Evaluation of post-mortem interval based on gingival tissue hypoxia inducible factor-1α gene expression

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The authors declare that they have no conflict of interest.

KEYWORDS

Post Mortem Interval, Hypoxia Inducible Factor-1, Gene Expression, Histopathological Analysis

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ABSTRACT

Purpose: In the realm of forensic science, the Post Mortem Interval (PMI) is a critical component that determines the time that has passed since the person's physiological death. Although techniques exist to precisely determine the PMI, the results are often unreliable. Hypoxia inducible factor-1 (HIF-1) is a transcriptional factor, and in hypoxic conditions, HIF-1α protein is expressed after proteosomal degradation and ubiquitination pathway involving von Hippel-Lindau protein (pVHL). The aim of the study was to assess HIF-1α mRNA expression in human gingival tissues at different PMIs.

Methods: Gum tissues were collected from cadavers at three definite intervals, namely short PMI (SPMI), medium PMI (MPMI), and long PMI (LPMI). The relative fold change in gene expression of HIF-1α was studied by RT PCR. Histopathological analysis of the tissue samples was done to determine the PMI.

Results: In the case of short PMI (SPMI), the relative fold change in gene expression of HIF-1α is 26.90 ± 23.62. However, in the medium PMI (MPMI) and long PMI (LPMI), the relative fold change in gene expression decreased to 6.32 ± 10.90 and 5.33 ± 8.12, respectively. Histopathological analysis of the post mortem samples revealed less necrosis in SPMI than LPMI. Inflammatory cell infiltration is more in SPMI than MPMI, with their notable absence in LPMI. Ulceration was prominent in LPMI. Destructive vasculitis was visible in SPMI and MPMI. Cystic changes were increased in MPMI and LPMI.

Conclusion: Combined gene expression of HIF-1α and histopathological analysis is a good option for determination of PMI.

INTRODUCTION

Post Mortem Interval (PMI) is one of the crucial factors in the field of forensic science as it determines the time that has elapsed from the physiological death of the person. The estimation of the PMI is considered to be a fundamental subject in the standard forensic practice.[1-3] In current practice, several factors are considered to determine the PMI accurately such as physical, biochemical, physicochemical, entomological, botanical and microbiological investigations.[4] According to the standard forensic practice, short PMI refers to the initial 24-hour period after death and it is evaluated based on the gross

post-mortem physical changes which include the temperature of the body, muscular and neuro-reactivity and livor mortis. However, the longer PMI (several days to years) is estimated depending on the stage of decomposition of the body, growth of the fly larvae (known as entomological analysis) along with the proper assessment of the bone radioisotope concentration.[5] Though these methods are utilized to determine the PMI accurately, there are several problems associated with the existing protocol. As the method gets influenced by the gender, age, pathological and physiological states of the deceased the results are often inaccurate.[6] Therefore, there is a need to develop a certain protocol of measurement based on the time-reliant degradation of the biologically available markers such as DNA or RNA and also the proteins. It was believed initially, that DNA is considered to be much more stable than RNA as it degrades rapidly based on temperature, ribonuclease which is ubiquitously present, growth of microorganisms, along with the environmental factors such as humidity and sunlight.[7] However, during the last few years, several literatures have reported works on RNA which could remain stable for a longer period of time and therefore, the quantification of the degradation of mRNA can be used as an excellent marker for the accurate estimation of PMI.[8] According to the study conducted by researchers Bauer et al., 2003 there is a significant correlation with the degradation of the fatty acid synthase-messenger RNA (FASN mRNA) with the PMI up to 5 days in the autopsy cases.[9] Several studies have also been conducted on the RNA integrity numbers (RIN) and quantitative PCR analysis with several tissues and cell states of the rat and humans. The findings of the study highlighted that β-actin, GAPDH, HIF-1α and 18S rRNA are the potential markers that can be utilized to estimate PMI upto several numbers of days. There are other studies also which pointed out that there is no such correlation between the degradation of RNA and the PMI estimation up to several years.[9, 10]

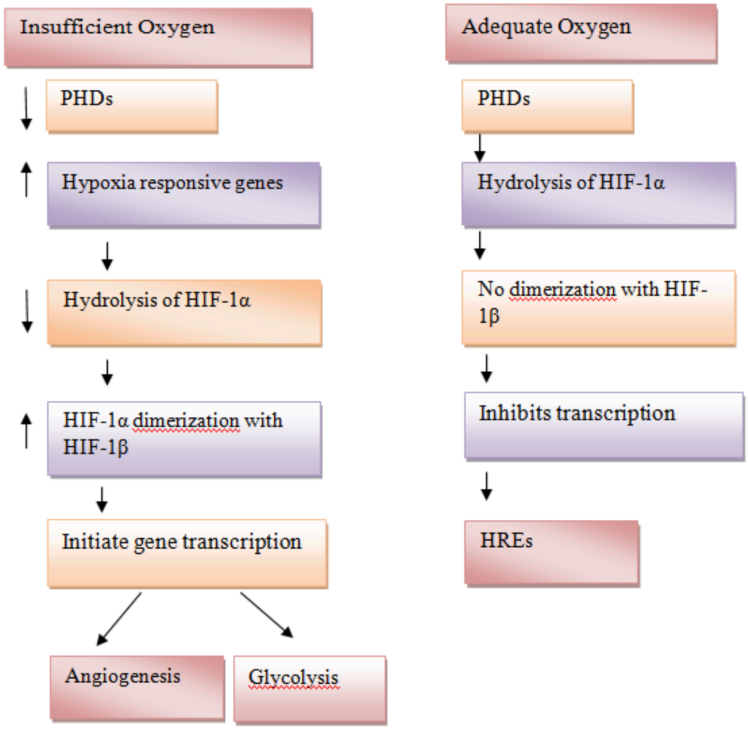
The study conducted by Tu et al.(2018) highlighted the stability of the circular RNAs (circRNAs) and miRNAs in mice models and also suggested certain markers that are suitable for the estimation of PMI miR-133a, miR-122, and 18S in heart tissue, circ-AFF1, LC-Ogdh, and miR-122 in liver and circ-AFF1, miR-133a and LC-LRP6 in skeletal muscle tissue.[7] Additionally, the study of Zhang et al. reported that U6, 18S RNA and GAPDH are considered to be the most accurate PMI markers of human tissues like the heart, kidney, skin, and brain [11]. The sample size of the study was 40 cadavers and the duration of PMI was between 1 to 72 hours. However, the study also emphasized that miRNA cannot be considered to be a stable endogenous control as mRNA because it lacks the cap structure at the 5' end and the 3' poly A tail. One of the most novel approaches in the field of forensic science is the study of the human thanatotranscriptome which determines the time and cause of death.[11] The approach includes all RNA transcripts from the awakened or functional internal parts of the dead body. In relation to this approach, the study of Javan et al. preliminary findings on the thanatotranscriptome study highlighted that RNA remains in a stable state within the internal organs of the cadavers whereas the pro-apoptotic genes such as the caspases gene expression were upregulated and the anti-apoptotic genes *BAG3* and *BCL2* remained downregulated in liver samples of humans.[11,12] As stated earlier, the existing approaches for the measurement of the PMI give controversial and sometimes inconclusive reports, therefore several strategies have been explored by the researchers. In addition to these existing methods, the histological and the immunohistochemical analyses of the varied post mortem tissues have become very important as they provide added insights in determining the PM time span.[13]

The key regulator of the hypoxic environment cellular adaptation is the transcription factor HIF1α protein. HIF-1 is composed of 2 sub units, HIF-1α and HIF-1β, whereas HIF-1α is an oxygen-sensitive subunit. Under hypoxic condition the HIF-1α protein is expressed after proteosomal degradation and ubiquitination pathway involving von Hippel-Lindau protein (pVHL) [14]. After death, the absence of blood flow leads to rapid cellular oxygen deprivation. So to estimate the time since death, the immune histochemical marker should be the one which is absent in physiologic condition and appears with hypoxia.[14] In HIF-1α, there are three domains. Near to the N terminal, there is Basic Helix Loop Helix DNA binding domain. Next to that is the Proline residue, which is necessary for stability of the factor. This domain is also known as Oxygen-Dependent Degradation Domain (ODD).

Oxygen -Dependent Degradation Domain is also the substrate for the enzyme Prolyl Hydroxylase enzyme (PHD). Among all these enzymes, PHD2 is most prominent. Beside it, near the C terminal, there is Arginine residue which regulates transcriptional activity. When PHD acts on Proline residue of HIF-1α under a normoxic condition, an oxygen molecule is utilized in oxidative decarboxylation of α-ketoglutarate (part of TCA cycle).[15] The products are hydroxyl prolyl residue, carbon dioxide and succinate. When both proline residues are decarboxylated, pVHL (Von Hippel Lindau) is attached to HIF-1α. In the next process under a normoxic condition, ubiquitin gets attached to the complex of HIF-1α and pVHL. Consequently, process of ubiquitylation takes place. When ubiquitylation process is complete, the complex is degraded by 26S proteosome[15] (Fig. 1).

On the basis of these observations, the aim of the study was to assess the HIF-1α and HIF-1α mRNA expression in human gingival tissues at different PMIs (Post Mortem Interval).

Figure 1. The fate of HIF-α in presence or absence of oxygen.



MATERIALS AND METHODS

Place of study

The autopsy samples were collected from Department of Forensic Medicine and Toxicology, N.R.S. Medical College & Hospital. The biopsy samples (as control) were collected from the Department of Periodontics, Dr. R. Ahmed Dental College and Hospital, Kolkata. The collected samples were sent to R&D Unit, Department of Biotechnology Heritage Institute of Technology, Kolkata for further processing and molecular biology study.

Period of study

The duration of the study was one year and six months.

Cadaver selection criteria

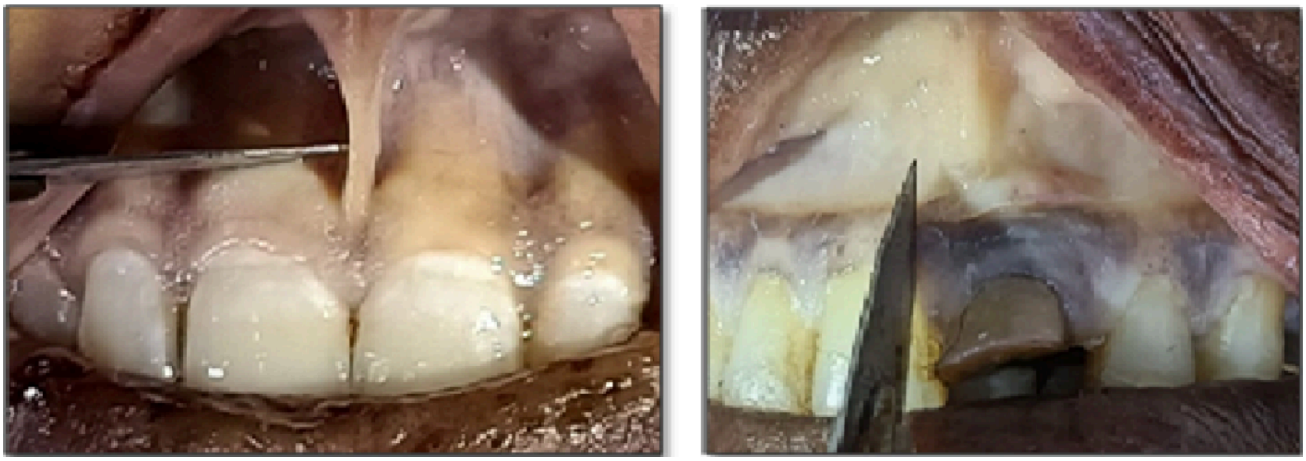
All samples were collected by a qualified dentist (SB) and a forensic expert (SC) from cadavers with no known medical history of periodontitis. Furthermore, only individuals without obvious oropharyngeal pathological lesions were selected. Consequently, cases of periodontitis were excluded, as significant alterations in HIF-1α levels-notably increased levels in diseased periodontal tissues-are typically associated with this condition. Biological changes—such as algor, livor, and rigor mortis, along with early autolysis (SPMI), putrefactive changes (MPMI), and advanced decay or mummification (LPMI)—occur significantly. Therefore, specific inclusion/

exclusion criteria were followed for each group, in addition to the general criteria provided below:
General Inclusion and Exclusion Criteria:
Inclusion Criteria: Cadavers aged 18 to 60 years, with a post-mortem interval ranging from 0 to 216 h.
Exclusion Criteria: Advanced putrefaction; direct, severe damage to the oral cavity, mucosa, or gingiva (e.g., due to acid burns or poisoning); premorbid history of lung-related disorders (e.g., tuberculosis, COPD).
Short Post-Mortem Interval (SPMI):
Inclusion Criteria: Existence of fresh symptoms, such as early secondary flaccidity, rigour in the muscles, and algor mortis. Death from trauma or natural causes does not substantially change the biochemistry of the body right away.[16]
Exclusion Criteria: Extended exposure to extremely high or low temperatures can skew cooling rates and biochemical indicators; prolonged resuscitation or medication therapy can alter early metabolic profiles.[16]
Medium Post-Mortem Interval (MPMI):
Inclusion Criteria: There should be enough soft tissue / insect colonisation (such as blowfly larvae) and obvious putrefactive alterations (such as bloating, marbling, and skin slippage).[16]
Exclusion Criteria: Primary tissue markers which were destroyed by animals; bodies recovered from water.[16]
Long Post-Mortem Interval (LPMI):
Inclusion Criteria: indications of mummification, skeletonization, or severe deterioration.[16]
Exclusion Criteria: insufficient context or environmental information on soil interaction.[16]

Sample grouping
The samples were divided into several groups according to the PMI (Post Mortem Interval, or time since death): The first group had a known PMI ranging from 0 to 72 h (short PMI – SPMI); the second group had a known PMI for 73-120 h (medium PMI–MPMI); the third group had a known PMI ranging from 121-216 h (long PMI – LPMI). Gingiva taken from healthy people during the frenectomy and crown lengthening procedure served as the control sample. There were no patients with gingival inflammation in this control group.

Collection of Gum tissue sample
Samples of gingival tissue of approximate size (0.5 – 1 cm2) were collected with sterile 22 no. surgical bard parker (b.p.) blade from the maxillary gingiva neighbouring to the central incisor at the time of medico-legal autopsies from the cadavers within the age range from 18 years to 60 years (Fig. 2).
All the bodies were stored in a government tertiary care hospital and medical college hospital morgue maintaining standard operating procedures. The storage system was comprised of specialized, refrigerated units designed to maintain body temperatures between 1°C and 5°C for 10 days with digital systems manage, display and monitor the temperature with alarm systems for power failure. Cases were mostly belonging to sudden death group excluding cases mentioned in exclusion criteria. After collection all the tissues were stored in homogenized conditions at -80°C.

Figure 2. Collection of gingival tissue from the cadavers.



Tissue Processing and RNA extraction
The reagent RNA isoplus (TAKARA, USA; Product code: 9108) is used to extract total RNA from the tissue sample that was collected. RNA isoplus makes it simple to extract total RNA from 50–100 mg of tissue sample. RNA isoplus reagent must be used to homogenise the tissue in a mortar and pestle treated with diethyl pyrocarbonate (DEPC) to make it endonuclease free. Chloroform is added to the RNAiso Plus solution after the tissues homogenization and the solution is then carefully mixed and centrifuged to separate it into three layers. Proteins, polysaccharides, fatty acids, cell debris, and a small amount of DNA are all present in the red organic solvent that makes up the lowest layer. A clear liquid containing RNA makes up the top layer, which is followed by a semi-solid containing DNA. An isopropanol precipitation method is used to extract the entire RNA. Following the RNA extraction, it is stored for the night at 4°C to dehydrate.[15]

Synthesis of cDNA and RT PCR study
After overnight dehydration, the dried RNA pellet was mixed with 60µl of de-ionized, nuclease-free water. To dissolve the RNA, it was then incubated in a hot water bath set at 56°C for ten minutes. Ten microlitres of RNA were removed from the solution and placed in a PCR tube in order to measure absorbance and determine the OD260/OD280 ratio. A nano-drop was used to measure the amount of RNA yield of each sample. By comparing the optical density (OD) values of various wavelengths (A260 and A280), the samples' purity was further examined. We determined the amount of water and RNA required to produce 1µg/ml of complementary DNA (c-DNA) using the OD260/OD280 ratio. 4 µL of the reverse transcriptase master mix (BIO-RAD, USA, Cat No. 1708841) was added within each PCR tube, along with calculated amount of RNA and water and it is mixed together in accordance with the kit's instructions. The PCR tubes were then put in PCR (T100 BIORAD, USA) to produce complementary c-DNA. After that, the c-DNAs were stored for RT PCR at -20°C.[15]
The iTaq SYBR green kit (BIO-RAD, USA; Cat No.: 1725121) was utilised to perform gene expression analysis of the gene HIF-1α using the synthesised cDNA in accordance with the comprehensive manufacturer's protocol. The RT-PCR (CFX-96 model, BIO-RAD, USA) was carried out using two microlitres of cDNA combined with eighteen microlitres of PCR master mix.[3] The gene expression of HIF-1α was determined by calculating the relative fold change in the gene expression of the housekeeping gene β-actin using RT-PCR (CFX-96 model, Bio-Rad, USA).[16]

The primer sequences of HIF-1α:
Forward Primer:
TATGAGCCAGAAGAACTTTTAGGC
Reverse Primer:
CACCTCTTTTGGCAAGCATCCTG

The PCR reaction condition followed is given below:
Stage 1: pre-heat: 95 °C for 10 min; Stage 2: Denaturation: 95 °C for 15 sec, Stage 3: Annealing: 60°C for 1 min.
Using RT-PCR (CFX-96 model, Bio-Rad, USA), the relative fold change in gene expression of the housekeeping gene β-actin was used to calculate the gene expression of HIF-1α. Using formula $2^{-(\Delta\Delta Ct)}$, the relative fold change in gene expression was determined. Since $\Delta\Delta Ct = \Delta Ct1 - \Delta Ct2$ in this case, meaning that ΔCt = Ct (target gene) - Ct (reference gene), $\Delta\Delta Ct = \Delta Ct$ (target sample) - ΔCt (Reference Sample). Cycle threshold is denoted here by Ct; because $\Delta\Delta CT$ equals 0 and 2^0 equals 1, the reference sample's gene expression is typically standardized to 1.[17]

Histopathological Study
The tissue samples were processed and stained with Hematoxylin and eosin (H&E) staining. The slides were observed under microscope.

Statistical analysis
The RT PCR analysis was done in triplicates and mean ± SD was calculated of the gene expression. The Statistical analysis was done as nonparametric data, Mann-Whitney U test one tailed (https://www.socscistatistics.com/tests/mannwhitney/) was performed and *p*-values were calculated. To confirm the results, the histopathological examinations were also conducted in duplicate.

RESULTS

RT PCR assay and statistical analysis:
In SPMI group we collected samples from 10 cadavers, similarly samples from 4 cadavers and

samples from 6 cadavers were collected in MPMI and LPMI groups respectively.

The 'Control sample' for the histopathological analysis was gingiva taken from healthy people during the frenectomy and crown lengthening procedure collected instantaneously served as the control set. From the same tissues we have extracted RNA using Trizol and synthesized cDNA and analyzed gene expression of HIF-1α. The gingiva taken from healthy people served as the molecular control to establish the basal expression. The gene expression was calculated using the formula $2^{-(\Delta\Delta Ct)}$, the relative fold change in gene expression was determined. Since ΔΔCt = ΔCt1 - ΔCt2 in this case, meaning that ΔCt = Ct (target gene) - Ct (reference gene), ΔΔCt = ΔCt (target sample) - ΔCt (Reference Sample). Cycle threshold is denoted here by Ct; because ΔΔCT equals 0 and 2^0 equals 1, the reference sample's gene expression is typically standardized to 1.

The relative fold change in gene expression of HIF-1α was calculated against the housekeeping gene β-actin, the relative fold change in HIF-1α gene expression among the control samples is 1 ± 0.32 (Mean ± SD); In case of short PMI (SPMI) the Mean ± SD of relative fold change in gene expression of HIF-1α calculated by $2^{-(\Delta\Delta Ct)}$ is 26.90 ± 23.62. However with the deterioration of tissue state and the unit cellular level as observed in the medium PMI (MPMI) and long PMI (LPMI) the mean relative fold change in gene expression came down to 6.32 ± 10.990 and 5.32 ± 8.12 respectively. Specific availability of cadavers at different time periods following our ethical guidelines and inclusion and exclusion criteria was extremely difficult and due to this samples size was relatively less. However, if we compare the number of cases of only one published paper so far available on this issue, we found they could get total of 10 cases throughout the different time-periods.[23] High standard deviations are common in gene expression studies, requiring the analysis of non-parametric data. This occurs because PCR fold changes in *HIF-1α* gene expression typically follow a log-normal distribution rather than a normal distribution, resulting in a long, skewed tail when plotted on a linear scale. While logarithmic transformation can normalize the data, the high raw variability—ranging from low-fold to over a hundred-thousand-fold changes -means a single high value can skew the mean, leading to a substantial standard deviation compared to the average. Statistical analysis was done as nonparametric data, Mann-Whitney U test (https://www.socscistatistics.com/tests/mannwhitney/) was performed and the *p*-values are given in the Table 1 and Fig. 3.

Table 1: Showing mean, standard deviation (SD), standard error of mean (SEM) of HIF-1α gene expression fold changes along with U, Z, *p*-values between different groups. * Represents distribution of two groups are significantly different, at the 0.05 significance level Null hypothesis is rejected.

In post mortem samples the surface keratin layer is largely absent, there was no hyperplasia, vascularity is indistinct but oedema degeneration and necrosis were markedly increased when compared with control samples (Fig. 4). In SPMI, necrosis is less (Fig 5) and in LPMI necrosis is more (Fig 7). Although inflammatory cells are more in SPMI but it was mild in MPMI (Fig 6) or may be absent in LPMI. Ulceration was extremely prominent in LPMI. Destructive vasculitis was only visible in SPMI and MPMI. Cystic changes were increased in MPMI and LPMI. The changes which usually occur in chronic cases like fibrosis, fibrinous deposits, giant cells, mononuclear inflammatory cells, granulomatous change, metaplastic change and calcification are absent in all intervals.

Table 1. Mean, standard deviation (SD), standard error of mean (SEM) of HIF-1α gene expression fold changes along with Mann-Whitney U, Z, Test Value *p*-values between different groups

Different groups	Mean ± SD ± SEM	Mann-Whitney U Test Value			
	HIF-1α gene expression fold changes	Group comparison	U	Z	p-value
SPMI	26.8934±23.6186±7.4688	SPMI:MPMI	6.00	-1.9799	0.0238*
MPMI	6.3238 ± 10.9907 ± 5.4953	SPMI:LPMI	7.00	-2.4947	0.0063*
LPMI	5.3278±8.1179±3.3141	MPMI:LPMI	6.00	-1.2792	0.1004

Figure 3. Mean of relative fold change in HIF-1α gene expression among the different samples collected at three different PMIs.

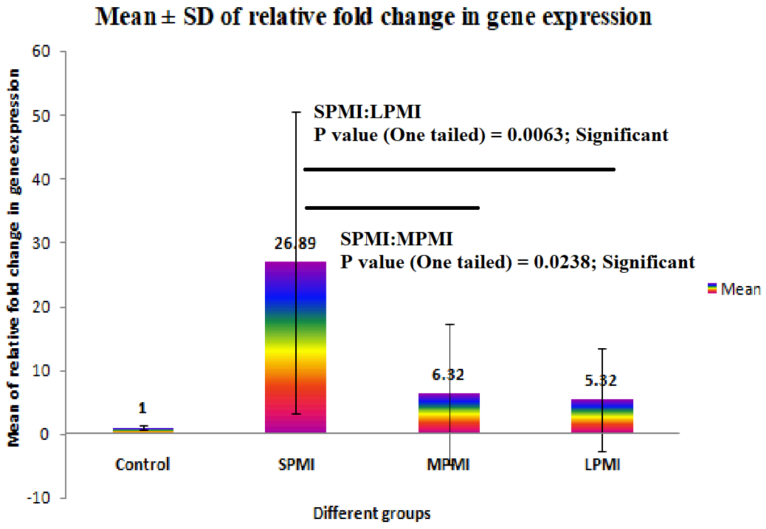


Figure 4. Histopathological Findings - Control Set: Normal (control) gingival tissue with normal keratin layer (a), normal stratified squamous layer (b) (400x), few inflammatory cells (c)(400x) and normal fibrovascular layer (d)(100x).

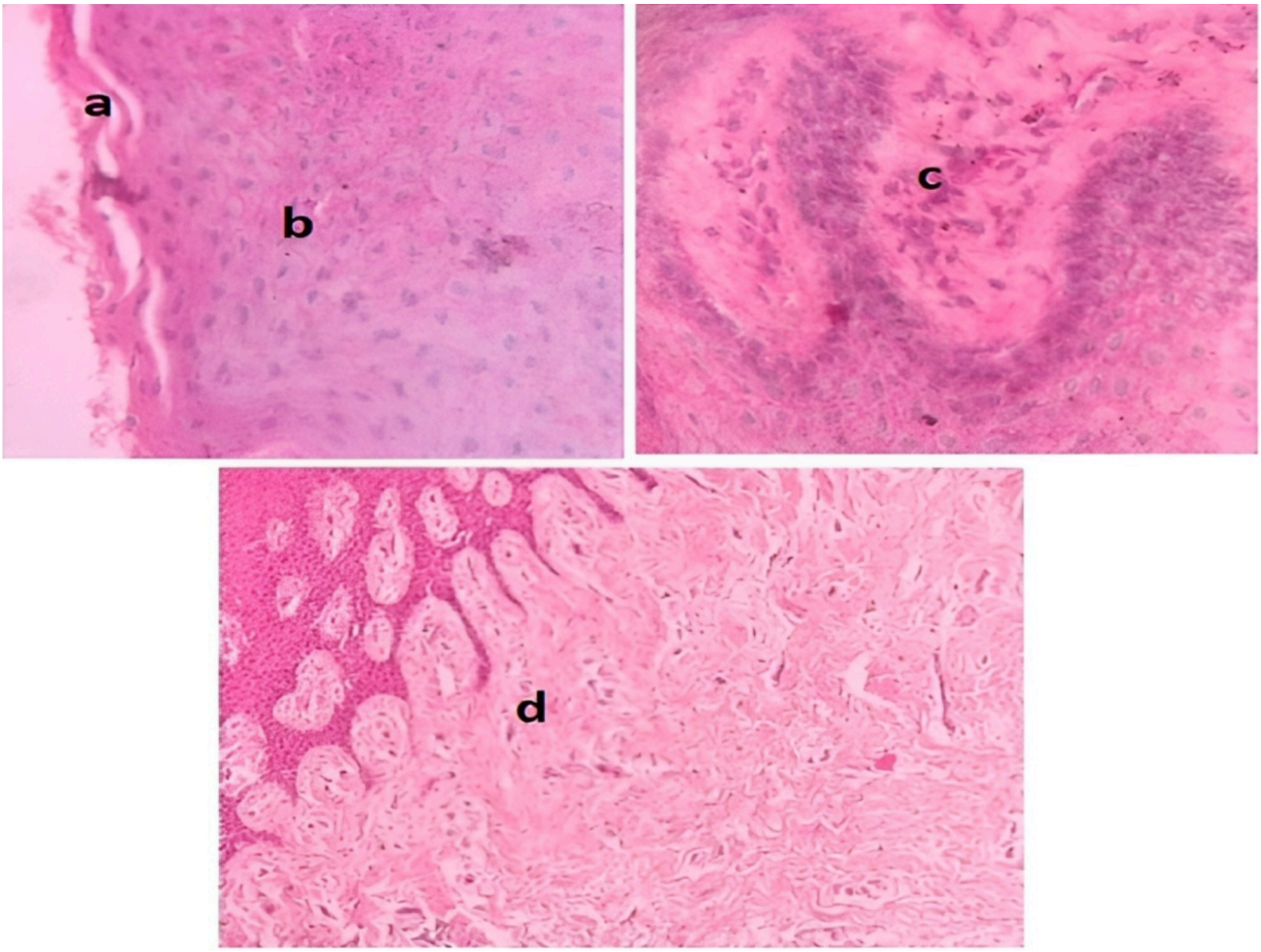


Figure 5. SPMI Group - SPMI: In SPMI, necrosis is less, cell is without nucleus (a), inflammatory cells (b) are more, destructive vasculitis (c) was visible in SPMI.400x

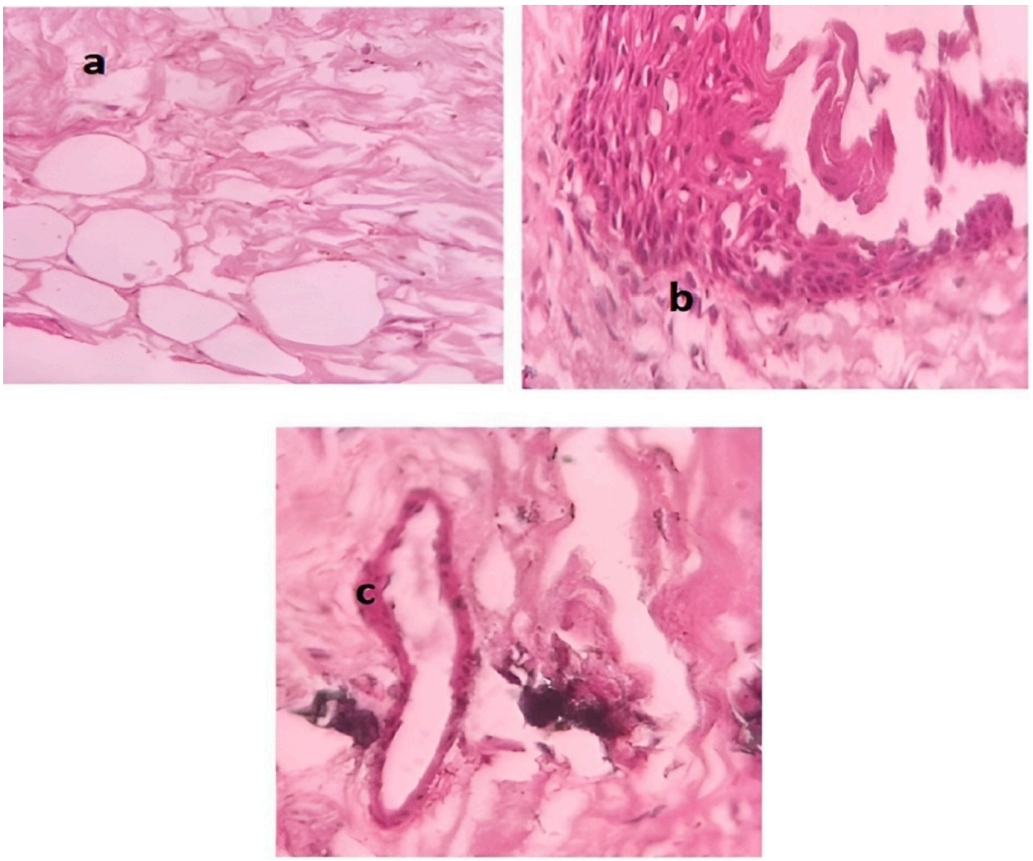


Figure 6. MPMI Group - MPMI: Inflammatory cells (a) was mild in MPMI, destructive vasculitis (b) was also visible in MPMI and cystic changes (c) were increased 400x

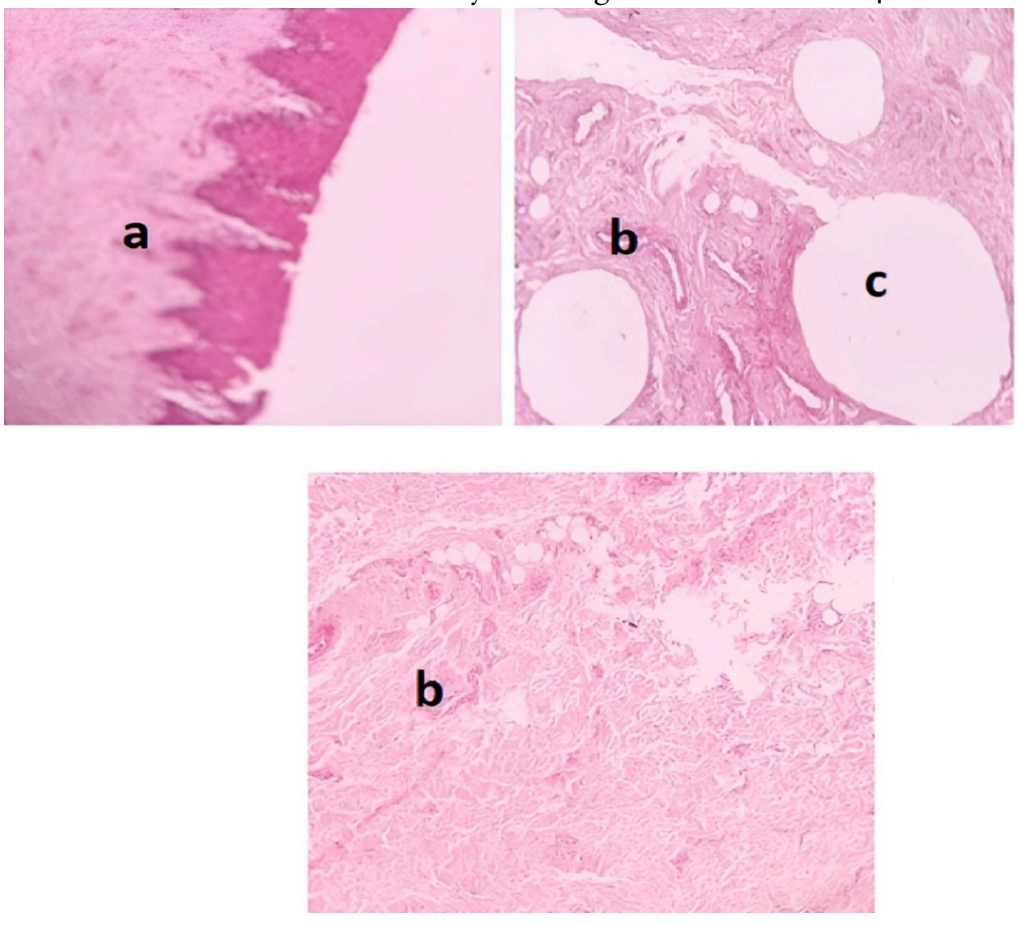
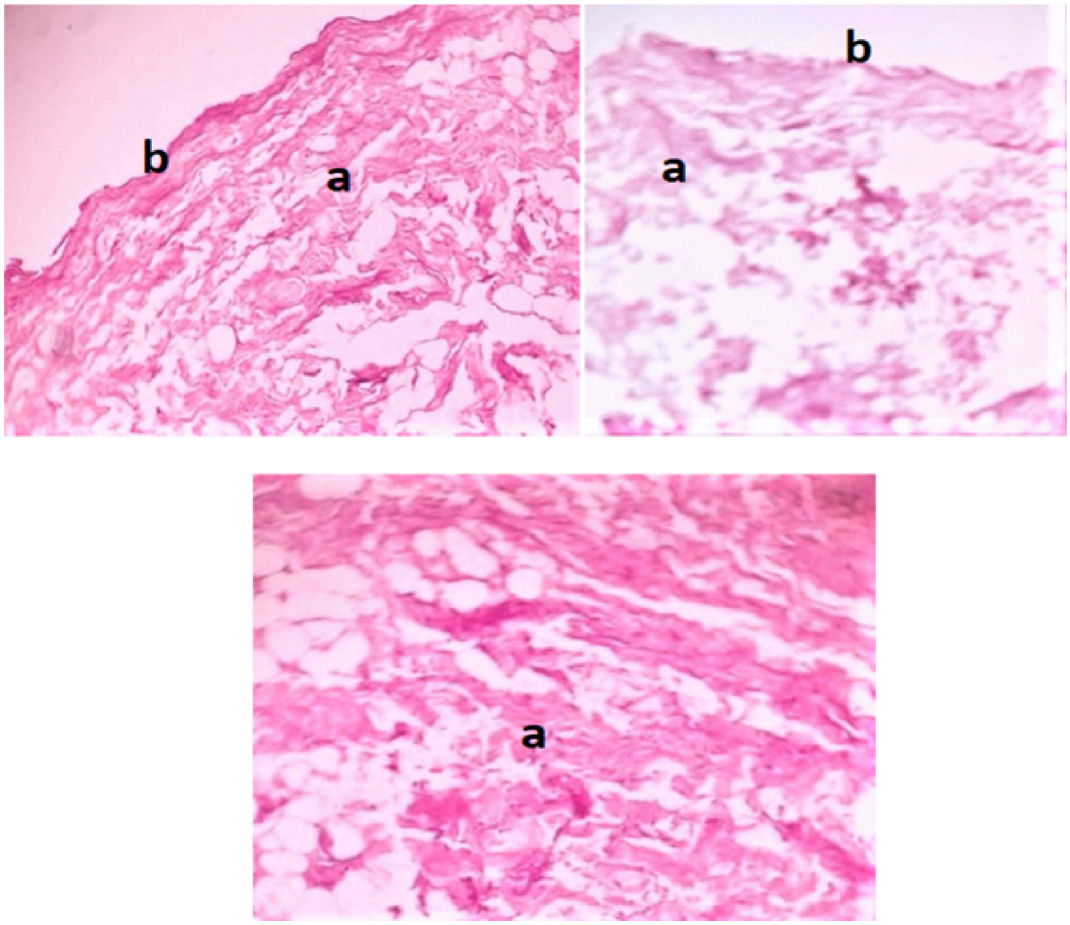


Figure 7. LPMI Group - LPMI: In LPMI necrosis is more (a), inflammatory cells are absent, ulceration (b) was extremely prominent in LPMI. 400x



DISCUSSION

The invention of hypoxia-inducible factor (HIF) in the mid-nineties of the last century by Gregg L. Semenza opened a new horizon of explaining pathophysiology of numerous pathological conditions and physiological adaptation in new dimensions [18]. This not only helped us to understand the pathological basis of a disease but also gave future direction for treatments. HIF was incidentally discovered during investigation on the regulatory mechanism of erythropoietin (EPO) gene which encodes EPO protein to promote erythropoiesis.[18] Hypoxia- inducible factor-1 (HIF-1) is a transcriptional factor of a dimer consisting of two proteins, HIF-1α and HIF-1β.[19] Under normal oxygen concentration (10-30 μ mol), HIF-1α is present in low quantity in THE cytoplasm and HIF-1β is present in high level in the nucleus of a cell. Evidence also suggests that HIF-1α also plays a vital role in the pathogenesis of cancer.[20] With an increase in the size of tumour, the inner part of it lacks perfusion favouring tumour cell death. But once HIF-1α is expressed in the hypoxic core of solid tumours where the partial pressure of oxygen may be less than 10 mm Hg, HIF-1α is not degraded by proteosomal enzymes but a varied number of transcriptional factors like vascular endothelial growth factor (VEGF) is released which not only helps for the survival of the tumour but also facilitates its growth.[20]

In our study, we have walked into a path not traversed by many researchers. Unnatural deaths, homicides, genocides, natural calamities and not to mention the mammoth loss of precious human lives in war necessitate post mortem investigations [21]. Time lapsed from death to the autopsy table, in other words the post mortem interval, is an integral part of post mortem investigations. These include post mortem changes like algor mortis, rigor mortis, livor mortis, nomogram methods, forensic entomology, thanatochemistry and many others. But no single methodology can clearly identify the time since death and it remains as elusive as before.[22, 23]

In 2018, Paolo Fais et al. in their landmark study postulated the gene expression of HIF-1α as a

potential biomarker for the post mortem interval.[24] The study aimed to investigate the immunohistochemical distribution and mRNA expression of hypoxia-inducible factor (HIF-1α) in post mortem gingival tissues to establish a correlation between the presence of HIF-1α and the time since death. They collected gingival tissues from 10 cadavers at different PMIs (1–3 days, 4–5 days and 8–9 days) and 3 controls were processed for immunohistochemistry with quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The results showed a time-dependent correlation of HIF-1α protein and mRNA with different times since death, which suggested that HIF-1α is a potential marker for post mortem interval (PMI) estimation.

In our study, we have taken samples of different known PMI. Among those, 10 samples of short PMI (0-3 days), 4 samples of medium PMI (4-5 days) and 6 samples of long PMI (6-9 days).They were compared with 5 biopsy samples of healthy gingival tissue collected during crown lengthening and gingival depigmentation surgeries in our department. We had been unable to maintain uniformity in sample size in all groups because medium and long PMI cadavers were scarce and patients with healthy gingiva reporting to our department for crown lengthening surgeries were inadequate in the stipulated time frame of our study. Samples of gingival tissue of approximate size (0.5 – 1 cm^2) were collected. The gene expression of HIF-1α was determined by calculating the relative fold change in the gene expression of the housekeeping gene β-actin. The relative fold change in gene expression of HIF-1α was calculated against the housekeeping gene β-actin, because the gene expression of β-actin is constant in all cell types across the organisms and its expression is not subjected to change in any experimental condition. We have done the relative fold change in gene expression of HIF-1α from the gingival tissue. Gingivae were used to investigate HIF-1a and HIF-1a mRNA expression due to the rapid, easy, and non-mutilating nature of the sampling technique. Furthermore, gingivae are typically shielded from labial tissues, reducing the influence of external elements that may cause tissue deterioration to occur quickly. Gingival tissue is therefore the most suitable option for protein and mRNA analysis since it is a more stable area for study. Gingival mRNA and immuno histochemical markers can be successfully analyzed within a range of 1 to 10 days (up to 240 hours), according to research on human cadavers. Signals are highest during the first 3 days (72 hours) and then gradually decline thereafter.[24]

The relative fold change in gene expression of HIF-1α was calculated against the housekeeping gene β-actin, the relative fold change in HIF-1α gene expression among the control samples is 1 ± 0.32 (Mean ± SD); In case of short PMI (SPMI) the Mean ± SD of relative fold change in gene expression of HIF-1α calculated by $2^{-(\Delta\Delta Ct)}$ is 26.90 ± 23.62. However with the deterioration of tissue state and the unit cellular level as observed in the medium PMI (MPMI) and long PMI (LPMI) the mean relative fold change in gene expression came down to 6.32 ± 10.990 and 5.32 ± 8.12 respectively (Fig. 3).

This is because immediately after death the value of HIF-1α gene expression increases due to the swift start of the hypoxic condition throughout the body. The cells within the body are experiencing a severe and sudden hypoxic state which can be considered as the last-ditch effort to adapt to the state of oxygen deprivation. However, with the deterioration both at the tissue and cellular level as observed in the medium PMI (MPMI) and long PMI (LPMI), the mean relative fold change in gene expression came down to 6.32 ± 10.990 and 5.32 ± 8.12 respectively. In the study by Paolo Fais et al**.** (2018), the medium PMI group had the highest elevation of gene expression of HIF-1α but in our study the short PMI group had the highest elevation in gene expression of HIF-1α.[24] Histopathological analysis of the post mortem samples revealed that the surface keratin layer is largely absent, there was no hyperplasia, vascularity is indistinct but oedema degeneration and necrosis were markedly increased. In SPMI, necrosis is less and in LPMI necrosis is more. Inflammatory cells are more in SPMI, mild in MPMI or may be absent in LPMI. Ulceration was extremely prominent in LPMI. Destructive vasculitis was only visible in SPMI and MPMI. Cystic changes were increased in MPMI and LPMI. The changes which usually occur in chronic cases like fibrosis, fibrinous deposits, giant cells, mononuclear inflammatory cells, granulomatous change, metaplastic change and calcification are absent in all intervals (Fig. 4 - 7; Table 2). However, the study has a few limitations. Firstly, the sample size was not

uniform because of the limited availability of samples in the stipulated time frame. Secondly, time frame and economic constraints had caused hindrance in increasing the sample size but the sample was anyway larger than that in the Paolo Fais et al**.** study (2018).

Table 2. Histopathological changes within the gingival tissues of different PMIs (in duplicate)

Sl. No.	Parameters	Con	Con	SPMI	SPMI	MPMI	MPMI	LPMI	LPMI
1	Epithelial keratin	++	++	+	-	-	-	-	-
2	Epithelial hyperplasia	+	+	-	-	-	-	-	-
3	Fibrosis	-	-	-	-	-	-	-	-
4	Vascularity	++	++	+	+	+	+	+	+
5	Oedema	-	-	+++	+++	++++	+++	+++	+++
6	Degenerative changes	-	-	++	+++	++++	+++	+++	+
7	Necrosis	-	-	+	+	+++	++	+++	++++
8	Fibrinous deposits	+	-	-	-	-	-	-	-
9	Inflammatory cells	+	+	++	+++	+	+	+	-
10	Giant cells	-	-	-	-	-	-	-	-
11	Ulceration	-	-	+	+	+	+	++++	++++
12	Large stellate fibroblast	-	-	-	-	-	-	-	-
13	Celullar stroma of mononuclear cells	-	-	-	-	-	-	-	-
14	Destructive vasculitis	-	-	++	++	++	+	-	-
15	Granulomatous change	-	-	-	-	-	-	-	-
16	Cystic change	-	-	+	+	+	+++	+	++
17	Metaplastic change	-	-	-	-	-	-	-	-
18	Calcification	-	-	-	-	-	-	-	-

The high time-dependent post-mortem stability of Hypoxia-Inducible Factor 1-alpha (HIF-1 α) in tissues such as the myocardium and gingiva justifies its inclusion as a marker for short PMI estimation (usually 1–3 days), which permits and frequently enhances the accuracy of conventional forensic techniques.[24] A molecular-level biomarker of the cellular response to post-mortem oxygen deprivation is HIF-1 alpha, whereas classical approaches are frequently impacted by environmental conditions. In the early stages (1–3 days), HIF-1 α gene expression is strongly correlated, observable in gingiva tissue even when outward body indications are unclear. Nonetheless, nomogram-based rectal temperature (mostly the Henssge nomogram) in conjunction with supravital responses and rigour mortis is also regarded as a very effective and trustworthy technique for calculating the PMI in the early post-mortem period, usually within 24 hours.[25] However, there are a few drawbacks; including the fact that its accuracy is impacted by environmental influences (such as fluctuating ambient temperature) and that precise body weight estimation and "cooling conditions" (correction factors) are necessary. After 12 to 15 hours, it becomes less accurate. Although it is subjective and less accurate, rigour mortis is a decent measure of PMI.[25] The "rule of 12" usually applies: it shows up in the first 12 hours, lasts for

12 hours, and then goes away in the following 12 hours.[26] The technique is less accurate than rectal cooling since it is greatly impacted by temperature, metabolic condition, and body muscle mass. It works best when used with other techniques, such AS the nomogram, to validate and refine the projected time range. Tissue responses that continue beyond a person's physical death are known as supravital reactions. For example, skeletal muscle may be electrically or mechanically excitable. During the first 12 to 15 hours after death, they are useful for narrowing down the PMI.[27] Thus, it can be said that the molecular approach to forensic odontology adds value by using Hypoxia-Inducible Factor 1-alpha (HIF-1 α) as a marker for short Post-Mortem Interval (PMI) assessment.

CONCLUSION

In this pilot study HIF-1α gene expression in gingival tissue along with histological changes allows preliminary indication for determination of PMI particularly in SPMI period. The findings are exploratory and require validation using a larger, better-controlled dataset. Future work incorporating larger sample numbers, environmental covariates and quantitative modelling of gene expression decay could substantially enhance the scientific impact and forensic applicability.

Key Points (3 key points)

This study was based on both HIF-1α gene expression in gingival tissue and its histological changes over time for determination of PMI.
Combined HIF-1α gene expression in gingival tissue and its histological changes appears to be a good option for determination of PMI.
These two parameters may be considered for accurate determination of PMI along with other conventional methods.

Authors' Contribution

Author SB carried out the experimental work along with DC. Author DC has written the first draft of the manuscript. Author SB and SC collected the gingival tissue from the cadavers. AR and SD guided the entire work. Author KP gathered the resources for the experimental work.

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Ethics approval and consent to participate

The study was approved by the Institutional Ethical Committee of Dr. R. Ahmed Dental College & Hospital, Kolkata, India dated 10th April 2023 and the study was completed with complete anonymization of the data in accordance with the Ethical Committee guidelines.

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