DEMONSTRATION OF MAST CELLS BY QUANTITATIVE ESTIMATION OF TNF-α AND IL-3 BY ELISA FROM LUNG TISSUE IN FATAL MECHANICAL ASPHYXIAL DEATHS

THESIS

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CERTIFICATE

This is to certify that the thesis titled **"Demonstration of mast cells by quantitative estimation of TNF-α and IL-3 by ELISA from lung tissue in fatal mechanical asphyxial deaths"** is the bonafide work of **Dr. Rahul Panwar** 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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DECLARATION

I hereby declare that thesis entitled "**Demonstration of mast cells by quantitative estimation of TNF-α and IL-3 by ELISA from lung tissue in fatal mechanical asphyxial deaths**" embodies the original work carried outby the undersigned.

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(Dr. Rahul Panwar)

CONTENTS

LIST OF ABBREVIATIONS

ELISA: Enzyme-linked Immunosorbent Assay

IL-3: Interleukin-3

TNF-α: Tumor Necrosis Factor Alpha

MC: Mast cell

TB: Toluidine blue

n: Number of samples

SD: Standard deviation

OD: Optical density

Conc.: Concentration

PB: Peribronchial tissue

PA: Perialveolar tissue

PA: Perialveolar tissue

SA-HRP: Streptavidin-horseradish peroxidase

TMB: Tetramethylbenzidine

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INTRODUCTION

INTRODUCTION

In forensic practice, the diagnosis of death attributed to asphyxia is mostly made on gross findings(1). A significant number of the cases handled by forensic pathologists involve asphyxia-related fatalities, and the incidence of asphyxia-related deaths is second only to the incidence of deaths from mechanical trauma (2).

In medico-legal cases, proving death by asphyxia solely on the basis of external features can be difficult. Death by acute asphyxiation is sometimes difficult for the forensic expert, especially in sensitive cases when histological, toxicological, and other auxiliary studies have not produced fruitful results. In circumstances of fatal pressure compression over the neck, it may be simple to determine that the cause of death was mechanical asphyxia. Still, drawing that judgment may be more challenging in cases of smothering, drowning, aspiration, and other similar scenarios

Several forensic experts have cited postmortem features, including cyanosis, pulmonary edema, and petechial hemorrhages, as crucial indicators of suffocation. These findings, however, have also been linked to non-asphyxial fatalities, according to certain reports (3). Since these results have not been shown to be definitive, it is important to look for biomarkers that can assist forensic experts in determining the cause of death in suspected situations.

Many researchers have linked the asphyxial fatalities with biomarkers, including macrophages, red splenic arteriolar hyaline, Tau protein, and pulmonary surfactant protein A (SP-A) (4–7).

Mast cells have a role in the mediation of allergy and hypersensitive inflammatory responses. They are dispersed all across the body's connective tissues. Microvascular reactions and tissue remodeling are mediated by mast cell degranulation linked to systemic hypoxia. It has been demonstrated that systemic hypoxia causes pulmonary vasoconstriction, which is mediated by perivascular mast cells. The discovery of femoral blood taken from asphyxia fatalities has raised the amount of mast cell tryptase, further supporting the significance of mast cells in systemic hypoxia (8).

Hypoxia-induced pulmonary vasoconstriction is a physiological response to acute hypoxia that improves the ventilation and perfusion balance in the lungs. Mast cells are the most

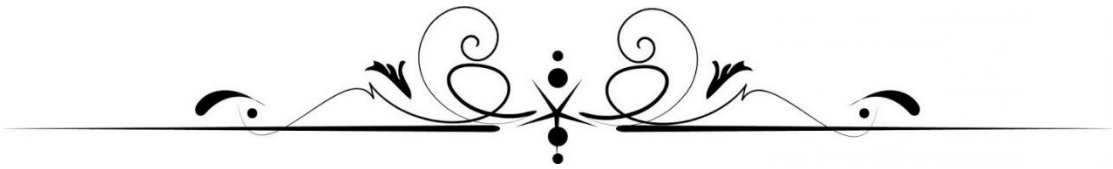
prolific source of proteolytic enzymes such as MC-specific proteases (tryptase, carboxypeptidase A, Chymase), biogenic amines, cytokines (most notably tumor necrosis factor), angiogenic growth factors under normoxic and hypoxic environments. Mast cells and matrix metalloproteinases play a critical role in remodeling pulmonary arteries during hypoxia (9).

Humans have a dramatic increase in perivascular mast cells due to oxygen deprivation, which is quickly recruited in the lungs within minutes (10). Studies on alveolar macrophages and mast cells in primary cell cultures revealed direct evidence of acute hypoxia-induced activation of macrophages (11).

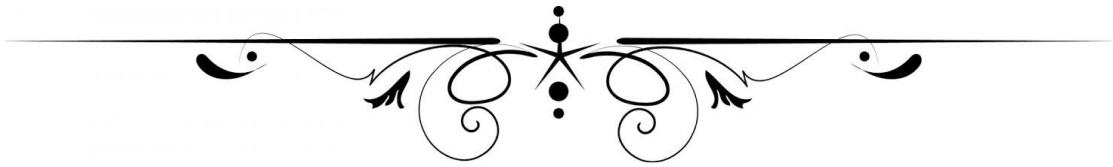
TNF-α is a cell signaling molecule (cytokine) that is a component of the acute phase response and is linked to systemic inflammation. Activated macrophages are the primary source of it. However, many other cell types can also produce it, such as T helper cells, natural killer cells, neutrophils, mast cells, eosinophils, and neurons. The TNF-α superfamily, which includes many transmembrane proteins with a homologous TNF-α domain, includes TNF-α as a member (12).

According to prior research, interleukin tests, such as IL-6 and IL-1, provide the sensitivity and specificity needed to identify newborn hypoxia (13). The pluripotent and hematopoietic cytokine IL-3, which is produced from T cells, is essential for the survival and growth of hematopoietic progenitor cells. The IL-3 gene, which is located on chromosome 5q31.1 in humans, encodes the interleukin 3 (IL-3) protein. Activated T cells, monocytes/macrophages, and stromal cells release IL-3 as a monomer. The main job of the IL-3 cytokine is to control the levels of different blood cell types. Both committed progenitors and early pluripotent stem cells are stimulated to proliferate and differentiate (14).

Based on these findings, we decided to investigate pulmonary mast cells' existence, quantity, and location in fatalities caused by fatal asphyxia using quantitative estimation by ELISA and special pathological stains. The association between mast cells and deadly asphyxia might aid forensic pathologists in making a differential diagnosis between fatal asphyxia and other causes of death when the court requires it. The present study is intended to study the role of mast cells in mechanical asphyxial deaths by estimating mast cells using histopathological techniques and measuring markers such as IL-3 and TNF-α concentrations using ELISA in asphyxial deaths.



REVIEW OF LITERATURE



REVIEW OF LITERATURE

ASPHYXIA

The word "asphyxia" comes from ancient Greek and, contrary to what is commonly said, really means "absence of the pulse" (sphygmós). There are several categories for "asphyxial" causes of death. To organize them, consider doing so into major categories: a) Suffocation is defined as breathing in polluted air. b) Mechanical "asphyxia" includes strangling by hand or ligature, hanging, and chest compression. c) Chemical "asphyxia" includes carbon monoxide and cyanide poisoning. Asphyxia has been hypothesized as a mechanism of death in all of these categories of causes of death (15).

In 1787, Farr released a concise textbook titled Elements of Medical Jurisprudence, briefly mentioning deaths caused by suffocation or strangulation (15).

The 1816 publication An Epitome of Juridical or Forensic Medicine; for the Use of Medical Men, Coroners, and Barristers by George Edward Male, MD, addresses hanging and strangling. Male asserts that the most common cause of death from hanging is asphyxia brought on by pressure from the cord (ligature) on the trachea; however, in other cases, the vertebrae of the neck are displaced (16).

In their three-volume textbook, Paris, and Fontblanque, which was released in 1823, they cite research on drowning and hanging that is identical to that of Male. Even though suffocation is the cause of death, it is impossible to exclude the fact that some factors, such as pressure on blood vessels, pressure on nerves, or fractures of the spine and odontoid process, might cause damage (17).

The first forensic medical textbook written in North America was titled Beck, released in 1823 (18). Alfred Swaine Taylor was England's foremost forensic medicine expert throughout the 19th century. Multiple editions of his medical law textbook were published. He extensively addresses "asphyxia" and notes that it was once thought to be equivalent to syncope in his first edition, which was released in 1843 (15). Syncope is defined as "the condition of seeming death brought on by the full stopping of circulation," In contrast, asphyxia is defined as "that state of appearing death which is caused by any cause, restricting the action of air on the blood during the process of breathing." A Treatise on Medical Jurisprudence, written by Americans Wharton and Stille, was released in 1855 (19). They described the postmortem manifestations of asphyxia.

At the University of Berlin, Casper served as a professor of forensic medicine. In 1864, the English version of his book A Handbook of Forensic Medicine was published. He asserts that suffocation death is caused by a negative poisoning of the blood because, when the atmospheric air is suddenly depleted of oxygen, which can occur in several ways, the blood is no longer able to vivify the nervous system and make it capable of performing its functions (20).

Casper listed the following 14 symptoms of "asphyxia":

1) In the bodies of persons who have died from suffocation, cadaveric stiffness happens under the same circumstances and lasts for the same amount of time compared to those who have died from any other cause.
2) The warmth in the internal organs lasts for a relatively long time.
3) Blood has a peculiar fluidity that is present in all types of suffocation without exception. Still, it is also undoubtedly present in cases of death from other causes, such as putrid fevers, narcotic poisoning, etc.
4) The blood's deep color.
5) Pulmonary apoplexy, or hyperemia of the lungs, is one of those symptoms that is seldom absent, but it is possible.
6) While the left side of the heart is either completely empty, which is uncommon, or only contains a few drachms of blood, the right side of the heart has hyperemia.
7) A clogged pulmonary artery is discovered.
8) Small capillary ecchymoses that resemble petechiae are observed underneath the pulmonary pleura in newborns and young children.
9) The mucous membrane of the larynx and trachea is more or less injected and cinnabar-red after every type of forceful suffocation, with the exception of that which proves deadly via neuroparalysis, which occurs instantly and leaves the distribution of the blood in the status quo.
10) Hyperaemia in the organs of the abdomen.
11) Organ hyperemia in the cranium. However, there are different degrees and frequent but minimal manifestations of this hyperemia.
12) Despite the fact that a person who is suffocating typically has a face that is more or less bluish-red, puffy, and has bulging eyes.

13) The protrusion of the tongue and clamping between the teeth or jaws, which are frequently used as indicators of suffocation-related deaths, are by no means exclusive to these types of deaths. Therefore, its look should not be given any significance.
14) As a last observation, the bodies of people who have been in any manner smothered frequently have the look of froth pouring out of the mouth. On the other hand, it is widely known that froth frequently seems to come from the lips after every type of death that is conceivable (including totally natural deaths) as a purely cadaveric occurrence, the result of the beginning of putrefaction. However, this phenomenon is not consistent.

Although Glaister said in his textbook of 1902, "asphyxia" was "consecrated by long use to denote disturbance with breathing, and it would only bring confusion were 'asphyxia' replaced for apnoea," he added. In 1953, Gordon, Price, and Turner released their textbook Medical Jurisprudence. They claimed that the term "asphyxia" was ambiguous. The term "asphyxia" was used to describe mechanical interference with breathing and the improper oxygenation of the blood in the lungs (anoxic anoxia). Visceral congestion, petechial hemorrhages, cyanosis, blood fluidity, and heart dilatation were all examined. They also spoke about biochemical modifications (21).

Gradwohl's Legal Medicine, which was initially published in 1954, discusses the most recent study of Gordon and Turner and recognizes the findings in "asphyxia" are not very specific. The second edition, edited by Francis Camps and released in 1968, once more acknowledged the vagueness of the "asphyxia" symptoms (22).

In 1974, Kay JM et al. investigated histamine, pulmonary histamine-forming capacity (PHFC), and lung mast cells (MC) in acute and chronic hypoxic pulmonary hypertension rats. They also examined pulmonary histamine-forming capability and lung mast cell hyperplasia. Six rats exposed to acute hypoxia in a decompression chamber that simulated 5,500 m of altitude did not exhibit any evidence of histamine depletion of perivascular MC, according to a histochemical analysis of lung tissue. Contrarily, intravenous administration of compound 48/80 to five rats within 2 minutes resulted in pulmonary MC degranulation and histamine depletion. The mean lung MC density rose from 2.97 & 0.48/mm2 in nine untreated controls to 7.65 & 1.23/mm2 in eight rats after chronic hypoxia for 20 days. Chronic hypoxia did not cause an increase in PHFC. In both control and chronically hypoxic rats, there was a linear, logarithmic relationship between right ventricular weight, a measure of pulmonary hypertension, and lung MC density. Data indicate that the perivascular lung

MC may be implicated in the modulation of the pulmonary pressor response to chronic hypoxia, even though histamine release from the lung MC during brief bouts of acute hypoxia has not been shown (11).

In 1987 Tamaki K et al. conducted a study, "Enzyme-linked immunosorbent assay for determination of plasma thyroglobulin and its application to postmortem diagnosis of mechanical asphyxia" they collected blood samples from mechanical asphyxia (n:14) and control group cause of death other than mechanical asphyxia. Cardiac blood samples were taken at medico-legal autopsies. The plasma was separated by centrifugation and kept frozen in 0.1% sodium azide until use. EDTA-infused samples of venous blood. The sandwich ELISA method was used. The sensitivity of the present ELISA was five ng/ml. All of the 15 patients who died for reasons other than mechanical asphyxia had plasma thyroglobulin levels below 200 ng/ml (99.8 +/- 37.9 ng/ml), compared to 12 out of the 14 victims of mechanical asphyxia who had levels over 200 ng/ml (2100 +/- 3450 ng/ml in 14 victims). Therefore, the current ELISA appears helpful for the postmortem identification of mechanical asphyxia (23).

Enzyme-linked immunosorbent test for plasma thyroglobulin following compression of the neck in autopsy cases was studied by Tamaki K et al. in 1990. They developed a very sensitive enzyme-linked immunosorbent test (ELISA) that they utilized to determine the plasma concentration of thyroglobulin (Tg) and make the postmortem diagnosis of the cause of death in cases with external compression of the neck. Within-assay and between-assay coefficients of variance were 2.4-6.6% and 6.8-12.0%, respectively. Tg levels determined by radioimmunoassay and ELISA were found to be significantly correlated (r = 0.996, P 0.001). All 36 cadavers without external neck compression or neck traumas had plasma Tg levels less than 200 ng/ml (73.6 51.9 ng/ml, M SD), despite these levels being somewhat greater than those in living bodies (16.7 11.8 ng/ml). However, the majority of the 42 victims of external neck compression-induced hypoxia had plasma Tg levels over 200 ng/ml, with the highest value coming in at 24,600 ng/ml. The cervical conditions in certain strangulation cases were evaluated, and in other cases, plasma levels of the supplementary thyroid hormones and thyroid-stimulating hormone were measured. According to their research, the thyroid gland was mechanically compressed during the agonal stage, resulting in Tg's release. Although thyroid conditions that result in excessive Tg levels are uncommon in autopsy practice

instances, they should be ruled out by regular thyroid histology at autopsy, making the discovery of a high Tg level more persuasive when presented as evidence (24).

A study on the immunohistochemical characterization of pulmonary giant cells and alveolar macrophages in terminal asphyxia was undertaken by Grellner et al. in 1996. Through the use of immunohistochemistry (APAAP technique) and semi-quantitative graduation, the characteristics of these alveolar cells were examined in a subset of fatalities with prolonged terminal oxygen deprivation. Twenty-two people who died from opiate overdoses, ten who died from deadly strangulations, and ten who died suddenly from cardiovascular causes were included in the study. Positive immunohistochemical reactions were observed for both the monoclonal antibodies PG-M1, a general marker of macrophages that detects nearly 100% of pulmonary macrophages and giant cells in all subgroups, and 25 F 9, a marker of late-stage inflammation that detects pulmonary macrophages/giant cells in 70%/50% of opiate-involved fatalities, 70%/20% of strangulations, and 40%/30% of control cases. The antigens did not stimulate these cell types for macrophages LN-4, 27E10, AMH152, or MIB1, which target early-stage inflammation, active macrophages, and macrophages, respectively (6).

In research titled "Cytokine Response in Cerebrospinal Fluid Following Birth Asphyxia," Sävman K et al. compare the levels of pro- and anti-inflammatory cytokines in the cerebrospinal fluid (CSF) of newborns who had suffered from asphyxiation to those of healthy controls. Twenty newborns who met the requirements for birth asphyxia and seven newborn control patients had their CSF samples taken. Using ELISA and a bioassay, the quantities of IL-1, IL-8, IL-10, tumor necrosis factor-alpha (TNF-α), and granulocyte/monocyte colony-stimulating factor (GM-CSF) were measured. Newborns that were asphyxiated (250, 35-543; median, interquartile range) had greater levels of IL-6 (pg/mL) than infants who were under control (0, 0-18). Additionally, there was a strong correlation between IL-6 and the severity of HIE and the final result. Additionally, there was a correlation between the level of HIE and IL-8, and the asphyxia group (170, 70-1440) had a greater IL-8 content (pg/mL) than the control group (10, 0-30) ($p = 0.009$). In conclusion, intrathecal levels of the proinflammatory cytokines IL-6 and IL-8 were correlated with the severity of HIE in asphyxiated newborns and were significantly higher in their CSF (25).

In forensic autopsy instances, the usefulness of pulmonary surfactant protein A (SP-A) as a realistic diagnostic marker of lethal mechanical asphyxia was assessed by Zhu BL et al. in their paper "Immunohistochemical evaluation of a pulmonary surfactant in fatal mechanical

asphyxia" published in 2000. Twenty-seven instances of asphyxia were studied histologically and immunohistochemically, while a control group of 16 cases of poisoning (9) and sudden accidental death (7) served as the comparison group. In both groups, there were histological signs of congestion, intra-alveolar and interstitial edema, localized atelectasis and emphysema, intra-alveolar hemorrhages, and in rare cases, pulmonary hemorrhages. About 60% of the time, the mechanically asphyxiated group had an intra-alveolar area with much more SP-A staining and many large aggregates than the control cases. These formations may be seen as collections of pulmonary surfactant freed from the alveolar wall due to increased secretion by vigorous forced breathing or mechanical asphyxia's overstimulation of the autonomic nervous system. Their research findings indicate that it is possible to identify mechanical asphyxia from other forms of hypoxia using immunohistochemistry SP-A detection (4).

In 2001 Vacchiano G et al., in their study "Is the appearance of macrophages in pulmonary tissue related to the time of asphyxia?" The authors examined 50 asphyxiated human lungs, focusing on the number of large cells and alveolar and interstitial macrophages. They contrasted 25 asphyxiated human lungs from histological specimens with 25 asphyxiated human lungs from histological specimens following a quick asphyxia (10–15 min) with a delayed asphyxia (30 min or more). Per section, alveolar and interstitial macrophages and large cells were taken into account and counted. Histological examinations of the injured lungs were used as controls. There were 27.74.4 macrophages per section in the pulmonary alveoli after acute suffocation. 68.27.1 alveolar macrophages were found in each area of those who died from delayed asphyxiation (p 0.001). The presence of interstitial macrophages was also common. There are no discernible changes in the quantity of polynuclear giant cells in human lungs that have been asphyxiated either quickly or slowly. As further histological evidence of gradual asphyxia, the amount of alveolar and interstitial macrophages in each slice can be used to distinguish between a slow asphyxia and an acute one (26).

In 2002 Ishida K et al., in their study "A quantitative RT-PCR assay of surfactant-associated protein A1 and A2 mRNA transcripts as a diagnostic tool for acute asphyxial death," In order to understand the molecular pathophysiology of SP-A, created a method for the quantitative RT-PCR detection of SP-A1 and SP-A2 mRNA transcripts. Using this method, the fatalities from acute mechanical suffocation (n=12) and drowning (n=9) were compared to the control groups (n=17) of fatalities from cardiac arrest (n=11) and death from brain injury (n=6). The

mean value of the SP-A1/A2 ratio was low in controls and much higher in drowning (6.72) and mechanical asphyxia (brain laceration, 2.56; acute myocardial infarction, 2.80). The evaluation of the molecular changes in SP-A linked to acute asphyxial death may be aided by studying the SP-A1/A2 ratio (27).

The microvascular inflammatory response to systemic hypoxia is mediated by mast cells, according to research done in 2003 by Dawn R. S. Steiner et al. Increased venular leukocyte-endothelial adhesion and emigration, vascular permeability, and reactive oxygen species (ROS) are indicative of an inflammatory response brought on by systemic hypoxia. Activated perivascular cells that produce the chemotactic gradient release mediators that normally cause inflammation to begin. These tests were designed to investigate potential mast cell involvement in microvascular inflammation by hypoxia. Using intravital microscopy on anesthetized rats, the mesenteric microcirculation was examined for vascular permeability, leukocyte adherence and emigration, mast cell degranulation, ROS levels, and leukocyte degranulation. These findings demonstrate the critical function of mast cells in hypoxia-persuaded inflammation and raise the possibility that changes in the ROS-nitric oxide balance contribute to mast cell activation during hypoxia (28).

Using enzyme-linked immunosorbent assays, Khalid Z. Matalka et al. conducted research in 2005 on the measurement of protein cytokines in tissue extracts. Following intraperitoneal injection of endotoxin-free PBS or lipopolysaccharide, mouse tissues were harvested. To improve cytokines extraction, 0.1% Igepal, a mild detergent, was added to the buffer. Following disruption, homogenization, and centrifugation of the tissues, the supernatants were collected and subjected to solid-phase immunoassays for analysis. Such outcomes In comparison to PBS alone, 0.1% Igepal dramatically increased the amount of TNF-alpha and IL-10 extracted from the liver (220%, p0.001), brain (358%, p0.05), and lungs (1600%, p0.01). However, compared to PBS alone, 0.1% of Igepal did not enhance IFN-gamma extraction from the liver, spleen, brain, lungs, skin, or kidneys (29).

A study on the effects of acute and chronic hypoxia, as well as a seven-day recovery from chronic hypoxia, on the location of pulmonary mast cells and the production of their MMP-13 in rats, was carried out in 2006 by Ludek Vajner et al. In rats that had been exposed to hypoxia for 4, 20, and 7 days (10% O2), the location of mast cells and the expression of the interstitial collagenase, MMP-13, were assessed. Toluidine blue staining was used to identify mast cells, while monoclonal antibodies were used to find MMP-13 expression.

Mast cell counts and MMP-13 expression significantly increased within the walls of alveolar arteries after four but not 20 days of hypoxia. In 20-day-treated rats, MMP-13 positive mast cells accumulated in the subpleural and conduit artery walls (30).

In 2006 Aly H et al., in their study "IL-1β, IL-6 and TNF-α and outcomes of neonatal hypoxic-ischemic encephalopathy" According to them, the levels of the inflammatory proteins interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha in CSF are correlated with the severity of brain damage and can predict neurological deficits in infants with hypoxic-ischemic encephalopathy. Prospective research was carried out, including 24 term babies with HIE diagnoses and 13 healthy controls. All newborns had blood and CSF samples taken from them during the first 24 hours of their lives as part of normal examinations for meningitis and sepsis. A neurological examination and the Denver Developmental Screening Test II (DDST II) were performed on the infants at 6 and 12 months of age. Compared to controls, infants with HIE showed significantly higher IL-1, IL-6, and TNF levels. IL-1 in the CSF was more significantly associated with the severity of HIE ($r=0.61$, $P=0.001$) than IL-6 ($r=0.45$, $P=0.004$) or TNF-α ($r=0.47$, $P=0.003$). Among the three cytokines under study, IL-1β had the greatest CSF/serum ratio, indicating that it was released locally in the brain following the first hypoxic damage. IL-1β in the CSF was the strongest predictor of abnormal neurological symptoms and abnormal DDST II at 6 and 12 months (sensitivity = 88%, specificity = 80%) (31).

Mast cell tryptase levels in postmortem serum—reference levels and confounders were the subjects of a 2007 research by Edston et al. They focused on the sample location, conjunctival petechial hemorrhage, prone position at the time of death as indicators of premortem hypoxia, and resuscitation efforts by external cardiac massage as some of the characteristics suspected of creating erroneously high tryptase concentrations. Sixty deaths were looked into, and tryptase levels in blood from the femoral vein were measured in 39 control cases who died suddenly (within minutes) from natural causes (sudden cardiac death and acute aortic dissection), 16 from prolonged asphyxia and five from anaphylaxis. Tryptase levels in the heart and femoral blood were tested in 44 of these instances. It was shown that 95% of the controls had levels < 44.3 g/l (femoral blood), SD 5.27 g/l. Tryptase concentrations were higher than that threshold in all but one of the cases of allergic fatalities. Compared to the controls, tryptase levels in femoral blood from anaphylactic fatalities were considerably higher ($p=0.007$). Additionally, tryptase levels in femoral blood were higher in

instances where suffocation had caused death (p=0.04). Blood from the heart and the femoral vessels had significantly different tryptase concentrations (p0.02) across the board (n=44). Premortem asphyxia tends to impact tryptase levels, femoral blood serum should be used for postmortem tryptase assays, and 44.3 g/l is considered the usual upper limit, or 95% coverage (32).

In 2008 Ikematsu K et al., in their study "Temporal expression of immediate early gene mRNA during the supravital reaction in mouse brain and lung after mechanical asphyxiation," wanted to know which immediate early genes (IEGs), such as c-fos, fos-B, and c-jun, were expressed after somatic death and at the site of the supravital response in mechanically asphyxiated mouse brain and lung. SLC, Inc. provided male BALB/c mice that were nine weeks old. These mice were given anesthesia before having their necks compressed by ligating them with a thread for 30 minutes, which caused them to pass away. The brain and lungs were dissected within 30 minutes or 60 minutes of death. Mice were beheaded and subsequently put to death in order to assess the IEG expression pattern in samples with and without hypoxia. After reverse transcription of total RNA, quantitative real-time PCR was carried out. They observed that in the mouse brain's forebrain, hippocampus, and cerebellum, the transcripts c-fos and fos-B levels were dramatically elevated due to mechanical hypoxia brought on by neck ligation. They also demonstrated that, depending on the cause of death and the types of organs being studied, differences in IEG expression occurred during supravital reactions after death. Additionally, after mechanical hypoxia, IEG expression rose significantly. These findings imply that the gene expression pattern after suffocation is distinct (33).

In their study titled "Histological findings and immunohistochemical surfactant protein A-SP-A expression in asphyxia: its application in the diagnosis of drowning," 120 cadavers from persons with a mean age of 48.73 years and a mean postmortem delay of 30 hours were investigated by Pérez C et al. in 2008. The cases were divided into three groups based on the scene, the cause and circumstances of death, and the autopsy results: (a) drowning (n=47); (b) other asphyxia (n=44); and (c) other causes (n=29). Histological analyses of H&E staining and immunohistochemistry surfactant protein A expression were performed on the upper and lower lobes of the lungs. Combining immunohistochemical SP-A expression with the presence and degree of congestion, hemorrhage, and edema may help distinguish asphyxia from other causes of death and drowning from other kinds of asphyxia. Even while the data

from the upper lobe may be more crucial for determining the specific cause of death, the results point to the need for both lobes to be investigated in order to make the diagnosis (34).

In 2009 Takahashi H et al. conducted a study, "Increase in dual specificity phosphatase 1, TGF-beta stimulated gene 22, domain family protein 3 and Luc7 homolog (S. cerevisiae)-like messenger RNA after mechanical asphyxiation in the mouse lung" Male BALB/c mice that were nine weeks old were purchased from SLC, Inc. Following anesthesia, the necks of these mice were strangulated with a thread for 30 minutes, which caused death, as previously described. Immediately upon death, 30 minutes later, and 60 minutes later, the lungs were dissected. The tissues were subsequently dissected as stated after control mice were decapitated without neck compression. They used serial gene expression analysis to examine the transcriptome profile of mechanical hypoxia and decapitation 60 minutes after death. When the results were compared, it became clear that mechanical asphyxia treatment significantly increased 11 genes in the mouse lung. The expression of three of these genes—dual specificity phosphatase 1 (Dusp1), TGF-beta stimulated gene 22, domain family protein 3 (TSC22d3), and Luc7 homolog (Saccharomyces cerevisiae)-like (Luc7l)—was significantly higher after suffocation than after decapitation, according to quantitative real-time PCR data. They discovered that quantifying the number of protein products in the lung might be useful in identifying asphyxia. These discoveries may also provide insight into the pathophysiology of asphyxia and help with asphyxia diagnosis, particularly hanging (35).

In their 2010 study "Mast Cell Survival and Mediator Secretion in Response to Hypoxia," Gulliksson M. et al. examined the impact of hypoxia in general on human mast cell endurance, mediator exudation, and reactivity. Mast cells isolated from human cord blood were cultured under three distinct conditions: normoxic (21% O2), hypoxic (1% O2), or hypoxic culture for 24 hours before stimulation in normoxic (21% O2). Mast cells did not degranulate due to hypoxia per se, but they did notice a rise in IL-6 release, and IL-6 produced by the autocrine system helped the mast cells survive. A23187-induced degranulation and cytokine release were unaffected by hypoxia. Contrarily, under hypoxia compared to normoxia, cytokine production after LPS or CD30 treatment was decreased but not blocked. According to their research, hypoxia does not impair mast cell survival, degranulation, or cytokine release. This may be significant for both host defense, where mast cells can respond to invaders in hypoxic tissue, and chronic inflammation, where mast cells' response is unrestricted by the hypoxia associated with the inflammation (36).

In 2010, Boskabadi H et al. published their paper, "Association between serum interleukin-6 levels and severity of neonatal hypoxia." Serum IL-6 levels in 45 randomly selected healthy newborns, and 37 consecutive uninfected neonates with perinatal hypoxia were measured at birth and 24 and 48 hours after giving birth. They found that children with asphyxia who later had hypoxic-ischemic encephalopathy had serum IL-6 concentrations that were 1.9 folds higher than those of normal infants and 43 folds higher than those of infants who later developed hypoxic ischemic encephalopathy ($p< 0.001$). The degree of hypoxic-ischemic encephalopathy at discharge and neurological developmental outcomes were associated with serum IL-6 levels. They concluded that newborns with hypoxia had elevated blood levels of IL-6, especially in those with poor outcomes (37).

In 2010 Turillazzi E et al., in their study "Tryptase, CD15, and IL-15 as reliable markers for the determination of soft and hard ligature mark vitality," observed that traditional macroscopic and histological results might not be accurate; vital signs are frequently missing and might be created after death. Using immunohistochemistry methods created a brand-new area of study into the problem of ligature marks. To determine if hanging signs and signals developed before or after the victim's death, researchers looked at the immunohistochemistry expression of a panel of cytokines and inflammatory cells in skin tissues from autopsy cases of hanging fatalities. They selected 21 instances where wide, soft materials were used for hanging and 28 instances where firm materials were used. The 21 cases that made up the control group were 7 cases of postmortem hanging (suspension) of bodies and 14 cases of sudden cardiac death (drug intoxication or suffocation as a cause of death in all the cases). Using antibodies against tryptase, fibronectin, TNF-α, IL-6, IL-8, IL-10, MCP-1, IL-15, IL-1ß, CD45, CD4, CD3, CD8, CD68, CD20, and CD15, skin tissues were examined immunohistochemically. They conclude that the variables tryptase, IL-15, and CD15 appear trustworthy in determining the liveliness of ligature signs for forensic reasons. This characteristic is especially true for soft markings, which are challenging to assess using traditional histological investigations and physical inspection (38).

Morphological characterization of astrocytes in the hippocampus during mechanical asphyxiation was studied in 2010 by Li DR et al. The morphology of astrocytes in the hippocampus and serum S100B levels were measured, and cases of mechanical asphyxia from neck compression were compared to cases of conventional autopsy findings and acute myocardial infarction/ischemia. Compared to asphyxiation brought on by other factors and

AMI, asphyxiation brought on by neck compression resulted in a larger drop in the number of intact astrocytes, as seen by S100 and GFAP-immunostaining, proving a connection between the rise in blood S100B levels. These findings imply that cerebral hypoxia and congestion, particularly in cases of mechanical asphyxia brought on by neck compression, are the main contributors to hippocampus astrocyte damage (39).

In 2014 Cecchi R et al., in their study "Markers of mechanical asphyxia: an immunohistochemical study on autoptic lung tissues," investigated if any of the antigens may be used as markers of asphyxia death by immunostaining the lungs of 62 autopsy cases acute mechanical asphyxia was present in 34 cases, while anti-P-selectin, anti-E-selectin, anti-SP-A, and anti-HIF-α antibodies were present in 28 control cases. The findings demonstrate that P- and E-selectin expression in the blood arteries of the lungs, which is induced by several trigger events other than hypoxia, cannot be interpreted as an indicator of asphyxia. As they seem connected to intense hypoxic stimulation, intra-alveolar granular deposits of SP-A may indicate that severe hypoxia was the cause of mortality when present in high amounts. The majority of mechanical asphyxia fatalities and CO intoxications exhibited HIF1-α expression, with the amount and concentration of positive-stained vessels rising with the duration of the hypoxia. Small, medium, and big size lung vessels contained HIF1- α (40).

Barbara Muciaccia et al. conducted an investigation into the involvement of mast cells in asphyxia in 2016. Through the immunodetection of the HIF1-alpha protein, a crucial regulator of cellular response to hypoxic conditions, they were able to demonstrate in an immunohistochemistry (IHC) experiment the responsiveness of lung tissue vasculature to hypoxia. Evidence linking asphyxia deaths to increased blood levels of tryptase enzymes produced by mast cells (MC) suggests that HIF1-alpha production and MC activation in hypoxic conditions may be related. This hypothesis motivated their inquiry into the potential involvement of pulmonary region mast cells in acute asphyxia fatalities. In order to evaluate peri-airway and peri-vascular MC as well as their numbers and features, the lung from 47 autopsy cases 35 asphyxia fatalities, 11 controls, and 1 anaphylactic death was processed by IHC analysis using anti-CD117 (c-Kit) antibody. According to the findings, a considerable rise in peri-vascular c-kit (+) MC was seen in various asphyxia deaths, including hanging, strangling, and aspiration deaths (8).

In a study done in 2016 by Zeng Y et al., it was shown that G6PC3, ALDOA, and CS induction coincides with mir-122 downregulation in mechanical asphyxia and can act as

hypoxia indicators. They analyzed the levels of expression of each miRNA in the human brain and heart samples from individuals who had mechanical asphyxia, (n:21) to those from instances of craniocerebral injury (n:18) and hemorrhagic shock cases (n:2) (n:18). In an additional 84 human specimens and rat model studies, they further confirmed differentially expressed miRNAs. Immediately following collection, RNA Later solution was applied to all samples. Reverse transcription and RNA purification were carried out initially, followed by Western blot analysis, 3'UTR reporter test, and quantification of gene expression (qRT-PCR assay). The hypoxia responses that control glucose and energy metabolism are shown by data to be mediated by mir-122 and its potential targets G6PC3, ALDOA, and CS, which can also function as hypoxia biomarkers. Compared to craniocerebral damage and hemorrhagic shock, they were shown to be considerably up-regulated in the brain and heart tissues in cases of mechanical asphyxia (41).

In research using Tau protein as a biomarker for asphyxia published in 2016 by Salama M et al., 20 (C57/bl6) mice of both sexes were used. Under isoflurane anesthesia, PND-7 saw right common carotid artery ligation in the HI group (10 mice). Mice were given 8% oxygen balanced with nitrogen for 20 minutes at 37 °C two hours after recovery. Age- and strain-matched naive (HI-free) mice were used as the control group. The anti-PHF Tau AT8 antibody toward pS202/pT205 was generated as a mouse monoclonal primary antibody and used to stain free-floating sections. The average postprocessing thickness was 20 m. Analysis of tau aggregates conducted one day after the HI model was detected revealed a significantly substantial elevation compared to the control group. P value < 0.001 was significant compared to the control group (5).

In 2017 Pérez IL et al., in their study, focused on the determination of vitality in skin wounds in ligature marks from suicide hanging; they observed Within a postmortem window of 19 to 36 hours, a total of 71 skin wounds were examined. Together and individually, the levels of Zn, Fe, Mg, and Ca and the expression of cathepsin D and P-selectin were examined by the immunohistochemistry or Immunohistochemical Semiquantitative Analysis. There were higher quantities of Ca and Mg than of Fe and Zn. Fe content increased with the severity of the damage, while Ca and Zn concentrations declined. Subcutaneous damage and low or medium Fe concentrations were connected with a large percentage of moderately negative expressions of both proteins. Finally, it is possible to distinguish between a damaged vital wound and non-injured skin through the combined analysis of metallic ions and proteins (42).

A study on how various cytokine patterns may control the severity of newborn hypoxia was carried out by Bajnok et al. in 2017. Blood samples were taken between 3 and 6 hours after birth, at 24 hours, 72 hours, one week, and one month after birth, all based on receiver operator curve analysis. Neonatals were split into two groups: mild and severe. Early on in asphyxia, it appears that the predominance of CD4+ IL-1β+ and CD4+ IL-1β+ CD49d+ cells may be used to predict how severe the insult would be. Both groups' intracellular TNF- levels in CD4 cells increased at all intervals relative to 6 hours. At one month, intracellular TNF-α levels were higher in the severe group. Plasma IL-6 levels increased in the severe group at 1 week and decreased in the moderate group at 1 month. Intracellular IL-6 concentrations peaked in both groups after 24 hours. The intracellular TGF-β levels increased in the moderate group after 24 hours (43).

In order to diagnose and predict the short-term projection of brain injury instigated by neonatal asphyxia, Zhang XH et al. in 2017 looked into the dynamic monitoring of neuron-specific enolase (NSE), high mobility cluster protein Bl (HMGBl), and tumor necrosis factor (TNF-α), as well as amplitude-integrated electroencephalogram (aEEG) monitoring, have therapeutic importance. The study included 60 full-term newborns delivered between January and December 2015. The newborns were divided into 3 groups: 30 control newborns admitted to the NICU without asphyxia, 7 newborns with severe asphyxia, and 23 newborns with moderate asphyxia. Before being sent to the NICU, neonates with asphyxia were given routine newborn resuscitation. The results of the aEEG and dynamic shifts in the levels of TNF-α, HMGBl, and NSE in the umbilical artery and peripheral blood were monitored and compared across the groups. The umbilical artery and serum TNF-α, HMGBl, and NSE thresholds at day 1 were significantly greater in the two asphyxia groups than in the control group, and the values were higher in the severe asphyxia group ($p < 0.05$). All the monitoring time points showed positive correlation coefficients between TNF-α and HMGB1, TNF-α and NSE, HMGB1 and NSE, and TNF-α and NSE. Additionally, serum TNF-α, HMGBl, and NSE levels were higher in the babies with abnormal aEEG findings at 6 hours postpartum than in the neonates with normal aEEG results ($p < 0.001$), 0.5516, 26.943, and 15.87, respectively (44).

In 2017, Feng X et al. investigated the expression of Glucose-Regulated Protein 78 and miR-199a in Rat Brain After Fatal Ligature Strangulation, 12 rats in the control group (n= 12) and 12 rats in the ligature strangulation group (n = 12). In a rat model of ligature strangulation,

the expressions of GRP78 and miR-199a in the cortex, hippocampi, and midbrain were examined using immunohistochemistry and Western blot analysis. They discovered that, compared to the control group, the ligature strangulation group had significantly higher levels of glucose-regulated protein 78 in the cortex and midbrain. The expression of GRP78 in the hippocampi did not differ noticeably between the two groups. The ligature strangulation group had substantially lower levels of miR-199a-3p in the cortex and midbrain ($P < 0.01$). However, miR-199a-5p did not suggestively diverge among the two groups in any brain regions. Conclusion: Ligature strangulation is a physiological and pathological process impacted by ER stress, and miR-199a upstream may have a significant regulatory impact on mechanical asphyxia (45).

To find a reliable asphyxia biomarker, Zeng Y et al. conducted a study in 2018 titled "DUSP1 and KCNJ2 mRNA upregulation can serve as a biomarker of mechanical asphyxia-induced death in cardiac tissue." They first examined 44 samples of human cardiac tissue from people who had died from mechanical asphyxia, head injury, hemorrhagic shock, or other causes, comparing the expression levels of 47,000 mRNAs in specimens from people who had died from mechanical asphyxia with the levels of the corresponding mRNAs in the specimen. They found that expression of the potassium voltage-gated channel subfamily J member 2 (KCNJ2) and dual-specificity phosphatase 1 (DUSP1) was higher in human heart tissues from the mechanical asphyxia group than in control tissues. DUSP1 and KCNJ2 may be linked to mechanical asphyxia-induced mortality as a result, and they may also be useful biomarkers of mechanical asphyxia death (46).

In a research titled "The Principal and Auxiliary Immunohistochemical Markers of Intravital Mechanical Strangulation Asphyxia," published in 2018, Bogomolov DV et al. examined 17 instances of mechanical strangulation asphyxia including 13 males and 4 women, ranging in age from 8 to 28 years. Standard histological and immunohistochemical procedures and polyclonal antibodies against total cytokeratin, fibrinogen, immunoglobulin-lambda, fibronectin, and CD-117 antigen were used in the unique laboratory research. The histology preparations were stained using toluidine blue and the Spielmeyer procedure. The CD-117 antigen in the pulmonary tissue indicates an upsurge in CD-117 positive mast cells in asphyxial fatalities and can also be used for such diagnoses. Alveolar hypoxia cases are characterized by the expression of this antigen (47).

Palmiere C et al. investigated the postmortem stability of numerous molecules (thyroglobulin, iodothyronines, calcitonin, and parathyroid hormone) in blood after death in their study "Postmortem biochemical investigation results in situations of fatal mechanical compression of the neck region," published in 2018. Their research revealed that while total and free T4 levels were falling and total and free T3 concentrations were increasing, postmortem levels of thyroglobulin, calcitonin and parathyroid hormone remained steady. Furthermore, their research found that antemortem mechanical stress applied to the neck area (hanging cases) is associated with greater thyroglobulin levels in peripheral (femoral) blood. However, specific cases can occur when these levels are not enhanced. Last but not least, their results demonstrated that postmortem serum specimens derived from blood drawn at different sampling locations might reveal higher thyroglobulin, total T3, and free T3 concentrations even in the absence of microscopically evident thyroid gland tissue damage (48).

Lung patterns of the inflammatory response to alveolar hypoxia and the importance of these patterns for the diagnosis of asphyxiation were the focus of a 2019 study by Ewgenija Gutjahr et al. The number of alveolar phagocytes, megakaryocytes, giant, and mast cells was counted using H&E and toluidine blue staining in specimens of suffocated human lungs after a short (n=13) and a long asphyxia terminal episode (n=15), as well as controls (sudden cardiovascular (n=11) and traumatic deaths (n=7)). CD68, late (25F9), and early (MRP-8/-14) stage inflammatory markers were immunohistochemically labeled to demonstrate macrophage activation. Despite the link between suffocation and severe lung edema, the results demonstrated the lack of specificity of macroscopic measures. There were no discernible changes in the number of inflammatory cells between case groups in the lungs. Compared to cardiovascular controls, MRP-8- and MRP-14-positive cells increased by five times, demonstrating an early stage of pre-existing monocyte activation (49).

In 2019, Zhang H et al. showed that under hypoxia, the mitochondrial proteins Cyto c and AIF are transported to the cytoplasm to start the apoptotic process. In order to determine if Cyto c and AIF are expressed in the cytoplasm, they developed the animal asphyxia model and the cell hypoxia model based on these phenomena. They followed up by using human samples to authenticate the results. They revealed that whereas the two proteins were expressed in the cytoplasm of mechanical asphyxia groups, they were only weakly demonstrable in the cytoplasm of other groups. This significant result offers hope for elucidating the specific processes of mechanical asphyxia (50).

In a study published in 2020, Umut Ocak et al. discovered that mast cell tryptase inhibition reduces neuroinflammation in rats after asphyxial cardiac arrest via the PAR-2/p38/NF-B pathway. Those who survive cardiac arrest experience neurological dysfunction, including cognitive deterioration. It has been demonstrated that mast cell tryptase increases microglial inflammation by activating microglial protease-activated receptor-2 (PAR-2). This study examined the underlying mechanism of PAR-2/p-p38/NF-B signalling during asphyxia-induced cardiac arrest in rats and the possible anti-neuroinflammatory advantages of mast cell tryptase inhibition. During suffocation-induced cardiac arrest, the levels of mast cell tryptase and PAR-2 were noticeably elevated in the brain. In resuscitated rats, mast cell tryptase inhibition by APC366 enhanced both short- and long-term neurological results (51).

Cases of mechanical suffocation significantly enhanced the expression of the miRNA-3185 (miR-3185) in cardiac tissues compared to cases of craniocerebral injury, sudden cardiac death, hemorrhagic shock, and poisoning, according to research by Han L. et al. published in 2020. They also pointed out that there is no correlation between postmortem duration, age, or temperature and miR-3185 expression. In contrast to earlier examples, they found that cardiac tissue samples collected from victims of mechanical asphyxia express CYP4A11, a possible miR-3185 target gene, at a low level. They contend that the miRNA-3185/CYP4A11 axis is linked to mechanically caused death and may add new information to research on asphyxial death (52).

Four biological indices found in human cardiac tissue were used by Han L. et al. in their paper titled "Model for the prediction of mechanical asphyxia as the cause of death" in 2021. In this study, 156 cadavers and autopsies were examined overall. 49 deaths by mechanical suffocation and 107 deaths from other causes, including poisoning, hemorrhagic shock, sudden cardiac death, and craniocerebral injury, were covered. Total RNA quality was thoroughly assessed, and the results showed that the average A260/A280 nm ratio for all samples was 2.06 0.03 (range 2.03 to 2.09), while the average A260/A230 nm ratio was 2.07 0.08. (varying between 1.89 and 2.17) Receiver operating characteristic curves for the Cq values of the four indices were produced based on the electrophoretic analysis, which showed separate bands for the 18S rRNA and 28S rRNA, suggesting good total RNA integrity without evident degradation (53).

In a 2022 study by Hu Y et al., they looked at the function of the ER stress-related protein CHOP in mechanical asphyxia. Eight groups (n=3) of different causes of death were included

in the study, along with rats with three sets of PMIs (0 h, 6 h, and 12 h), simulating the real-world situation. The PMI and cause of death for the human samples were categorized. The samples in the short-term PMI groups have a PMI of 6.3442.792 (n=32), whereas those in the long-term PMI group have a PMI of 25.567.999 (n=32). Each group has eight samples from each of the following causes of death: mechanical asphyxia, hemorrhagic shock, brain damage, and other causes of death. As a result, they found that over a brief postmortem interval (PMI), the ER stress-related protein CHOP significantly increased in the brain tissue of DMA samples. Additionally, it was demonstrated in human samples that the degree of CHOP expression might serve as a potential biomarker of DMA within a particular PMI (54).

AIM & OBJECTIVES

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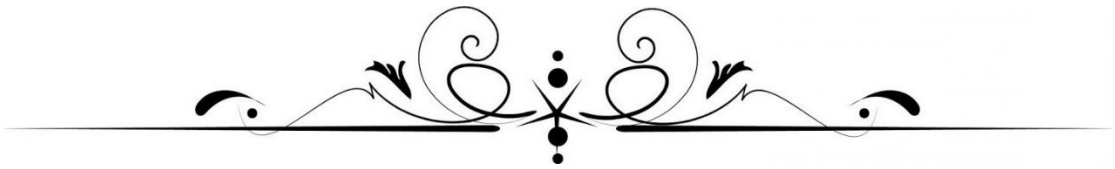
Aim:

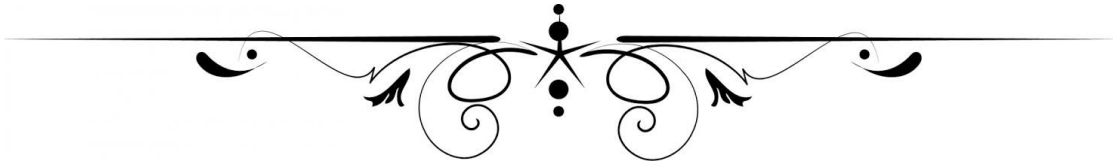
To study the role of mast cell degranulation in fatal mechanical asphyxia deaths.

Objectives:

1. To estimate TNF- α by quantitative ELISA from lung tissue in fatal mechanical asphyxia and non asphyxial deaths.
2. To estimate IL-3 by quantitative ELISA from lung tissue in fatal mechanical asphyxia and non-asphyxial deaths.
3. To demonstrate/ evaluate mast cells by toluidine blue in the lung tissue samples of fatal mechanical asphyxia and non-asphyxial deaths.
4. To compare the numerical values of TNF-α and IL-3 in fatal mechanical asphyxia and non asphyxial deaths.



MATERIALS & METHODS



MATERIALS AND METHODS

The study was carried out at the Departments of Forensic Medicine and Toxicology, Biochemistry and Pathology, and Lab Medicine at the All India Institute of Medical Sciences (AIIMS), Jodhpur. The study began with the Institutional Ethics Committee's (IEC's) approval (certificate number: AIIMS/IFC/2021/3315). 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 study stage, and complete confidentiality was maintained.

Study design

The proposed study was an autopsy-based prospective study.

Study duration

Ninety (90) medico-legal autopsy cases were included in the study during the period of January 2021 to July 2022**.**

Sample size

The anticipated feasible sample size for the current study was 90 (45 cases: 45 controls) for a period of 18 months (as per the availability of cases observed from the previous trend). Informed consent of the relatives of the deceased was taken. Human lung tissue samples were taken from human cadavers at the mortuary of AIIMS, Jodhpur. The collected lung tissue samples were sent for histopathological examination and quantitative ELISA separately. We collected lung tissues from the hilar region of each lung for quantitative ELISA and the perihilar and peripheral regions of each lung for histopathology; both the tests and tissue preservation were done according to the test requirements. Routine macromorphological, histological, and toxicological results were used to determine the cause of death, and cases with primary pulmonary diseases were excluded from the study. We analyzed IL-3 and TNF-α by ELISA, with two samples from each case (1 from the right lung and 1 from the left). Therefore from these 90 cases, we studied 180 human lung tissue samples over 18 months.

The cases were classified into two categories. The first category comprised cases of asphyxia [total n= 45: hanging (n=35), drowning (n=8), and smothering (n=2)]. The second category served as a control group/category (CC), consisting of deaths due to head trauma, traffic accident, or non-asphyxial causes. [total n=45: road traffic accidents (n=14); railway traffic accidents (n=4), sudden unexpected death (n=8), head injury (n=4), fall from height (n=3), poisoning (n=7), electrocution (n=3), physical assault (n=2)]. The study did not include cases of pulmonary pathology like diffuse alveolar damage (DAD), bronchiolitis, pulmonary hemorrhage, and pulmonary edema in cases of poisoning.

Information regarding the deceased

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

Inclusion criteria

Cases of fatal mechanical asphyxia deaths (Hanging, strangulation, smothering, aspiration, drowning)

Exclusion criteria

1. Death due to anaphylactic shock.
2. Any other condition in which mast cells are increased as information collected from previous medical history.

Histopathological examination

Tissue pieces from each case's right and left human lung were received from the perihilar location and peripheral location in 10% neutral buffered formalin. Representative sections were taken from the tissues and processed in an automated Leica tissue processor (Image 1). Microtome was used to slice tissue into thin sections 4-5 microns thick, and an automated slide stainer (Leica) was used to apply Hematoxylin & Eosin (H&E) stain to the slides. Representative sections were stained for toluidine blue manually to highlight mast cells by the following steps:

First, a toluidine blue working solution (pH 2.0-2.5) was prepared from the Toluidine blue stock by the following steps:

Toluidine blue stock solution

Toluidine blue O - 1g + 100 mL of 70%alcohol = Mixed to dissolve

Sodium chloride (1%)

0.5g sodium chloride + 50mL distilled water = Mixed to dissolve (this solution was made fresh each time). pH adjusted to 2.0-2.5 using glacial acetic acid/HCl

Toluidine blue working solution (made fresh each time and discarded after use)

45 mL of 1% sodium chloride + 5mL toluidine blue stock solution, pH 2.0-2.5
Mixed well, and the pH of this solution was adjusted to 2.0-2.5.

Staining procedure

1. Sections are deparaffinized and hydrated with distilled water.
2. Stain sections for 2 to 3 minutes in a toluidine blue working solution.
3. Wash in distilled water; 3 changes.
4. Dehydrate quickly through 95% and 2 changes of 100% alcohol (10 dips each)
5. Clear in xylene, 2 changes, 3 minutes each
6. Coverslip with mounting media

The toluidine blue stained slides were examined on a microscope (Nikon, model Eclipe Ci-L) (Image 2) on 40X (field area 0.332mm^2). The peribronchial, perialveolar, and perivascular locations were seen for the presence and number of mast cells in 5 fields, and the mean mast cell count per field was calculated.

Image 1: Tissue processed in automated Leica tissue processor.

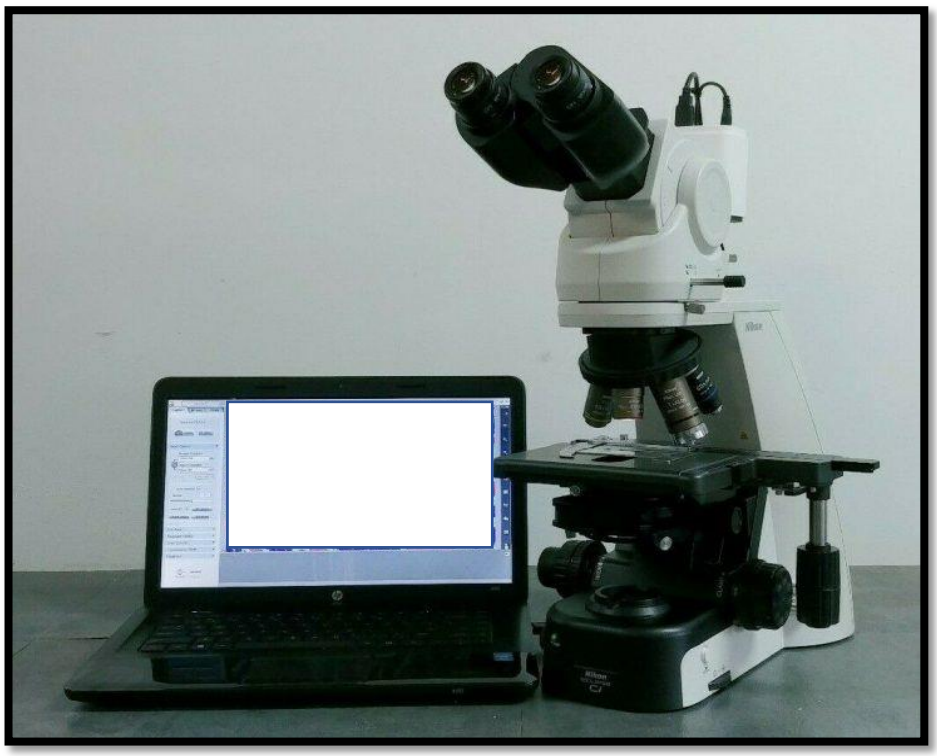


Image 2: The toluidine blue stained slides were examined on microscope (Nikon, model Eclipe Ci-L)

Cytokine analysis by ELISA

Principal of the present ELISA method

Sandwich enzyme immunoassay serves as the test principle used in this kit (G-Biosciences) (Image 4). Interleukin-3 (IL-3) and tumor necrosis factor alpha (TNF-α) specific antibodies have been pre-coated on the microtiter plate. A biotin-conjugated antibody specific for IL-3 and TNF-α is added to the appropriate microtiter plate wells after adding standards or samples. Each microplate well is then filled with and incubated with Avidin conjugated to Horseradish Peroxidase (HRP). Only the wells containing IL-3, TNF-α, biotin-conjugated antibody, and enzyme-conjugated Avidin will demonstrate a change in color once the TMB solution is introduced. The sulphuric acid solution is added to stop the enzyme-substrate reaction, and the change is detected spectrophotometrically at a wavelength of 450nm + 10nm (Figure 1).

The optical density (OD) of the samples is then compared to the standard curve to measure the concentration of Interleukin-3 (IL-3) and Tumor Necrosis Factor Alpha (TNF-α) in the samples.

Sample collection and storage

Each human lung in the case and control groups had tissue samples removed from the hilar area, which were then placed in PBS buffer and kept at -80°C.

Tissue homogenates

- Before homogenization, tissue was weighed at 750µg after being properly cleansed in ice-cold PBS to eliminate excess blood.
- Using a glass homogenizer set up on ice, the tissue was minced into small pieces and homogenized in freshly prepared PBS lysis buffer.
- An ultrasonic cell disrupter was used to sonicate the resultant suspension until the solution was clear.
- The homogenates were then centrifuged for 30 minutes at 12000 rpm.
- Immediately test the supernatant after collection, or aliquot it and preserve it at -80°C.

ELISA Summary

Equilibrated the kit at room temperature, add 100µL of sample to each well

80 minutes at 37°C (Incubation)

Discard the liquid in the plate, add 200 µL of Wash Buffer,

3 x assay buffer (Washing)

Appropriate amount: add 100 µL Biotinylated antibody working solution

50 minutes at 37°C (Incubation)

Discard the liquid in the plate, add 200 µL of Wash Buffer,

3 x assay buffer (Washing)

Appropriate amount: add 100 µL SA-HRP (Streptavidin-horseradish peroxidase)

50 minutes at 37°C (Incubation)

↓

Discard the liquid in the plate, add 200 µL of Wash Buffer,

5 x assay buffer (Washing)

↓

Appropriate amount: add 90 µL TMB(Tetramethylbenzidine) substrate solution

↓

20 minutes at 37°C (Incubation)

↓

Appropriate amount: add 50 µL stop solution to each well

↓

Read plate at 450nm immediately (Reading)

↓

Results (Calculation)

Flowchart depicting the steps of sandwich ELISA for estimation of TNF-α and IL-3 in asphyxial and non-asphyxial deaths.

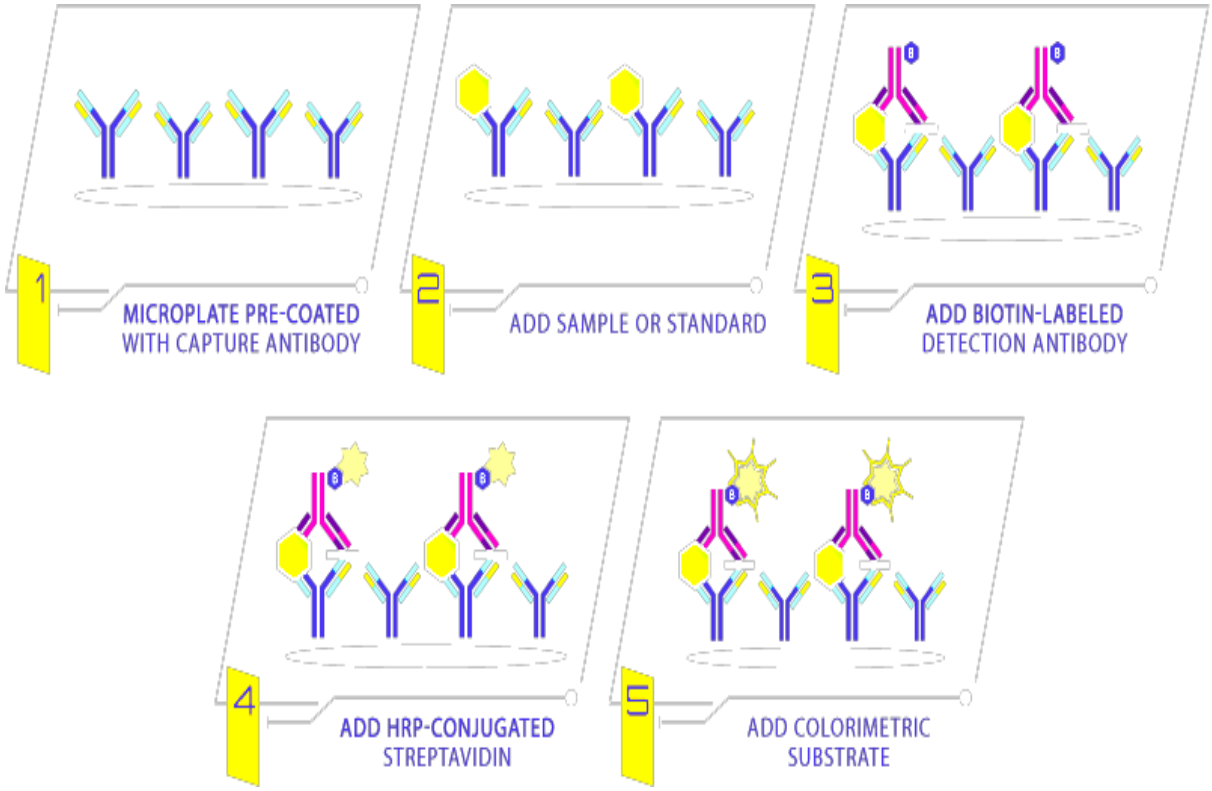


Figure 1: Schematic illustrating the sandwich ELISA procedure.
(Source: Raybiotech)

Materials required

SAMPLE	EQUIPMENTS	REAGENTS
➢ Human lung tissue	➢ Weighing scale ➢ Centrifuge machine (Image 8) ➢ Homogeniser ➢ Micropipette (Image 3) ➢ Microplate washer (Image 5) ➢ Microplate Spectrophotometer (Image 6) ➢ Orbital Incubator Shaker (Image 7)	➢ Biotinylated antibody ➢ Streptavidin-horseradish peroxidase ➢ Tetramethylbenzidine

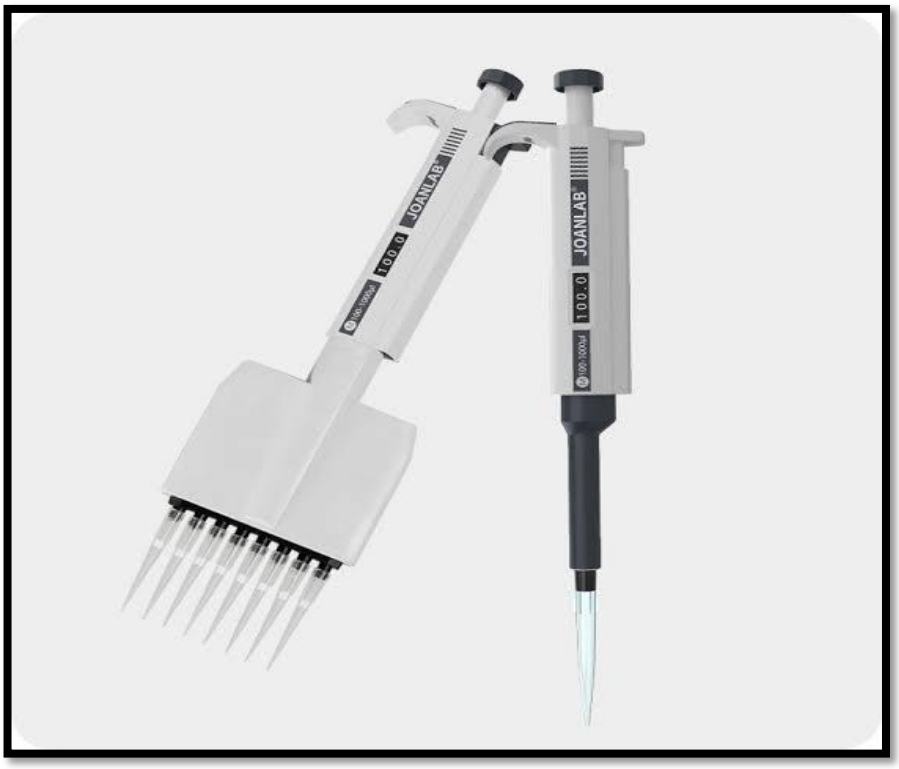


Image 3: Multichannel micropipette and one channel micropipette

Image 4: Sandwich ELISA kit (G-Biosciences)

Image 5: ELx50 ELISA Microplate Washer

Image 6: Eon™ Microplate Spectrophotometer

(Wavelength 200nm to 999nm)

Image 7: The Thermo Scientific MaxQ 6000 Orbital Incubator Shaker

Image 8: The Thermo Scientific Fresco™ 21 Microcentrifuge

Statistical considerations

Data were recorded in Microsoft office excel sheet 2019 and were analysed and processed using Statistical Package for Social Sciences (IBM SPSS v26.0) software. Descriptive statistics (minimum, maximum, mean and standard deviation) were calculated for the continuous variables. IL-3 and TNF-α optical density (OD) and concentration (Conc.) values were estimated by using sandwich ELISA in Asphyxia and Non-asphyxial cases and were calculated using the Unpaired T-Test. To determine if there are real differences in the results of the peri-bronchial, perialveolar, and perivascular zones in Toluidine blue stained human lung tissue samples, the Mann-Whitney U test was performed. The Kruskal Wallis test was used to determine the differences in mast cell mean rank between asphyxial (Cases) and non-asphyxial (Control) samples.

RESULTS

RESULTS

The study comprised ninety (90) autopsy cases. The age of the cases ranged from 2 to 68 years (Figure 2), with a mean age of 33.23 years. 74 cases were of males, and 16 were of females. For the histopathological analysis, we used lung tissue samples from each lung's peribronchial, perialveolar, and perivascular areas, and for the ELISA test, we used the hilar region of each lung.

Descriptive statistics of TNF-α and IL-3 in the form of concentrations and optical density are given in Table 1, and for mast cells in histopathological examination with toluidine blue stain, given in Table 4, which shows statistically significant results.

According to statistics comparing the asphyxial cases and control groups, the asphyxial group had more mast cells expression than the control group. Additionally, quantitative ELISA demonstrated significantly more IL-3 and TNF-α concentrations in cases of hanging, drowning, and smothering compared to the control group. Increased mast cells were noted in the peri-alveolar, peri-bronchial and perivascular locations in the lung sections of asphyxia deaths. Atelectasis and vascular congestion were found in the lungs of both asphyxial and non-asphyxial deaths.

The Mann-Whitney U test was used to ascertain whether there are genuine differences between the findings of the peri-bronchial, perialveolar and perivascular zones given in Table 5. Mast cell concentration in the peri-bronchial, perialveolar and perivascular regions of the asphyxia group showed statistically significant changes by histopathological examination using toluidine blue stain analysis, peri-bronchial ($Z = -5.92$, $p < 0.01$), perialveolar ($Z = -6.78$, $p < 0.01$), perivascular ($Z = -7.40$, $p < 0.01$).

Table 1: Descriptive statistics of TNF-α, IL-3 in the form of concentrations and optical density in Deaths Associated with Fatal Asphyxia performed on both Cases and control groups.

	Asphyxial samples			**Non-asphyxial samples**		
	n	Range	Mean ± SD	n	Range	Mean ± SD
TNF-α (OD)	90	0.33 – 3.33	0.76 ± 0.43	90	0.11 – 0.84	0.34 ± 0.16
TNF-α (Conc.)	90	211.17-3201.55	499.75 ± 479.41	90	53.85 – 445.40	208.08 ± 81.23
IL 3 (OD)	90	1.51 – 3.96	2.85 ± 0.59	90	0.33 – 3.28	1.64 ± 0.83
IL 3 (Conc.)	90	776.46–2209.35	1558.50 ± 350.53	90	86.27 – 1810.62	849.73 ± 484.99

n = Number of samples from each lung; SD = Standard deviation; OD = Optical density; Conc. = Concentration

Table 1 shows the descriptive statistics (minimum, maximum, mean and standard deviation) for TNF-α and IL-3 in the form of concentrations and optical density in deaths associated with asphyxia (Case) and non-asphyxial (Control) groups.

Table 2: Estimation of IL-3 and TNF-α (OD) value by ELISA in Asphyxia and Non-asphyxial cases were calculated using the Unpaired T-Test.

Parameter		N	Mean ± SD	Significance (P)
IL 3 (OD)	Asphyxia	90	2.85 ± 0.59	<0.01
IL 3 (OD)	Non- asphyxia	90	1.64 ± 0.83	<0.01
TNF-α (OD)	Asphyxia	90	0.76 ± 0.43	<0.01
TNF-α (OD)	Non- asphyxia	90	0.34 ± 0.16	<0.01

n = Number of samples from each lung; SD = Standard deviation; OD = Optical density;

Table 2 shows the mean change of IL-3 and TNF-α (OD) value estimated using ELISA, which shows the Mean ± SD in asphyxial and non-asphyxial cases. In asphyxial cases, the average IL-3 optical density (OD) was found to be 2.85 ± 0.59. The average TNF-α optical density (OD) was 0.76 ± 0.43. In contrast, in non-asphyxial cases, the average IL-3 optical density (OD) was found to be 1.64 ± 0.83, and the average TNF-α optical density (OD) was

0.34 ± 0.16. The mean change in IL-3 and TNF-α (OD) values are found to significant (<0.01) in asphyxial cases as compared to non-asphyxial cases.

Table 3: Estimation of IL-3 and TNF-α (Conc.) value by ELISA in Asphyxia and Non-asphyxial cases were calculated using the Unpaired T-Test.

Parameter		N	Mean ± SD	Significance
IL 3 (Conc.)	Asphyxia	90	1558.50 ± 350.53	<0.01
IL 3 (Conc.)	Non- asphyxia	90	849.73 ± 484.99	<0.01
TNF-α (Conc.)	Asphyxia	90	499.75 ± 479.41	<0.01
TNF-α (Conc.)	Non- asphyxia	90	208.08 ± 81.23	<0.01

n = Number of samples from each lung; SD = Standard deviation; Conc. = Concentration

Table 3 Shows IL-3 and TNF-α (Conc.) value estimated using sandwich ELISA, which shows the Mean ± SD in asphyxial and non-asphyxial cases. In asphyxial cases, the average IL-3 concentration (Conc.) was found to be 1558.50 ± 350.53, and the average TNF-α concentration (Conc.) was 499.75 ± 479.41. In contrast, in non-asphyxial cases, the average IL-3 concentration (Conc.) was found to be 849.73 ± 484.99, and the average TNF-α concentration (Conc.) was 208.08 ± 81.23.

The mean change in IL-3 and TNF-α (Conc.) values are found to significant (<0.01) in asphyxial cases as compared to non-asphyxial cases.

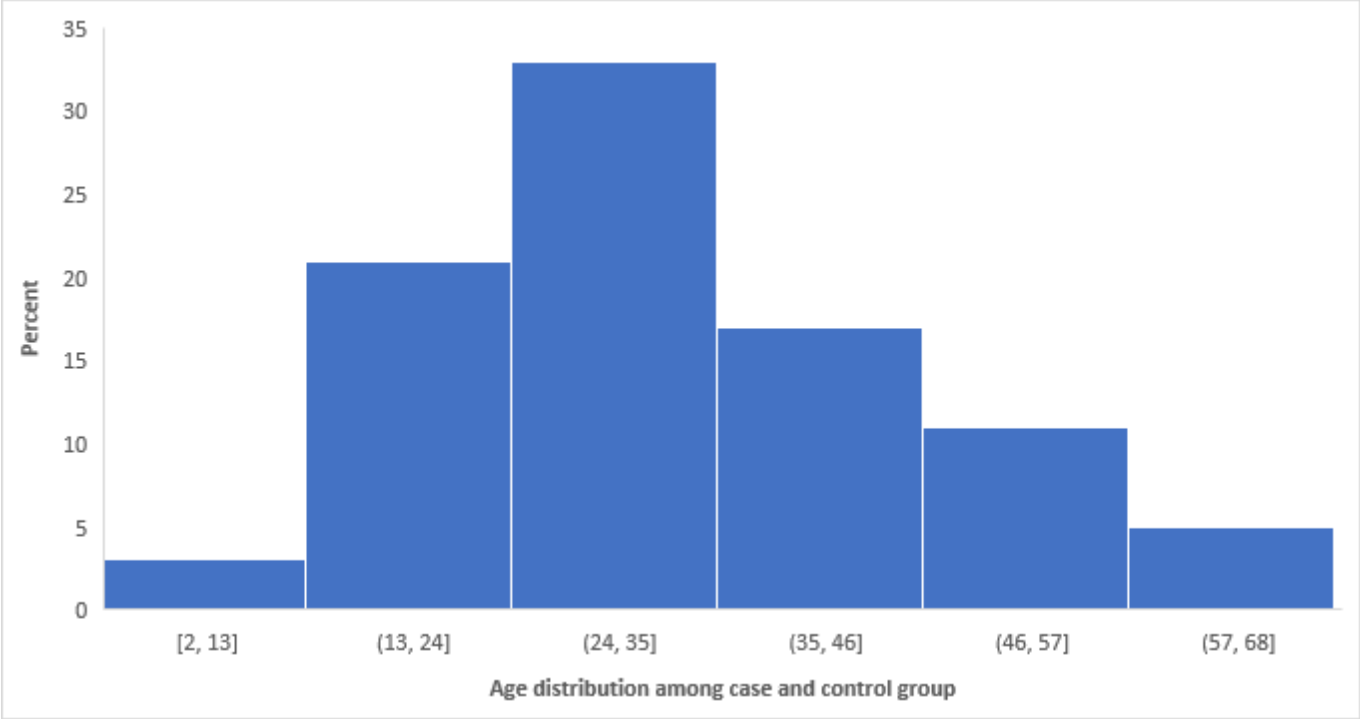


Figure 2: Bar chart showing age distribution among asphyxial (case) and non-asphyxial (control) group.

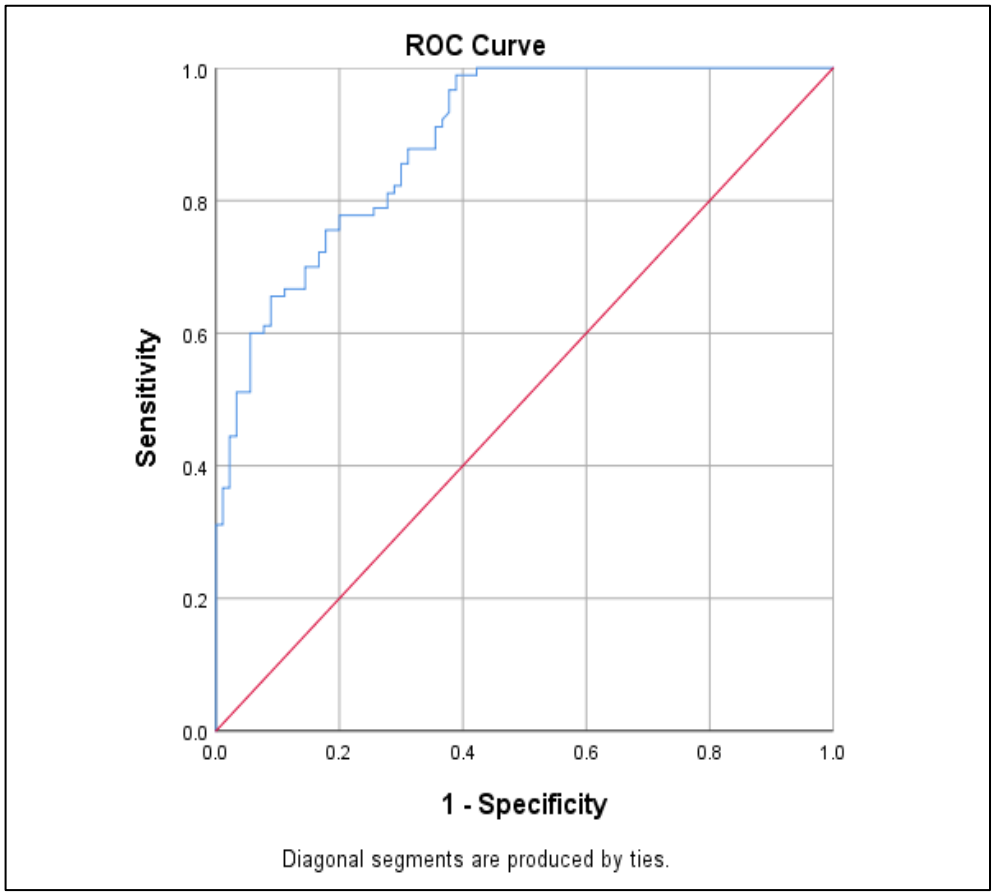


Figure 3: The ROC curve for TNF-α concentration (Conc.) in cases of asphyxia/non-asphyxia

ROC analysis revealed that TNF-α concentration (Conc.) was a stronger predictor of asphyxial/non-asphyxial deaths (AUC=0.89) (Figure 3)

The optimal cut-off value using the ROC curve for estimation of the asphyxial component using TNF-α concentration (Conc.) in our study is found to be 455.20 with a sensitivity of 31% and specificity100%, Youden index of 0.31.

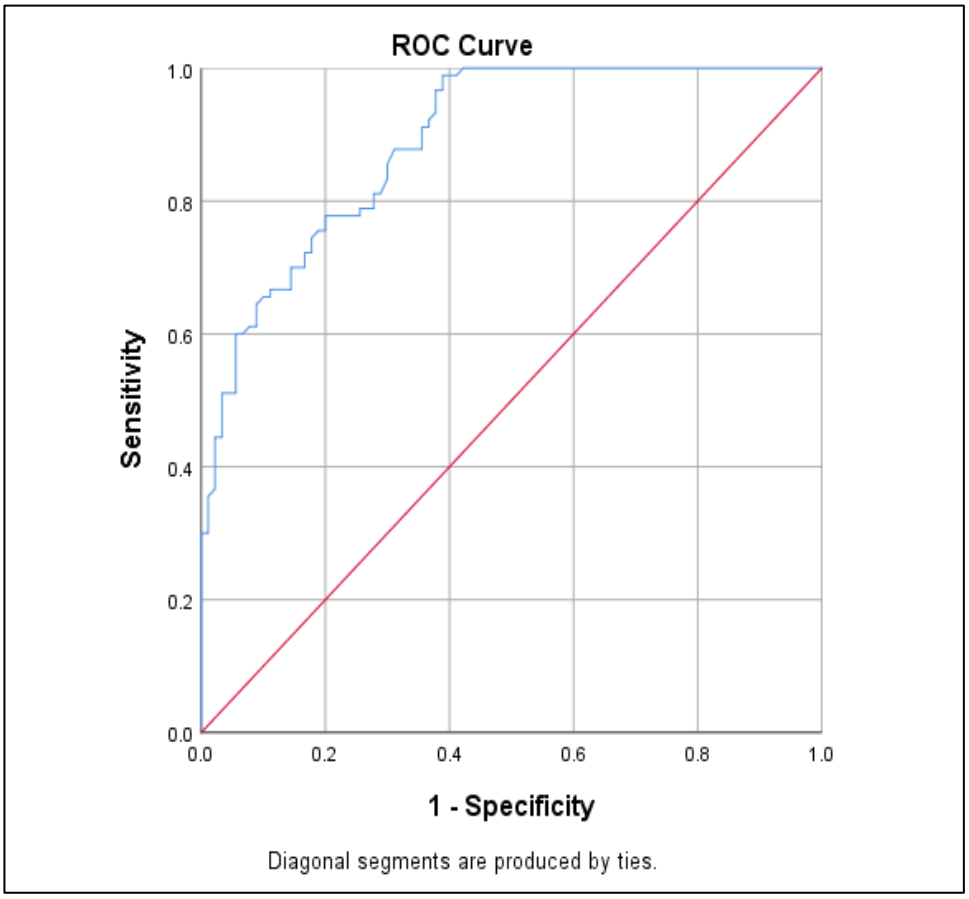


Figure 4: The ROC curve for TNF-α optical density (OD) in cases of asphyxia/non-asphyxia

ROC analysis revealed that TNF-α optical density (OD) was a stronger predictor of asphyxial/non-asphyxial deaths (AUC=0.89) (Figure 4)

The optimal cut-off value using the ROC curve for estimating asphyxial components using TNF-α optical density (OD) in our study is 0.85 with a sensitivity of 30% and specificity of 100%, Youden index of 0.30.

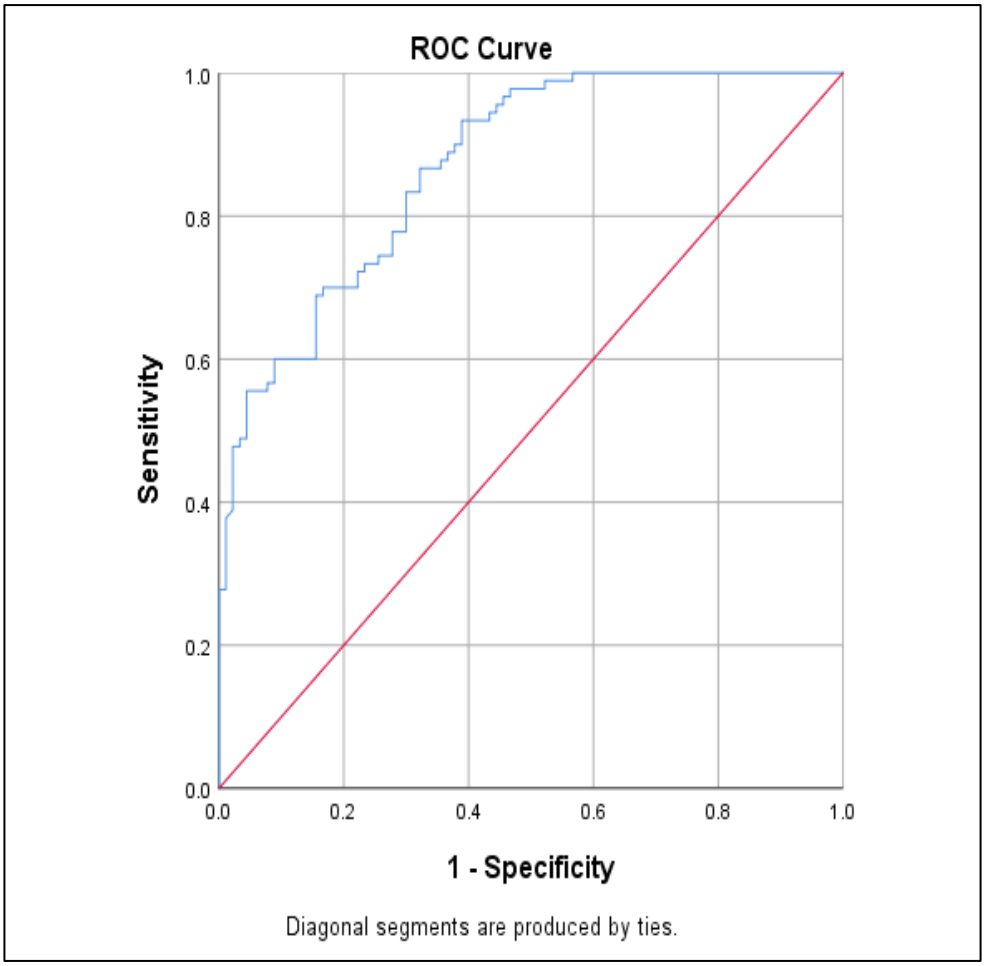


Figure 5: The ROC curve for IL-3 concentration (Conc.) in cases of asphyxia/non-asphyxia

ROC analysis revealed that IL-3 concentration (Conc.) was a stronger predictor of asphyxial/non-asphyxial deaths (AUC=0.87) (Figure 5)

The optimal cut-off value using the ROC curve for estimating the asphyxial component using IL-3 concentration (Conc.) in our study is 1700.62 with a sensitivity of 37% and specificity of 99%, Youden index 0.38.

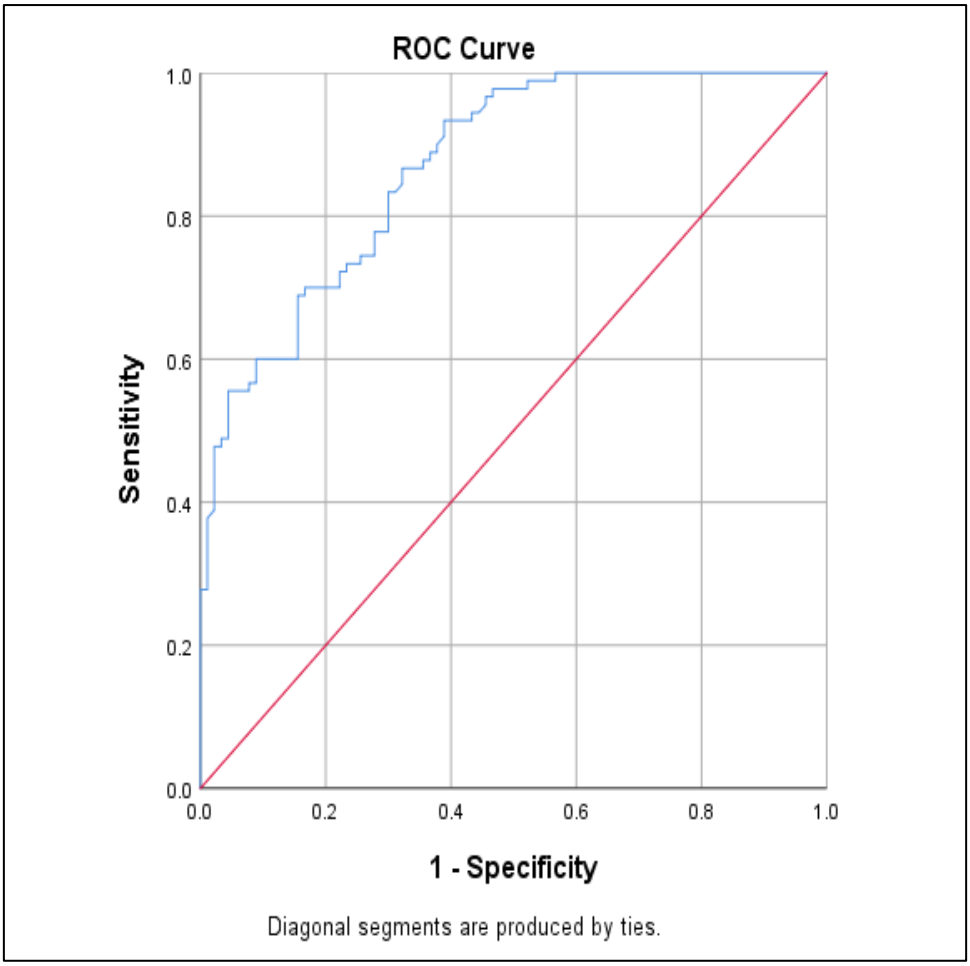


Figure 6: The ROC curve for IL-3 optical density (OD) in cases of asphyxia/non-asphyxia

ROC analysis revealed that IL-3 optical density (OD) was a stronger predictor of asphyxial/non-asphyxial deaths (AUC=0.87) (Figure 6)

The optimal cut-off value using the ROC curve for estimation of the asphyxial component using IL-3 optical density (OD) in our study is found to be 3.09 with a sensitivity of 37% and specificity of 99%, Youden index 0.38.

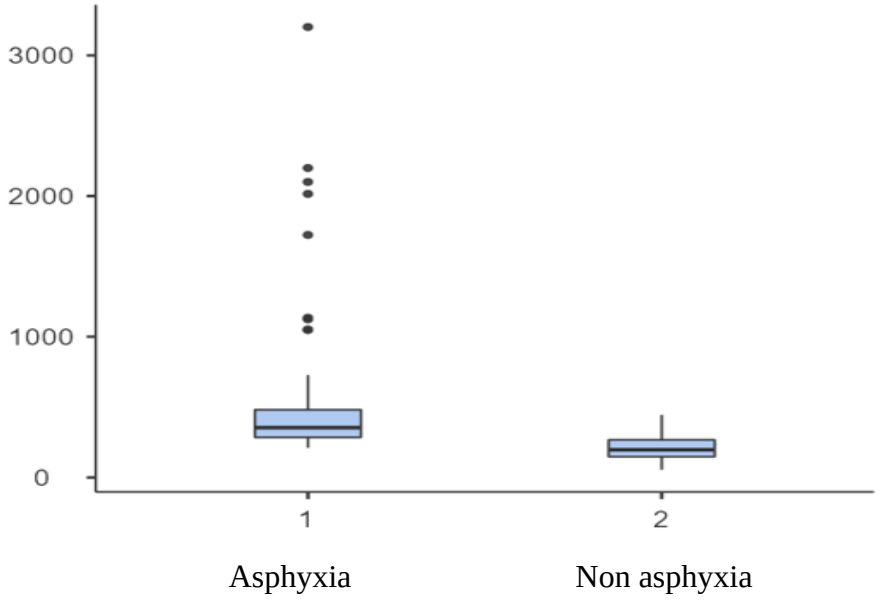


Figure 7: Box and Whisker plots of the TNF-α concentration (Conc.) in asphyxial and non-asphyxial cases.

TNF-α concentrations in asphyxial and non-asphyxial controls are depicted in Figure 7 as box and whisker plots, and the plots demonstrate that the asphyxial case group has a considerably greater concentration than the non-asphyxial group. The whisker displays the standard deviation, while the box displays the mean concentration.

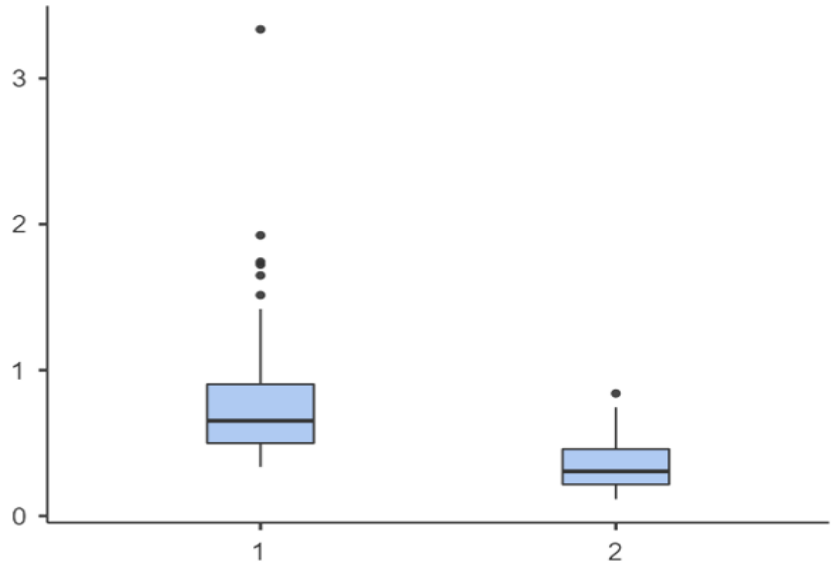


Figure 8: Box and Whisker plots of the TNF-α optical density (OD) in asphyxial and non-asphyxial cases.

TNF-α optical density in asphyxial and non-asphyxial controls are depicted in Figure 8 as box and whisker plots, and the plots demonstrate that the asphyxial case group has a considerably greater optical density than the non-asphyxial control group. The whisker displays the standard deviation, while the box displays the mean concentration.

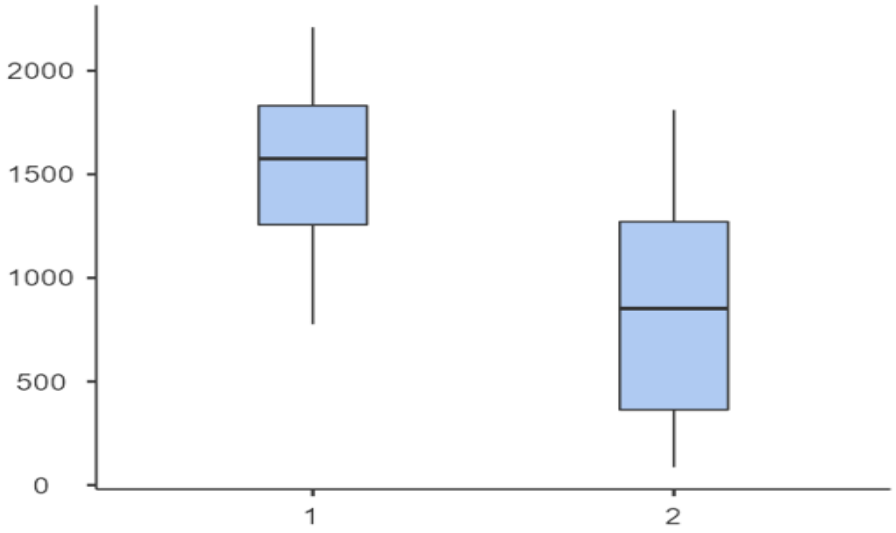


Figure 9: Box and Whisker plots of the IL-3 concentration (Conc.) in asphyxial and non-asphyxial cases.

IL-3 concentrations in asphyxial and non-asphyxial controls are depicted in Figure 9 as box and whisker plots. The plots demonstrate that the asphyxial case group has considerably greater concentrations than the non-asphyxial control group. The whisker displays the standard deviation, while the box displays the mean concentration.

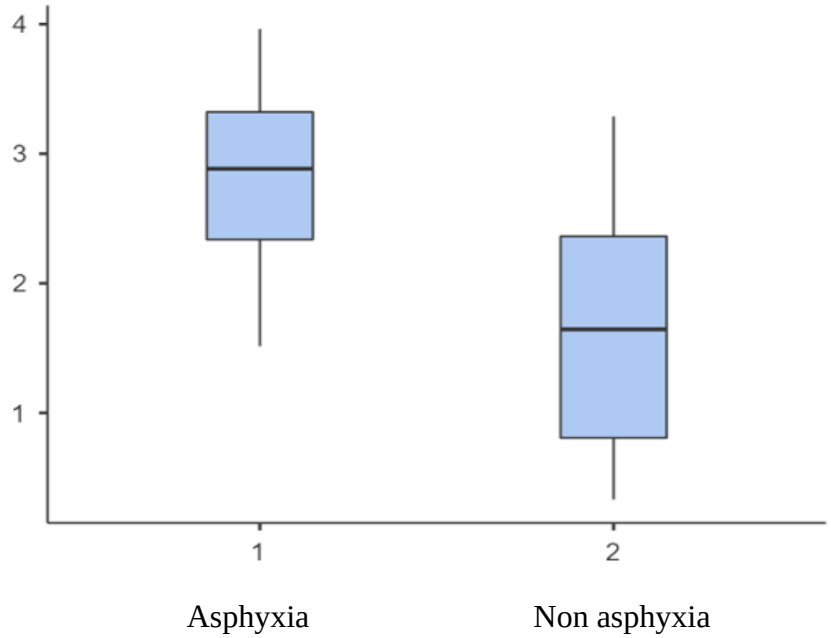


Figure 10: Box and Whisker plots of the IL-3 optical density (OD) in asphyxial and non-asphyxial cases.

IL-3 optical density in asphyxial and non-asphyxial controls are depicted in Figure 10 as box and whisker plots. The plots demonstrate that the asphyxial case group has a considerably greater optical density than the non-asphyxial control group. The whisker displays the standard deviation, while the box displays the mean concentration.

Table 4: Descriptive statistics of Toluidine Blue stained Mast cells in Deaths Associated with Fatal Asphyxia performed on both Cases and control groups.

	Asphyxial samples			**Non-asphyxial samples**		
	n	Range	Mean ± SD	n	Range	Mean ± SD
TB PB	45	0.0 – 0.6	0.18 ± 0.18	45	0.0 – 0.0	0.00 ± 0.00
TB PA	45	0.0 – 0.8	0.25 ± 0.19	45	0.0 – 0.2	0.00 ± 0.04
TB PV	45	0.0 – 2.0	0.54 ± 0.43	45	0.0 – 0.2	0.02 ± 0.06

n = Number of samples; SD = Standard deviation; PB = Peribronchial tissue; PA = Perialveolar tissue; PA = Perialveolar tissue; TB = Toluidene blue staining;

According to the asphyxial and control groups' descriptive statistics in Table 4, the histopathological analysis of lung tissues revealed a substantial difference between the case and control groups.

Table 5. Differences in mast cell visualization between asphyxial (Cases) and non-asphyxial (Control) samples were calculated using the Mann-Whitney U test.

	TB PB	TB PA	TB PV
Z-score	-5.92	-6.78	-7.40
p-value	< 0.01	< 0.01	< 0.01

TB = Toluidene blue staining; PB = Peribronchial tissue; PA = Perialveolar tissue; PV = Perivascular

The Mann-Whitney test is a method of determining whether there are differences in mast cell visibility between the asphyxia and non-asphyxia groups were analysed using the U test given in Table 5.

Table 6: Differences in mast cell mean rank between asphyxial (Cases) and non-asphyxial (Control) samples were calculated using the Kruskal Wallis test.

	Mean Rank		
	MC PB	MC PA	MC PV
Asphyxia	58.50	61.92	64.57
Non-asphyxia	32.50	29.08	26.43
P (sig.)	<0.01	<0.01	<0.01

Mc = Mast cell ; PB = Peribronchial tissue; PA = Perialveolar tissue; PV = Perivascular

The Kruskal Wallis test is used to compare dependent variables that are assessed at least on an ordinal level across three or more groups. Table 6 displays the mean rank of asphyxial and non-asphyxial groups in parabronchial, perialveolar, and perivascular areas.

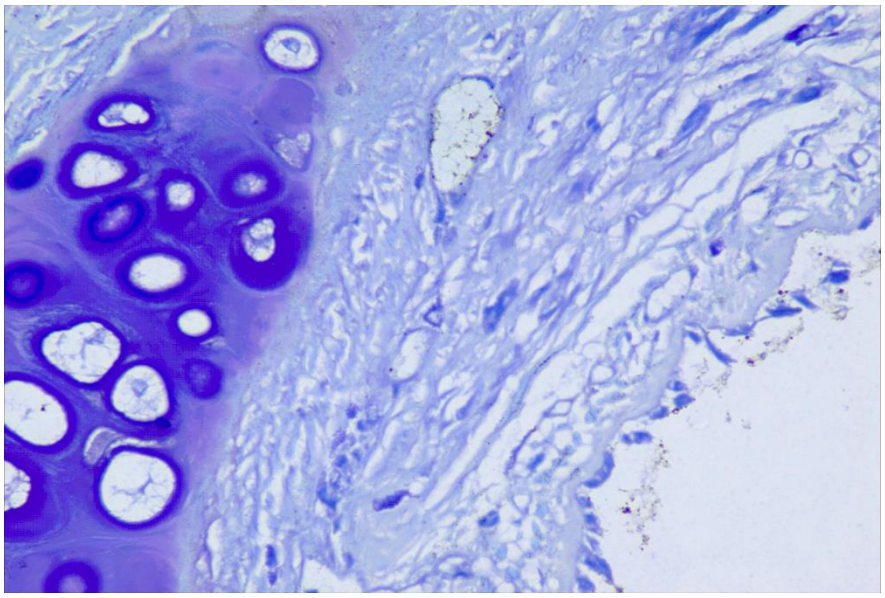


Figure 11: Photomicrographs of high power view of toluidine blue stained sections showing peribronchial mast cells (40X).

Mast cells are seen in Figure 11, between the respiratory epithelium on the right and the bronchial cartilage on the left of the picture.

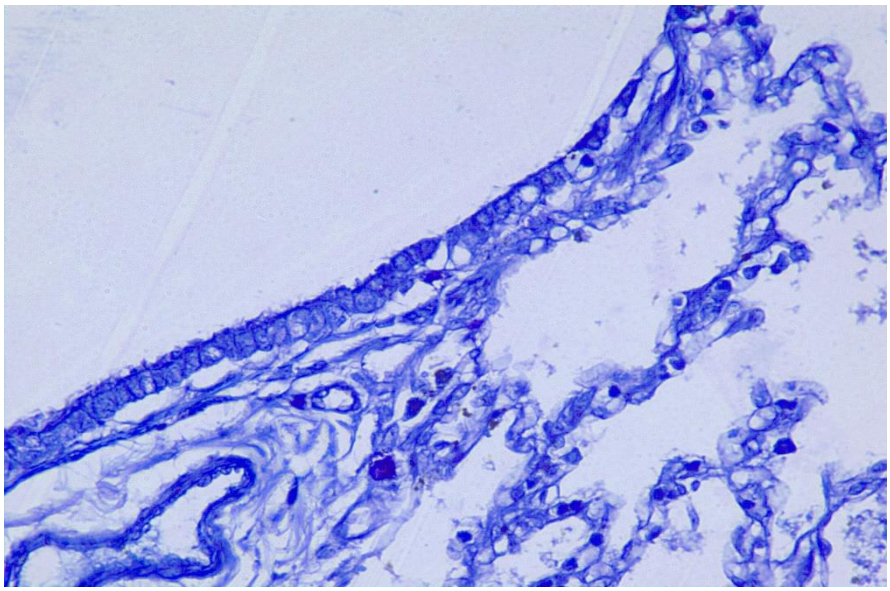


Figure 12: Photomicrographs of high power view of toluidine blue stained sections showing peribronchial mast cells (40X).

Subepithelial mast cells are seen in Figure 12, with epithelium visible in the upper portion of the picture.

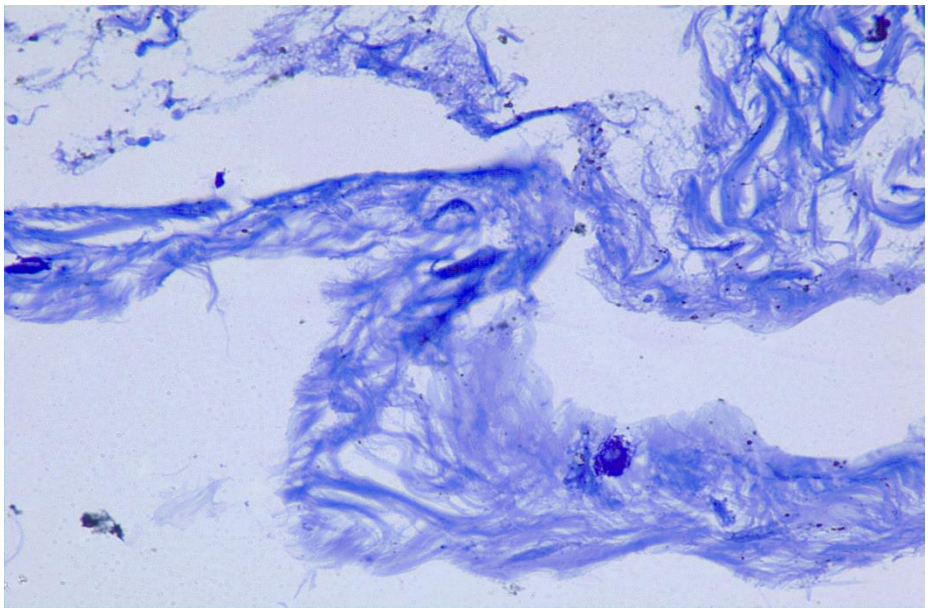


Figure 13: Photomicrographs of high power view of toluidine blue stained sections showing perivascular mast cells (40X).

The mast cell is seen in a medium vessel's perivascular region in Figure 13, (mast cell is seen in the lower part of the image).

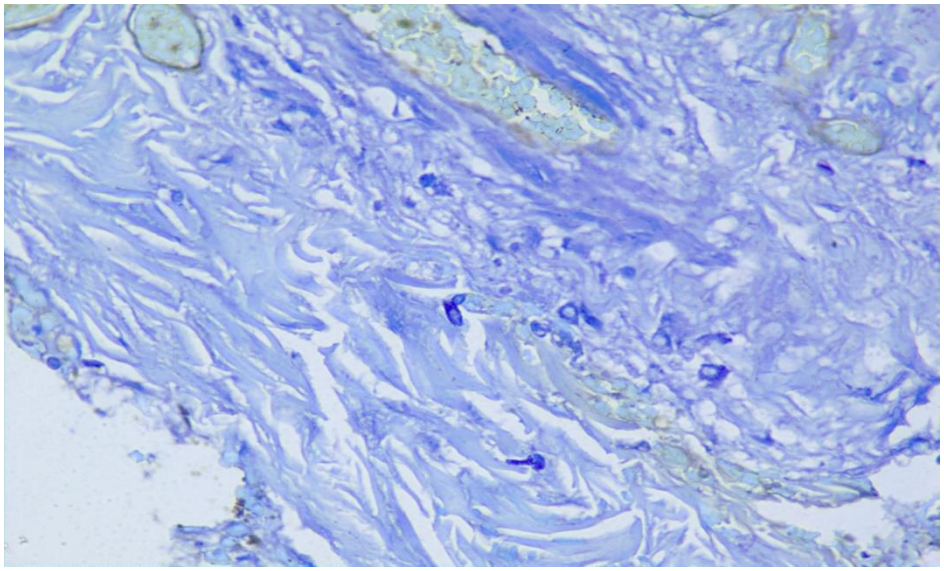


Figure 14: Photomicrographs of high power view of toluidine blue stained sections showing perivascular mast cells (40X).

In Figure14, a medium-sized vessel can be seen at the top of the picture, and two to three mast cells can be seen in the perivascular region in the picture's lower half.

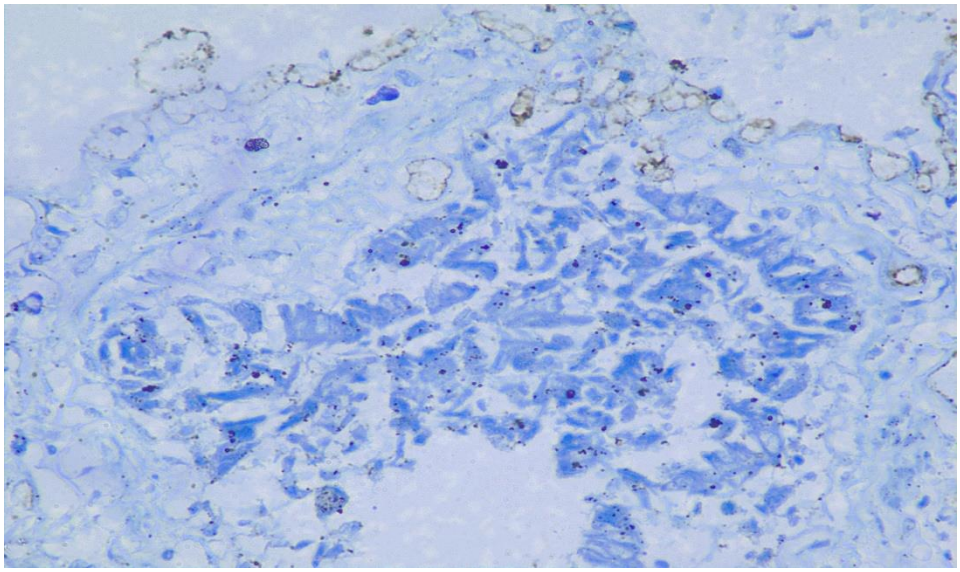


Figure 15: Photomicrographs of high power view of toluidine blue stained sections showing perialveolar mast cells, with one mast cell seen in each image, highlighted by toluidine blue stain (40X).

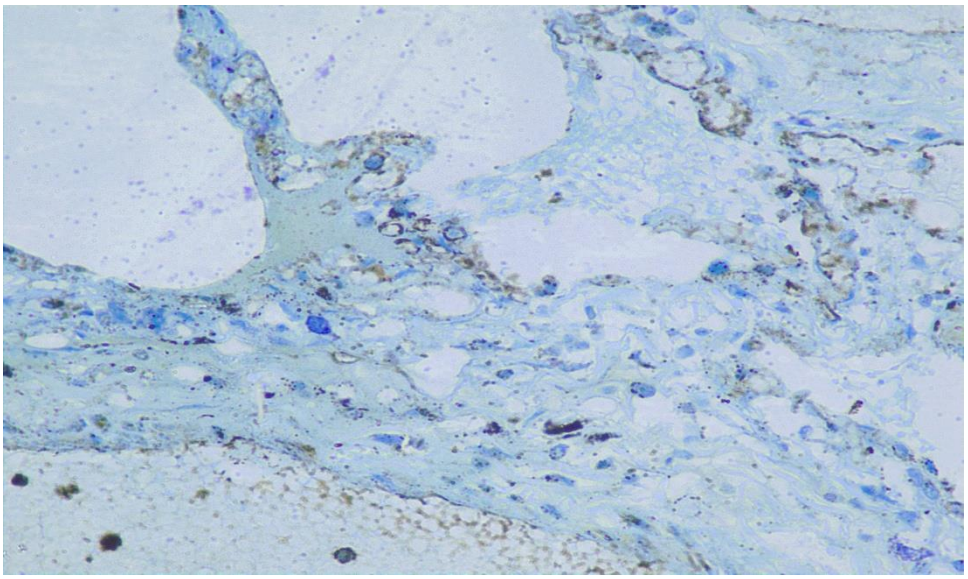


Figure 16: Photomicrographs of high power view of toluidine blue stained sections showing perialveolar mast cells, with one mast cell seen in each image, highlighted by toluidine blue stain (40X).

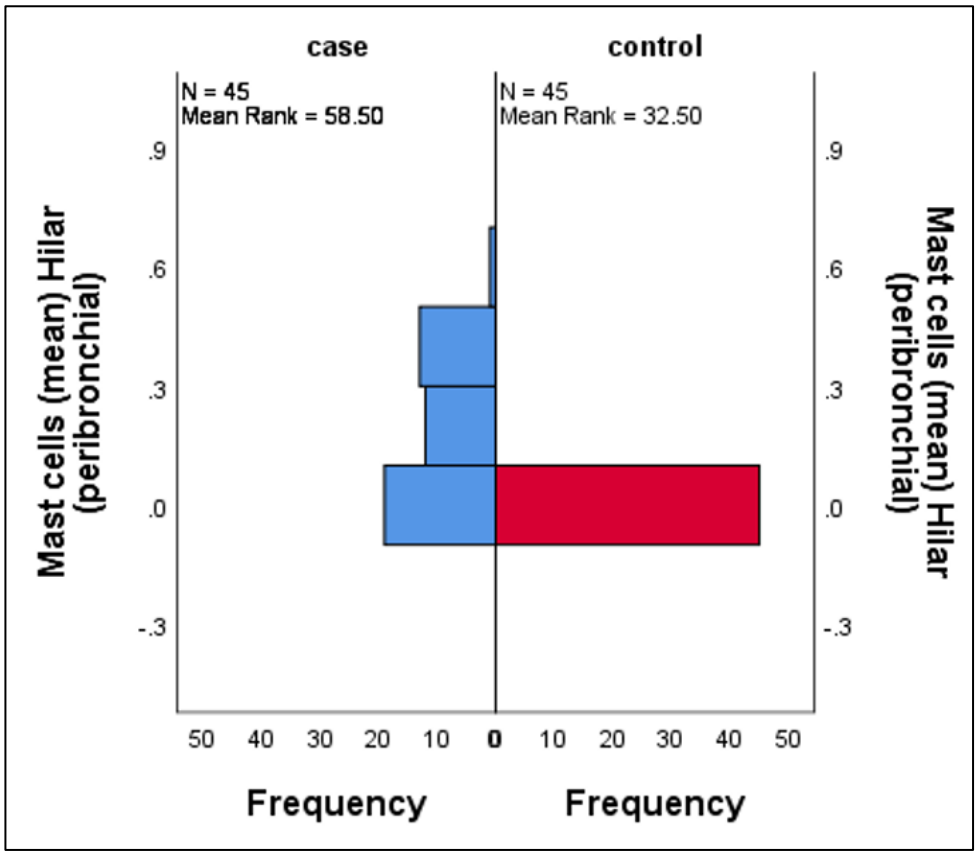


Figure 17: Expression level of mast cells in peribronchial regions of asphyxial (case) and non-asphyxial (control) cases as per the Mann Whitney U test.

According to the Mann-Whitney test, Figure 17 depicts the degree of mast cell expression in the peribronchial area of asphyxial and non-asphyxial patients. Compared to non-asphyxial instances, asphyxial cases have a considerable increase in mast cell expression.

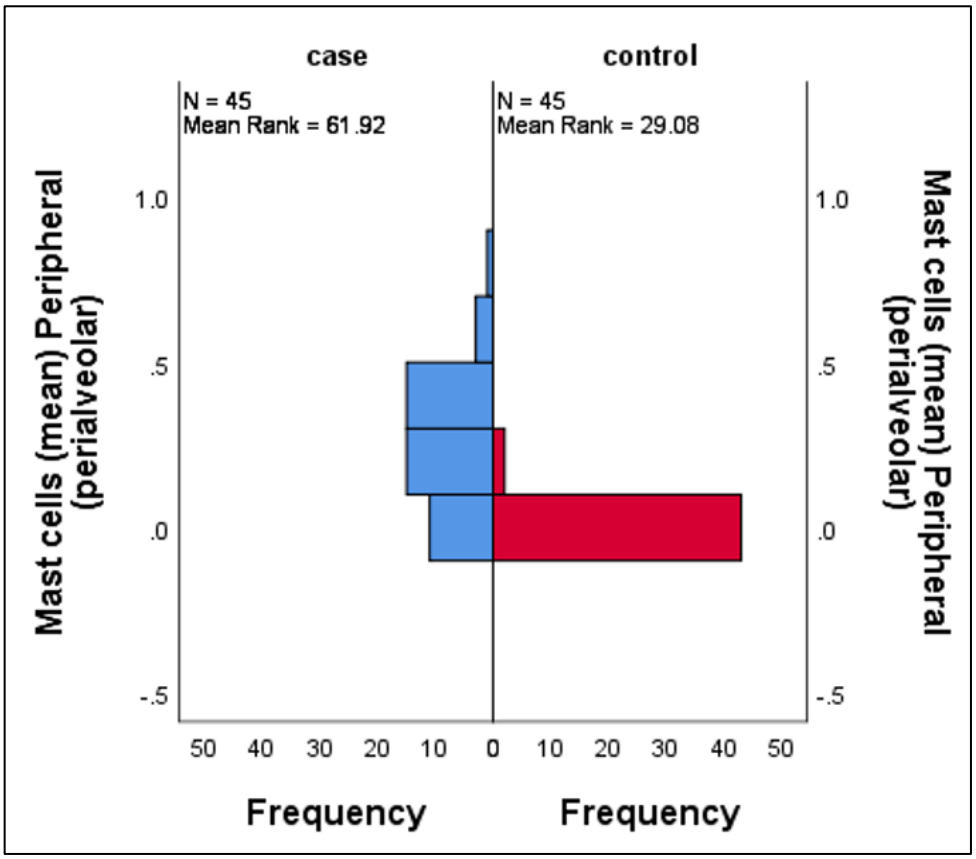


Figure 18: Expression level of mast cells in perialveolar regions of asphyxial (case) and non-asphyxial (control) cases as per the Mann Whitney U test.

According to the Mann-Whitney test, Figure 18 depicts the degree of mast cell expression in the perialveolar area of asphyxial and non-asphyxial patients. Compared to non-asphyxial instances, asphyxial cases have a considerable increase in mast cell expression.

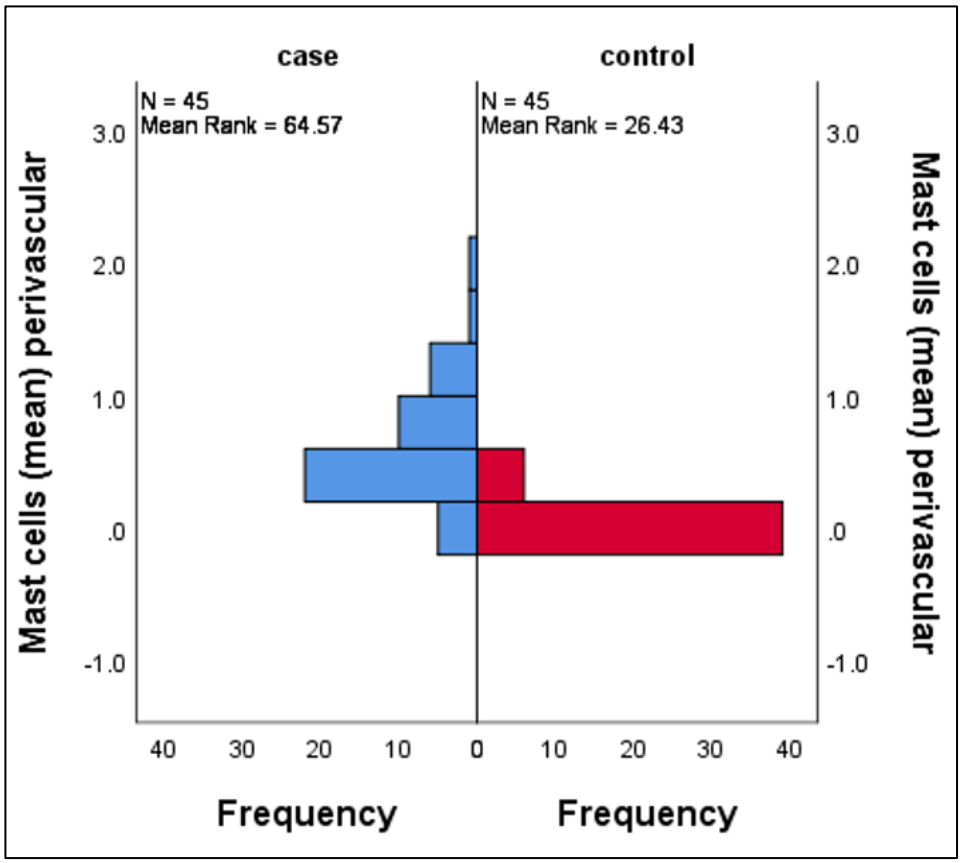


Figure 19: Expression level of mast cells in perialveolar regions of asphyxial (case) and non-asphyxial (control) cases as per the Mann Whitney U test.

According to the Mann-Whitney test, Figure 19 depicts the degree of mast cell expression in the perivascular area of asphyxial and non-asphyxial patients. Compared to non-asphyxial instances, asphyxial cases have a considerable increase in mast cell expression. Compared to the peribronchial and perivascular regions, the perivascular area has the greatest mast cell expression in cases of asphyxia.

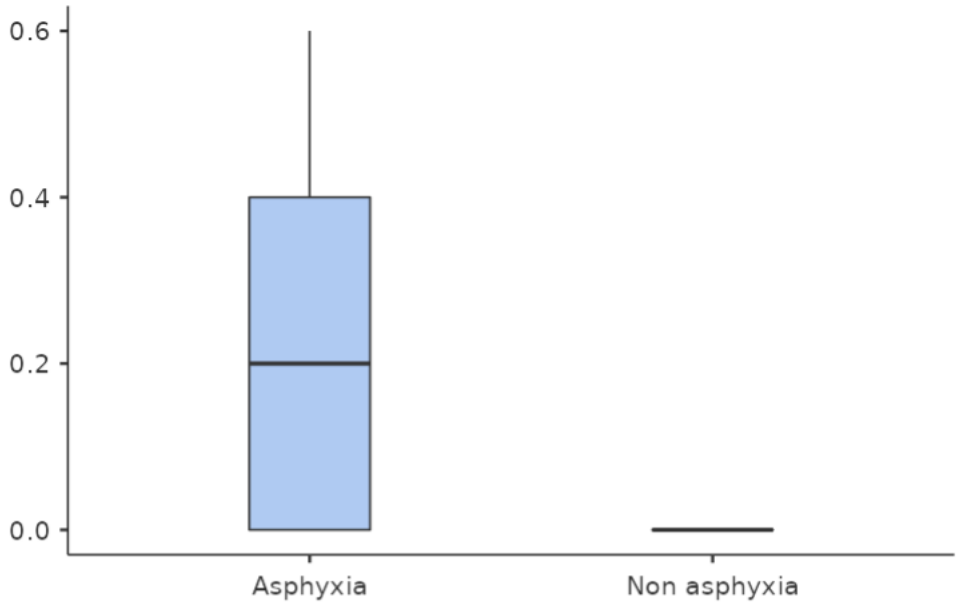


Figure 20: Box and Whisker plots of the expression level of mast cells (TB MC) in peribronchial regions of asphyxial and non-asphyxial cases.

The expression level of mast cells in peribronchial regions of asphyxial cases and non-asphyxial controls is depicted in Figure 20, as box and whisker plots. The plots demonstrate that the asphyxial case group has considerably increased mast cell expression compared to the non-asphyxial control group. The whisker displays the standard deviation, while the box displays the mean concentration.

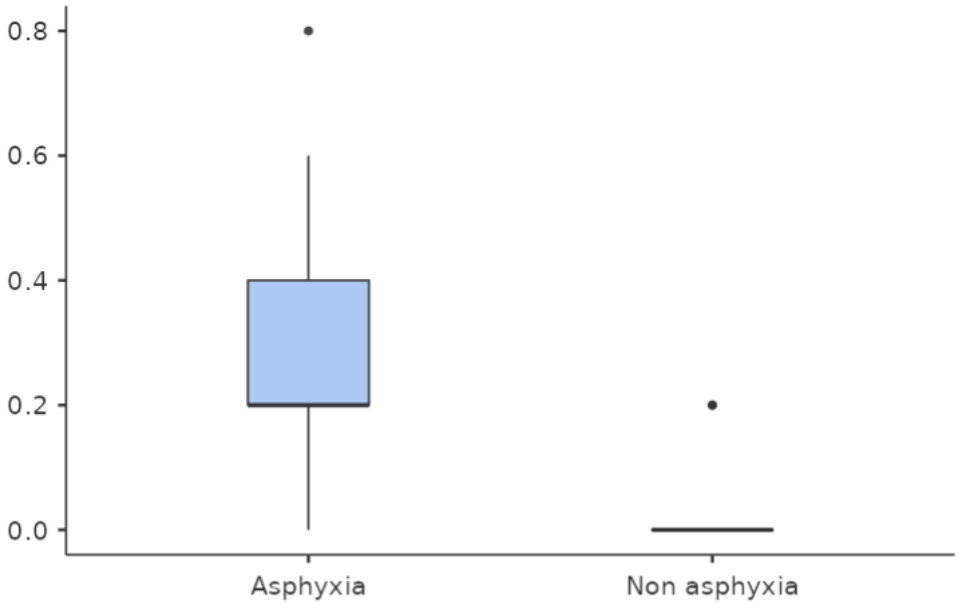


Figure 21: Box and Whisker plots of the expression level of mast cells (TB MC) in perialveolar regions of asphyxial and non-asphyxial cases.

The expression level of mast cells in perialveolar regions of asphyxial cases and non-asphyxial controls is depicted in Figure 21, as box and whisker plots. The plots demonstrate that the asphyxial case group has considerably increased mast cell expression compared to the non-asphyxial control group. The whisker displays the standard deviation, while the box displays the mean concentration.

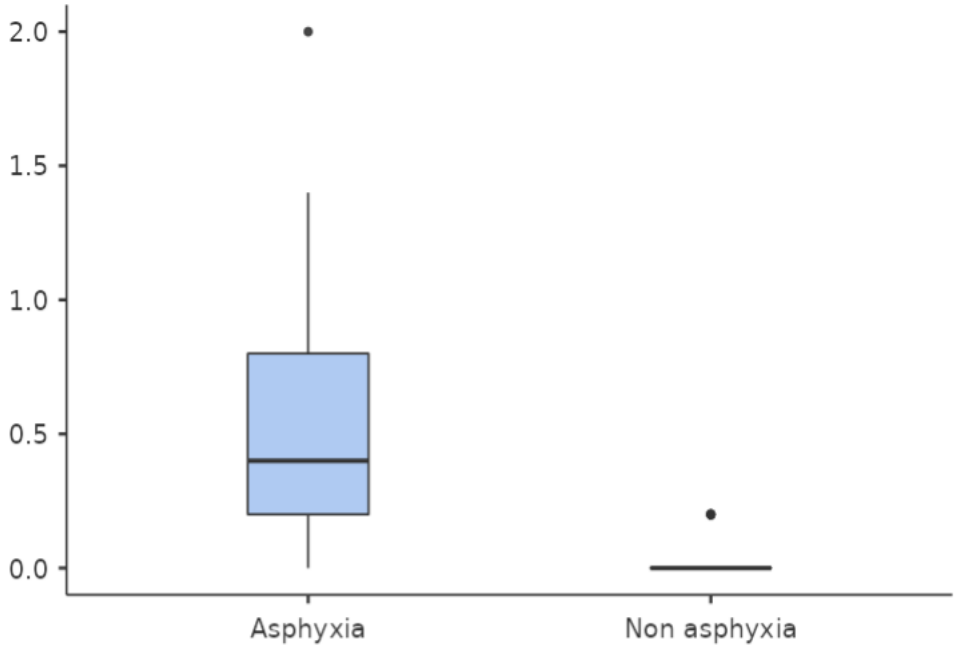


Figure 22: Box and Whisker plots of the expression level of mast cells (TB MC) in perivascular regions of asphyxial and non-asphyxial cases.

The expression level of mast cells in perivascular regions of asphyxial cases and non-asphyxial controls is depicted in Figure 22, as box and whisker plots. The plots demonstrate that the asphyxial case group has considerably increased mast cell expression compared to the non-asphyxial control group. The whisker displays the standard deviation, while the box displays the mean concentration.

DISCUSSION

DISCUSSION

Current and prior translational research on clinical asphyxia has attempted to elucidate the function of mast cells in tissue remodelling modules. In the lungs of chronically hypoxic mice, researchers identified a varied population of mast cells (MC) (55).

In this context, the literature from the clinical specialties is mostly available for perinatal brain injury and birth asphyxia (56).

Forensic pathologists initially neglected mast cells (MCs) for reasons related to their critical anaphylaxis role and suspected involvement in drug-related fatalities. Similarly, relevant research in forensic medicine and pathology is forthcoming, in which different indicators of asphyxiation have been examined using a variety of techniques, including immunohistochemistry (4,5,40)

Nonetheless, there is currently inadequate evidence for the function of MCs in acute hypoxic pulmonary vasoconstriction. Additionally, in the previous studies, the sample size was smaller. Thus, we decided to examine a larger sample size (n = 45 cases and 45 controls) using quantitative ELISA IL-3 and TNF-α quantification as well as histopathological examination with toluidine blue stain.

All the forms of mechanical asphyxia induce a state of decreased oxygen levels at the tissue level. Studies have been done in the past, showing that mast cell degranulation occurs due to tissue hypoxia induced by inflammation, even in chronic inflammatory conditions. Tucker et al. examined how chronic hypoxia affected microcirculation in six different species. They discovered a substantial shift in the concentration of mast cells in the lungs of severely hypoxic animal models. This has also been demonstrated in the endothelium and pulmonary artery smooth muscles (36,57). Serine proteases are crucial in the activation/deactivation of signaling peptides and contribute significantly to the etiology of systemic inflammation. In this context, the most important proteases are Dipeptidyl-peptidase 4, coagulation factors (like fibrinogen, prothrombin, thromboplastin, etc.), and mast cell chymase. So during the early stages of hypoxia, the release and activation of soluble MC-mediators involved in tissue remodeling and the degranulation of mast cells (MC) appear to be significant events (28,36,58).

Ultimately, mast cells are generated from pluripotent stem cells. Two distinct developmental pathways arise from stem cells, one myeloid and one lymphoid. The last stage of mast cell maturation occurs in peripheral organs, where mast cell progenitors (MCPs) are found (59). Mature mast cells have a protracted half-life and persist even after their main purpose of degranulation has been achieved (60). Degranulation happens when an antigen interacts with the IgE/FcRI complex on the surface of mast cells. With a very high affinity, to the point that binding is irreversible. It is activated when the antigen binds to the IgE/FcRI complex (61,62).

The Human Markers of mast cells are FcεRI (Fc epsilon RI), CD117/C-Kit, CD23, CD203c, HLA-DR, IL-3R alpha/CD 123. While in the murine models, MCs express ENPP-3/CD203c and CD200 R3 (63,64). CD117 is a transmembrane type III tyrosine kinase growth factor receptor expressed primarily in bone marrow stem/progenitor cells. MCPs consistently exhibit the high-affinity IgE receptor FC epsilon receptor (FcRI) and C-KIT/CD117 (SCF receptor). Various techniques have been employed to detect mast cells, ranging from basic methods like special stains (Toluidine blue and Giemsa) to advanced techniques like flow cytometry, immunohistochemistry, etc. (8,65,66).

The formation of marrow progenitor cells (tryptase+, c-kit+) and mast cells (tryptase+, c-kit+) within the artery's vasa vasorum wall occurs in the presence of idiopathic pulmonary arterial hypertension, according to the findings of Montani and colleagues. These results are consistent with our observations, which show that the vascular system aggregates peri-vasal mast cells as a consequence of the stimulation and migration of mast cells to respond to hypoxic conditions in the circulating blood (67).

In their study, Vacchiano G et al. investigated 50 asphyxiated people before contrasting 25 asphyxiated people's lung histological specimens after long asphyxia (30 min or more) with 25 asphyxiated people's lung histological specimens after quick asphyxia (10–15 min). Each section's alveolar, interstitial, and giant cells were considered and assigned numbers. On the histopathological evaluation of injured lungs, controls were performed. Following acute suffocation, there were 27.74.4 macrophages per section in the pulmonary alveoli. 68.27.1 alveolar macrophages were found in each area of those who died gradually from asphyxiation (p 0.001), which shows very significant values of mast cells in asphyxial deaths (26). In our study; also, we examined peribronchial, perialveolar, and perivascular areas of the lungs in the asphyxial and non-asphyxial groups by histopathology with toluidine blue stain.

Muciaccia et al. show unambiguously that even brief durations (a few minutes) of oxygen deprivation cause enormous numbers of mast cells (MCs) to be quickly recruited to the lungs in humans (acute asphyxia/hypoxia); Mast cells (MCs) are not regularly dispersed all over the tissue once recruited, with a larger amount of mast cells found in the peribronchial and perialveolar areas than the rest of the tissue. The increase in peri-bronchial and perialveolar mast cells (MCs) after acute asphyxia/hypoxia may be initiated by hypoxic stimulation through the artery walls rather than the blood (8).

During our research, we discovered that, despite including typical sequences of events such as hanging, drowning, or smothering, as seen in cases of asphyxia, the mast cell count in the peribronchial, perialveolar, and perivascular region of the asphyxial group was significantly advanced than in the non-asphyxial group, yielding statistically significant results ($p<0.01$).

Mast cells and their connection to suffocation have attracted the attention of several researchers. Their objective was to understand how mast cells contributed to asphyxia-related mortality. Many significant studies employed histopathology of the lung tissue as the method of choice, demonstrating mast cells, macrophages, and giant cells with various stains such as H&E, toluidine blue, and Giemsa. In their work, Vacchiano G et al. proposed a relationship between the onset of hypoxia and the development of macrophages in lung tissue. The authors investigated fifty asphyxiated human lungs. As a check, the wounded lungs' histological analyses were performed. After acute asphyxia, the pulmonary alveoli had 27.74.4 macrophages per section. Each region of individuals who passed away from delayed asphyxiation included 68.27.1 alveolar macrophages ($p<0.001$) (26). A seven-day recovery from chronic hypoxia and the properties of acute and chronic hypoxia were studied in relation to the location of pulmonary MC and the MMP-13 production of these cells in rats. Toluidine blue staining was used to identify mast cells. Mast cell counts significantly increased within the walls of the prealveolar arteries after 4 but not 20 days of hypoxia. Alveolar hypoxia induces patterns of inflammatory lung responses, and their importance for the asphyxiation analysis was the subject of research by Gutjahr E et al. H&E, and toluidine blue staining was used to count the alveolar phagocytes, megakaryocytes, giant, and mast cells in samples of buried human lungs. The number of lung inflammatory cells varied significantly between the case and cardiovascular control groups (49). In this study, we also used the Toluidine blue staining to determine the mean mast cell count in the case and control group's peripheral, perialveolar, and peribronchial regions.

Many asphyxia and mast cell-related investigations used immunohistochemistry as their method of choice, and it proved to be an effective one. Tryptase, CD15, and IL-15 are investigated by Turillazzi et al. as trustworthy indicators for assessing the viability of soft and hard ligature marks. They found that predictable macroscopic and histological results might be unreliable and that vital signs were frequently absent or could develop after death. Their technique of choice to show the ligature mark's life was immunohistochemistry. TNF-α, IL-6, IL-8, IL-10, MCP-1, IL-15, IL-1ß, CD3, CD8, CD68, CD45, CD4, CD20, and CD15 antibodies were employed in an immunohistochemical analysis of skin tissues. Tryptase, CD15, and IL-15 appear to be trustworthy factors that precisely determine the liveliness of ligature signs for forensic purposes (38).

In their paper "Markers of mechanical asphyxia: an immunohistochemistry investigation on autoptic lung tissues," Cecchi R et al. looked into whether any antigens might be utilized as indicators of asphyxia death by immunostaining the lung tissue. The findings indicate that P- and E-selectin expression in the blood arteries of the lungs, which is induced by a variety of trigger events beyond hypoxia, cannot be interpreted as an indicator of asphyxia (40). In their study, Barbara Muciaccia et al. demonstrated the responsiveness of lung tissue vasculature to hypoxia by using immunodetection of the HIF1-alpha protein, a critical regulator of cellular response to hypoxic circumstances. An IHC study employing the anti-CD117 (c-Kit) antibody to assess peri-airway and peri-vascular MC as well as their numbers and features, revealed a substantial increase in peri-vascular c-Kit (+) MC in several asphyxia fatalities, including hanging, strangling, and aspiration deaths (8). The Spielmeyer method and toluidine blue were used to stain the histology preparations. The study's findings are consistent with the notion that mechanical strangulation asphyxia may be identified even if there aren't any additional intravital death signs by the anti-fibrinogen antibody reaction in the stromal tissue around the constriction mark. Such diagnoses may also be made using the CD-117 antigen in the pulmonary tissue, which shows an upsurge in CD-117 positive MC in asphyxial deaths (5). The selection of the markers for our investigation was greatly aided by the immunohistochemical studies that identified several significant interleukins and TNF-α mast cells.

In several earlier research, real-time PCR was also employed to identify the significant asphyxia markers in fatal mechanical asphyxial fatalities.

Numerous important investigations into the estimate of cytokines in asphyxial situations have been carried out, including cytokines' function in the pathogenesis of brain damage and their connection to the neurological consequences of asphyxiated newborns. They postulate that the levels of the inflammatory protein's interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) in cerebrospinal fluid (CSF) are correlated with the degree of brain damage and can foretell neurological abnormalities in newborns with hypoxia ischemic encephalopathy (HIE). All newborns were given blood and CSF samples within the first 24 hours of life. In comparison to IL-6 ($r = 0.45$, $P = 0.004$) and TNF- α ($r = 0.47$, $P = 0.003$), the presence of IL-1β in the CSF was observed to be more strongly linked with the severity of HIE ($r = 0.61$, $P = 0.001$). The greatest CSF/serum ratio was seen in IL-1β. The cerebrospinal fluid (CSF) of babies who suffered from asphyxia was compared to healthy controls to determine the quantities of pro- and anti-inflammatory cytokines present by the authors. CSF samples were collected from seven newborn control patients, 20 newborns that satisfied the criteria for birth asphyxia, and 20 newborns. Asphyxiated newborns had significantly greater levels of the proinflammatory cytokines IL-6 and IL-8 in their CSF, according to the ELISA technique, which was employed to gather the data(25). Another investigation by Gulliksson M. et al. looked into the effects of hypoxia generally on the survival, secretion, and reactivity of human mast cells. Human cord blood mast cells were extracted, and the cells were cultivated in three different environments: normoxic (21% O2), hypoxic (1% O2), or hypoxic culture for 24 hours before stimulation in normoxic (21% O2). They concluded that hypoxia alone did not cause mast cells to degranulate. Still, they did see an increase in IL-6 release, and IL-6 generated by the autocrine system assisted the mast cells in surviving (36). Serum interleukin-6 levels and the severity of newborn hypoxia are examined by H. Boskabadi et al. They discovered that newborns with asphyxia had blood IL-6 concentrations that were 43-fold greater than those of infants who later acquired hypoxic-ischemic encephalopathy and 1.9-fold higher than those of infants with normal serum IL-6 concentrations ($p < 0.001$). The clinical significance of dynamic measurements of seric tumor necrosis factor (TNF-α), high mobility cluster protein Bl (HMGBl), neuron-specific enolase (NSE), and amplitude-integrated electroencephalogram was investigated by Zhang XH et al. to assist in the diagnosis and predict the short-term prognosis of brain damage caused by neonatal asphyxia. TNF-α, HMGBl, and NSE levels in the umbilical artery and peripheral blood were monitored and compared across groups along with the results of the aEEG. Compared to the control group, the umbilical artery and serum TNF-α, HMGBl, and NSE thresholds at day 1 were considerably higher in the two asphyxia groups, with the values being higher in the severe

asphyxia group (p 0.05) (37,44). The severity of newborn hypoxia may be regulated by certain cytokine patterns, according to research done in 2017 by Bajnok et al. Blood samples were taken between 3 and 6 hours after birth, at 24 hours, 72 hours, one week, and one month after birth, all based on receiver operator curve analysis. Neonates were split into two groups: mild and severe. Early on in asphyxia, the predominance of CD4+ IL-1β+ and CD4+ IL-1β+ CD49d+ cells may be used to predict how severe the insult would be. At all time intervals relative to 6 h in both groups, CD4 cell intracellular TNF-α levels increased. TNF-α intracellular levels were higher in the severe group at one month (43). As per literature majority of the studies on TNF-α are related to neonatal asphyxia which showed a positive correlation and very few studies are there which investigate TNF-α expression in mechanical asphyxia. In the present study we evaluate the role of TNF-α in death due to mechanical asphyxia compared to non-asphyxial deaths.

However, the majority of researchers are solely concerned with birth asphyxia fatalities and perinatal brain damage, and in certain cases, studies were done on mice and pigs. There were a relatively small number of studies on mechanical asphyxial fatalities in humans, such as hanging, strangling, drowning, and smothering, that employed quantitative ELISA to show the importance of mast cells.

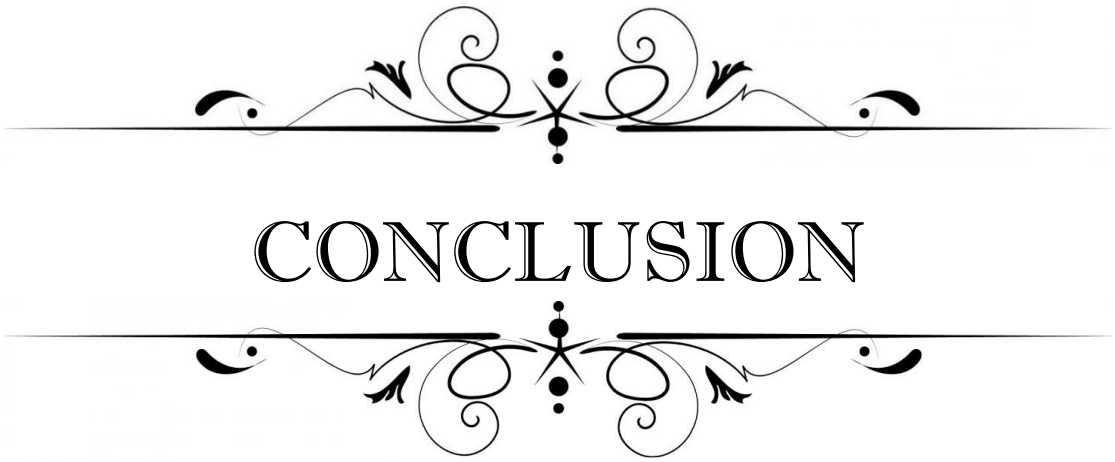
Histopathology, immunohistochemistry, real-time PCR, and ELISA have all been widely used to study the connection between hypoxia and mast cells up to this point. Numerous studies also revealed several significant indicators, which were highly useful in comparing asphyxia-related deaths to those caused by other causes. These indicators are crucial in diagnosing the cause of death if the deceased's external examination at autopsy did not show any macroscopical signs (such as a strangulation mark, cyanosis, petechial hemorrhage, and pulmonary edema). Additionally, recent research shows that prolonged hypoxia is sufficient to expand mast cells (MC).

However, previous studies typically used samples from blood, CSF, cardiac tissue, and brain tissue; only a few studies in the adult population have used samples from human lung tissue. To corroborate the importance of our findings, we took samples from each hilar area of the lung and used ELISA and histopathological staining with toluidine blue to analyze them.

The sandwich ELISA method which was employed in our study to estimate TNF-alpha concentration also showed a positive correlation in asphyxial deaths, hence it can be used as a

biomarker in asphyxial deaths which helps forensic pathologists to confirm the cause of death.

In contrast to non-asphyxial fatalities, our work demonstrates for the first time, as far as we are aware, that interleukin-3 (IL-3) can be employed as a marker for the identification of traumatic asphyxial deaths. There was a dearth of research demonstrating the use of interleukin-3 (IL-3) as a marker for asphyxia. Quantitative ELISA estimated the interleukin-3 (IL-3) concentration in this investigation, and the findings showed a substantial difference between asphyxial and non-asphyxial fatalities. Despite the fact that the majority of studies in this field focused on neonatal hypoxia mortality or neonatal hypoxic brain damage.



CONCLUSION

CONCLUSION

- In this study, we looked at the expression of IL-3 and TNF-α in terms of concentration and optical density in cases of asphyxial and non-asphyxial deaths.
- The findings of this study were helpful in identifying the cause of death in situations where hanging, drowning, strangling, and suffocation was suspected, but no substantial gross external evidence was visible.
- The quantitative estimation of IL-3 and TNF-α concentrations using lung tissue sample in asphyxia and non-asphyxial cases was assessed by sandwich ELISA.
- The statistical examination of the ELISA data revealed statistically significant results ($p < 0.01$), and the Receiver Operating Characteristic Curve (ROC) revealed statistically significant areas under the curve for TNF-α (AUC=0.89) and IL-3 (AUC=0.87).
- The peribronchial, perialveolar, and perivascular portions of the lung were examined under a microscope using Toluidine Blue stain for the histopathological investigation of asphyxia and non-asphyxial cases for mast cell quantification using lung tissue samples.
- Five fields were examined for the presence and quantity of mast cells in the peribronchial, perialveolar, and perivascular regions, and the mean mast cell count per field was determined.
- Mast cell estimate in asphyxial instances revealed a considerable increase in mean mast cell count, with the perivascular area showing the greatest mean mast cell count when compared to the peribronchial, and perialveolar, regions.
- When comparing instances of asphyxia to the control group, the findings of the histological investigation of lung tissues revealed statistically significant outcomes ($p < 0.01$).
- The findings of this study demonstrated that histological examination and quantitative determination of IL-3 and TNF-α concentrations by ELISA of lung tissue could aid in detecting the asphyxial component and cause of death in suspected autopsy cases with allegations of death due to asphyxia.

REFERENCES

REFERENCES

1. Saukko P, Knight B. Knight's forensic pathology. CRC press; 2015.

2. Pan H, Lu Y, Zhang H, Xue A, Chen L. Death from a special mechanical asphyxia during suspect restraint: a case report. Romanian J Leg Med. 2014;22:81–4.

3. Shkrum MJ, Ramsay DA. Forensic pathology of trauma. Springer Science & Business Media; 2007.

4. Zhu BL, Ishida K, Fujita MQ, Maeda H. Immunohistochemical investigation of a pulmonary surfactant in fatal mechanical asphyxia. Int J Legal Med. 2000 Aug 1;113(5):268–71.

5. Salama M, Mohamed WMY. Tau protein as a biomarker for asphyxia: A possible forensic tool? Appl Transl Genomics. 2016 Jun;9:20–2.

6. Grellner W, Madea B. Immunohistochemical characterization of alveolar macrophages and pulmonary giant cells in fatal asphyxia. Forensic Sci Int. 1996 Jun 14;79(3):205–13.

7. Kotani H, Miyao M, Manabe S, Ishida T, Kawai C, Abiru H, et al. Relationship of red splenic arteriolar hyaline with rapid death: a clinicopathological study of 82 autopsy cases. Diagn Pathol. 2012;7(1):1–8.

8. Muciaccia B, Sestili C, De Grossi S, Vestri A, Cipolloni L, Cecchi R. Are mast cells implicated in asphyxia? Int J Legal Med. 2016;130(1):153–61.

9. Dunham-Snary KJ, Wu D, Sykes EA, Thakrar A, Parlow LRG, Mewburn JD, et al. Hypoxic Pulmonary Vasoconstriction: From Molecular Mechanisms to Medicine. Chest. 2017 Jan;151(1):181–92.

10. Chao J, Wood JG, Blanco VG, Gonzalez NC. The systemic inflammation of alveolar hypoxia is initiated by alveolar macrophage–borne mediator (s). Am J Respir Cell Mol Biol. 2009;41(5):573–82.

11. Kay J, Waymire J, Grover R. Lung mast cell hyperplasia and pulmonary histamine-forming capacity in hypoxic rats. Am J Physiol-Leg Content. 1974 Jan;226(1):178–84.

12. Idriss HT, Naismith JH. TNF alpha and the TNF receptor superfamily: structure-function relationship(s). Microsc Res Tech. 2000 Aug 1;50(3):184–95.

13. Boskabadi H, Moradi A, Zakerihamidi M. Interleukins in diagnosis of perinatal asphyxia: A systematic review. Int J Reprod Biomed. 2019;17(5):303.

14. Aglietta M, Pasquino P, Sanavio F, Stacchini A, Severino A, Fubini L, et al. Granulocyte-macrophage colony stimulating factor and interleukin 3: Target cells and kinetics of response in vivo. Stem Cells. 1993;11(S2):83–7.

15. Milroy CM. A Brief History of "Asphyxia." Acad Forensic Pathol. 2015;5(2):254–65.

16. Male GE. An Epitome of Juridical Or Forensic Medicine: For the Use of Medical Men, Coroners, and Barristers. T. and G. Underwood; 1816.

17. Paris JA, Fonblanque JSM. Medical jurisprudence. Vol. 3. W. Phillips; 1823.

18. Beck TR, Dunlop W. Elements of medical jurisprudence. John Anderson [and three others]; 1825.

19. Wharton F. A treatise on medical jurisprudence. Kay & Brother; 1860.

20. Casper JL. A Handbook of the practice of forensic medicine v. 3 1864. Vol. 3. New Sydenham Society; 1864.

21. RHODES WF, Gordon I, PRICE TW, TURNER RA. Medical Jurisprudence. By I. Gordon ... R. Turner ... and T.W. Price ... Third edition. Pp. lv. 944. E. & S. Livingstone: Edinburgh & London; 1953. 8 p.

22. Book Reviews: LEGAL MEDICINE, by R. B. H. Gradwohl, The C. V. Mosby Company, St. Louis, 1954, 1,093 pp., $20.00 - Silas Warner, 1954 [Internet]. [cited 2022 Dec 8]. Available from: https://journals.sagepub.com/doi/abs/10.1177/003288555403400209

23. Tamaki K, Sato K, Katsumata Y. Enzyme-linked immunosorbent assay for determination of plasma thyroglobulin and its application to postmortem diagnosis of mechanical asphyxia. Forensic Sci Int. 1987;33(4):259–65.

24. Tamaki K, Katsumata Y. Enzyme-linked immunoabsorbent assay for plasma thyroglobulin following compression of the neck. Forensic Sci Int. 1990 Feb 1;44(2):193–201.

25. Sävman K, Blennow M, Gustafson K, Tarkowski E, Hagberg H. Cytokine response in cerebrospinal fluid after birth asphyxia. Pediatr Res. 1998;43(6):746–51.

26. Vacchiano G, D'Armiento F, Torino R. Is the appearance of macrophages in pulmonary tissue related to time of asphyxia? Forensic Sci Int. 2001 Jan;115(1–2):9–14.

27. Ishida K, Zhu BL, Maeda H. A quantitative RT-PCR assay of surfactant-associated protein A1 and A2 mRNA transcripts as a diagnostic tool for acute asphyxial death. Leg Med. 2002 Mar 1;4(1):7–12.

28. Steiner DRS, Gonzalez NC, Wood JG. Mast cells mediate the microvascular inflammatory response to systemic hypoxia. J Appl Physiol. 2003 Jan;94(1):325–34.

29. Matalka KZ, Tutunji MF, Abu-Baker M, Baker YA. Measurement of protein cytokines in tissue extracts by enzyme-linked immunosorbent assays: application to lipopolysaccharide-induced differential milieu of cytokines. Neuroendocrinol Lett. 2005;26(3):231–6.

30. Vajner L, Vytášek R, Lachmanová V, Uhlík J, Konrádová V, Novotná J, et al. Acute and chronic hypoxia as well as 7-day recovery from chronic hypoxia affects the distribution of pulmonary mast cells and their MMP-13 expression in rats. Int J Exp Pathol. 2006;87(5):383–91.

31. Aly H, Khashaba MT, El-Ayouty M, El-Sayed O, Hasanein BM. IL-1β, IL-6 and TNF-α and outcomes of neonatal hypoxic ischemic encephalopathy. Brain Dev. 2006;28(3):178–82.

32. Edston E, Eriksson O, van Hage M. Mast cell tryptase in postmortem serum—reference values and confounders. Int J Legal Med. 2007 Jul;121(4):275–80.

33. Ikematsu K, Takahashi H, Kondo T, Tsuda R, Nakasono I. Temporal expression of immediate early gene mRNA during the supravital reaction in mouse brain and lung after mechanical asphyxiation. Forensic Sci Int. 2008 Aug 6;179(2):152–6.

34. Pérez Cárceles MD, Sibón A, Vizcaya MA, Osuna E, Luna A, Martínez Díaz F. Histological findings and immunohistochemical surfactant protein A-SP-A-expression in asphyxia, Its application in the diagnosis of drowning. Histol Histopathol. 2008;

35. Takahashi H, Ikematsu K, Tsuda R, Nakasono I. Increase in dual specificity phosphatase 1, TGF-beta stimulated gene 22, domain family protein 3 and Luc7 homolog (S. cerevisiae)-like messenger RNA after mechanical asphyxiation in the mouse lung. Leg Med. 2009;11(4):181–5.

36. Gulliksson M, Carvalho RF, Ullerås E, Nilsson G. Mast cell survival and mediator secretion in response to hypoxia. PLoS One. 2010;5(8):e12360.

37. Boskabadi H, Afshari JT, Ghayour-Mobarhan M, Maamouri G, Shakeri MT, Sahebkar A, et al. Association between serum interleukin-6 levels and severity of perinatal asphyxia. Asian Biomed. 2010;4(1):79–85.

38. Turillazzi E, Vacchiano G, Luna Maldonado A, Neri M, Pomara C, Rabozzi R, et al. Tryptase, CD15 and IL-15 as reliable markers for the determination of soft and hard ligature marks vitality. Histol Histopathol. 2010;

39. Li DR, Ishikawa T, Quan L, Zhao D, Michiue T, Zhu BL, et al. Morphological analysis of astrocytes in the hippocampus in mechanical asphyxiation. Leg Med. 2010;12(2):63–7.

40. Cecchi R, Sestili C, Prosperini G, Cecchetto G, Vicini E, Viel G, et al. Markers of mechanical asphyxia: immunohistochemical study on autoptic lung tissues. Int J Legal Med. 2014;128(1):117–25.

41. Zeng Y, Lv Y, Tao L, Ma J, Zhang H, Xu H, et al. G6PC3, ALDOA and CS induction accompanies mir-122 down-regulation in the mechanical asphyxia and can serve as hypoxia biomarkers. Oncotarget. 2016;7(46):74526.

42. Pérez IL, Falcón M, Gimenez M, Diaz FM, Pérez-Cárceles MD, Osuna E, et al. Diagnosis of vitality in skin wounds in the ligature marks resulting from suicide hanging. Am J Forensic Med Pathol. 2017;38(3):211–8.

43. Bajnok A, Berta L, Orbán C, Veres G, Zádori D, Barta H, et al. Distinct cytokine patterns may regulate the severity of neonatal asphyxia—an observational study. J Neuroinflammation. 2017 Dec 12;14(1):244.

44. Zhang XH, Zhang BL, Guo SM, Wang P, Yang JW. Clinical significance of dynamic measurements of seric TNF-α, HMGBl, and NSE levels and aEEG monitoring in neonatal asphyxia. Eur Rev Med Pharmacol Sci. 2017;21(19):4333–9.

45. Feng X, Zhang D, Gong Q, Zhang Z, Quan L. Expression of glucose-regulated protein 78 and miR-199a in rat brain after fatal ligature strangulation. Am J Forensic Med Pathol. 2017;38(1):78–82.

46. Zeng Y, Tao L, Ma J, Han L, Lv Y, Hui P, et al. DUSP1 and KCNJ2 mRNA upregulation can serve as a biomarker of mechanical asphyxia-induced death in cardiac tissue. Int J Legal Med. 2018;132(3):655–65.

47. Bogomolov DV, Fetisov VA, Denisova OP, Zbrueva YV, Semenov GG. The principal and auxiliary immunohistochemical markers of intravital mechanical strangulation asphyxia. Sud Med Ekspert. 2018;61(2):11–3.

48. Palmiere C, Tettamanti C, Scarpelli MP, Rousseau G, Egger C, Bongiovanni M. Postmortem biochemical investigation results in situations of fatal mechanical compression of the neck region. Leg Med. 2018;30:59–63.

49. Gutjahr E, Madea B. Inflammatory reaction patterns of the lung as a response to alveolar hypoxia and their significance for the diagnosis of asphyxiation. Forensic Sci Int. 2019;297:315–25.

50. Zhang H, Hu Y, Wang H, Tian L, Li W, Han L, et al. Cytoplasmic upregulation of Cyto c and AIF serve as biomarkers of mechanical asphyxia death. Am J Transl Res. 2019;11(7):4568.

51. Ocak U, Eser Ocak P, Huang L, Xu W, Zuo Y, Li P, et al. Inhibition of mast cell tryptase attenuates neuroinflammation via PAR-2/p38/NFκB pathway following asphyxial cardiac arrest in rats. J Neuroinflammation. 2020;17(1):1–17.

52. Han L, Zhang H, Zeng Y, Lv Y, Tao L, Ma J, et al. Identification of the miRNA-3185/CYP4A11 axis in cardiac tissue as a biomarker for mechanical asphyxia. Forensic Sci Int. 2020;311:110293.

53. Han L, Li W, Hu Y, Zhang H, Ma J, Ma K, et al. Model for the prediction of mechanical asphyxia as the cause of death based on four biological indexes in human cardiac tissue. Sci Justice. 2021;61(3):221–6.

54. Hu Y, Tian L, Ma K, Han L, Li W, Hu L, et al. ER stress–related protein, CHOP, may serve as a biomarker of mechanical asphyxia: a primary study. Int J Legal Med. 2022;136(4):1091–104.

55. Williams A, Heath D, Kay JM, Smith P. Lung mast cells in rats exposed to acute hypoxia, and chronic hypoxia with recovery. Thorax. 1977;32(3):287–95.

56. Golubnitschaja O, Yeghiazaryan K, Cebioglu M, Morelli M, Herrera-Marschitz M. Birth asphyxia as the major complication in newborns: moving towards improved individual outcomes by prediction, targeted prevention and tailored medical care. EPMA J. 2011;2(2):197–210.

57. Tucker A, McMurtry IF, Alexander AF, Reeves JT, Grover RF. Lung mast cell density and distribution in chronically hypoxic animals. J Appl Physiol. 1977;42(2):174–8.

58. Krystel-Whittemore M, Dileepan KN, Wood JG. Mast cell: a multi-functional master cell. Front Immunol. 2016;620.

59. Valent P, Akin C, Hartmann K, Nilsson G, Reiter A, Hermine O, et al. Mast cells as a unique hematopoietic lineage and cell system: from Paul Ehrlich's visions to precision medicine concepts. Theranostics. 2020;10(23):10743.

60. Qiu C, Zhong L, Huang C, Long J, Ye X, Wu J, et al. Cell-bound IgE and plasma IgE as a combined clinical diagnostic indicator for allergic patients. Sci Rep. 2020;10(1):1–9.

61. de Castro RO. Regulation and function of syk tyrosine kinase in mast cell signaling and beyond. J Signal Transduct. 2011;2011.

62. MacGlashan Jr D. IgE receptor and signal transduction in mast cells and basophils. Curr Opin Immunol. 2008;20(6):717–23.

63. Nishida K, Yamasaki S, Ito Y, Kabu K, Hattori K, Tezuka T, et al. FcεRI-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane. J Cell Biol. 2005;170(1):115–26.

64. Metcalfe DD, Pawankar R, Ackerman SJ, Akin C, Clayton F, Falcone FH, et al. Biomarkers of the involvement of mast cells, basophils and eosinophils in asthma and allergic diseases. World Allergy Organ J. 2016;9(1):1–15.

65. Mutsaddi S, Kotrashetti VS, Nayak RS, Pattanshetty SM. Comparison of histochemical staining techniques for detecting mast cells in oral lesions. Biotech Histochem. 2019;94(6):459–68.

66. Walls AF, Amalinei C. Detection of mast cells and basophils by immunohistochemistry. In: Basophils and Mast Cells. Springer; 2020. p. 263–80.

67. Montani D, Perros F, Gambaryan N, Girerd B, Dorfmuller P, Price LC, et al. C-kit–positive cells accumulate in remodeled vessels of idiopathic pulmonary arterial hypertension. Am J Respir Crit Care Med. 2011;184(1):116–23.

ANNEXURES

ANNEXURES

Ethical clearance certificate

1. Information sheet in English

2. Information sheet in Hindi

3. Consent form in English

4. Consent form in Hindi

5. Data collection sheet

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

No. AIIMS/IEC/2021/ 3480 **Date: 12/03/2021**

ETHICAL CLEARANCE CERTIFICATE

Certificate Reference Number: **AIIMS/IEC/2021/3315**

Project title: **"Demonstration of mast cells by quantitative estimation of TNF-α and IL-3 by ELISA from lung tissue in fatal mechanical asphyxial deaths"**

Nature of Project:	**Research Project Submitted for Expedited Review**
Submitted as:	**M.D. Dissertation**
Student Name:	**Dr. Rahul Panwar**
Guide:	**Dr. Raghvendra Singh Shekhawat**
Co-Guide:	**Dr. Tanuj Kanchan, Dr. Naveen Sharma, Dr. Kamla Kant Shukla & Dr. Meenakshi Rao**

Institutional Ethics Committee after thorough consideration accorded its approval on above project.

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.

Please Note that this approval will be rectified whenever it is possible to hold a meeting in person of the Institutional Ethics Committee. It is possible that the PI may be asked to give more clarifications or the Institutional Ethics Committee may withhold the project. The Institutional Ethics Committee is adopting this procedure due to COVID-19 (Corona Virus) situation.

If the Institutional Ethics Committee does not get back to you, this means your project has been cleared by the IEC.

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

Dr. Praveen Sharma
Member Secretary

Member secretary
Institutional Ethics Committee
AIIMS,Jodhpur

Basni Phase-2, Jodhpur, Rajasthan-342005; **Website:** www.aiimsjodhpur.edu.in; **Phone:** 0291-2740741 Extn. 3109
E-mail : ethicscommittee@aiimsjodhpur.edu.in; ethicscommitteeaiimsjdh@gmail.com

Annexure 1: Information sheet

Information Sheet for the relatives of the deceased

Title of Thesis: - Demonstration of mast cells by quantitative estimation of TNF-α and IL-3 by ELISA from lung tissue in fatal mechanical asphyxial deaths.

Name of PG Student: - Dr. Rahul Panwar Contact No.: - +91 9521160603

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 demonstrate of mast cells by quantitative estimation of TNF-α and IL-3 by ELISA from lung tissue in fatal deaths mechanical asphyxia.

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 collection and study of tissues from lung.

4. Will confidentiality of my deceased relative 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 पत्र

रो गयों के लए सूचना पत्र

शोध कार्य का शीर्षक- घातक यांत्रिक श्वासावरोध मौतों में फेफड़ों के ऊतकों से ए लसा द्वारा **TNF-α** और **IL-3** के मात्रात्मक आकलन द्वारा मस्ट को शकाओं का प्रदर्शन.

पीजी छात्र का नाम: डॉ. राहुल पंवार

संपर्क नंबर - + 91-9521160603

इससे पहले क आप तय करें क आप इस अध्ययन में भाग लेना चाहते हैं या नहीं, आपके लए यह समझना आवश्यक है क शोध क्यों कया जा रहा है और इसमें क्याशा मल होगा।कृपया निम्न ल खत जानकारी को ध्यान से पढ़ने के लए समय निकालें और यदि आप चाहें तो दूसरों के साथ इस पर चर्चा करें।

1. अध्ययन का उद्देश्य क्या है?

अध्ययन का उद्देश्य - घातक यांत्रिक अलैं गक मौतों में फेफड़ों के ऊतकों से ए लसा द्वारा TNF-α और IL-3 के मात्रात्मक आकलन द्वारा मस्ट को शकाओं का प्रदर्शन ।

2. क्या होगा अगर मैं इस अध्ययन में भाग नहीं लेना चाहता या यदि मैं बाद में वापस लेना चाहता हूं?

इस अध्ययन में भागीदारी स्वैच्छिक है। यह पूरी तरह से आप पर निर्भर है क आप भाग लेते हैं या नहीं। आप कसी भी समय और कसी भी कारण या बिना कसी कारण के अध्ययन से हट सकते हैं। कृपया शोधकर्ता को बताएं क आप अध्ययन से हटना चाहते हैं।

3. इस अध्ययन में क्या शा मल है?

इस अध्ययन में फेफड़े के ऊतक का संग्रह और अध्ययन शा मल होगा।

4. क्या मेरे मृतक रिश्तेदार की गोपनीयता की रक्षा की जाएगी?

आपके मृतक के बारे में जानकारी पूर्ण गुमनामी के अधीन होगी।

Annexure 3: Consent form

All India Institute of Medical Sciences Jodhpur, Rajasthan

Informed Consent Form

Title of Thesis: - Demonstration of mast cells by quantitative estimation of TNF-α and IL-3 by ELISA from lung tissue in fatal mechanical asphyxial deaths

Name of PG Student: - Dr. Rahul Panwar Contact No: - +91 9521160603

Case Number (Post Mortem Number): - _____Date: - _____I,_____son/Mr. daughter/Mr. _____

_____R/O_____

_____give my full, independent, voluntary consent for the research work upon my dead relative _____, which is " Demonstration of mast cells by quantitative estimation of TNF-α and IL-3 by ELISA from lung tissue in fatal mechanical asphyxial deaths". 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, collection of body 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

अनुलग्नक **4:** सहमति फॉर्म

अ खल भारतीय आयु र्वज्ञान संस्थान जोधपुर, राजस्थान

सू चत सहमति प्रपत्र

शोध कार्य का शीर्षक:- घातक यांत्रिक श्वासावरोध मौतों में फेफड़ों के ऊतकों से ए लसा द्वारा **TNF-α** और **IL-3** के मात्रात्मक आकलन द्वारा मस्ट को शकाओं का प्रदर्शन

पीजी स्टूडेंट का नाम: डॉ. राहुल पंवार संपर्क नंबर - + 91-9521160603

तारीख_____

मैं, _____ पुत्र/श्री,पुत्री/श्री _____,
निवासी_____
मेरी पूर्ण, स्वतंत्र, स्वैच्छिक सहमति देता/देती हूँ क मेरे मृत परिजन के देह को शोध कार्य के लए इस्तेमाल कर सकते हैं, जिसका शीर्षक है " घातक यांत्रिक अलैं गक मौतों में फेफड़ों के ऊतकों से ए लसा द्वारा TNF-α और IL-3 के मात्रात्मक आकलन द्वारा मस्ट को शकाओं का प्रदर्शन । ", इसकी प्र क्रया और प्रकृति को मेरी भाषा में पूरी तरह समझाया दिया गया है और मैं पुष्टि करता/करती हूँ क मुझे प्रशन पूछने का अवसर मला ।

मैं समझता/समझती हूं क इस अध्ययन में ऊतक का संग्रह और अध्ययन आधारित अध्ययन है। मैं उपरोक्त अध्ययन के लए फेफड़े के ऊतक का संग्रह और अध्ययन के उपयोग क मंजूरी देता/देती हूँ।

दिनांक: _____ हस्ताक्षर / अंगूठे का निशान

स्थान: _____ रोगी का _____

यह साबित करने के लए क मेरी उपस्थिति में उपरोक्त सहमति प्राप्त हुई है।

तारीख: _____

स्थान: _____ पीजी छात्र के हस्ताक्षर

1. साक्षी 2. साक्षी

हस्ताक्षर: हस्ताक्षर:

नाम: नाम:

पता: 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: -

Case no.	Autopsy details	Asphyxia /Non - asphyxia	Qty. of sample	Sample given yes / no & date	TB mast cells (peri alveolar)	TB mast cells (peri bronchial	TB mast cells (peri vascular)	TNF-α OD	TNF-α Conc.	IL-3 OD	IL-3 Conc.

*TB – Toluidine Blue

S. No.	Autopsy details	Asphyxia/ Non asphyxia	Age	Sex	Quantity of sample		Histopathology Results			TNF-α OD		TNF-α Conc.		IL-3 OD		IL-3 Conc.	
					Hilar	Peripheral	Mast cells (mean) Hilar (peribronchial)	Mast cells (mean) Peripheral (perialveolar)	Mast cells (mean) perivascular	Right Lung Sample	Left Lung Sample	Right Lung Sample	Left Lung Sample	Right Lung Sample	Left Lung Sample	Right Lung Sample	Left Lung Sample
1	Hanging	Asphyxia	50	Male	2	2	0.2	0.4	1	0.411	0.514	245.933	291.787	2.444	2.296	1318.299	1232.306
2	Hanging	Asphyxia	25	Male	2	2	0	0	0.2	1.742	0.753	2100.42	401.064	3.724	3.564	2070.1754	1971.822
3	Hanging	Asphyxia	14	Female	2	2	0	0	0	0.645	0.679	349.911	365.638	2.504	3.018	1353.536	1653.228
4	Hanging	Asphyxia	42	Male	2	2	0	0	0.2	0.429	0.646	254.051	350.275	2.004	3.001	1061.72	1643.602
5	Hanging	Asphyxia	35	Male	2	2	0	0.2	0	0.671	0.95	361.977	509.22	3.153	3.535	1731.987	1955.02
6	Drowning	Asphyxia	4	Male	2	2	0.4	0.4	0.8	0.738	0.668	393.344	360.545	3.314	3.437	1826.206	1897.614
7	Drowning	Asphyxia	30	Male	2	2	0	0	0.2	0.487	0.411	280.017	246.339	2.559	1.605	1385.798	828.711
8	Electrocaustion	Non asphyxia	40	Male	2	2	0	0	0	0.285	0.28	186.08	183.526	1.678	1.732	871.357	903.211
9	Physical assault	Non asphyxia	30	Male	2	2	0	0	0	0.364	0.242	224.792	162.403	2.715	3.288	1476.691	1810.629
10	RTA	Non asphyxia	55	Female	2	2	0	0	0	0.152	0.19	99.967	129.626	0.504	0.618	186.914	252.954
11	Hanging	Asphyxia	25	Male	2	2	0	0.2	0.4	0.644	0.655	349.501	354.386	3.261	2.753	1795.286	1498.861
12	Railway accident	Non asphyxia	27	Male	2	2	0	0	0	0.464	0.461	269.845	268.212	2.532	2.077	1369.93	1104.6
13	Fall from height	Non asphyxia	61	Male	2	2	0	0	0	0.404	0.561	243.135	312.506	2.34	2.797	1258.034	1524.238
14	Poisoning	Non asphyxia	20	Female	2	2	0	0.2	0.2	0.267	0.222	176.517	150.428	1.867	2.118	981.911	1128.111
15	Sudden unexcepted death	Non asphyxia	18	Female	2	2	0	0	0	0.19	0.58	129.626	320.646	0.618	2.825	252.954	1540.515
16	Head injury	Non asphyxia	59	Male	2	2	0	0	0	0.606	0.594	332.493	326.824	2.796	2.922	1523.888	1597.28
17	RTA	Non asphyxia	37	Male	2	2	0	0	0	0.311	0.214	199.265	145.83	1.612	0.955	832.969	449.968
18	RTA	Non asphyxia	52	Male	2	2	0	0	0	0.295	0.153	190.966	100.517	1.537	1.447	789.039	736.709
19	Sudden unexcepted death	Non asphyxia	35	Male	2	2	0	0	0	0.305	0.557	196.222	310.609	1.577	2.75	812.842	1497.227
20	Sudden unexcepted death	Non asphyxia	25	Male	2	2	0	0	0	0.316	0.152	201.689	99.414	0.332	1.81	86.277	948.424
21	Sudden unexcepted death	Non asphyxia	50	Male	2	2	0	0	0	0.45	0.214	263.39	146.019	1.366	1.203	689.745	594.651
22	Electrocaustion	Non asphyxia	39	Male	2	2	0	0	0.2	0.203	0.171	139.007	115.974	0.96	1.097	452.652	532.694
23	Hanging	Asphyxia	22	Male	2	2	0	0.2	0.4	0.586	0.357	323.398	221.284	2.511	1.993	1357.328	1055.303
24	Sudden unexcepted death	Non asphyxia	18	Male	2	2	0	0	0	0.295	0.198	191.271	135.207	0.417	0.618	136.041	252.954
25	RTA	Non asphyxia	32	Male	2	2	0	0	0	0.152	0.154	99.414	101.788	0.876	0.66	403.53	277.457
26	Poisoning	Non asphyxia	35	Male	2	2	0	0	0	0.307	0.335	197.173	210.79	1.781	1.796	931.389	940.256
27	Hanging	Asphyxia	32	Male	2	2	0.4	0.4	2	0.389	0.803	236.091	473.308	1.791	1.515	937.573	776.496
28	Hanging	Asphyxia	34	Male	2	2	0.4	0.4	0.8	1.514	1.071	1723.501	594.617	3.78	3.461	2102.9239	1911.499
29	Smothering	Asphyxia	24	Female	2	2	0.2	0.2	0.4	1.419	0.518	1135.009	293.544	3.519	2.599	1945.336	1408.901
30	Drowning	Asphyxia	33	Male	2	2	0	0.2	0.4	0.424	0.923	252.129	493.085	2.066	3.254	1097.716	1791.143
31	Railway accident	Non asphyxia	2	Female	2	2	0	0	0	0.421	0.389	250.517	236.091	2.308	2.137	1239.073	1139.429
32	Drowning	Asphyxia	22	Male	2	2	0	0	0	1.077	0.713	599.175	381.573	3.759	3.573	2090.6432	1977.364
33	Drowning	Asphyxia	17	Male	2	2	0.2	0.2	0.4	0.879	0.958	467.431	514.347	3.238	3.195	1781.926	1756.665
34	Drowning	Asphyxia	17	Male	2	2	0	0	0.2	0.409	1.21	245.122	728.157	2.036	3.609	1080.214	2002.9239
35	Hanging	Asphyxia	24	Male	2	2	0.2	0.4	0.8	0.715	0.719	382.478	384.578	2.63	2.882	1426.694	1573.944
36	Hanging	Asphyxia	23	Female	2	2	0.2	0.4	1	0.497	0.89	284.323	473.308	2.973	2.856	1627.15	1559.067
37	Hanging	Asphyxia	24	Male	2	2	0.4	0.6	1.2	0.504	0.379	287.529	231.46	2.313	2.175	1242.34	1161.306
38	Hanging	Asphyxia	26	Male	2	2	0.4	0.4	0.2	1.649	0.818	1049.218	433.76	2.316	1.768	1243.974	924.155
39	Hanging	Asphyxia	17	Female	2	2	0.4	0.4	0.4	0.904	0.405	481.548	243.406	3.679	2.627	2043.8596	1425.061
40	Hanging	Asphyxia	26	Male	2	2	0	0	0.2	0.951	1.098	510.101	617.178	3.118	3.102	1711.918	1702.35
41	Hanging	Asphyxia	26	Male	2	2	0	0.2	0.4	0.984	1.723	531.424	2015.44	3.74	3.849	2079.5321	2143.2748
42	Hanging	Asphyxia	40	Male	2	2	0	0.2	0.6	0.62	0.591	338.505	325.755	3.023	3.127	1656.495	1716.76
43	Hanging	Asphyxia	27	Male	2	2	0.2	0.2	0.4	0.627	0.689	341.705	370.393	3.418	3.307	1886.646	1822.064
44	Hanging	Asphyxia	55	Male	2	2	0.2	0	0.2	0.901	0.491	479.852	281.556	2.987	2.573	1635.143	1393.674
45	Hanging	Asphyxia	36	Female	2	2	0.4	0.4	0.6	1.392	1.924	1050.415	2199.32	3.81	3.718	2120.4678	2066.6666
46	Drowning	Asphyxia	65	Male	2	2	0	0	0.2	0.646	0.394	350.64	238.325	2.932	2.278	1603.056	1221.746
47	Hanging	Asphyxia	28	Male	2	2	0.2	0.4	0.6	0.647	0.363	350.685	224.046	2.111	2.181	1124.202	1156.04
48	Drowning	Asphyxia	25	Male	2	2	0.2	0.2	0.4	0.726	0.567	387.887	314.935	3.438	2.897	1898.489	1582.812
49	Hanging	Asphyxia	22	Male	2	2	0.4	0.4	0.8	0.458	0.646	266.974	350.275	2.887	2.589	1577.094	1402.892
50	Smothering	Asphyxia	4	Male	2	2	0.2	0.2	0.4	0.943	1.027	505.092	561.348	3.096	2.834	1698.908	1546.291
51	Hanging	Asphyxia	22	Male	2	2	0	0.4	0.8	0.875	1.015	465.002	552.838	3.346	2.856	1844.816	1559.067
52	Head injury	Non asphyxia	30	Male	2	2	0	0	0	0.609	0.257	333.657	171.233	0.773	0.784	343.615	350.265
53	Electrocaution	Non asphyxia	46	Male	2	2	0	0	0	0.542	0.291	303.826	189.329	2.129	2.304	1134.703	1236.856
54	RTA	Non asphyxia	22	Male	2	2	0	0	0	0.214	0.404	145.641	243.135	1.066	0.77	514.667	341.806
55	RTA	Non asphyxia	17	Male	2	2	0	0	0	0.497	0.404	284.235	243.135	2.366	2.353	1273.26	1265.151
56	RTA	Non asphyxia	34	Male	2	2	0	0	0	0.332	0.516	209.537	292.578	0.467	2.541	165.036	1375.005
57	Railway traffic accident	Non asphyxia	40	Male	2	2	0	0	0	0.569	0.405	315.864	243.451	2.562	2.181	1387.256	1165.215
58	Hanging	Asphyxia	28	Male	2	2	0.4	0.6	1.2	0.336	0.409	211.174	245.031	1.796	2.608	940.315	1414.034
59	RTA	Non asphyxia	57	Female	2	2	0	0	0	0.316	0.278	201.689	182.423	1.602	1.588	827.019	818.909
60	Sudden unexcepted death	Non asphyxia	34	Male	2	2	0	0	0	0.191	0.464	130.397	269.845	0.655	2.419	275.065	1303.714
61	RTA	Non asphyxia	55	Male	2	2	0	0	0.2	0.342	0.316	214.094	201.442	2.528	2.036	1367.479	1080.68
62	Poisoning	Non asphyxia	19	Male	2	2	0	0	0	0.411	0.494	246.249	282.83	2.686	2.719	1459.831	1479.142
63	Hanging	Asphyxia	39	Male	2	2	0.4	0.4	0.8	0.39	0.398	236.365	240.144	2.329	2.302	1251.383	1235.856
64	Hanging	Asphyxia	38	Male	2	2	0.4	0.4	0.8	0.405	0.514	243.315	291.787	2.245	2.296	1202.436	1232.306
65	RTA	Non asphyxia	68	Male	2	2	0	0	0	0.252	0.248	167.549	165.991	1.17	1.71	575.107	890.376
66	Hanging	Asphyxia	36	Male	2	2	0.4	0.4	1.2	0.763	0.58	406.056	320.957	3.349	2.662	1846.216	1445.538
67	Sudden unexcepted death	Non asphyxia	29	Male	2	2	0	0	0.2	0.467	0.144	270.992	91.586	0.968	0.591	457.552	237.553
68	Hanging	Asphyxia	24	Male	2	2	0.4	0.6	1.2	0.914	0.592	487.573	326.022	3.325	2.771	1832.565	1509.245
69	Fall from height	Non asphyxia	52	Male	2	2	0	0	0	0.745	0.402	396.752	241.867	2.52	2.062	1363.045	1095.557
70	Hanging	Asphyxia	25	Male	2	2	0.2	0.2	0.4	0.745	0.548	396.947	306.687	3.049	2.789	1671.488	1519.513
71	Sudden unexcepted death	Non asphyxia	35	Male	2	2	0	0	0	0.649	0.681	351.598	366.335	2.547	2.779	1378.797	1513.621
72	Head injury	Non asphyxia	45	Male	2	2	0	0	0	0.124	0.171	67.935	115.974	0.501	0.45	184.755	155.177
73	Head injury	Non asphyxia	32	Male	2	2	0	0	0	0.201	0.35	137.22	217.893	1.108	1.88	539.111	989.379
74	RTA	Non asphyxia	64	Male	2	2	0	0	0	0.336	0.305	211.27	196.222	2.201	2.463	1176.591	1329.325
75	Poisoning	Non asphyxia	27	Female	2	2	0	0	0	0.124	0.549	67.935	306.907	0.584	0.534	233.469	204.474
76	RTA	Non asphyxia	49	Male	2	2	0	0	0	0.153	0.223	100.517	151.223	1.01	1.306	481.822	654.624
77	Poisoning	Non asphyxia	17	Female	2	2	0	0	0	0.115	0.228	53.854	154.37	0.619	0.601	253.713	243.387
78	Poisoning	Non asphyxia	36	Female	2	2	0	0	0.2	0.536	0.648	301.583	351.415	1.607	1.938	830.286	1023.216
79	Railway traffic accident	Non asphyxia	42	Male	2	2	0	0	0	0.254	0.472	169.518	273.064	1.986	2.722	1051.569	1480.542
80	Physical assault	Non asphyxia	32	Male	2	2	0	0.2	0.2	0.291	0.449	189.329	262.902	2.709	2.889	1473.074	1577.794
81	Poisoning	Non asphyxia	53	Male	2	2	0	0	0	0.266	0.235	175.869	158.457	0.655	1.032	275.065	494.598
82	RTA	Non asphyxia	52	Male	2	2	0	0	0	0.391	0.21	236.821	143.098	0.913	3.096	425.057	1698.908
83	Fall from height	Non asphyxia	30	Male	2	2	0	0	0	0.84	0.264	445.402	174.895	0.641	1.387	266.606	701.938
84	RTA	Non asphyxia	18	Male	2	2	0	0	0	0.245	0.201	164.006	137.617	0.534	0.618	204.474	252.954
85	Hanging	Asphyxia	43	Female	2	2	0	0	0	0.729	0.519	389.138	294.071	3.244	2.999	1785.018	1642.26
86	Hanging	Asphyxia	42	Female	2	2	0.2	0.2	0.4	0.382	0.411	232.747	246.339	2.365	1.605	1272.502	828.711
87	Hanging	Asphyxia	27	Male	2	2	0	0.2	0.4	0.789	0.545	419.18	305.234	1.857	2.768	976.077	1507.437
88	Hanging	Asphyxia	33	Female	2	2	0	0.2	0	0.37	0.394	227.348	238.325	2.277	2.16	1221.221	1153.08
89	Hanging	Asphyxia	30	Male	2	2	0	0	0.2	1.415	3.336	1123.675	3201.55	3.717	3.962	2066.0818	2209.3567
90	Hanging	Asphyxia	40	Male	2	2	0.6	0.8	1.4	0.679	0.606	365.638	332.404	3.018	2.835	1653.228	1546.583