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# EVALUATION OF GINGIVAL TISSUE SAMPLES FOR PREDICTING THE TIME OF DEATH USING HISTOLOGICAL AND BIOCHEMICAL TESTS

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**Abstract**

Thanatochemistry, also known as the chemistry of death, is utilized to estimate the postmortem interval (PMI) and plays a crucial role in forensic investigations. The integration of biochemical analysis alongside traditional methods has gained significance, as it enables the detection of early postmortem changes in tissue specimens. This study aimed to establish a correlation between histological alterations and enzymatic activity in gingival tissue samples at various time intervals: immediately postmortem, 1 hour, 5 hours, 24 hours, and 48 hours after death.

Observed histological changes included the loss of epithelial architecture, chromatin clumping, nuclear vacuolation, karyopyknosis, eosinophilia, and widened intercellular junctions. Additionally, two enzymes—acid phosphatase (indicative of the autolytic phase) and ammonia (associated with the putrefactive

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phase)—were analyzed using a UV spectrometer. The results demonstrated a progressive increase in ammonia levels (1.13±0.24 to 26.6±2.09) and a gradual decline in acid phosphatase activity (5.61±0.67 to 1.25±0.53) over the 48-hour period. The observed cellular changes in gingival tissue were also found to correspond with postmortem intervals.

These findings highlight the potential of enzymatic changes as reliable indicators of time since death when correlated with histological analysis. Future studies replicating this experiment under diverse taphonomic conditions and incorporating additional enzymes may further establish the forensic value of gingival tissue in determining the time of death with greater accuracy.

**Keywords**

Gingival Tissue, Postmortem Interval, Forensic Odontology, Histological Alterations, Biochemical Variations, Ammonia.

## ОЦЕНКА ОБРАЗЦОВ ДЕСНЕВОЙ ТКАНИ ДЛЯ ПРОГНОЗИРОВАНИЯ ВРЕМЕНИ СМЕРТИ С ПОМОЩЬЮ ГИСТОЛОГИЧЕСКИХ И БИОХИМИЧЕСКИХ ТЕСТОВ

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**Аннотация**

Танатохимия, также известная как химия смерти, используется для оценки посмертного интервала (ПМИ) и играет важную роль в судебно-медицинских расследованиях. Интеграция биохимического анализа с традиционными методами приобрела большое значение, поскольку позволяет выявлять ранние посмертные изменения в образцах тканей. Целью данного исследования было установить корреляцию между гистологическими изменениями и ферментативной активностью в образцах десневой ткани в различные временные интервалы: сразу после смерти, через 1 час, 5 часов, 24 часа и 48 часов после смерти.

Наблюдаемые гистологические изменения включали потерю эпителиальной архитектуры, комкование хроматина, ядерную вакуолизацию, кариопикноз, эозинофилию и расширение межклеточных стыков. Кроме того, с помощью УФ-спектрометра были проанализированы два фермента - кислая фосфатаза (свидетельствующая об аутолитической фазе) и аммиак (связанный с гнилостной фазой). Результаты продемонстрировали прогрессивное увеличение уровня аммиака (от 1,13±0,24 до 26,6±2,09) и постепенное снижение активности кислой фосфатазы (от 5,61±0,67 до 1,25±0,53) в течение 48-часового периода. Наблюдаемые клеточные изменения в десневой ткани также соответствовали посмертным интервалам.

Эти результаты подчеркивают потенциал ферментативных изменений как надежного индикатора времени, прошедшего с момента смерти, при сопоставлении с гистологическим анализом. Будущие исследования, повторяющие этот эксперимент в различных тафономических условиях и с включением дополнительных ферментов, могут еще более точно установить судебно-медицинскую ценность десневой ткани для определения времени смерти.

**Ключевые слова**

Десневая ткань, посмертный интервал, судебная одонтология, гистологические изменения, биохимические изменения, аммиак.

**Introduction**

Determining the postmortem interval (PMI), or time of death, is one of the most complex and crucial aspects of forensic science **[1]**. PMI estimation helps establish how long an individual has been deceased. Following death, a series of physicochemical changes occur, leading to the gradual breakdown of soft tissues **[2]**. Histological analyses of the gingival epithelium suggest that decomposition

begins within ten hours postmortem, with distinct cellular changes observed during this process **[3]**.

Research has emphasized the significance of oral epithelial cells in forensic investigations. These cells can be easily collected using simple techniques, such as brushing, and have been utilized for DNA profiling and gender determination **[3, 4]**. A growing approach in PMI estimation involves assessing chemical changes within human tissues over time, a field known as **Thanatochemistry [4]**. This method provides a quantitative approach to PMI determination, making it a more objective alternative to traditional techniques.

Existing PMI determination methods can generally be classified into two phases:

1. **Early postmortem phase**, which involves soft tissue decomposition.
2. **Late postmortem phase**, characterized by changes in the hard tissue matrix **[5]**.

Recent advancements have underscored the importance of analyzing biochemical markers using techniques such as **nuclear magnetic resonance (NMR) and mass spectrometry**. These methods provide insights into postmortem metabolic shifts while reducing examiner biases associated with traditional approaches **[6]**.

The organic components of the body undergo biochemical transformations after death, primarily divided into:

- **Autolysis (self-destruction)**, initiated by endogenous enzymes such as acid phosphatase.
- **Putrefaction**, which results in ammonia production as a byproduct of protein, nucleotide, and tissue breakdown.

Both **acid phosphatase and ammonia** have independently been recognized as potential biomarkers for PMI estimation **[7]**. The evaluation of these enzymes is particularly relevant in gingival tissue, as acid phosphatase is localized within gingival cells **[8]**, while ammonia, being volatile, accumulates due to postmortem amino acid degradation **[9]**.

Previous PMI studies have focused on various body tissues beyond blood, including:

- **Cardiac tissue [10]**
- **Skeletal muscle [11]**
- **Pineal gland [12]**
- **Pancreas [13]**
- **Brain [14]**

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Thus, the objective of the present study was to examine the histological and biochemical changes in gingival tissues at different postmortem intervals: immediately, 1 hour, 5 hours, 24 hours, and 48 hours after death.

**Materials and Methods**

**2.1. Sample Collection**

This study was approved by the institute's ethics committee, and written informed consent was obtained from all participants. A total of **twenty gingival samples** were collected from individuals who met the inclusion and exclusion criteria.

**Inclusion criteria:**

- Systemically healthy male and female participants aged **20–40 years**.
- Patients undergoing **therapeutic or cosmetic dental surgery**.

**Exclusion criteria:**

- **Smokers, pregnant individuals, diabetics**, and those with any systemic conditions or medications that could affect gingival health.
- Biopsy sites exhibiting clinical inflammation were also excluded, ensuring that only **non-inflamed gingival tissues** were selected.

**2.2. Processing of Gingival Tissue Samples**

Gingival biopsy samples were collected from **10 subjects** at the **attached gingiva** using a **4 mm punch biopsy kit**. Each subject provided **two samples**:

1. **Histological analysis** – Ten samples were processed for microscopic evaluation.
2. **Biochemical analysis** – The remaining ten samples were divided into two parts:
   - One portion was analyzed for **acid phosphatase** levels.
   - The other portion was assessed for **ammonia concentration**.

The **time of excision** was documented for each sample. The **first sample was immediately fixed** in **10% formalin**, serving as a control. The remaining tissue samples were stored in sealed containers at **room temperature** and fixed in **10% formalin** at the following intervals:

- **1 hour**
- **5 hours**
- **24 hours**
- **48 hours**

**2.3. Histological Evaluation**

Gingival samples were fixed according to the designated time intervals, with **immediately fixed samples serving as controls**. Each specimen was processed, sectioned, and stained with **hematoxylin and eosin (H&E)** for microscopic

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examination under a **compound microscope** (Lawrence Mayo, Bangalore, INDIA). Tissue samples were assessed at **10X and 400X magnifications** to identify **epithelial and connective tissue changes**.

**Epithelial changes were evaluated based on:**

- **Chromatin clumping** – Fragmentation and dispersion of chromatin, with an absent nucleolus.
- **Nuclear vacuolation** – Formation of vacuoles within the nucleus.
- **Karyopyknosis** – Irreversible chromatin condensation in necrotic or apoptotic cells.

**Connective tissue changes were assessed based on:**

- **Eosinophilia** – One of the earliest indicators of cell death, staining bright pink.
- **Vacuolation** – Formation of vacuoles within the tissue.

The degree of these changes was recorded to determine the **initiation and progression of decomposition** in gingival tissues. For statistical analysis, the observed changes were scored from **0 to 3**, based on their distribution within the microscopic field. The detailed scoring system is presented in **Table 1**.

Table 1. Scoring Criteria for Changes in the Gingival Post Biopsy

**Epithelium**

| Feature | Score 0 | Score 1 | Score 2 | Score 3 |
|---|---|---|---|---|
| **Chromatin Clumping** | Absent | Changes limited to superficial 1/3 | Changes in superficial and middle 1/3 | Changes seen in all layers |
| **Vacuolation** | Absent | Changes limited to superficial 1/3 | Changes in superficial and middle 1/3 | Changes seen in all layers |
| **Karyopyknosis** | Absent | Changes limited to superficial 1/3 | Changes in superficial and middle 1/3 | Changes seen in all layers |

**Connective Tissue**

| Feature | Score 0 | Score 1 | Score 2 | Score 3 |
|---|---|---|---|---|
| **Eosinophilia** | Absent | Occasional specks | Small defined patches | Large homogenous areas |
| **Vacuol** | A | Less than 10% of | 10–50% of | Greater than |

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| Feature | Score 0 | Score 1 | Score 2 | Score 3 |
|---|---|---|---|---|
| ation | bsent | connective tissue cells | connective tissue cells | 50% of cells |

### 2.4. Biochemical Evaluation

Gingival tissue samples were thoroughly rinsed with water and immersed in saline solution. Ten samples were immediately frozen at −20°C, while another ten were frozen at 1-hour, 5-hour, 24-hour, and 48-hour intervals. The collected tissue samples were homogenized at 2% weight by volume (wt/v) using 0.1 M phosphate buffer, 2 mM ethylenediaminetetraacetic acid (EDTA), and 0.2% Triton X-100. The resulting homogenates were centrifuged under cooling conditions, and the supernatants were analyzed for biochemical markers.

Acid phosphatase and ammonia levels were measured using a biochemical analyzer (Robonik Prietest TOUCH, Mumbai, India), based on visible and ultraviolet spectrophotometry. The readings were taken at wavelengths of 409 nm for acid phosphatase and 340 nm for ammonia, following the manufacturer's guidelines.

### 3. Statistical Analysis

Sample size estimation was conducted using G*Power v3.1.9.2, with an effect size (f) of 70%, study power of 80%, and a 5% margin of error, resulting in a required total of 20 samples. The Kruskal-Wallis non-parametric test was employed to assess statistically significant differences in enzyme levels at 0 h (immediate), 1 h, 5 h, 24 h, and 48 h.

### 4. Results

At 1 hour post-mortem, all samples exhibited chromatin clumping, with initial cytoplasmic vacuolation restricted to the superficial and spinous layers. Nuclear vacuolation, karyopyknosis, and chromatin clumping (Photomicrographs 2 and 3) were observed throughout the epithelial thickness. Loss of epithelial architecture, increased intercellular spaces, and eosinophilia were inconsistently noted but became evident by the fifth hour, alongside nuclear vacuolation throughout the epithelium (Photomicrograph 1).

By the eighth hour, eosinophilia increased significantly (Photomicrograph 5), and the epithelial structure displayed prominent intercellular spaces

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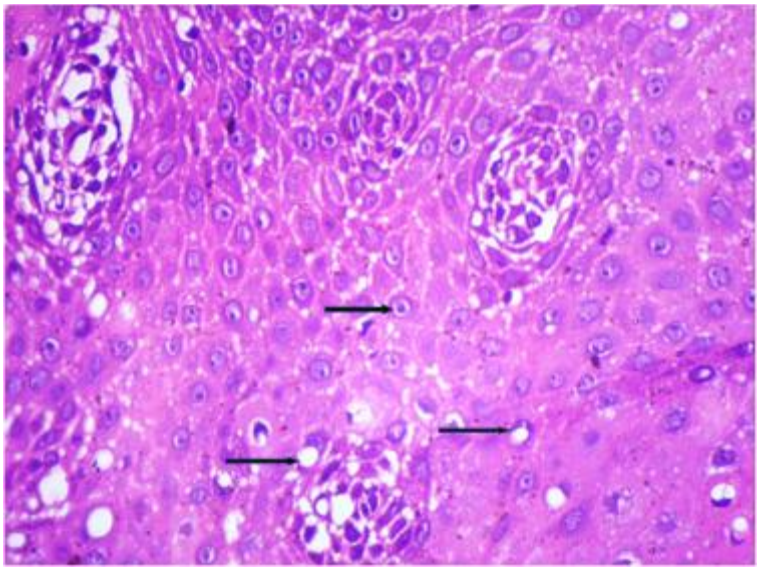
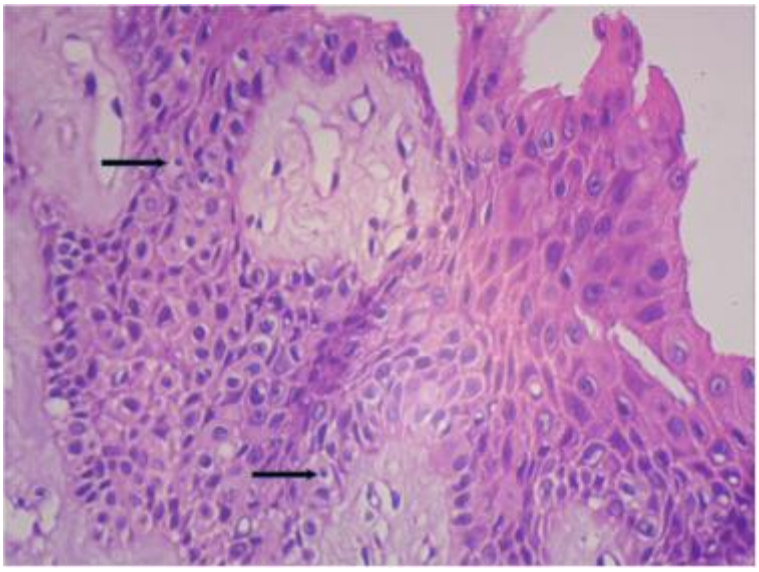
(Photomicrograph 4). Structural changes became more pronounced at 24 hours and remained visible at 48 hours (Photomicrograph 6).

Enzymatic assays revealed a decline in acid phosphatase levels from **5.61±0.67 mmol/L at 0 h** to **1.25±0.53 mmol/L at 48 h**. Conversely, ammonia levels increased from **1.13±0.24 mmol/L at 0 h** to **26.6±2.09 mmol/L at 48 h** (Table 2). Statistical analysis using the Kruskal-Wallis test confirmed a significant decrease in acid phosphatase levels ($p < 0.05$) and an increase in ammonia levels ($p < 0.05$) over time.



Photomicrograph. 1. Nucleolar vacuolation observed histologic section of gingival epithelial tissue 1hrs post removal tissues (H&E 400X).



Photomicrograph. 2. Multiple cells in gingival epithelium demonstrating karryopyknosis 5 h after removal. (H&E 400X).

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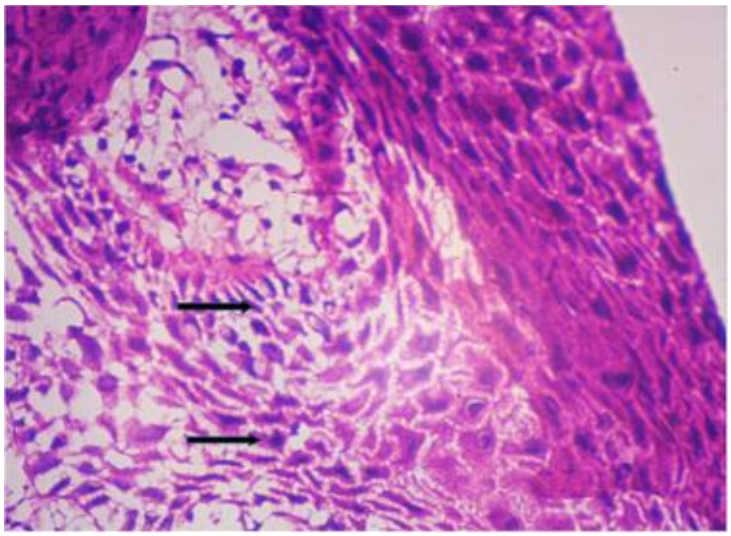
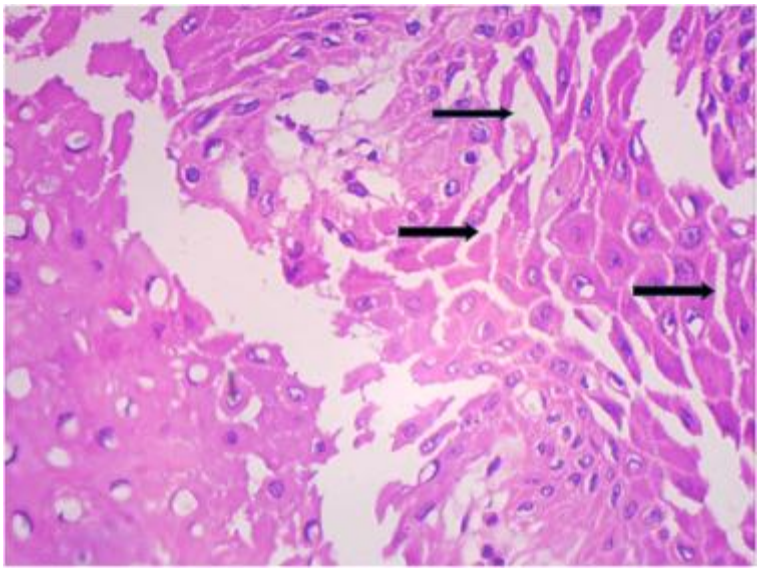


Photomicrograph. 3. Prominent Chromatin clumping and increased intercellular spaces 5 h post removal. (H&E 400X).



Photomicrograph. 4. Photomicrograph of gingival epithelium showing widened intercellular spaces at 48 hrs post removal (H&E 400X).

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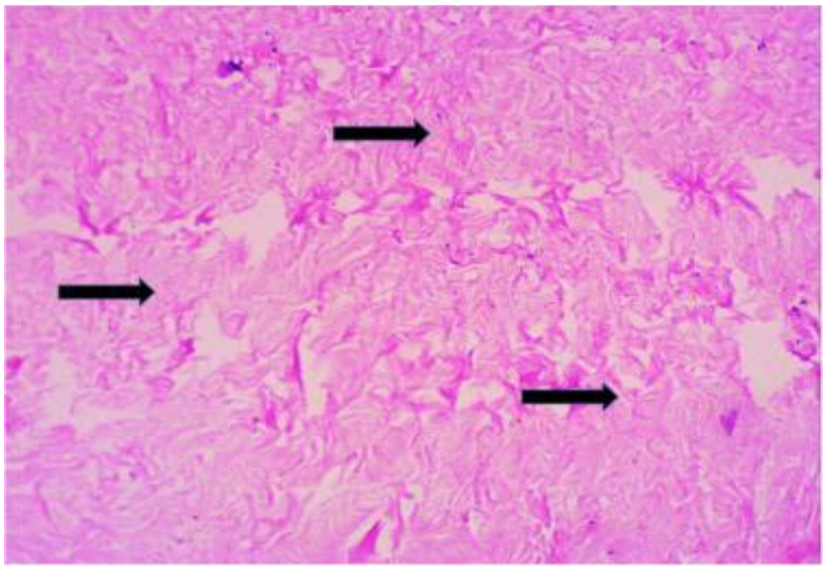
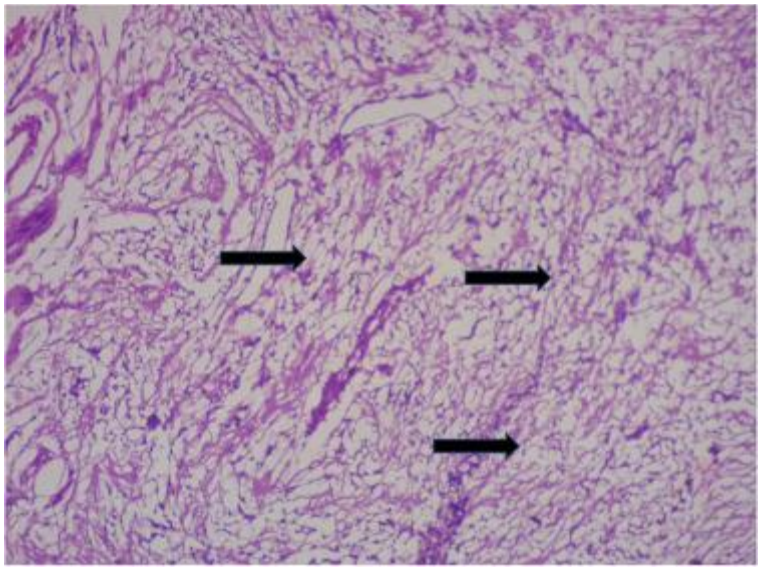


Photomicrograph. 5. Intense Eosinophilia observed in the gingival connective tissue 24 h post removal. (H&E 400X).



Photomicrograph. 6. Complete loss of architecture and prominent edematous changes seen in the gingival connective tissue 48hrs post removal. (H&E 200X).

**Table 2. Bio-enzyme Levels of Gingival Tissues at Different Time Intervals**

| Time (h) | Acid Phosphatase (Mean ± SD) | Ammonia (Mean ± SD) |
|---|---|---|
| **0 h** | 5.61 ± 0.67 | 1.13 ± 0.24 |
| **1 h** | 5.30 ± 0.43 | 8.26 ± 0.99 |
| **5 h** | 4.00 ± 0.87 | 9.80 ± 1.40 |
| **24 h** | 2.03 ± 0.37 | 17.75 ± 1.35 |

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| Time (h) | Acid Phosphatase (Mean ± SD) | Ammonia (Mean ± SD) |
|---|---|---|
| 48 h | 1.25 ± 0.53 | 26.60 ± 2.09 |

| Statistical Analysis | Acid Phosphatase (0–48 h) | Ammonia (0–48 h) |
|---|---|---|
| Kruskal-Wallis Value | 24.69 | 27.14 |
| p-value | 0.00006 | 0.00002 |

**Notes:**

† SD – Standard Deviation

‡ h – Hours

This version enhances readability and organizes the data in a structured format for better clarity. Let me know if you need any modifications!

## 5. Discussion

Determining the time of death is a crucial aspect of forensic science. While traditional methods for post-mortem interval (PMI) estimation continue to be widely used, newer techniques are being developed to improve reliability and reproducibility. One such approach, *Thanatochemistry*, involves the chemical analysis of tissues and bodily fluids, offering precise quantification for PMI determination.

This study aimed to examine changes in biopsied gingival tissues by correlating histological and biochemical/enzymatic alterations with potential applications for PMI estimation. We focused on two enzymes: **acid phosphatase**, an intracellular enzyme associated with autolysis, and **ammonia**, a byproduct of putrefaction [7]. Our findings, summarized in *Table 1* and *Graph 1*, indicate a progressive increase in ammonia concentration from 0 to 48 hours, whereas acid phosphatase levels showed a continuous decline over the same period. These biochemical changes were time-dependent and exhibited clear differentiation across the time intervals analyzed.

The most immediate biochemical and histological changes following death result from **vascular stasis**, leading to anaerobic glycolysis and the accumulation of acidic byproducts. This acidic environment triggers the release of lytic enzymes,

resulting in autolysis or enzymatic degradation of cellular components. However, these changes are not immediately apparent at the histological level.

A **twenty-fold increase** in ammonia concentration over time in gingival samples was observed in our study. This increase is likely due to the breakdown of amino acids and tissue degradation, with ammonia accumulation occurring as it is no longer metabolized by the liver. The predictable **ten-fold rise** in ammonia levels after five hours, continuing until 48 hours, suggests its potential as a PMI marker [7]. The gradual increase in ammonia concentration may be attributed to the gingiva's rich vascularization. Given the limited research on the relationship between ammonia levels in gingival tissue and PMI, further studies are warranted to establish its forensic significance.

Acid phosphatase levels remained relatively stable during the first five hours post-mortem but declined nearly **five-fold** between 24 and 48 hours. As an intracellular enzyme present in gingival tissue, acid phosphatase is a marker of **cellular degradation** [8]. Previous studies have linked increased acid phosphatase activity to destructive processes affecting alveolar bone and surrounding structures [15,16]. This suggests that acid phosphatase not only serves as a potential PMI indicator but may also provide insights into pre-existing periodontal disease in the deceased.

Traditional PMI estimation methods—such as **rigor mortis, algor mortis, and putrefaction**—are subjective, labor-intensive, and require specialized expertise. Additionally, they typically become evident only **six hours post-mortem** [17]. Since biochemical and histological changes precede visible morphological alterations, early-stage histological analysis may offer a more precise PMI estimation. While a few studies have examined histological changes in ante-mortem versus post-mortem gingival tissues [18,19], no standardized histological method has been established for PMI determination.

In our study, ante-mortem gingival tissue samples were examined at regular intervals. By the **fifth hour**, all stained sections exhibited features of autolysis, with **chromatin clumping** being the most prominent change in the prickle layer. **Nuclear vacuolation, karyopyknosis, and cytoplasmic vacuolation** were observed as early as one hour post-mortem, while **eosinophilia, widening of intercellular junctions, and prominent intercellular spaces** became noticeable by the fifth hour. Differences between the **5-hour, 24-hour, and 48-hour** intervals were minimal, suggesting that PMI estimation using histological changes is most reliable after the **fifth hour**. However, early and late-stage histological alterations were distinct enough to categorize PMI within broader timeframes.

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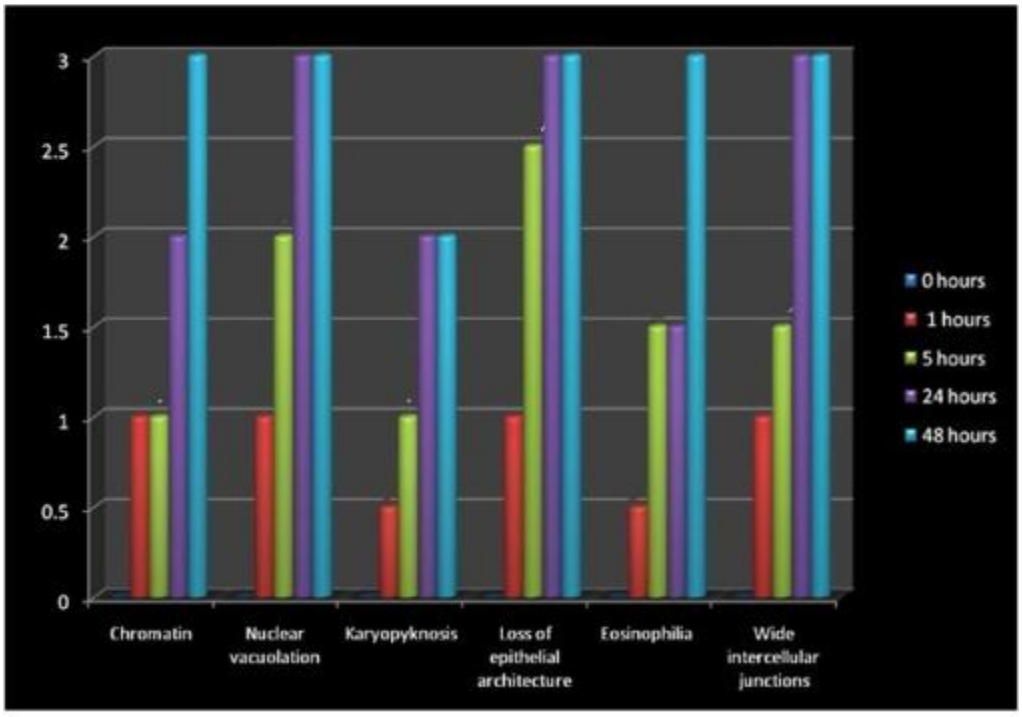
Previous research comparing ante-mortem and post-mortem histological changes suggests that **decomposition progresses more rapidly in ante-mortem tissues** [18–20]. This reinforces the potential value of integrating **biochemical and histological analyses** to improve PMI accuracy in post-mortem samples.

Several factors, including **specimen storage, processing, and staining protocols**, can influence observed changes. However, in this study, we controlled for these variables by maintaining standardized storage conditions, preventing specimen dehydration, and following established fixation protocols to minimize bias in autolysis rates.

To our knowledge, this is the **first study** to investigate both **histological and biochemical changes** in ante-mortem gingival tissue samples for PMI estimation. The histological changes observed align with findings from previous research on ante-mortem and post-mortem gingival tissues. Based on our results, the **time interval between 5 and 48 hours** appears to reflect the most significant biochemical and histological changes, making it a potentially valuable timeframe for early PMI estimation.




Graph 1. Distribution of histological changes of gingival tissues at various intervals. X axis denotes the changes in the epithelium. Y axis denotes the time in hours. Colours depict the time intervals of the samples with 0 being immediate.

## 6. Conclusion

Early post-mortem histological changes in gingival tissues, combined with ammonia and acid phosphatase enzyme analysis, may enhance the accuracy of

time-of-death estimation. Future research should focus on replicating this study using real cadavers under controlled taphonomic conditions while incorporating additional enzymatic markers to further refine PMI estimation.

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