere due to different climatic regions and is also affected by different variables such as the amount of adipose tissue under the skin,

air currents, and humidity, size of the body, existence of coverings, and clothing. So, all these factors should be taken into account while calculating PMI using algor mortis.

Rigor mortis

The depletion of adenosine triphosphate (ATP) from anoxic tissue/muscles, which is induced by the breakdown of actin-myosin filaments in the muscle fibres, is the cause of rigor mortis (RM)[32]. RM starts immediately after death; it was first described by the Nysten. It usually developed sequentially starting from eyelid, jaw and neck followed by the limbs. This sequence is known as the "march of rigor" or Nysten's Law[34]. RM simultaneously developed in all the voluntary and involuntary muscles of the body, it develops more rapidly in small muscles like around the eyes, mouth, etc. than in large muscles such as muscles of the lower limbs[29]. RM develops in the muscles of the face in 2-4 hours after death, extends to the limbs in 6-12 hours, and lasts for 24-36 hours[35,36]. The relaxation following rigor mortis is determined to be caused by the dissipation of the actin-myosin complex that has developed due to proteolysis of post-mortem muscles. It passes off in the same sequences as it appears[37]. Age, body muscular mass, temperature, the presence of infections, existing ante-mortem diseases, the degree of muscular activity immediately before death, and climatic conditions are all factors that influence the onset and persistence of RM[33,35,38,39].

Livor Mortis

After the circulation stops, livor mortis or post-mortem lividity immediately begins. Gravitational pull causes blood to settle in the dependent areas of the body, resulting in a purplish-blue colouring of the skin[25,34,38]. After 20-30 minutes, Liver Mortis emerges as dull red patches that combine into larger patches to form a consistent pattern of red-purple discoloration that appears between 6 to 12 hours. Due to the dissolution of blood cells and the seepage of haemoglobin, the discoloration becomes ‘fixed' after about 10-12 hours[40]. Traditionally, fixed post-mortem staining was employed to indicate a time since the death of more than 12 hours[41]. The movement of the body causes a secondary pattern of post-mortem staining to form even after 24 hours. This method required a new strategy and objective to calculate the time since death accurately, which led to the creation of colorimetric methods[42].

Post-mortem Decomposition

Decomposition is the process in which soft tissue of the body is destroyed after death and it occurs by the actions of endogenous enzymes and bacteria. Autolysis and putrefaction are the two mechanisms of decomposition:

- Autolysis is the process that begins soon after the death, in which the breakdown of tissue occurs by the leakage of hydrolytic endogenous enzymes. The changes of autolysis mainly occur microscopically instead of macroscopic. Grossly the pancreas is the first organ that shows prominent features of autolysis, decomposition of the non-gravid uterus and prostrate takes much longer[43,44].
- Putrefaction is the process that occurs due to the leakage of the content of cells which can provide culture to grow microbes like fungi, protozoa, and bacteria, which do the degradation and disintegration of surrounding tissues[45,46]. The first obvious sign of putrefaction is a greenish colouring of the skin at the right iliac fossa, followed by liquefactive necrosis or gas production, both of which produce bloating. There are five phases of decomposition; Fresh, Early decomposition, Advanced decomposition, Skeletonization, Extreme decomposition[47].

1. Fresh stage

This phase starts immediately after death and as late as seven days in which autolysis occurs. In this stage rigor mortis, algor mortis, and livor mortis occur. Other than the collecting of blowfly eggs in areas of tissue dehiscence and cavities, there is no insect activity visible. In living patients, egg depositions are also documented especially in debilitated, bedridden, and immobile subjects[48]. There PMI estimation based upon blowfly eggs is confounded and not reliable.

2. Early decomposition stage

This stage begins with hair loss and skin slippage, it usually starts from 24 hours after death up to five days. The greyish-green discoloration of the body due to the formation of sulph hemoglobin and marbling (purple-brown discoloration of superficial vein visible over skin as a network) starts 2nd day after death, some part of the body stays still pinkish and also maggots start to grow on the body. Due to the superficial position of the caecum with bloating of the abdomen, greenish staining of the skin appears as early as the second day after death at the right iliac fossa. On the second day following death, brownish discoloration with drying of skin of the extremities, notably over the nose, ears, and fingers, usually appears. Bloating and purging is appreciable on the stage and oozing out of the decomposition fluid

from the natural orifices with a foul smell. Usually, the bloating disappears after the 2nd week after the death due to the release of abdominal decomposition gases. By the 2nd week, the body becomes blackish green, and later on, it becomes brownish-black with leathery hard skin. Between the 10th day and the end of the first month following death, maggot activity appears beneath the leathery hard skin[49–51].

3. Advanced decomposition stage

This stage, also known as late decay or black putrefaction, begins with a shrinking abdominal cavity and loose skin, as well as a significant maggot infestation. These alterations normally develop between the 4th and 10th day after death. In this phase, the body becomes less than half due to loss of skin and soft tissue. Drying up of outer skin and shrinkage or loss of internal organs due to autolysis. Usually, pupa is present over the clothing and body presence of molds, it is seen in a period of 2nd month to 9th month after the death. Decomposition is influenced by environmental factors; for example, if the body is buried or kept in a high-humidity environment, autolysis and maggot activity increase, causing skin shrinking and the mummification phase to escape, resulting in skeletonization[51–53].

4. Skeletonization stage

This phase, called the dry remains stage, is associated with shrinkage of skin and tissues. It result in half of the skeletal elements being exposed out and that could show soft tissue still attached[53,54]. These tissues appear at ligamental and muscular attachments at the end of long bones and between the vertebras[55]. This phase is seen usually between the 2nd and 9th months after the death leading to exposing dry bones and greasy material left behind and can last for years depending on environmental conditions like being buried or left in a high humidity environment.

5. Extreme decomposition stage

Extreme decomposition is exhibited in this phase, with skeletal elements eroding solely in the settings in which they are exposed to the environment; this is caused by the process of bone bleaching, and it is usually seen six months following exposure. In the literature, it is documented that this phase starts as early as 2nd month to 2.5 years after death. Further, the skeletal bones go under the degeneration of cortical structures which can lead to loss of metaphyseal of bones and cancellous part of bone exposed out. It is commonly seen 1 to 1.5 years after death, and it has been reported as beginning as early as the 4th month after death[52].

Differential decomposition involving mummification or adipocere formation is also documented in the literature.

Mummification

This is a phenomenon in which shrinkage of tissue occurs due to a dry and hot environment. The skin of the deceased look dry, dark, leathery in appearance, and the body appears parched[56].

Adipocere

The adipocere is the process that occurs in the deceased body in a high moisture environment with lack of oxygen which favours the formation of adipocere and leads to the formation of waxy material of yellowish to grey colour due to anaerobic bacterial hydrolysis and hydrogenation of body fat, converting into fatty acids and soaps[53,56,57]. Clostridium perfringens is the primary organism that leads to adipocere formation by causing aggregation of fatty acid crystals and loss of epidermis. As per literature it starts as early as the 3rd week to the 4th week after death[57].

Supra-vital reactions

The processes that occur in the body after somatic death are known as supra-vital reactions[58]. Maximum force after stimulation with the same current intensity decreases as TSD increases, yet relaxation time increases due to weaker muscular contraction, and relaxation time has an exponential relationship with maximum force[58]. Madea defines the time since death into 4 stages for this method as follows[58]

S.no.	Stages	Remark
I.	Latency period	Stoppage of circulation
II.	Survival period	The tissue continues to breathe aerobically until it is depleted.
III.	Resuscitation period	Loss of tissue function, but by using external stimuli they can be re-activated.
IV.	Supra-vital period	The tissue's ability to heal has been destroyed.

In 2002, Henssge et al. claimed to have measured the maximum force of reaction using a sensitive force sensor in response to a definite stimulation[58]. Also, the maximal force decreases in proportion to the period since the death. The super-sensitivity of tissue in the

immediate post-mortem phase, known as Zsako's phenomenon, should also be considered[58,59]. Supra-vital reactions are affected by various factors such as climate, temperature, diseases, and the presence of drugs. It can be only used after a few hours since death for estimating PMI and has no use in cases of damaged and burned bodies.

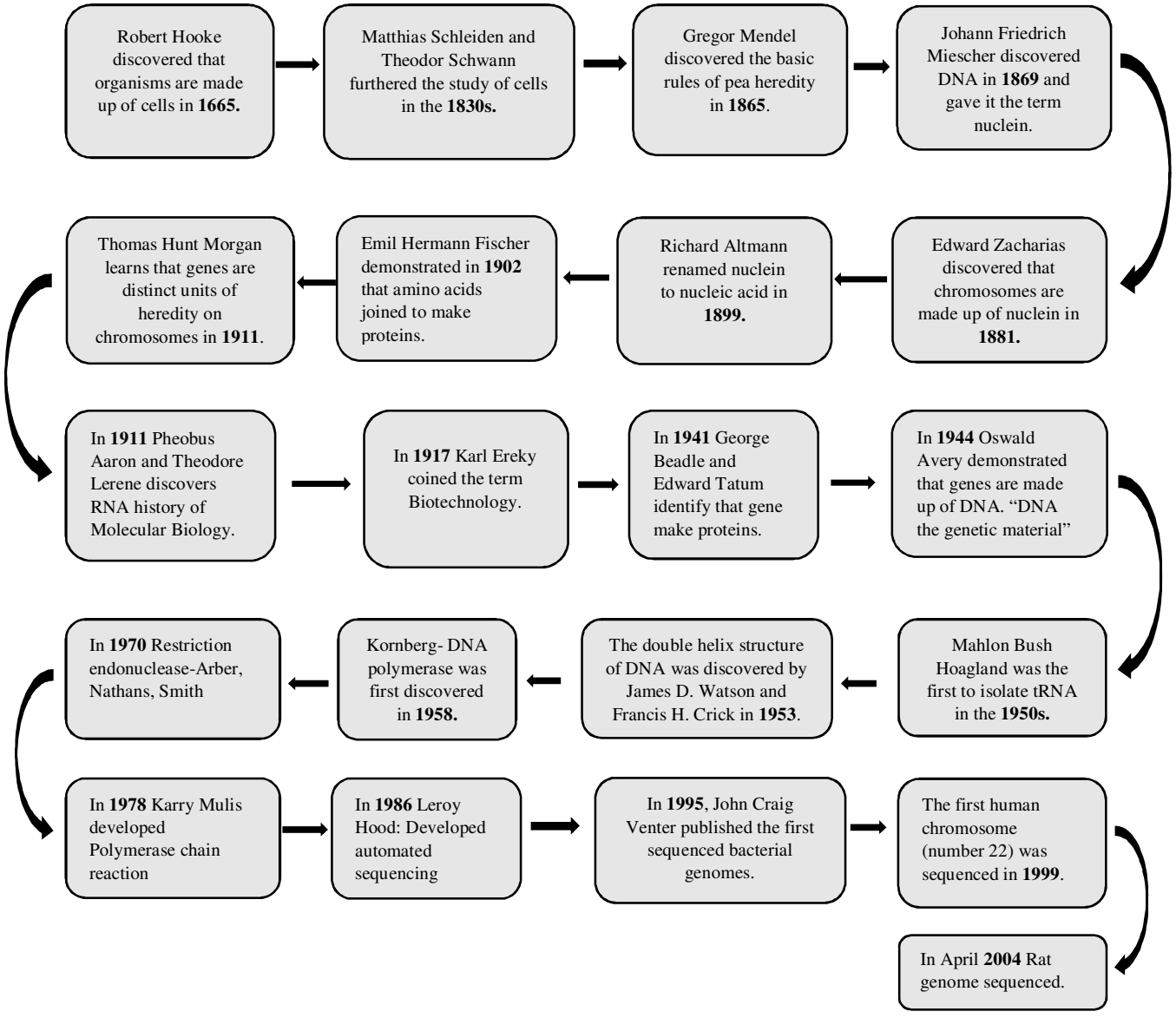
Biochemical assessment

In the last 65 years, various biochemical methods have been proposed for the estimation of PMI. Biochemical changes showed immediately or shortly after death by body fluids such as spinal fluids, blood, urine, pericardial fluid, vitreous humour, and aqueous humour of eyes[60,61]. In blood, chloride concentration has been studied for estimating PMI. It decreases with increasing TSD. According to Jetter(1959), the chloride content decreases at a rate of eighty to ninety mEq/L every 24 hours. Schleyer (1963) later stated that decreases occur at a rate of 0.30 to 1 mEq/L per hour. Querido (1990) then discovered a twofold logarithmic association between PMI and plasma chloride levels[60–63]. In comparison to cerebral fluid and blood, vitreous humour has become the most researched material for calculating PMI because it is highly protected, topographically isolated, and autolytic changes are slower. Potassium is the most investigated parameter in the vitreous humour. After death the concentration of potassium in the vitreous humour increases. In 1983, Aggarwal et al. reported that potassium concentration increased linearly with increasing PMI, while Zhou et al. (2007) reported that the increase was unaffected by humidity, environmental temperature, sex, or age[1,46]. In 1988, Montbrun et al. calculated PMI by looking at the percentage of creatinine concentration, aspartic aminotransferase activity, and non-protein nitrogen on total soluble protein in bird muscles. They found that creatinine and non-protein nitrogen percentages were positively correlated, while aspartic aminotransferase was negatively correlated[64]. They de-arranged biochemical profiles due to factors such as environmental factors, survival period, cause of death, pre-existing diseases or disorders, and also the analyte under investigation's qualities[64,65]. In literature a total of 388 biochemical markers were found on which studies were conducted out of which only a few markers such as sodium, potassium, chloride, magnesium, hypoxanthine, urea, and cardiac troponin T had sufficient investigation with consideration. Eighteen markers were found not suitable and poorly investigated for application, meanwhile, 6 were found suitably researched but for practical use not suitable and did not have sufficient information of 364 biochemical markers[66].

Molecular Biology and assessment

In the last 15 years, within the field of forensic genetics, molecular revolution has taken place. Molecular biology mainly focuses on nucleic acid (DNA & RNA), a constituent of genes and proteins. In the 1950s the history of molecular biology began with the union of various genetics, biochemistry, virology, and microbiology. In 1984, the first forensic DNA analysis was used by Sir Alec Jeffreys[67].

History of Molecular Biology and Biotechnology



Flowchart 3: Timeline mentioning the principal contributors in the history of Molecular biology and forensic genetics[67]

In recent times, forensic genetics have led to various recent advances in the determination of time since death. The degradation of DNA, proteins, and mRNA are evaluated and can be used to analyse the PMI. It was found that due degradation and temporal correlation of RNA transcript are most relevant. In literature various studies demonstrate the linear correlation between degradation and time since death, it was found that the correlation was tissue and temperature-dependent[68].

The author looked at several studies from 1979 to 2020 to see if there is a link between the rate of RNA breakdown and RNA stability when calculating PMI. The following table summarises this research in chronological sequence, with study, year, species, tissues and organs, sample size, temperature, time frame examined, detection methods, statistical models (if provided), and remarks underlined.

Table 1. At various points, there is a correlation between RNA degradation and the post-mortem interval (PMI).

Study	Year	Species	Tissues and Organs	Sample Size	Temp. (° C)	Time Frame Assessed	Detection methods	Statistical Analysis	Remarks/ Conclusion
NABER et al.[69]	1979	Human	Brain	28	16°C	0-42 hours	Nucleic acid extraction and determination	Comparison	No significant decrease was found during PMI 6-25 hours in the nucleic acid or protein content of brain tissue.
Johnson et al.[70]	1986	Human and Rat	Brain	344	2°C -4°C	0 to 48 hours	RNA Preparation and Northern Blot Analysis	Relative proportion	No relation between RNA degradation and PMI. At all PMI human cortical RNA revealed high molecular weight peptides.
Noguch et al.[71]	1990	Rat	Brain	-	-	0, 4, 8, 16, 24 or 48 hours	Northern blot analysis	Student's t-test	With the increase in PMI, the amount of AVP mRNA and rRNA decrease and the amount of RNA did not alter with PMI, and the half-life of mRNA in the rat postmortem seemed to be approximately 16 hrs.

Burke et al.[72]	1991	Human	Brain	10	136°C	-	PCR	Pearson's correlation	Significant negative correlations were found between hypoxia and PNMT mRNA, And between storage and PMI.
Leonard et al.[73]	1993	Human	Brain	13	-70°C	0-42.3 hours	Northern Blot Analysis and PCR	-	The utilization of the human postmortem brain for oligo-dT priming, construction, and in vitro expression investigations will be compromised if it is stored at -70°C for more than 5 years.
Eastwood et al.[74]	1994	Human	Brain	12	-	84 hours	Northern analysis, Immuno-cytochemistry	Correlations	There were found correlations between the abundance of synaptophysin mRNA and synaptophysin interconnected hippocampal subfields, and found to be similarly affected by age as well as by fixation time.

Pardue et al.[75]	1994	Rat	Brain	30	37 °C	0-24 hr	Northern Analysis, Slot-Blot Hybridization, mRNA Quantitation	Mann-Whitney U test, t-test	Carefully evaluated the effect of PMI on mRNA degradation when analyzing levels of inducible hsp70 mRNAs
Harrison et al.[76]	1995	Human and Rat	Brain	59	-	0-96 hour	Standard method	Comparison and linear regression	On mRNA and protein products, the PMI had just a minor impact. At 96 hours following death, RNA content was reduced by 40%, and pH was found to be strongly related.
Marchuk et al.[77]	1997	Rabbit	Ligament, tendon and cartilage	-	4°C	0, 24, 48, 72, and 96 h	Northern blot analysis and RT–PCR	Comparison	Up to 96 h postmortem observed no degradation of rRNA.
Johnston et al.[78]	1997	Human	Brain	89	-70°C to -75°C	2 to 24 months	RT-PCR	Logs of the dependent variables	pH significantly correlated to levels of GAPDH.
Cummings et al.[79]	2001	Human	Brain	19	-	1 h 10 min to 14 h	RT-PCR and Agarose gel electrophoresis	Ct method	With extended postmortem delays, mRNA expression can be retained.
Fitzpatrick et al.[80]	2001	Bovine	Reproductive tissues	-	Room temp.	0 to 96 hours	RT-PCR and Northern analysis	-	The yield and purity of RNA are unaffected by a PM delay of up to 24h

Thorsell et al.[81]	2001	Rat	Brain	3-6	-	0,1,4 hour	RNase protection assay (RPA)	ANOVA and Tukey's HSD posthoc test	Estimates of NPY mRNA levels obtained using established methods are influenced by postmortem processes.
Paepe et al.[6]	2002	Human (perinatal)	Lungs	25	Room temp.	Within 41 hours	RT-PCR and Agarose gel electrophoresis	t-test	Live-born and non-macerated stillborn infants' perinatal lung tissues are acceptable for routine mRNA and protein gene expression.
Hynd et al.[82]	2003	Human	Brain	Review article	-	-	RT-PCR, cDNA microarrays, western blotting, immune-histochemistry.	Correlation	The majority of nucleic acids and proteins are quite stable after death. Variability can disclose crucial biochemical and functional information.
Malik et al.[5]	2003	Human	Retina and Retinal Pigment Epithelium	191	4°C	5, 12, 24, 29, 36, 48, and 72 hours	Electrophoresis , RT-PCR	-	RT-PCR electrophoresis of retinal tissue revealed bands of similar intensity from actin, GAPDH, and RHO at each interval. After 5

									hours, the RT-PCR gene products showed degradation in RPE band patterns.
Preece et al.[83]	2003	Human	Brain	90 + 81 (control)	-70°C	-	TaqMan real-time RT-PCR	ANOVA	In Alzheimer's disease brains, PMI was linked to brain pH, but not in controls.
Kuliwaba et al.[4]	2005	Human	Trabecular bone	18	4°C	0 and 30	RT-PCR	Pearson's correlation coefficient	After 48 h bone-specific mRNAs begins to degrade.
Catts et al.[84]	2005	Balb/c mice	Brain	40	Ambient temp.	0, 6, 12, 18, 24, 36 and 48 h	Microarrays and Real-time PCR	Tukey's posthoc test using ANOVA	Increased PMI was linked to increased RNA degradation and lower tissue pH.
Stan et al.[85]	2006	Human	Brain	114	-	-	Agarose gel electrophoresis	Tukey's posthoc test using ANOVA	There was no link found between protein levels and the aforementioned variables. RIN and pH have a good relationship.
Gopee et al.[86]	2007	Mice	Skin	3	Room temp.	0–60 min	Real-time quantitative PCR.	ANOVA and Student's t-test	No statistical differences in the relative gene expressions of Ccnd1, Hif1α, cMyc, and

									Cyr61 as a function of PMI.
Seear et al.[87]	2007	Fish-Atlantic salmon (Salmosalar)	Brain, kidney, liver and muscle	2004	Room temp.	1, 4, 8, and 24 h	qRT-PCR, microarray analysis, Northern blot analysis, Agarose gel electrophoresis	-	Total RNA of brain stays constant for 8h and small fall at 24h postmortem and kidney tissue indicated degradation at 8h and liver at 24h PMI.
Popova et al.[88]	2008	Human	Brain	-	-	-	RNA purification, reverse transcription, amplification and labeling, fragmentation and hybridization	Correlations	Negative correlations emerge from the compensatory increase in stable and 3'-end probed transcripts, whereas positive correlations indicate the 5'-end to the 3'-end direction of mRNA degradation.
Birdsill et al.[89]	2010	Human	Brain	79	-	-	RT-PCR	Pearson correlations. And T-tests	RNA degrades progressively with increasing PMI.
Koppelkamm et al.[90]	2011	Human	Heart, Brain, and skeletal muscle	136	70°C	0 to 175 min	RT-PCR	Pearson correlation (R).	When compared to skeletal and cardiac muscle, the brain's RNA quality reveals much lower integrities.

Sampaio-Silva et al.[91]	2013	Balb/c mice	8 organs (skin, spleen, heart, femoral quadriceps, pancreas, liver, stomach, and lungs)	5+15	21°C	0-20 Hour	Quantitative real-time PCR	Linear regression and Pearson correlation	Actb, Gapdh, Ppia, and Srp72 are four quadriceps muscle genes that have been determined to have a strong relationship with PMI.
Montanini et al.[92]	2013	Human	Retinal tissue	11	4°C	T ≤12 h and T >12 h	PCR amplification	Pearson's/chi-squared test	Short T2 was linked to high RNA quality retrieved from the retina/RPE complex ($p = 0.043$) and effective tissue-specific gene expression ($p = 0.007$).
Herrera et al.[93]	2013	Human	Myocardial tissue, Pericardial fluid, and Blood	30	4°C	5-24 Hour	RT-qPCR	Mean, median, standard deviation and range, Spearman's rank correlation coefficient, Mann–Whitney U	Good RNA integrity showed by all the samples and up to 24h after death, all remain stable. In any of the five zones of the myocardium investigated during the PMI, gene expression of proteins linked to ischemic myocardial

								test, One-way (ANOVA) test	damage shows no significant changes.
Young et al.[94]	2013	Pig	Tooth pulp	8	-	0-140 days	RT-qPCR	Δ Ct method	RNA degradation behaved in a non-linear fashion for PMI estimation.
Wen-Can et al.[95]	2014	Rat	Heart, liver, lung, spleen, brain, kidney, back skin, and leg muscle.	6	25	1-168 hour	RT-qPCR	Δ Ct method	The linear regression of 18S-rRNA was superior to the 18S-rRNA/miR-1 regression.
Linden et al.[96]	2014	Human	Heart, liver, and kidney	24 PM and 218 tissue samples	-	0- >45 h	RT-qPCR	Differences in RIN values and Cq values	GAPDH expression and RIN levels were substantially greater in fresh frozen post-mortem samples.
Schober et al.[97]	2014	Human	Brain	8	-	0-132 hour	Quantitative RT-PCR	Ct method	42 mRNAs expressed shows significant >2-fold differences of downregulated (n=24) and upregulated (n=18) genes.

Hansen et al.[98]	2014	Human	Blood and muscle (psoas major)	45	Room temp.	1 to more than 14 days	rt-PCR	Cq value	In postmortem muscle tissue DNA was more stable than RNA.
Lv et al.[99]	2014	Rat	Spleen	21	4°C or 25°C	0, 12, 24, 36, 48, 72, 96, 120, 144,168, 192, 216, 240, 264, 288, and 312 h	RT-qPCR	Ct values	GAPDH1 and ACTB1 fluctuated little like cubic curves as PMI increased, however, GAPDH2 and ACTB2 decreased significantly.
Kraus et al.[100]	2014	Human	Brain	15	-	-	qPCR	unpaired t-tests	Within PMI of up to 27 h the expression levels of the newly identified 5, universal lncRNA are stable.
Nagy et al.[101]	2015	Human	Brain	-	4°C	0-96 hour	Real-Time Quantitative PCR	1-way and 2-way ANOVA, Pearson correlation coefficients	The threshold for methylated histone alterations was found to be between 72 and 96 hours, which matched the results from histone proteins at 72 hours.
Ma et al.[102]	2015	Rat	Brain	270 + 36 (additional)	4, 15, 25, and 35°C	0-144 h	quantitative RT-PCR	DCt method	At higher temp. with increasing PMI the RNA integrity degrades

Poór et al.[103]	2015	Human	Dental pulp	62	22–25 °C	0-121days	RT-PCR	Uni-variate linear regression	Estimation of PMI of tooth incubation time between 20 and 42 days after the extraction.
Blair et al.[104]	2016	Human	Brain	5	4°C	1-24 hour	Western blot, RT-PCR, and immunohistochemistry	-	Immuno-staining profiles and unique degradation patterns in different protein species were detected in western blots after a PMI of over 50 hours.
Philips et al.[105]	2016	Human	Pancreas	236	-	-	RNA extraction	Logistic regression	High-quality RNA obtained from organ donors dying of anoxia with normal lipase levels.
Lv et al.[106]	2016	Rat	Brain	13	4 ,15,25 and 35 °C	-	quantitative RT-PCR	ΔCt method	With PMI, β-actin (ΔCt) was showed the best correlation coefficient.
Matthews et al.[107]	2016	Human	Bladder tissue	15	Room temp.	4 hr to 11.1 hr	-	Correlation	The shorter PMI did not connect with an increase in RNA quantity or quality.
Walker et al.[108]	2016	Human	18 different tissues	389	-	3.25-29.2 hour	RT-qPCR	Graphpad Prism v.6	Each tissue has a high degree of variety. The yields were lowest in

									the tissues having a lot of fibrous material or fat (skin, cervix, and prostate). The liver, submandibular gland, and pancreas were the tissues with the highest yields.
Ali et al.[109]	2017	Human	Skin	12	24°C and 40°C	0, 1, 2, 3, 4, and 5 days	Real-Time Fluorescent Quantitative PCR	The Pearson correlation	With increasing the time interval, the expression levels of LCE1C were decreased, whereas no statistical significance was found with changing the surrounding temperatures.
Kim et al.[110]	2017	Mice	Brain, lung, muscle (quadriceps femoris), and Liver	4	21-23°C	0, 1, 6, 12, 24, 48, 72, and 96 h	qRT-PCR	ΔCt method	rRNA with cell death-related cleavage sites was rapidly eliminated during postmortem RNA degradation. In both mice and human autopsy tissues, the degradation rates between the two domains in mammalian

									28S rRNA were significantly proportionate to increasing PMI, with a significant linear association.
Tao et al.[111]	2018	Rat	Heart	91	10, 25 and 35 °C	0, 1, 3, 6, 12, 24 and 36h	real-time fluorescent quantitative PCR (qPCR)	ΔCt method	Cdc25b was discovered to be the most sensitive marker for estimating early PMI, and Rpl27 was discovered to be a good endogenous control.
Peng et al.[112]	2019	Mice	Brain and heart tissues	87	37 °C	0-48h	Real-time quantitative PCR	dCq method	The quantity of HAF in brain tissue increases after death, and it is significantly linked with 0–48 h PMI.

Cardiac Troponin I and N-terminal pro-B-type natriuretic peptide

The N-terminal pro-B-type natriuretic peptide (NT-proBNP) and cardiac troponin I (cTnI) are the leading cardiac biomarkers as per currently available literature. Numerous studies have been conducted to determine the levels of cardiac biomarker proteins in various samples, including antemortem and post-mortem blood levels, pericardial fluids, cerebral fluids, and non-human heart tissue[7,8]. The first commercial cTnI enzyme immunoassay was approved by the Food and Drug Administration (FDA) in 1995[9]. At the N-terminus of cardiac protein, 31 amino acid residues distinguish it from its skeletal counterpart (sTnI)[10,11].

Sabucedo et al. published a study in 2003 that used the protein marker cardiac troponin I to assess the postmortem delay. The investigation was conducted on six human cadavers, and the methodology was developed and optimized using a bovine model. The study found a pseudo-linear association between percent cTnI degraded and the log of time since death ($r>0.95$), as well as a qualitative degradation band pattern that may be utilized to estimate the post-mortem period using a simple comparative analysis with a conventional human heart. The early post-mortem interval (0–5 days) can be determined using the degradation-banding pattern of tissue cTnI[8].

Michaud et al. did a study on post-mortem NT-proBNP measurement on 96 autopsy cases in 2007. The goal of the study was to determine the post-mortem stability of NT-proBNP and to measure the amounts of NT-proBNP in the heart of people who have had myocardial ischemia. It highlighted the relationships between different autopsy specimens (e.g., pericardial fluid, vitreous humour, serum and blood). The findings suggested that NT-proBNP levels are much greater in those who have had a heart attack and that there are good connections between NT-proBNP levels in different samples of pericardial fluid, femoral blood, and serum[113].

In 2016, Tettamanti et al. conducted a study on 16 forensic autopsy cases to determine the levels of NT-proBNP, troponin I, and troponin T in sepsis-related deaths from femoral blood. The levels of serum troponin T, troponin I, and NT-proBNP were all higher in sepsis-related mortality, according to the study[114].

In 2017, Palmiere et al. published an overview of a study on post-mortem intervals utilizing cardiac troponins and NT-proBNP. The researchers compared antemortem serum levels of these markers to post-mortem levels detected in pericardial fluid and post-mortem serum samples collected from various sampling locations. The findings showed that when

measuring cardiac troponins in post-mortem samples, the time since death should always be taken into account[7].

High-mobility group box 1 and B cell lymphoma 2

Autophagy is a phenomenon in which degradation of dysfunctional organelles, and removes long-lived proteins and generates substrate for ATP production during periods of fasting and other types of cellular stress. High-mobility group box 1 (HMGB1) sustains autophagy and promotes cytosolic localization by directly interacting with the autophagy proteins Beclin 1 displacing B cell lymphoma 2 (Bcl2)[13].HMGB1 has been characterized as the best non-histone chromosomal protein which regulates autophagy in a localization-dependent manner[12,13] and it is an effector of autophagy in human cells, interfering with the binding of BCL2 to Beclin[15].

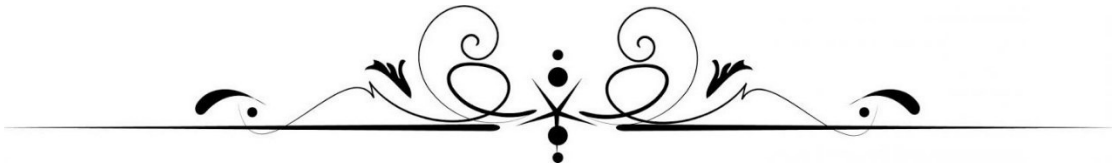
HMGB1 is secreted by mature dendritic cells, active macrophages, natural killer cells, and discharged from necrotic cells, according to Wang et al. in 1999, and it mediates the response to inflammation, infection, and damage. Kikuchi et al. in the year 2009 conducted a study on HMGB1 to the estimation of postmortem interval by analyzing the serum levels of 90 male Wistar rats. The study shows that HMGB1 has been reported to follow a serial increase in concentration with increasing time after death[12].

Peter A. Noshy in the year 2020 conducted a study with an aim to estimate of the PMI by exploring the post-mortem changes in apoptosis-related genes are expressed differently in the livers of mice. B cell lymphoma 2 (Bcl2) is the apoptosis-related gene. The process of programmed cell death or apoptosis is increased after death due to anoxia caused by the absence of blood flow in tissues. Bcl2 has been reported in the liver of mice to follow a decrease in relative expression with increasing time after death[16].

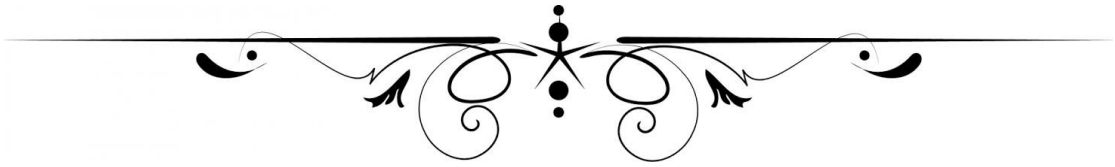
Welson et al. in the year 2020 conducted a study to evaluate post-mortem interval in relation to oxidative stress markers, histopathological examination, HMGB1 genetic expression, and BCL2 immunohistochemical analysis in major organs of the 42 adult male rats at room temperature and were obtained at 0, 12, 24, 48, 72, 96, and 120 h. The result reveals that at 48 h after death HMGB1 showed enhanced post-mortem gene expression with a peak and BCL2 expression began to decline 24 h and at 96 h after death become negative[14].

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Elghamry et al. in the year 2018 conducted a study to estimate post-mortem interval by studying the degradation of RNA taking skin and heart tissue GAPDH mRNA levels. However, this study was a non-human study done in 78 female albino rats. The study demonstrated no significant correlations between the heart GAPDH mRNA level and the time interval (up to 72 hours). However environmental factors were the limiting variables in this study[115].



MATERIALS AND METHODS



Materials and Methods

Ethical considerations

This study conformed to the guidelines of the **Institutional Ethics Committee (IEC) of All India Institute of Medical Sciences (AIIMS), Jodhpur** (letter no. AIIMS/IEC/2020/2021; dated 01/01/2020).

It was conducted following the principles of the Declaration of Helsinki for medical research and written informed consent was taken from the family member of each deceased before enrolling in the study.

The study was verbally explained to the family member of each deceased, for discussion of any of their queries. The subjects retained the right to withdraw consent at any stage of the study and complete confidentiality was maintained.

Study setting

This study was conducted at the Mortuary Complex of the Department of Forensic Medicine and Toxicology and Biochemistry laboratory at the Department of Biochemistry, All India Institute of Medical Sciences, Jodhpur

Study design

The proposed study was an autopsy-based prospective study.

Study duration

The study was conducted over a period of 18 months (January 2020 to June 2021).

Study participants

This study is the only human-based study till now. It is the first of its kind on human cadaveric heart tissue samples within a time frame of up to 24 hours since the death.

Sample size

The sample size for the present study was considered to be 16. Consideration of such a sample size is following the fact that only a few studies on animal models are existent relevant to our context. The reference sample size present in previous studies is 6 – 16, so we took the upper end. We analyzed 5 genes at 3 different time points, so we got 15 samples (5x3) per case. Therefore 16 cases, we studied 240 samples (16x15) in 18 months.

Information regarding deceased:

After taking informed consent from the relatives/authorized/nominated person for the participation in the study, all the relevant questions regarding the study were asked to be filled in proforma designed for the study taking care that the investigation is not hampered in any way.

Selection criteria

Inclusion criteria:

i. All medico-legal autopsies with known time since death performed in the mortuary of the Department of Forensic Medicine and Toxicology, All India Institute of Medical Sciences, Jodhpur.
ii. Cases with no previous history of cardiac illness.
iii. Cases with no gross cardiac abnormality detected at the time of autopsy.

Exclusion criteria:

i. Death due to cardiac disease.
ii. Death due to acute pulmonary embolism, pulmonary hypertension, sepsis, a chronic obstructive pulmonary disease with cor-pulmonale or respiratory failure, hyperthyroidism, acute or chronic kidney injury.
iii. All the autopsies in which time since death is not known.
iv. Known cases of cancer or inflammatory disorders.

Material Required:

SAMPLE	EQUIPMENTS	REAGENTS
➢ Cadaver heart tissue	➢ Weighing scale ➢ Centrifuge machine ➢ NanoDrop apparatus ➢ Homogeniser ➢ Gradient PCR ➢ RT-PCR	➢ Phosphate-buffered solution ➢ RNA later solution ➢ Trizol reagent ➢ Chloroform ➢ Isopropanol ➢ SYBR green dye ➢ cDNA synthesis kit ➢ Gene-specific oligonucleotides primers

Cadaver sample and homogenization:

The dissection of the heart was performed on those cases that fulfilled the inclusion criteria, using a sterile scalpel, scissors, forceps, and 10g of right ventricular heart tissue was harvested from each case. After collection, tissue was transported in the universal container containing chilled 1% phosphate-buffered saline (PBS) within 5 minutes to the biochemistry laboratory and homogenized for further nucleic acid extraction. If the nucleic acid extraction was not done immediately, the sample so obtained was immersed in RNA Later solution and then stored at -80 °C.

Sample processing and RNA isolation

The samples were immediately processed for nucleic acid extraction. 100 mg cardiac tissue sample was taken and RNA was isolated. This was labelled as the first sample. Thereafter, 100 mg cardiac tissue samples were harvested 6 hours and 12 hours after the first sample and processed respectively. Total RNA was isolated using Trizol (RNA-XPress™ Reagent, HiMedia Laboratories) as per the manufacturer's instructions. Firstly, the tissue sample was washed with 1% chilled PBS, chopped finely and for 100mg of the right ventricle, heart tissue was homogenized by using 750µl of trizol and incubated for 5 min at room temperature. The homogenate was mixed with 200µl of chloroform, then vigorously shaken for 15 seconds, incubated on ice for 15 minutes, and centrifuged at Relative Centrifugal Force (RCF) 12,000 x g for 15 minutes at 4°C. The upper aqueous layer was transferred in a new tube (~400µl) and equal volume (400µl) chilled isopropanol was added to the aqueous layer, then vigorously shaken for 15 seconds, incubated on ice for 20 minutes, and then centrifuged at RCF 12,000 x g for 12 minutes at 4°C. The resulting supernatant was discarded and the pellet left at the base of Eppendorf, 1ml of 75% chilled ethanol was added to it, vortexed for 5-10 seconds, and then centrifuged at RCF 12,000 x g for 5 minutes at 4°C. The same step was performed twice. The pellet was then dissolved in DEPC treated water (30-100µl) by gentle pipetting.

The quantity and quality of isolated RNA were measured using a multimode plate reader (BioTek Instruments, Inc., Vermont, USA). Extracted RNA samples were quantified at 260/280 and 260/230 ratios of ≥ 1.8 and ≥ 2 respectively, and were considered acceptable for further processing and stored at -80°C until all sample collection was done[16,17,115].

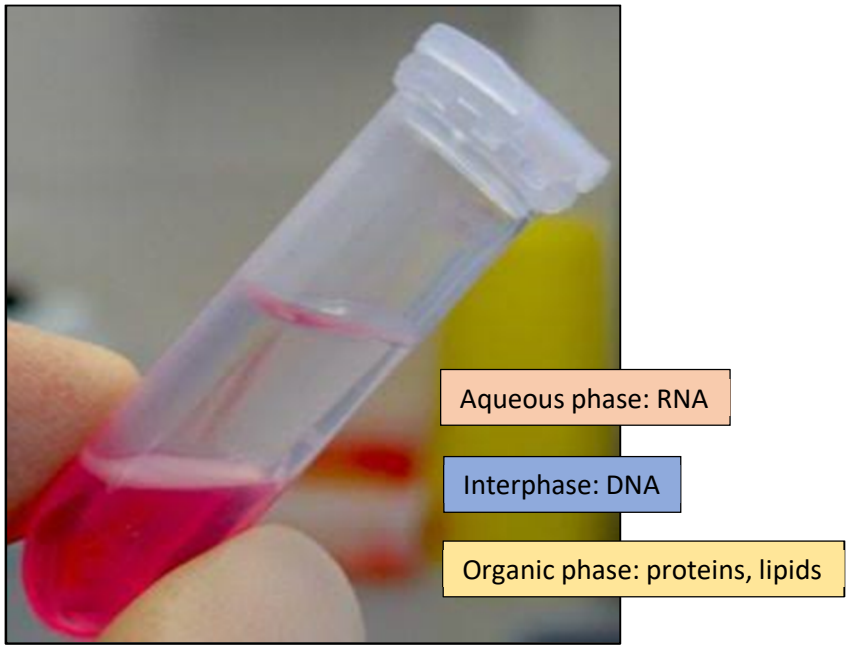


Image 1: The homogenate after incubation with chloroform separated into Aqueous phase, Interphase, and Organic phase.

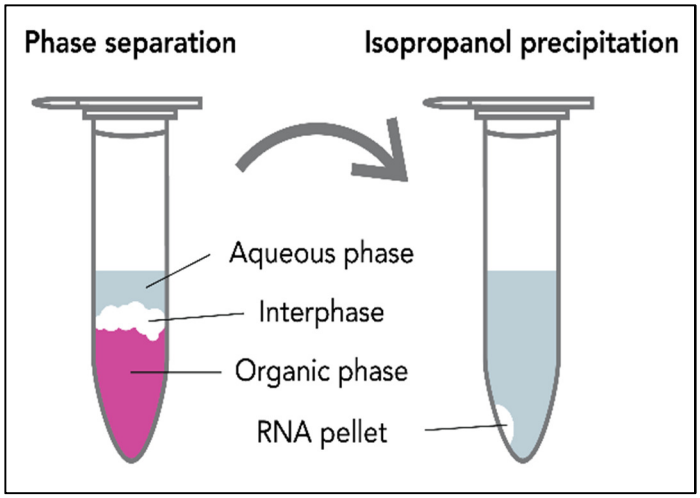


Image 2: Isopropanol precipitation: Aqueous phase transferred to a fresh tube and isopropanol added to it.

Generation of cDNA and quantitative real-time PCR

For the generation of cDNA, 500 ng RNA of each sample of different time intervals was reversed transcribed using BIO-RAD iScript cDNA Synthesis Kit (Catalog n. 1708891) as per the manufacturer's protocol. The total volume of the reaction mixture was 10 µL, the

complete reaction mix was incubated in a thermal cycler following the protocol, and reverse transcription was done at 46°C for 20 minutes[16,115].

Image 3: RNA isolated in separate tubes.

Reverse-transcription reaction components for cDNA conversion

Reagents	**Volume**
5x miScript HiFlex Buffer	2 µl
RNase-free water	Variable
miScript Reverse Transcriptase Mix	1 µl
Template RNA	Variable (upto 1µg)
Total Volume	10 µl

All the mRNA primers for cTnI, NT-proBNP, HMGB1, Bcl2, and GAPDH (housekeeping gene) were designed using NCBI BLAST and purchased from Sigma[17]. The primer sequences used for qRT-PCR of the genes are as follows-

cTnI

forward 5'-GCAAGAAAAAGTTTGAGAGC-3' &

reverse 5'-TTTTTCAGCTCAGAGAGAAG-3',

NT-proBNP

forward 5'-ATTAAGAGGAAGTCCTGGC-3' &

reverse 5'-AAATGAGTCACTTCAAAGGC -3'

HMGB1

forward 5'- TACGAAAAGGATATTGCTGC-3' &

reverse 5'- CTCCTCTTCCTTCTTTTTCTTG-3'

Bcl2

forward 5'- GATTGTGGCCTTCTTTGAG-3' &

reverse 5'- GTTCCACAAAGGCATCC-3'

GAPDH

forward 5'- ACAGTTGCCATGTAGACC-3' &

reverse 5'- TTGAGCACAGGGTACTTTA-3'

The SsoAdvanced Universal SYBR® Green PCR Kit (Biorad, cat. no. 1725270) was used as per the manufacturer's instructions. The kit included 2x QuantiTect SYBR Green PCR Master Mix, 10x miScript Universal Primer, 10x miScript Primer Assay, and RNase-free water. In the case of mRNA detection, the miScript Universal Primer was used as per the manufacturer's instructions. All the reagents and cDNA samples were thawed at 4°C after removing from -20°C and a reaction master mix was prepared according to the following specifications:

Image 4: Preparation of Master mix for PCR

Reaction setup for real-time PCR

Reagents	**Volume**
2x QuantiTect SYBR Green PCR Master mix	5 µl
10x miScript Universal Primer	1 µl
10x miScript Primer Assay	1 µl
RNase free water	Variable
Template cDNA	≤1 µl
Total volume	10 µl

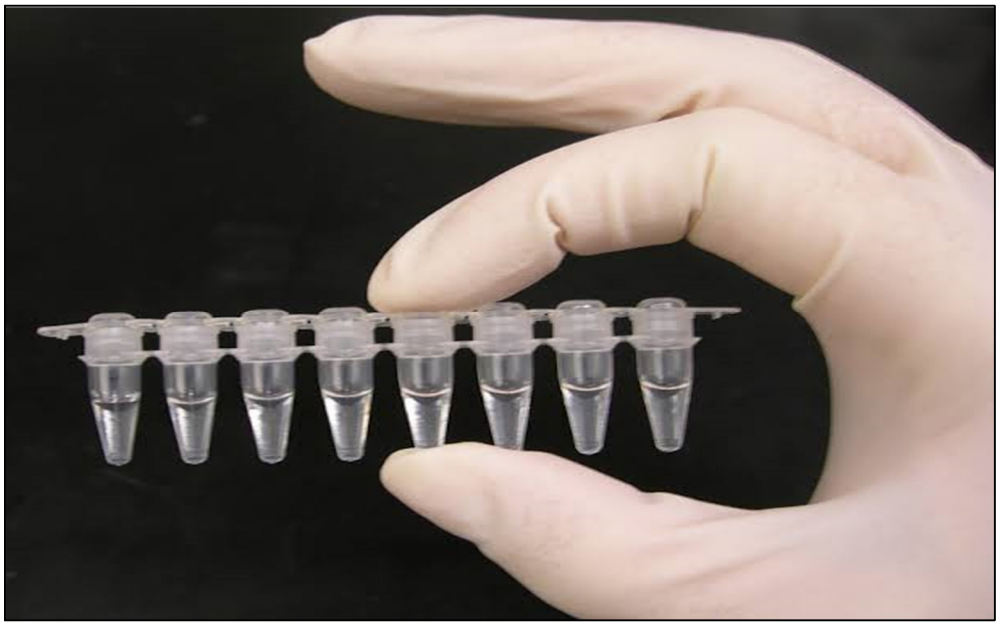


Image 5: PCR tubes with the reaction mixture.

The template cDNA was dispensed into the individual wells of the PCR strips and then a thoroughly mixed reaction mix was added into the cDNA containing wells of PCR strips. To detect mRNA levels in the samples; real-time PCR reaction was carried out by placing the reaction strips in an automated and temperature-controlled cycle of denaturation, annealing, and elongation using a thermal cycler. The thermal cycle parameters were set as follows:

Steps	Temperature	Time
Initial activation step	95 °C	15 min
3-step cycling Denaturation Annealing	 94 °C 55 °C	 15s 30s
Extension Cycle number	70°C 40 cycles	30s

All quantitative real-time polymerase chain reaction (qRtPCR) amplification and expression analyses were performed using CFX96 Real-Time System and CFX Manager Software (Bio-Rad, California, USA). All Real-time PCR was performed using SYBR Green qPCR Kit according to the manufacturer's instructions using Thermo Scientific DyNAmo Color Flash. Melt curves (55°C-65°C) were generated to confirm the specific gene amplification and each sample was run at least in duplicates. For each primer used to optimize the annealing temperature gradient, PCR was done. Real-time PCR was performed under the following conditions: PCR initial activation at 95 °C for 2 minutes followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 56°C (for all genes) for 60 seconds. The Ct values of cDNA were normalized by using the mean cycle threshold (Ct) values of the GAPDH gene which was used as an internal housekeeping gene ($\Delta Ct = CtcDNA_{target} - CtcDNA_{Housekeeping\ gene}$[115]. For gene expression, data were analyzed using the $\Delta\Delta Ct$ method for relative quantification.

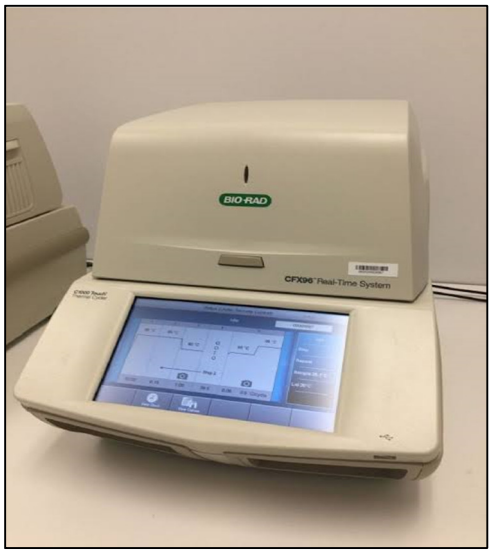
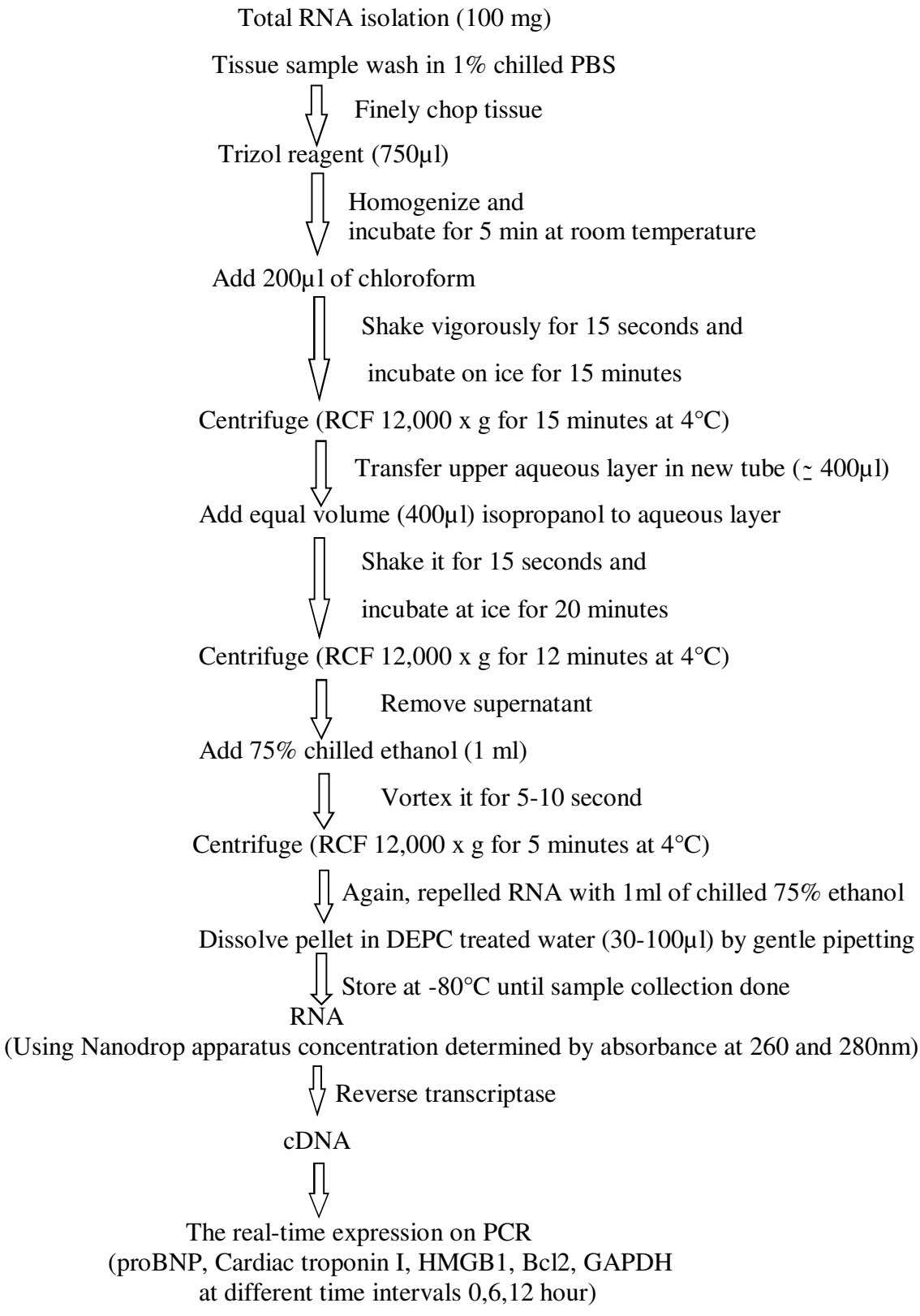


Image 6: Bio-Rad CFX 96 Thermal cycler (real-time PCR System)

Flowchart:



Flowchart 4: Flowchart depicting the sample collection, processing, RNA isolation, and real-time PCR for heart tissue for estimation of PMI.

Data analysis:

Livak's method was used for FCE analysis and GAPDH was used for the normalization of target gene expression. In the current study, we normalized the mean cycle threshold (Ct) values for cardiac-specific genes (NT-proBNP, cTnI) and autophagy genes (HMGB1, Bcl2) with the GAPDH gene as an internal housekeeping gene for fold change expression by using the ΔΔCt method for relative quantification and exploring the pattern of gene fold expression at a different time interval.[115,116]

Steps followed for ΔΔCt analysis

• The average of the Ct values for the housekeeping gene and the gene being tested (T) in all the samples was obtained through RT-qPCR.

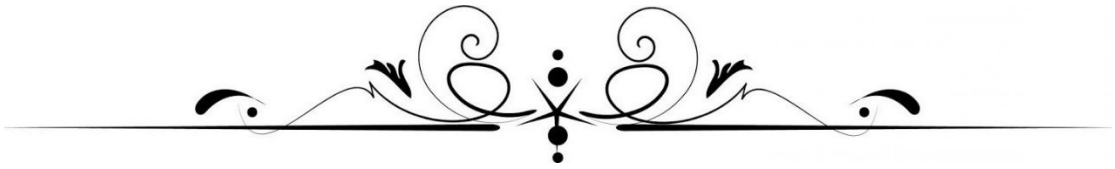
• The differences between Tested Experimental and housekeeping gene and Control and housekeeping Gene were calculated, through which ΔCt values for the experimental (ΔCTE) and control (ΔCTC) were obtained respectively.

• The difference between ΔCTE and ΔCTC was used to calculate the Double Delta

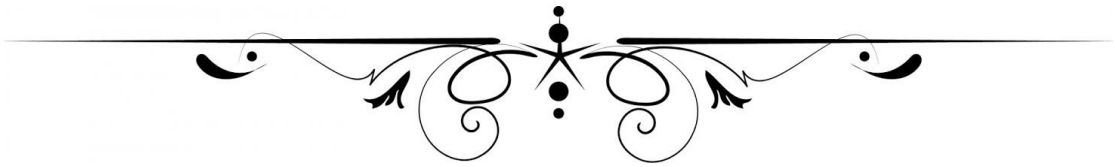
Ct Value (ΔΔCt).

• Since all the calculations were made in logarithm base 2, and every cycle there is twice as much DNA, therefore the value of 2^-ΔΔCt was calculated to get the FCE of the gene

The study data were analyzed by using an independent t-test in the latest version (1.0.0.1406) of Statistical Package for the Social Sciences (SPSS) software.



RESULTS



Results

The data obtained after the completion of qRT-PCR was expressed in cycle threshold value (Ct). The datasheet includes the Ct value of cardiac-specific biomarkers of the assessed gene (AG) NT-proBNP, cTnI, HMGB1, Bcl2, and internal housekeeping gene GAPDH at different time points. The Ct values of RNA of NT-proBNP, cTnI, HMGB1, and Bcl2 were normalized by using the mean cycle threshold (Ct) values of the GAPDH gene which was used as an internal housekeeping gene (HG) from the same sample of the same time point.

Formula template of ΔΔCt analysis(115)**:**

$$\Delta Ct_{(sample)} = \Delta Ct_{(AG)} - \Delta Ct_{(HG)}$$

$$\Delta\Delta Ct = \Delta Ct_{(experimental)} - \Delta Ct_{(control)}$$

$$FCE = 2^{-\Delta\Delta Ct}$$

ΔCt Value (Experimental)	**ΔCt Value (Control)**	**Delta Delta Ct Value**	**Fold Change Expression**
ΔCTE	**ΔCTC**	**ΔΔCt**	**FCE**
$\Delta Ct_{(E)} = \Delta Ct_{(AG)} - \Delta Ct_{(HG)}$	$\Delta Ct_{(C)} = \Delta Ct_{(AG)} - \Delta Ct_{(HG)}$	ΔCTE- ΔCTC	$2^{-\Delta\Delta Ct}$

In total, 240 samples were processed and analyzed. These were then grouped into the following seven groups 0-6 hrs, 6-9 hrs, 9-12 hrs, 12-15 hrs,15-18 hrs,18-21 hrs, 21-24 hrs, where 0 hour was taken as the time of death. Data were statistically evaluated by the ΔΔCt method. The t-test for independent variables was applied on the delta Ct values to obtain the p values. Statistical significance was considered for p values <0.05.

Cardiac troponin I (cTnI)

PCR was done for the cTnI gene and the following graphs for gene amplification, melt curve and melt peak were obtained.

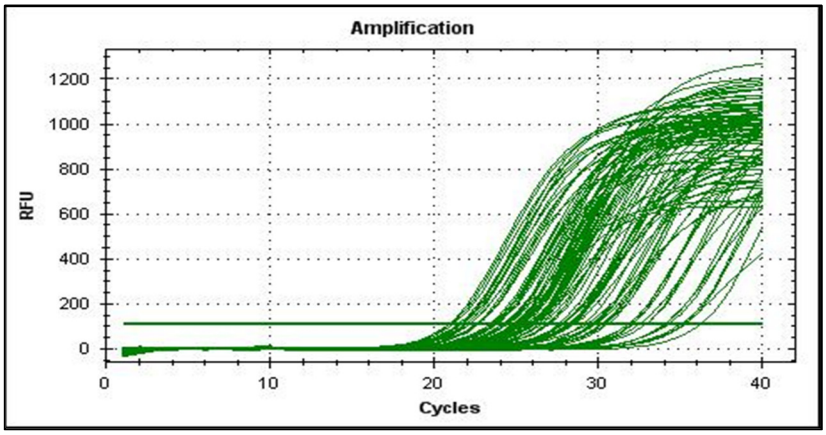


Figure 1: Gene amplification curve for cTnI gene.

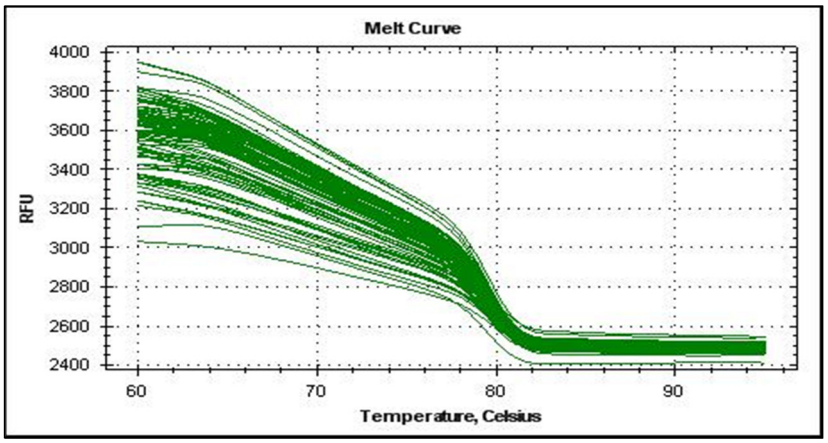


Figure 2: Melt curve for cTnI gene.

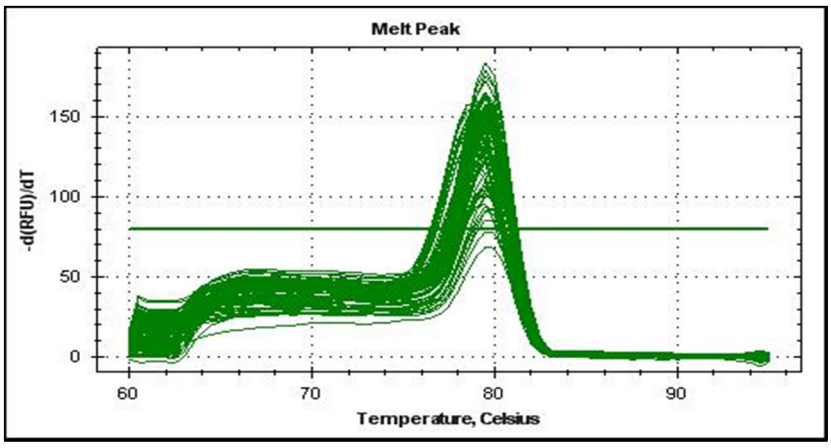


Figure 3: Melt peak curve for cTnI gene.

On plotting the average Ct value of cTnI graphically at different time intervals, a continuous increase in the levels of cardiac troponin I up to 15-18 hours and then decrease up to 24 hours of PMI was observed (Figure 4). The average ct value of cardiac troponin I at different time groups are shown in table 2 below.

Groups	Group 1 (0-6 h)	Group 2 (6-9 h)	Group 3 (9-12 h)	Group 4 (12-15 h)	Group 5 (15-18 h)	Group 6 (18-21 h)	Group 7 (21-24 h)
Average ΔCt value	-1.57	-1.52	-1.45	-1.42	-1.33	-1.56	-1.79

Table 2: Average ct value of cardiac troponin I at different time groups.

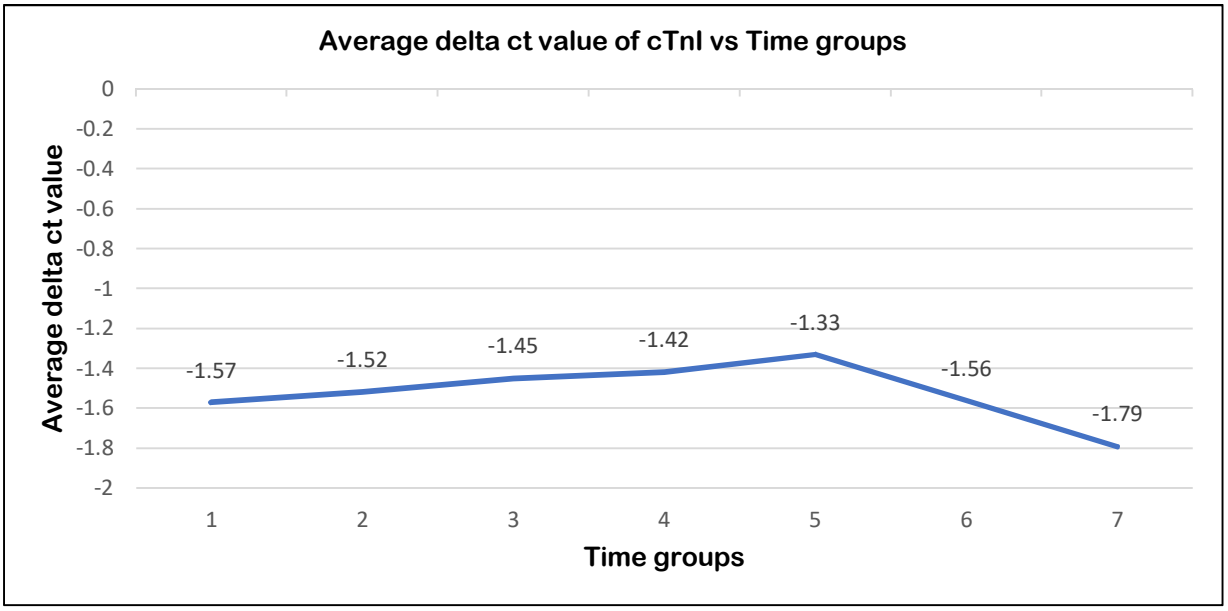


Figure 4: The ΔCt value of the cTnI gene of the cardiac muscle at different time groups

The fold change expression (FCE) of cardiac troponin I by ΔΔCt method at different time intervals showed that the FCE of cTnI was almost stable till 15 hours of PMI and then after 15 hours, it showed the downregulation up to 24 hours after the death. The values of delta delta ct (ΔΔCt) and FCE with PMI are shown in table 3 and figure 5.

ΔΔCt Value $\Delta\Delta Ct = \Delta Ct_{(E)} - \Delta Ct_{(C)}$	$\Delta Ct_{(G2)} - \Delta Ct_{(G1)}$	$\Delta Ct_{(G3)} - \Delta Ct_{(G2)}$	$\Delta Ct_{(G4)} - \Delta Ct_{(G3)}$	$\Delta Ct_{(G5)} - \Delta Ct_{(G4)}$	$\Delta Ct_{(G6)} - \Delta Ct_{(G5)}$	$\Delta Ct_{(G7)} - \Delta Ct_{(G6)}$
Delta delta ct value	0.05	0.07	0.03	0.09	-0.23	-0.23
Fold Change Expression (2^-ΔΔCt)	1.03	1.04	1.02	1.06	0.85	0.85

Table 3: Values of ΔΔCt and fold change expression of cTnI at different time groups.

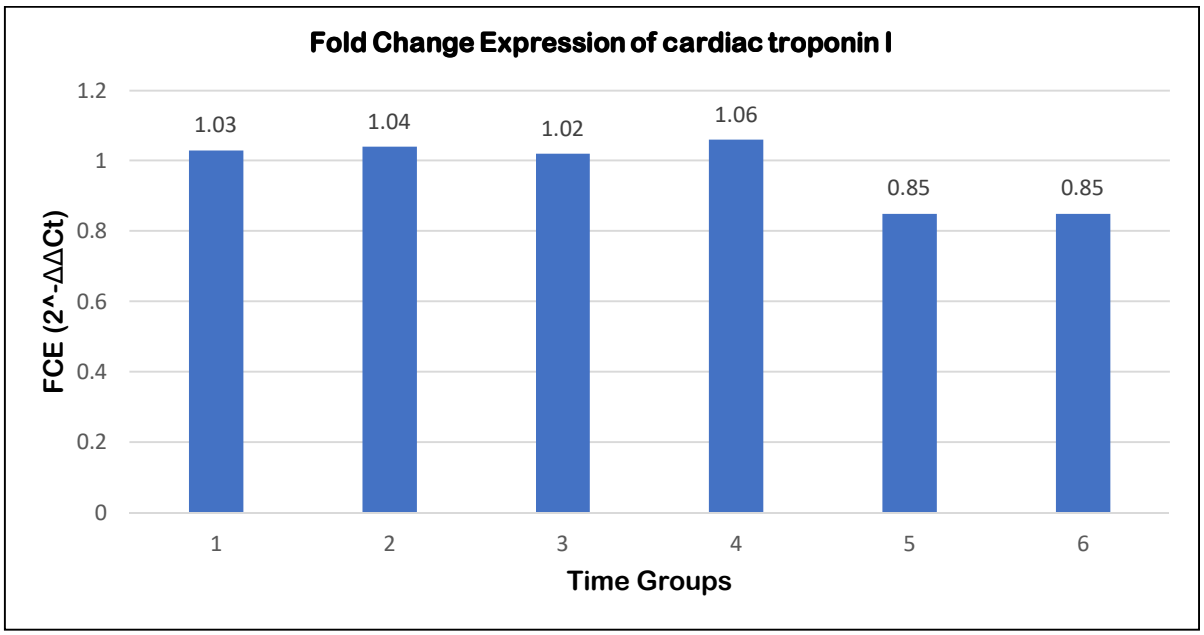


Figure 5: Fold change expression by ΔΔCt of cTnI value with PMI

N-terminal pro-B-type natriuretic peptide (NT-proBNP)

PCR was done for the NT-proBNP gene and the following graphs for gene amplification, melt curve and melt peak were obtained.

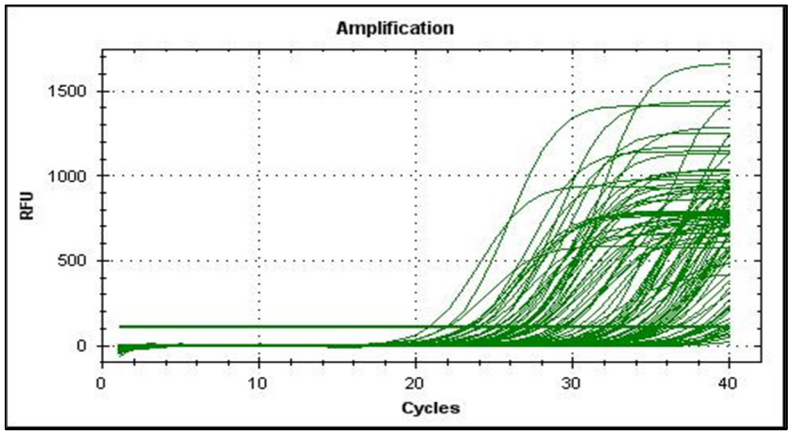


Figure 6: Gene amplification curve for NT-proBNP gene.

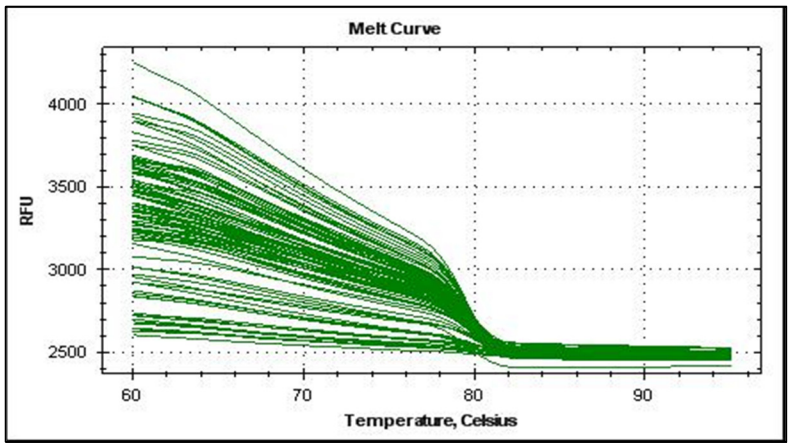


Figure 7: Melt curve for NT-proBNP gene.

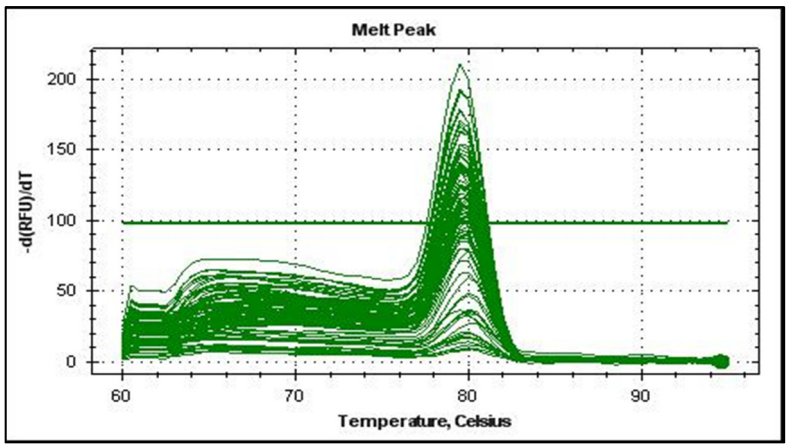


Figure 8: Melt peak curve for NT-proBNP gene.

The plotting of average Ct value of NT-proBNP graphically at different time intervals showed that there was a linear increase in the levels of NT-proBNP up to 12 to15 hours of PMI followed by a drop at 15 to18 hours of PMI, and continuously decreased up to 24 hours after death (Figure 9). The average Ct value of NT-proBNP is shown in table 4 below.

Groups	Group 1 (0-6 h)	Group 2 (6-9 h)	Group 3 (9-12 h)	Group 4 (12-15 h)	Group 5 (15-18 h)	Group 6 (18-21 h)	Group 7 (21-24 h)
Average ΔCt value	1.62	2.21	2.84	3.42	3.14	2.03	0.55

Table 4: Average Ct value of NT-proBNP at different time groups.

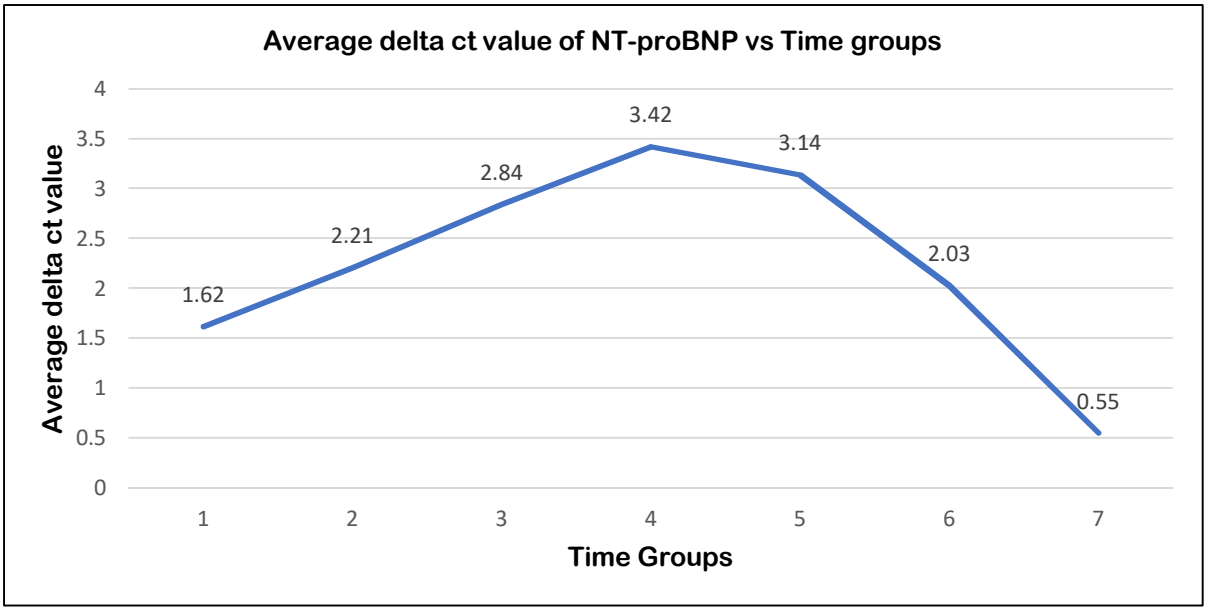


Figure 9: The ΔCt value of the NT-proBNP gene of the cardiac muscle in the time interval

The FCE of NT-proBNP by ΔΔCt method at different time intervals showed that FCE was stable till 12 hours after death and then after 12 hours of PMI, it showed the downregulation up to 24 hours after the death. The values of delta delta Ct (ΔΔCt) and FCE are shown in table 5 and figure 10.

ΔΔCt Value $\Delta\Delta Ct = \Delta Ct_{(E)} - \Delta Ct_{(C)}$	$\Delta Ct_{(G2)} - \Delta Ct_{(G1)}$	$\Delta Ct_{(G3)} - \Delta Ct_{(G2)}$	$\Delta Ct_{(G4)} - \Delta Ct_{(G3)}$	$\Delta Ct_{(G5)} - \Delta Ct_{(G4)}$	$\Delta Ct_{(G6)} - \Delta Ct_{(G5)}$	$\Delta Ct_{(G7)} - \Delta Ct_{(G6)}$
Delta delta ct value	0.59	0.63	0.58	-0.28	-1.11	-1.48
Fold Change Expression (2^-ΔΔCt)	1.50	1.54	1.49	0.82	0.46	0.35

Table 5: Values of ΔΔCt and fold change expression of NT-proBNP at different time groups.

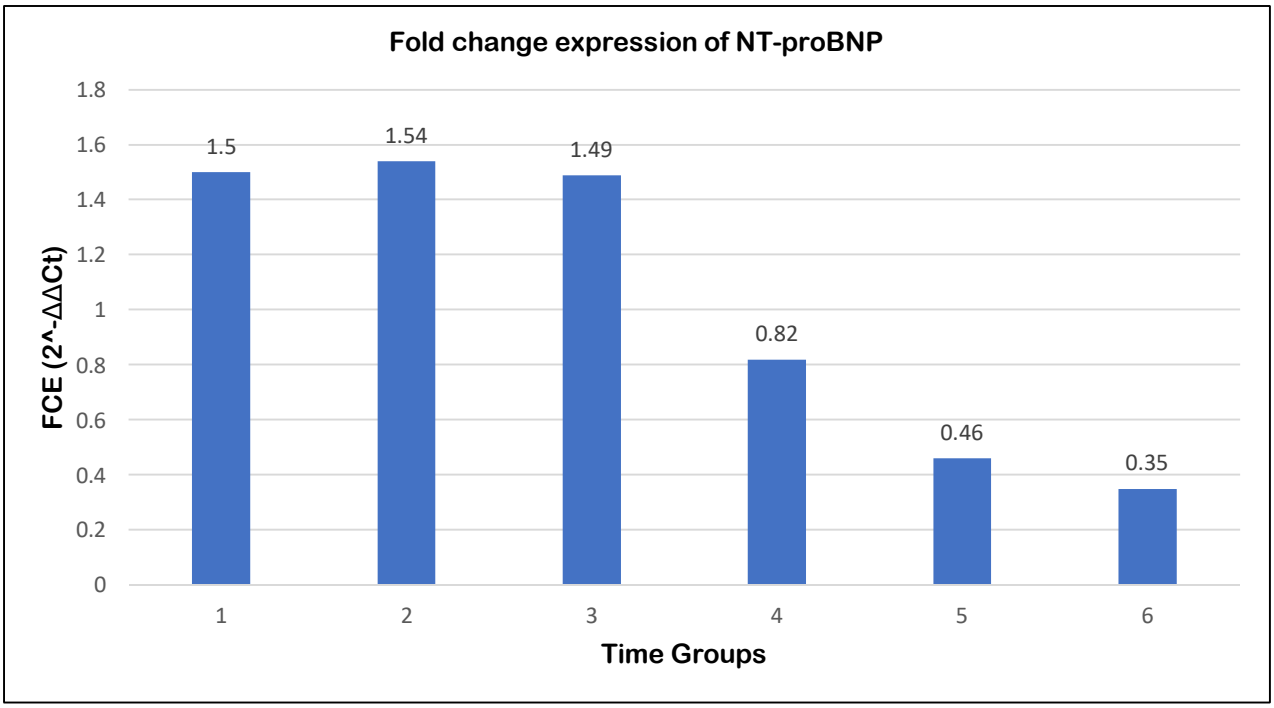


Figure 10: Fold change expression by ΔΔCt of NT-proBNP value with PMI

High-mobility group box 1 (HMGB1)

PCR was done for the HMGB1 gene and the following gene amplification, melt curve and melt peak was obtained.

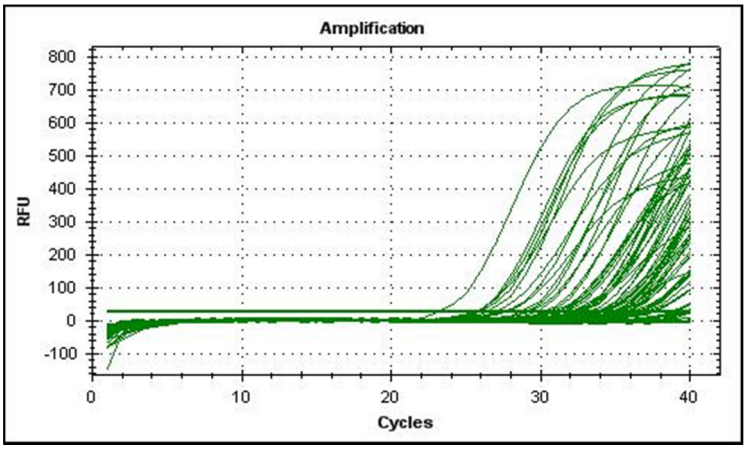


Figure 11: Gene amplification curve for HMGB1 gene.

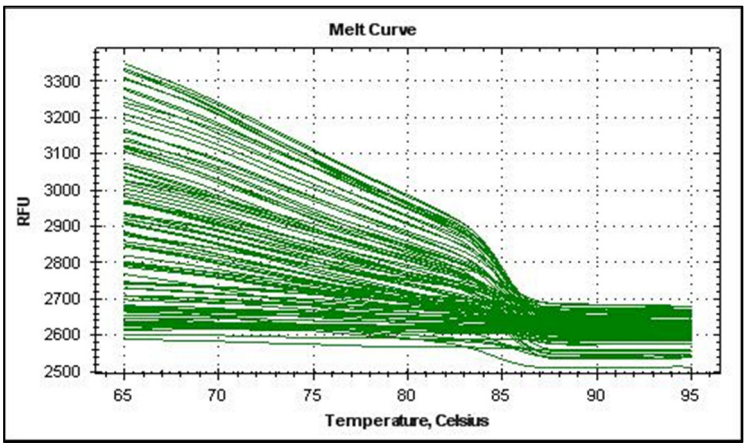


Figure 12: Melt curve for HMGB1 gene.

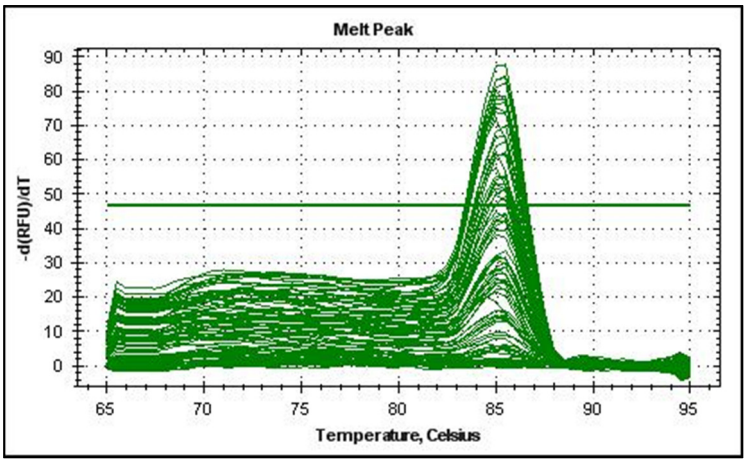


Figure 13: Melt peak curve for HMGB1 gene.

On plotting the average Ct value of the HMGB1 gene graphically at different time intervals, it showed that there was a linear decrease in the levels of HMGB1 up to 24 hours after death (Figure 14). The average Ct value of HMGB1 is shown in table 6 below.

Groups	Group 1 (0-6 h)	Group 2 (6-9 h)	Group 3 (9-12 h)	Group 4 (12-15 h)	Group 5 (15-18 h)	Group 6 (18-21 h)	Group 7 (21-24 h)
Average ΔCt value	6.57	4.71	3.65	2.52	1.35	1.00	0.95

Table 6: Average Ct value of HMGB1 at different time groups.

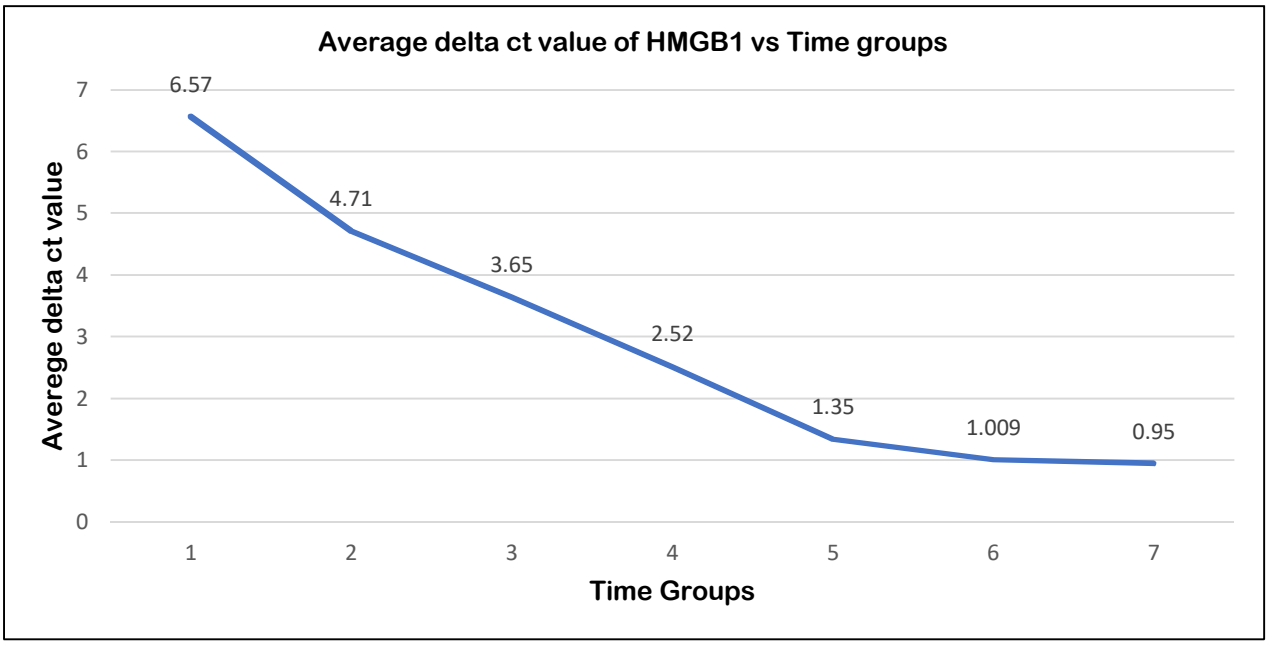


Figure 14: The ΔCt value of the HMGB1 gene of the cardiac muscle in the time interval

The fold change expression of HMGB1 by ΔΔCt method at different time intervals showed that FCE was upregulated in the first 6-9 hours after death and then almost stable till 15-18 hours and then again showed upregulation up to 24 hours after the death. The values of ΔΔCt and fold change expression are shown in table 7 and figure 15.

ΔΔCt Value $\Delta\Delta Ct = \Delta Ct_{(E)} - \Delta Ct_{(C)}$	$\Delta Ct_{(G2)} - \Delta Ct_{(G1)}$	$\Delta Ct_{(G3)} - \Delta Ct_{(G2)}$	$\Delta Ct_{(G4)} - \Delta Ct_{(G3)}$	$\Delta Ct_{(G5)} - \Delta Ct_{(G4)}$	$\Delta Ct_{(G6)} - \Delta Ct_{(G5)}$	$\Delta Ct_{(G7)} - \Delta Ct_{(G6)}$
Delta delta ct value	-1.86	-1.06	-1.13	-1.17	-0.34	-0.06
Fold Change Expression (2^-ΔΔCt)	0.27	0.47	0.45	0.44	0.78	0.95

Table 7: Values of ΔΔCt and fold change expression of HMGB1 at different time groups.

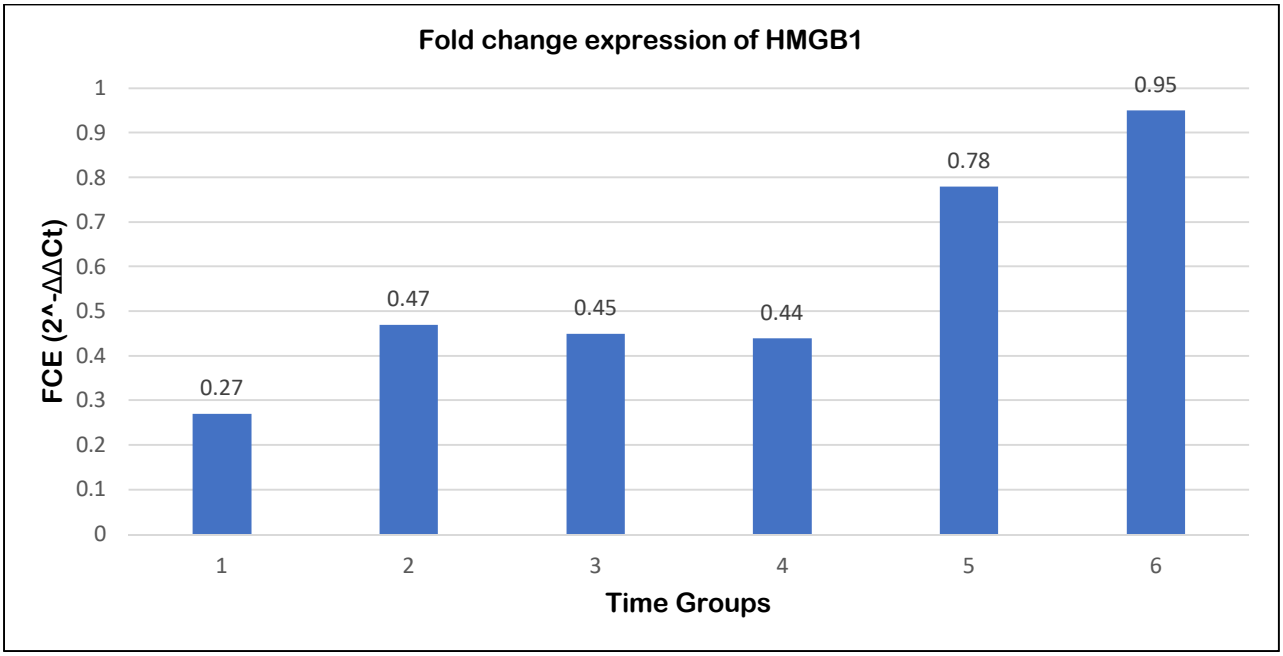


Figure 15: Fold change expression by ΔΔCt of HMGB1 value with PMI

B cell lymphoma 2 (Bcl2)

Bcl2 shows no Ct value at any point of time after death, so no fold change gene expression was observed at any point of time.

Comparison of gene expression with PMI.

GENE	Heart tissue
NT-proBNP	FCE is stable till 12 hours PMI and then shows downregulation up to 24 hours PMI.
cTnI	FCE is almost stable till 15 hours of PMI and then shows downregulation up to 24 hours PMI.
HMGB1	FCE is upregulated in the first 6-9 hours after death and then almost stable till 15-18 hours and then again shows upregulation up to 24 hours PMI.
Bcl2	No gene expression was observed at any point of time
GAPDH	Internal Housekeeping gene

Table 8: Gene expression with PMI.

Independent t-test

Independent t-test was applied on the delta Ct values of NT-proBNP, cTnI, and HMGB1with GAPDH and the result was found to be significant (p-value <0.0001 respectively) at different times intervals.

Gene	t- value	p-value	Significant or not
CTnI	-35.27705	< .00001	Significant
NT-proBNP	-26.00456	< .00001	Significant
HMGB1	-34.27705	< .00001	Significant
BCL2	Nil	Nil	Nil

Table 9: P and T value of different genes.

Pattern and percentage of RNA degradation and gene expression with PMI

The percentage degradation of cTnI, NT-proBNP, and HMGB1 as drawn from Ct value w.r.t PMI is shown in fig. 16, 17, and 18.

Cardiac troponin I (cTnI)

The result indicates that cTnI degraded about 15% in the first 6 hours of PMI and then degrade almost 13% to 17% every next 3 hours till 24 hours of PMI at room temperature.

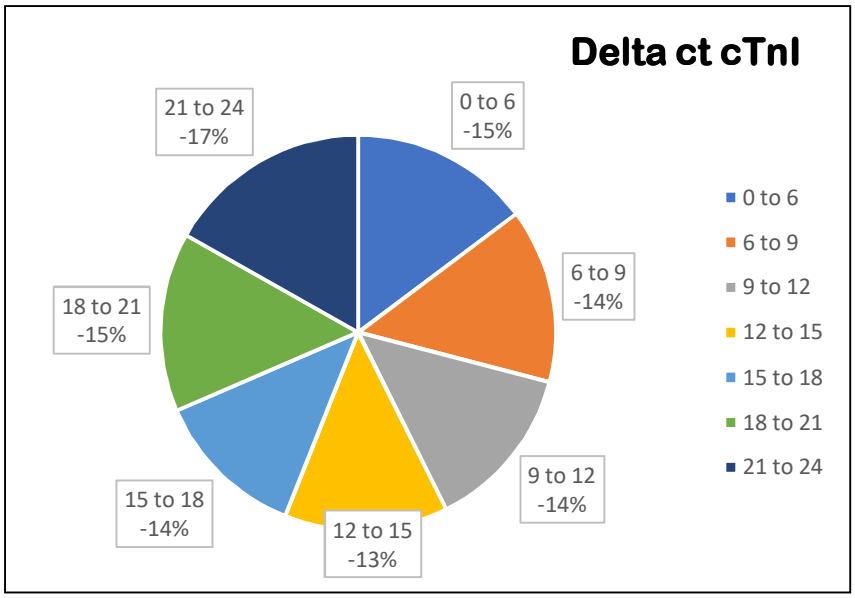


Figure 16: Pie chart showing ΔCt value of cTnI w.r.t PMI

N-terminal pro-B-type natriuretic peptide (NT-proBNP)

The result indicates that NT-proBNP degraded about 10 % in the first 6 hours after death and then degrade almost 13% to 22% in every next 3 hours till 21 hours of PMI, and 3% at 21 to 24 hours of PMI at room temperature.

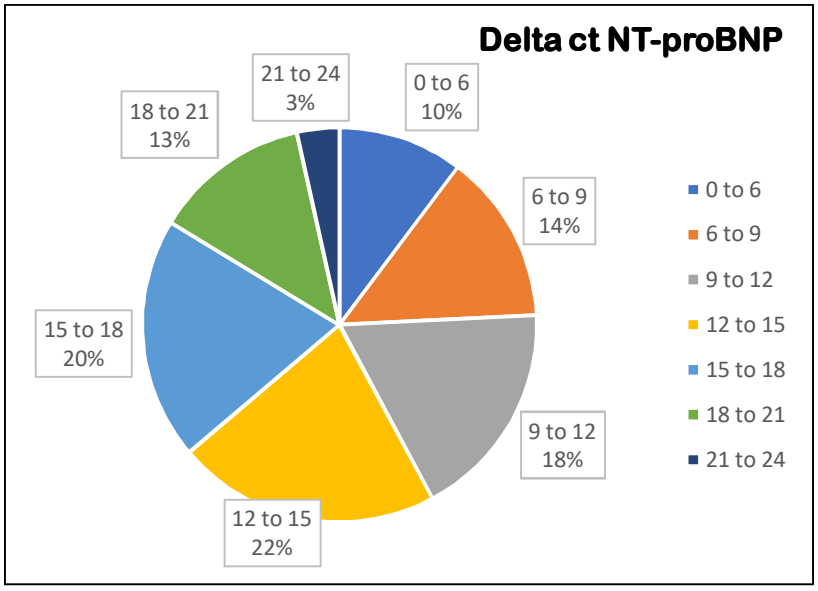


Figure 17: Pie chart showing ΔCt value of NT-proBNP w.r.t PMI

High-mobility group box 1 (HMGB1)

The result indicates that HMGB1degraded about 32% in the first 6 hours after death and then degrade almost 12% to 21% in every next 3 hours till 15 hours of PMI, and 5% to 7% at in every next 3 hours till 24 hours of PMI at room temperature.

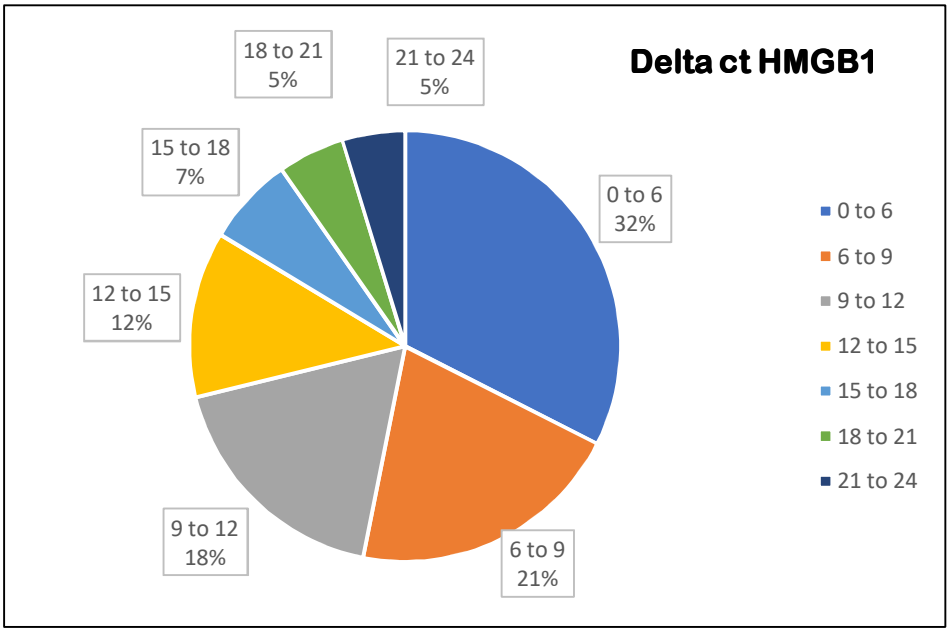
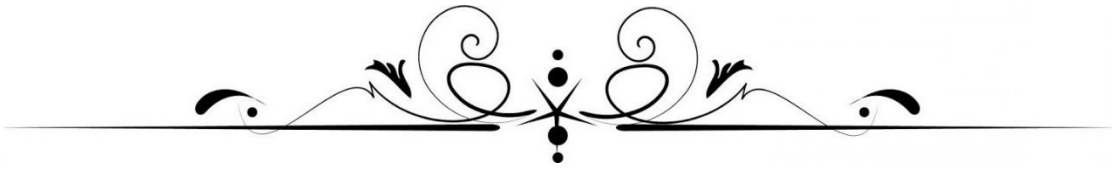
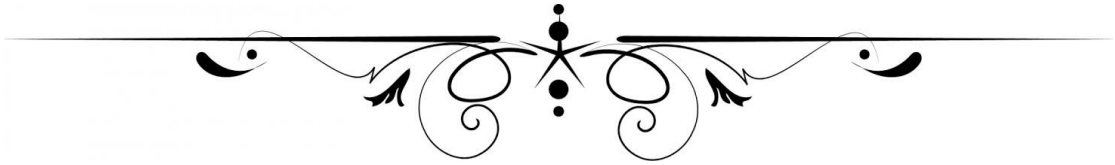


Figure 18: Pie chart showing ΔCt value of HMGB1 w.r.t PMI



DISCUSSION



Discussion

One of the most persistent questions in forensic medicine is how to estimate the time since death. If it is accurately estimated, then a lot of controversies can be resolved. The goal of this work was to find out how to calculate PMI by looking at gene expression in cardiac-specific and autophagy genes. Our study is the first of its kind in human cadavers for estimating PMI using cardiac-specific (NT-pro-BNP and cTnI) and autophagy (HMGB1 and Bcl2) genes.

In the present study, human cadaveric heart tissue was used and the approach behind it was to assess the FCE of the cardiac muscle-specific genes (cTnI, NT-proBNP) and autophagy genes (HMGB1 and Bcl2) at different time intervals (0,6,12 hours) to calculate the time since death. The gene expression of cardiac-specific and autophagy genes was normalized using the mean cycle threshold (Ct) values for NT-proBNP and cTnI genes with the GAPDH gene as an internal housekeeping gene. For FCE, the ΔΔCt method for relative quantification was used and explored the pattern of FCE at a different time interval[115]. Sustained research work has been carried out in the past to estimate the PMI by analyzing the cardiac biomarkers cTnI and NT-proBNP, however very few studies have been carried out on HMGB1 and Bcl2 on the animal model. Blood serum and pericardial fluid have been used as samples in these studies to compare the antemortem and postmortem values of these biomarkers. The analysis shows a concordant trend of postmortem cTnI with PMI in sudden cardiac death up to 12 hours but no successful correlation could be drawn between the antemortem and postmortem levels of the same[7,117,118]. There is no reported study on human cTnI and ProBNP. The only reported study to estimate the PMI using protein marker cardiac troponin I was conducted on non-human (bovine) cadaver heart tissue on six samples and it showed the proportion of cTnI and the log of time since death have a pseudo linear connection ($r > 0.95$)[8]. The ΔCt value of the cTnI gene of the cardiac muscle showed almost equal degradation at equal time intervals correlated with PMI within 0 to 12 hours at room temperature[119]. Our study is the only human-based study till now and the result indicates that cTnI degraded about 15% in the first 6 hours of PMI and then degrade almost 13% to 17% every next 3 hours till 24 hours of PMI at room temperature and continuous increase in the gene expression of cardiac troponin I up to 15-18 hours and then decreases up to 24 hours of PMI was observed. The FCE of cardiac troponin I by ΔΔCt method at different time intervals shows that the FCE of cTnI was almost

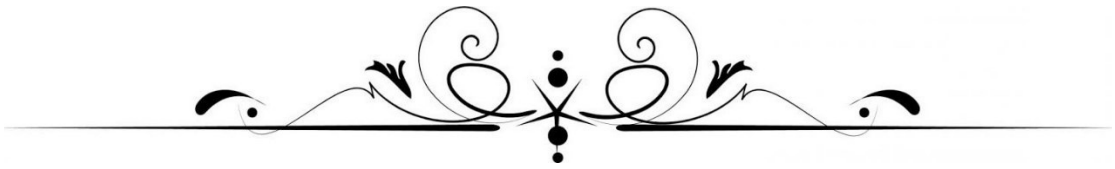
stable till 15 hours of PMI and then after 15 hours, it showed the downregulation up to 24 hours after death.

The NT-proBNP degraded about 10 % in the first 6 hours after death and then degrade almost 13% to 22% in every next 3 hours till 21 hours of PMI, and 3% at 21 to 24 hours of PMI at room temperature, and also showed that there was a linear increase in the levels of NT-proBNP up to 12 to15 hours of PMI followed by a drop at 15 to18 hours of PMI, and continuously decreased up to 24 hours after death. The FCE of NT-proBNP by ΔΔCt method at different time intervals showed that FCE was stable till 12 hours after death and then after 12 hours of PMI it showed the downregulation up to 24 hours after death.

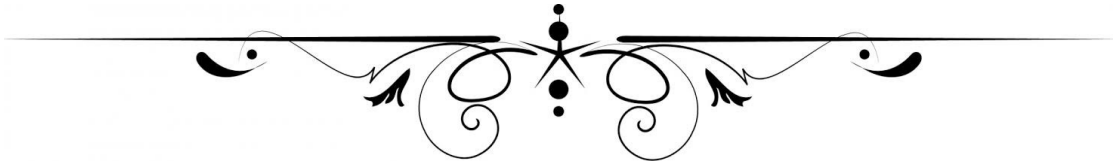
Preliminary animal studies have been suggestive that HMGB1 can be a useful marker for estimating PMI[12,16]. The role of HMGB1 in autophagy and carcinogenesis has been also extensively studied. During autophagy, it is released and facilitates the process of autophagy. Usually, the amount of HMGB1 increases with an increase in the number of dying cells[12]. The current study result indicates that HMGB1degraded about 32% in the first 6 hours after death and then degrade almost 12% to 21% in every next 3 hours till 15 hours of PMI, and 5% to 7% in every next 3 hours till 24 hours of PMI at room temperature and also showed that there was a linear decrease in the levels of HMGB1 up to 24 hours after death. The fold change expression of HMGB1 by ΔΔCt method at different time intervals showed that FCE was upregulated in the first 6-9 hours after death and then almost stable till 15-18 hours and then again showed upregulation up to 24 hours after death.

The postmortem gene expression of apoptosis-related gene Bcl2 was obtained in the heart tissue of a human cadaver using RtPCR and normalized the mean cycle threshold (Ct) values with the GAPDH. Bcl2 is known to be an important regulator of apoptosis. After death, tissue anoxia occurs due to the absence of blood flow which promotes programmed cell death or apoptosis. As Bcl2 is an anti-apoptotic gene, it inhibits the programmed cell death via a binding outer mitochondrial membrane and prevents the release of mitochondrial cytochrome c into the cytosol, which is essential for the formation of the apoptosome resulting in inhibition of apoptosis. In the past, there was one non-human study conducted on the liver of mice that showed that the Bcl2 gene is time-dependent in expression level from 3 to 24 hours after death[16,120]. The current study observes that Bcl2 shows no gene expression in the heart tissue after at any point of time till 24 hours after death, which is possibly due to its anti-apoptotic action.

The current study is significant since it is the first of its kind on human cadaveric tissue samples for estimation of postmortem interval using cardiac-specific and autophagy genes, whereas earlier reports are on an animal model, which showed promising results if used for the estimation of time since death. The profound difference between the earlier reports and current study findings is that the animal model study was conducted in test conditions in the laboratory where they had full control over the time of death to analysis as well as the surrounding environment. On the contrary, the current study as mentioned earlier is done in human cadavers coming for medico-legal autopsies, where we had no control over the conditions of or cause of death as well as the surrounding environment. This makes the current report more applicable in real-life scenarios[120,121]. The objectivity and reliability of this scientific method provide a stronger value in comparison to physical methods and when applied properly it can be more reliable as compared to other methods, where until now there is only a rough estimate of time of death based on algor mortis, rigor mortis or other gross shreds of evidence. The uniqueness of exploring such new methods lies in their accuracy and objectivity in estimating PMI and these genes may be a robust marker for estimation of time since death in medico-legal cases.



CONCLUSION



Conclusion

- In this study, we determined the patterns, gene expression, and serial estimation of cardiac tissue-specific genes (NT-proBNP, cTnI) and autophagy genes (HMGB1, Bcl2) to determine PMI.
- The Ct value was obtained was assessed using RT-PCR.
- The Ct values of genes NT-proBNP, cTnI, HMGB1, and Bcl2 were normalized by using the mean cycle threshold (Ct) values of the GAPDH gene which was used as an internal housekeeping gene from the same sample of the same time point.
- The data was analyzed using the value of average delta ct (ΔCt) value of assessed gene and housekeeping gene, and delta delta ct (ΔΔCt) value to calculate the fold change expression at the different 7-time group (0-6 hours, 6-9 hours, 9-12 hours, 12-15 hours,15-18 hours,18-21 hours, 21-24 hours).
- The cTnI degraded about 15% in the first 6 hours of PMI and then degrade almost 13% to 17% every next 3 hours till 24 hours of PMI at room temperature.
- The continuous increase in the Ct values of cTnI up to 15-18 hours and then decreases up to 24 hours of PMI was observed.
- The FCE of cTnI was almost stable till 15 hours of PMI and then after 15 hours, it showed the downregulation up to 24 hours after death.
- The NT-proBNP degraded about 10 % in the first 6 hours after death and then degrade almost 13% to 22% in every next 3 hours till 21 hours of PMI, and 3% at 21 to 24 hours of PMI at room temperature.
- The linear increase in the Ct values of NT-proBNP up to 12 to15 hours of PMI was followed by a drop at 15 to18 hours of PMI and continuously decreased up to 24 hours after death.
- The FCE of NT-proBNP was stable till 12 hours after death and then after 12 hours of PMI it showed the downregulation up to 24 hours after death.
- The HMGB1degraded about 32% in the first 6 hours after death and then degrade almost 12% to 21% in every next 3 hours till 15 hours of PMI, and 5% to 7% at in every next 3 hours till 24 hours of PMI at room temperature.
- The linear decrease in the Ct values of HMGB1 up to 24 hours after death.
- The FCE was upregulated in the first 6-9 hours after death and then almost stable till 15-18 hours and then again showed upregulation up to 24 hours after death.

- The Bcl2 shows no gene expression in the heart tissue after at any point of time till 24 hours after death.
- Independent t-test was applied on the delta Ct values of NT-proBNP, cTnI, and HMGB1with GAPDH and the result was found to be significant (p-value <0.0001 respectively) at different times intervals.
- The current study is significant since it is the first of its kind on human cadaveric tissue samples for estimation of PMI using cardiac-specific and autophagy genes

Using the major mRNA markers identified here, we found fresh possibilities for molecular techniques to early post-mortem interval assessment. These results indicated that pattern, fold change expression of genes cTnI, NT-proBNP, and HMGB1 can be used to determine PMI and these all genes are feasible for PMI estimation. This makes the current report more applicable in real-life scenarios However, Bcl2 was not useful for determining PMI because it shows no expression till 24 hours after death. The objectivity and reliability of this scientific method provide a stronger value in comparison to physical methods and when applied properly it can be more reliable as compared to other methods, where until now there is only a rough estimate of time of death based on algor mortis, rigor mortis or other gross shreds of evidence. The uniqueness of exploring such new methods lies in their accuracy and objectivity in estimating PMI and these genes may be a robust marker for estimation of time since death in medico-legal cases. More studies involving a larger sample size will better establish the practicality of post mortem interval.

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ANNEXURES

Annexures

Ethical clearance certificate

ICMR Award Letter

ICMR Financial Grant Letter

1. Information sheet in English
2. Information sheet in Hindi
3. Consent form English
4. Consent form in Hindi
5. Data collection sheet

Ethical clearance certificate

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर
All India Institute of Medical Sciences, Jodhpur
संस्थागत नैतिकता समिति
Institutional Ethics Committee

No. AIIMS/IEC/2020/2021 **Date: 01/01/2020**

ETHICAL CLEARANCE CERTIFICATE

Certificate Reference Number: **AIIMS/IEC/2019-20/944**

Project title: **"A pilot study to analyse post-mortem interval by serial estimation of expression of autophagy and cardiac muscle specific genes"**

Nature of Project:	**Research Project**
Submitted as:	**M.D. Dissertation**
Student Name:	**Dr.Sahil Thakral**
Guide:	**Dr.Puneet Setia**
Co-Guide:	**Dr.Purvi Purohit, Dr.Vikas P Meshram & Dr. Arvind Sinha**

This is to inform that members of Institutional Ethics Committee (Annexure attached) met on **23-12-2019** and after through consideration accorded its approval on above project. Further, should any other methodology be used, would require separate authorization.

The investigator may therefore commence the research from the date of this certificate, using the reference number indicated above.

Please note that the AIIMS IEC must be informed immediately of:

- Any material change in the conditions or undertakings mentioned in the document.
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.

The Principal Investigator must report to the AIIMS IEC in the prescribed format, where applicable, bi-annually, and at the end of the project, in respect of ethical compliance.

AIIMS IEC retains the right to withdraw or amend this if:

- Any unethical principle or practices are revealed or suspected
- Relevant information has been withheld or misrepresented

AIIMS IEC shall have an access to any information or data at any time during the course or after completion of the project.

On behalf of Ethics Committee, I wish you success in your research.

Dr. Praveen Sharma
Member secretary
Institutional Ethics Committee
AIIMS,Jodhpur

Enclose:
1. Annexure 1

Page 1 of 2

Basni Phase-2, Jodhpur, Rajasthan-342005, **Website:** www.aiimsjodhpur.edu.in, **Phone:** 0291-2740741 Extn. 3109
Email: ethicscommittee@aiimsjodhpur.edu.in

ICMR Award letter

INDIAN COUNCIL OF MEDICAL RESEARCH
Serving the nation since 1911

भारतीय आयुर्विज्ञान अनुसंधान परिषद
स्वास्थ्य अनुसंधान विभाग, स्वास्थ्य और परिवार कल्याण मंत्रालय, भारत सरकार

Indian Council of Medical Research
Department of Health Research, Ministry of Health and Family Welfare, Government of India

No.3/2/June-2020/PG-Thesis-HRD (28) **Dated: 02.09.2020**

Dr. Sahil Thakral
Department of Forensic Medicine and Toxicology,
All India Institute of Medical Sciences,
Jodhpur-342005, Rajasthan
Registration No. MD20JUN-0157

Subject: - Award of ICMR Financial Support for the MD/MS/DM/MCh/MDS dissertation /thesis for June 2020 batch-reg.

Dear Dr. Sahil Thakral,

This is with reference to your application seeking financial assistance from the ICMR for MD/MS/DM/MCh/MDS dissertation/thesis entitled **"A pilot study to analyse post-mortem interval by serial estimation of expression of autophagy and cardiac muscle specific genes."**

I am glad to inform you that, based on the recommendation of Expert Committee, Director General, ICMR, has approved your application / thesis for the financial support of **Rs. 50,000/- (Fifty thousand only)** as stated above, which will be disbursed in two /three installments. Initial amount of **Rs. 30,000/-** will be released after receipt of the Undertaking as per the guidelines and remaining amount of **Rs. 20,000/-** on receipt of the electronic copy and summary of work done of your dissertation / thesis duly approved by the University/ Institute along with one publication in an indexed Journal. Mandatory requirement to avail this opportunity is to submit an Undertaking duly forwarded through the Guide, to the undersigned, enabling us to release the first Installment.

Kindly also submit the Guide details as well as the MANDATE FORM (available on ICMR website) along with a photocopy of a Cancelled Cheque (Please ignore, if already submitted) latest by 21st September, 2020 for receiving e-payment for purpose of verification of the concerned bank account where money is to be remitted.

Yours faithfully

(Ishwar Likhar)
Administrative Officer
(For Director General)

Copy to:

1. Guide:- Dr. Puneet Setia, Assistant Professor, Dept. of Forensic Medicine and Toxicology, All India Institute of Medical Sciences, Jodhpur-342005, Rajasthan

वी. रामलिंगस्वामी भवन, पोस्ट बॉक्स नं. 4911,
अंसारी नगर, नई दिल्ली - 110 029, भारत
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ICMR Financial Grant letter

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फैक्स /FAX : 011-26588662, 011-26589791, 011-26589258

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भारतीय आयुर्विज्ञान अनुसंधान परिषद
INDIAN COUNCIL OF MEDICAL RESEARCH
स्वास्थ्य अनुसंधान विभाग (स्वास्थ्य एवं परिवार कल्याण मंत्रालय)
वी रामलिंगस्वामी भवन, अन्सारी नगर, नई दिल्ली - 110029
DEPARTMENT OF HEALTH RESEARCH (MINISTRY OF HEALTH & FAMILY WELFARE)
V. RAMALINGASWAMI BHAWAN, ANSARI NAGAR, NEW DELHI-110029

No.3/2/June-2020/PG-Thesis-HRD (28) **Dated:** 26.10.20

Dr. Sahil Thakral
Department of Forensic Medicine and Toxicology,
All India Institute of Medical Sciences,
Jodhpur-342005, Rajasthan
Registration No. MD20JUN-0157

Subject: Payment of Rs 30,000/- under ICMR - MD/MS/DM/MCH thesis financial assistance for the project entitled. :-" **A pilot study to analyse post-mortem interval by serial estimation of expression of autophagy and cardiac muscle specific genes**"

Sir/Madam,

The Director General, ICMR sanctions the payment of 1st installment of Rs. 30,000/-(Rupees thirty thousand only) as the award under ICMR MD/MS/MCH thesis financial assistance.

A RTGS for the amount of Rs. 30,000/- will be sent to you in due course. The grant has been sanctioned as laid down in ICMR rules.

Second installment Rs. 20,000/- will be given after receiving the copy of publication as mentioned in award letter.

Yours faithfully,

(Ishwar Likhar)
Administrative Officer
For Director General

Copy to:

1. Account-1, ICMR along with a formal bill of Rs 30,000/- for payment of 1st installment at an early date from allocation made under the scheme (2020-21), Div of HRD The expenditure may be met related to "17-P" Human Resource Development Plan.
2. Guide:- Dr. Puneet Setia, Assistant Professor, Dept. of Forensic Medicine and Toxicology, All India Institute of Medical Sciences, Jodhpur-342005, Rajasthan

Annexure 1: Information sheet

Information Sheet for the relatives of the deceased

Title of Thesis: **A pilot study to analyses post-mortem interval by serial estimation of expression of autophagy and cardiac muscle-specific genes**
Name of PG Student: Dr. Sahil Thakral Contact No.: +91 8285484704

Before you decide whether or not you wish to participate in this study, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with others if you wish.

1. What is the purpose of the study?
The purpose of the study is to determination of the PMI from gene expression in human cadaver at different time intervals.

2. What if I don't want to take part in this study or if I want to withdraw later?
Participation in this study is voluntary. It is completely up to you whether or not you participate. You may withdraw from the study at any time and for any reason or no reason. Please tell the researcher that you wish to withdraw from the study.

3. What does this study involve?
This study will involve the collection of cadaver's heart tissue.

4. Will the confidentiality of my deceased relatives be protected?
The information about your deceased will be subjected to absolute anonymity.

Thank you for taking the time to consider this study.
If you wish to take part, please sign the attached consent form.
This information sheet is for you to keep.

अनुलग्नक 2: सूचना पत्रक

मृतक के रिश्तेदारों के लिए सूचना पत्रक

थीसिस का शीर्षक:- **ऑटोफैगी और कार्डियक मांसपेशी-विशिष्ट जीन की अभिव्यक्ति के सीरियल आकलन द्वारा पोस्टमार्टम अंतराल का विश्लेषण।**

पीजी छात्र का नाम:- डॉ. साहिल ठकराल संपर्क संख्या:- +91-8285484704

□ह त□ करने से पहले कि आप इस □ ध्य□न में भाग लेना चाहते हैं □ा नहीं, □ह समझना आपके लिए महत्वपूर्ण ह□कि □ह शोध क्योंकि □ाजा रहा ह□और इस में क्या शामिल होगा। कृप□ा निम्नलिखित जानकारी को ध्यानपूर्वक पढ़ें और दूसरों के साथ चर्चा करें, □दि आप चाहें ।

1. इस □ ध्य□न का उद्देश्य क्या ह?

   इस □ ध्य□न का उद्देश्य विभिन्न सम□ □ंतरालों पर मानव क□ेवर में जीन □ भिव्यक्ति और ऑटोफ□ी जीन से पीएमआई का निर्धारण करना ह।

2. क्या होगा □दि मैं इस □ ध्य□न में भाग नहीं लेना चाहता हूं □ा □दि मैं बाद में वापस लेना चाहता हूं?

   इस □ ध्य□न में भागीदारी स्व□ि□छक ह। □ह पूरी तरह आप पर निर्भर ह□कि आप भाग लेते हैं □ा नहीं। आप किसी भी सम□ और किसी भी कारण से □ा किसी कारण से □ ध्य□न से वापस ले सकते हैं। कृप□ा शोधकर्ता को बताएं कि आप □ ध्य□न से हट ना चाहते हैं।

3. इस □ ध्य□न में क्या शामिल ह?

   इस □ ध्य□न में शव से ह्द□ का ऊतक लेना शामिल हैं।

4. क्या मेरे मृत रिश्तेदार की गोपनी□ता सुरक्षित रहेगी ?

   आपके मृतक रिश्तेदार के बारे में जानकारी पूर्णत: गोपनी□ रहेगी।

इस □ ध्य□न पर विचार करने के लिए सम□ निकालने के लिए आपका धन्यवाद।

□दि आप भाग लेना चाहते हैं, तो कृप□ा संलग्न सहमति फॉर्म पर हस्ताक्षर करें।

□ह जानकारी पत्र आपके रखने के लिए ह।

Annexure 3: Consent form

All India Institute of Medical Sciences
Jodhpur, Rajasthan

Informed Consent Form

Title of Thesis: **A pilot study to analyses post-mortem interval by serial estimation of expression of autophagy and cardiac muscle-specific genes**

Name of PG Student: Dr. Sahil Thakral Contact No.: +91 8285484704

Case Number (Post Mortem Number): Date

I,_____son/Mr.,daughter/Mr._____
resident_____give my full, independent, voluntary consent for the research work upon my dead relative _____, which is to study the role of expressions of various genes (NT-proBNP, cTnI, GAPDH, HMGB1, Bcl2) in estimating post-mortem interval. Its process and nature have been explained in my own language with my complete satisfaction and I confirm that I have the opportunity to ask questions.

I understand that after giving proper advice, the collection of heart tissue samples of my relative's body has been approved by me and I have the right to leave the study at any time without any reason.

I understand that medical information related to my deceased relative and any of his/her medical records can be seen by the person responsible for the regulatory authorities. I allow these people to access the records of my deceased relatives.

Date: _____ Signature / thumb impression

Place: _____ Relationship with the deceased:

To prove that the above consent has been received in my presence.
Date: _____

Place: _____ Signature of PG student

1.Witness **2. Witness**

_____ _____

Signature Signature
Name: Name:
Address: Address:

<u>अनुलग्नक 4: सहमति फॉर्म</u>

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर, राजस्थान

<u>सूचित सहमति प्रपत्र</u>

थीसिस का शीर्षक:- **ऑटोफैगी और कार्डियक मांसपेशी-विशिष्ट जीन की अभिव्यक्ति के सीरियल आकलन द्वारा पोस्टमार्टम अंतराल का विश्लेषण।**

पीजी छात्र का नाम: डॉ. साहिल ठकराल संपर्क संख्या : +91 8285484704 प्रकरण संख्या (पोस्टमार्ट मनंबर): दिनांक

मैं,_____पुत्र/श्री,पुत्री/श्री _____निवासी _____मेरे मृतसम्बन्धी _____परशोध-कार्य के लिए मेरी पूर्ण, स्वतंत्र, स्वेच्छिक सहमति देताहूँ/देतीहूँ जोकि "इस अध्ययन का उद्देश्य विभिन्न समय अंतरालों पर मानव कंकालेवर में जीन अभिव्यक्ति और ऑटोफैगी जीन से पीएमआई का निर्धारण करना है। इसकी प्रक्रियाऔर प्रकृति मुझे मेरी अपनी भाषा में मेरी पूर्ण संतुष्टि से समझाया गयाहैऔर मैं पुष्टि करता हूँ/करती हूँ कि मुझे प्रश्न पूछने का अवसर मिला है।

मैं समझता हूँ/समझती हूँ कि उचित सलाह देने के बाद मेरे रिश्तेदार के शरीर के नमूनों का संग्रह मेरे द्वारा अनुमोदित किया गया हैऔर मुझे किसी भी कारण दिए बिना किसी भी समय अध्ययन से बाहर निकलने का अधिकार है।

मैं समझता हूँ / समझती हूँ कि मेरे मृतक रिश्तेदार किसे सम्बंधित चिकित्सीय सूचना और उसके किसी भी मेडिकल रिकॉर्ड को नियामक प्राधिकरणों के जिम्मेदार व्यक्ति द्वारा देखा जा सकता है। मैं इन लोगों के लिए मेरे मृतक रिश्तेदार के अभिलेखों तक पहुंच की अनुमति देता हूँ / देती हूँ ।

तारीख : _____ हस्ताक्षर /अंगूठे का छाप

जगह: _____ मृतक के साथ संबंध:

यह प्रमाणित करने के लिए कि मेरी उपस्थिति में उपरोक्त सहमति प्राप्त की गई है।

तारीख : _____

जगह: _____ पीजी छात्र के हस्ताक्षर

1. साक्षी

_____

हस्ताक्षर:
नाम:
पता:

2. साक्षी

_____

हस्ताक्षर
नाम:
पता :

Annexure 5: Data collection sheet

Data case no:

Name:

Age:

Sex: -

PM No.:

History:

Height:

Weight:

Time of death:

Cause of death:

History of medical illness:

History of cardiac disease:

History of Cancer/ Inflammatory disease:

Time of samples:

Time of sample processing	PMI duration	Gene expression				
		NT-proBNP	CnT I	HMGB1	BCL2	GAPDH
0 hour						
6 hours						
12 hours						