Section A -Research paper

Forensic Methods for Postmortem Interval Estimation: A Literature review

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Abstract:

Background: Estimation of the postmortem interval (PMI) within the context of a medicolegal death investigation is simultaneously one of the most common and most difficult to answer questions within forensic medicine. It plays a role in nearly every aspect of a death investigation including implicating or excluding suspects, determining the time of assault versus the time of death, whether there was a period of survivability or neglect throughout the chain of events leading to the death of an individual, and may even be useful in determining the identity of the deceased. **Aim:** The current work provides a review of the different methods applied for postmortem interval estimation in the field of forensics. **Conclusion:** Researchers have pointed out many techniques for estimating the post-mortem interval including regular biochemical examination, immunohistochemical staining, histopathological analysis and Molecular techniques. A combination of both numerous types of evidence and numerous methods of estimation should be jointly evaluated in order to provide more accurate postmortem interval estimation. Efforts to improve the precision and reliability of estimating PMI are ongoing, including the use of artificial intelligence in PMI estimation.

Keywords: Postmortem interval, Methods, Death.

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Introduction:

Throughout the history of forensic medicine, the postmortem interval (PMI) has been one of the most commonly and thoroughly investigated problems. The importance of an accurate PMI in the context of a medicolegal death investigation cannot be understated due to its utility and application toward investigative determinations including inclusion or exclusion of suspects, determination of time of assault versus time of death, and preliminary victim identification. However, despite its importance, the question of postmortem interval estimation is often answered with a low degree of accuracy as compared to the rates of certainty within other forensic disciplines **(1).**

Determination of postmortem interval is done with various methods depending upon the condition of the dead body and the circumstances in which it is found **(2).**

The longer the interval of time between death and examination of the body, the more difficult and the more important postmortem interval estimation becomes as it is a crucial precursor to a thorough case investigation. In some cases, there may be a period of survivability between the initial injury and the actual death itself. Understanding the relationship between such events and being able to clearly discern time since death is crucial for a medicolegal death investigation **(1).**

This necessitates a twofold approach to the investigative practices surrounding postmortem interval estimation. First, antemortem and perimortem changes and injuries such as wound pattern analysis, infection, or other injury must be assessed to determine what, if any, effect this may have on the period of survivability as it relates to the time since death. And second, the postmortem changes must be assessed to provide a preliminary estimation of the time since death **(3).**

Traditional methods for estimating PMI are based on changes undergone by a corpse after death, including early changes involving physical processes (e.g., body cooling and hypostasis), metabolic processes (supravital reactions), autolysis (loss of selective membrane permeability, diffusion), and physicochemical processes (rigor mortis).They produce relatively reliable estimates of time since death for the early post-mortem interval (EPMI), typically only within the

first 24 h after physiological death **(4).**

After this stage, the focus shifts almost exclusively to loss of soft tissue and, later, to changes to the skeletal structures. These methods provide only very rough estimates of PMI, particularly after EPMI **(5).**

In general, post-mortem changes happen in a predictable order of increasing stages of degeneration. However, there are significant variances because of a wide range of influencing factors originating from both the environment and the human body itself. Changes caused by the human body itself are referred as intrinsic factors while changes due to environment are extrinsic factors **(6).**

Due to the medico-legal limitations related to the assessment of the classical triad of postmortem changes (rigor mortis, livor mortis, and algor mortis), methods from various fields, such as entomology, chemistry and biochemistry, histopathology and immunohistochemistry, and molecular biology have been used to assess the PMI. At the same time, tissues, and organs, mainly the heart, liver, kidney, blood, and other body fluids have been the subject of research **(7).**

Methods of postmortem interval estimation:

- **Forensic entomology**

Forensic entomology is the study of insects/arthropods in criminal investigation. Right from the early stages insects are attracted to the decomposing body and may lay eggs in it **(8).**

Depth knowledge and experience of the biodiversity, biology, ecology, and behavior of forensic insects found on a human corpse or at a crime site, can provide information about time since death, location of the crime, and in some cases cause of death **(9).**

A new term arises relevant to forensic entomology study, which is an entomological postmortem interval that is defined as the time taken from the colonization of flesh-eating insects to their last developmental or adult stage. This entomological timeline is utilized when the traditional markers or rate methods of postmortem intervals have weaned off and remains are putrefied or unrecognizable **(2).**

With the death of an individual when biological functions are stopped, these microorganisms behave contrarily along with the invasion of degrading microbes from the environment. Human cadaver becomes a rich source of nutrients due to autolysis of cells, which attracts various invading microorganisms as well as macro-organisms. At different stages of degradation, the succession of microorganisms differs significantly **(Table 1)** which can be explored for accurate PMI estimation **(10).**

Table (1): Stages of decay and ecological succession information of certain Fly species **(11).**

	Fresh	Bloated	Decay	Post Decay
Approximat Days After Death (DAD)	**1-4**	**3-6**	**5-20**	**+19**
Appearance on body	**Natural**	**Bloated**	**Deflated Odiferous**	**Mummified or Skeletal**
Calliphora Vomitoria larva	**Yes**	**Yes**	**Yes**	**No**
Calliphora Vomitoria larva	**Yes**	**Yes**	**Yes**	**No**
Sarcophaga Carnaria Larvae	**Yes**	**Yes**	**Yes**	**No**
Musca Domestica Larvae	**NO**	**Yes**	**Yes**	**No**
Piophila Nigriceps Larvae	**No**	**No**	**Yes**	**No**
Location of Larvae	**Orifices Wound**	**Orifices Wound**	**Throughout body**	**None**
Pupae are present	**No**	**No**	**Yes**	**Possible**

With advances in the lab techniques, DNA identification of insects is also useful in determining the time since death and species identification of the insects **(2).**

Even if forensic entomology does not provide a precise PMI estimate, it can help to provide important insights into cases involving human remains **(12).**

- **Thanatochemistry**

There is also a huge literature on chemical methods proposed for estimating the time since death **(13).**

Recent research attempting to improve PMI estimates have considered more predictable and quantifiable biological markers and processes associated with human decomposition, particularly from the field of thanatochemistry, which focuses on post-mortem chemical changes exhibited by the decomposing body. This field is mostly based on the quantification of volatile byproducts of different organic decay processes **(5).**

These methods have been shown to be more accurate in PMI estimation, since the effect of external conditions is less relevant than in the currently used traditional methods. Moreover, these changes begin immediately after death, so can be used to estimate the PMI during the first hours after death since most victims are found in this period **(14).**

Every kind of death results in changes in metabolites in body tissues and fluids due to lack of oxygen, altered circulation, enzymatic reactions, cellular degradation, and cessation of anabolic production of metabolites. Metabolic changes may provide markers determining the time since death, which is challenging in current analytical and observation-based methods **(7).**

The biochemical assessment has been useful for estimating PMI from vitreous humor, synovial fluid, pericardial fluid, urine, and cerebrospinal fluid. Numerous factors, however, need to be accounted for when examining the PMI based on biochemistry including, but not limited to, age, gender, biological background, lifestyle, cause of death, and a whole range of other intrinsic and extrinsic factors **(15).**

Previous tests were performed mainly on blood and spinal fluid in the cerebrospinal tract. However, vitreous humor over time has been the most studied measure of time after death. This

was due to the fact that the vitreous jaws were sparse and well-covered, leading to slow autolytic changes associated with blood and spinal fluid in the brain. The most reliable and proven vitreous analytes are sodium (Na+), chloride (Cl−) and potassium (K+) **(16, 17).**

After death, there is steady potassium leak through the cell membrane to approach equilibrium with the plasma in the postmortem period, which helps to estimate the PMI. Numerous workers have demonstrated the linear relationship between increase in vitreous potassium concentration and increasing PMI. However, the sodium and chloride levels have no role in estimating PMI **(18).**

The study of the relationship between the concentrations of the different vitrous humor substances (mainly K^+ and hypoxanthine) has intensified and several studies using different conditions as correctional factors to minimize the error of estimation have been carried out **(19).**

Similar to vitreous, cerebrospinal fluid (CSF) is a well-protected fluid and is relatively acellular compared to blood. The literature in using CSF in determining PMI is rather scant. One study that compared CSF and vitreous biochemistry in determining PMI showed vitreous K+ remains the best in determining PMI **(16).**

Human beings have always involved themselves in various activities to ensure their wellbeing and survival. In doing so, the human body has directed the release of different free radicals or reactive substances which are reactive chemicals with an unpaired electron in an outer orbit. Reactive oxygen species (ROS) comprise both free radical and non-free radical oxygen containing molecules. There are also reactive nitrogen, iron, copper, and sulfur species which could attribute to increased ROS formation and oxidative stress and impair the redox balance **(14, 20).**

Oxidative stress is a phenomenon resulting from an imbalance between the production and accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in cells and tissues and the ability of biological system to eliminate these reactive products. The level of oxidative stress is quantified by the oxidative stress markers **(21).**

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are defined as unstable species containing oxygen (O_2) and nitrogen that react quickly with other molecules in the cells. They can be provided from extracellular or intracellular sources. Extracellular sources include ultraviolet (UV) light, ionizing radiation, heavy or transition metals, and others (revised later). In contrast, intracellular sources comprise mitochondria, peroxisomes, endoplasmic reticulum (ER), and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidases (NOXs) **(22).**

The reactive nitrogen and oxygen species (RNS/ROS) play a twofold role as both toxic and beneficial compounds to the organism's system. At lower concentrations, they have beneficial effects and indulged in different physiological processes such as redox regulation, mitogenic responses, cellular signaling pathways, and an immune function while at a higher level, these reactive species generate nitrosative and oxidative stress **(20).**

Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are molecules capable of independent existence, containing at least one oxygen atom and one or more unpaired electrons. This group includes oxygen free radicals, e.g. superoxide anion radical, hydroxyl radical, hydroperoxyl radical, singlet oxygen, as well as free nitrogen radicals **(23).**

Excessive ROS production determines structural modification of cellular proteins and the alteration of their functions, leading to cellular dysfunction and disruption of vital cellular processes. High ROS levels cause lipid, protein, and DNA damage. In particular, ROS can break the lipid membrane and increase membrane fluidity and permeability. Protein damage involves site-specific amino acid modification, peptide chain fragmentation, cross-linked reaction products aggregation, electric charge alteration, enzymatic inactivation, and proteolysis susceptibility. Finally, ROS can damage DNA through oxidizing deoxyribose, breaking strand, removing nucleotides, modifying bases, and crosslinking DNA-protein **(24).**

❖ Reactive Nitrogen Species (RNS):

Reactive Nitrogen Species (RNS) are a group of highly reactive nitrogen-containing molecules that are generated as by-products of nitrogen metabolism. These molecules are crucial in various physiological and pathological processes. Nitric oxide (NO) is the most well-known and significant member of the RNS family **(25).**

Nitric oxide (NO) is a diatomic molecule that consists of 1 nitrogen atom (N, 7 electrons) and 1 oxygen atom (O, 8 electrons). So, it contains 1 unpaired electron which makes it a free radical **(26).**

It is produced in response to homeostatic, inflammatory, or mitogenic stimuli. It can produce hydroxyl radicals as well as nitrogen dioxide radicals **(14).**

According to **Griendling et al. (27),** NO is involved in numerous physiologic conditions including neurotransmission, vascular smooth muscle relaxation and autoimmunity, as well as in numerous pathological conditions, including neurodegeneration and neuroinflammation, and usually is accompanied by elevated nitrosative/oxidative stress **(28).**

Nitric oxide is soluble in water and fat, and it therefore diffuses readily through the cytoplasm and plasma membrane. If human blood plasma is exposed to $NO^{\bullet}$, ascorbic acid and uric acid concentrations become depleted and lipid peroxidation is triggered **(29).**

❖ Lipid Peroxidation and Malondialdehyde (MDA) Formation:

Lipid peroxidation can be described generally as a process under which oxidants such as free radicals or non-radical species attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) results in the generation of toxic lipid aldehyde species, including 4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA), and acrolein **(30).**

Among the aldehydes produced by lipid peroxidation (LPO), malonaldehyde (MDA) has gained most attention, since MDA is produced at high levels during LPO, so that it is commonly used as a measure of oxidative stress **(31).**

Malondialdehyde (MDA) is an aldehyde formed at the end of the oxidation of polyunsaturated fatty acids, such as arachidonic acid, through enzymatic processes involving the biosynthesis of thromboxane A2 (TXA2) or by nonenzymatic processes through endoperoxides produced during lipid peroxidation **(fig.1)** (**32**).

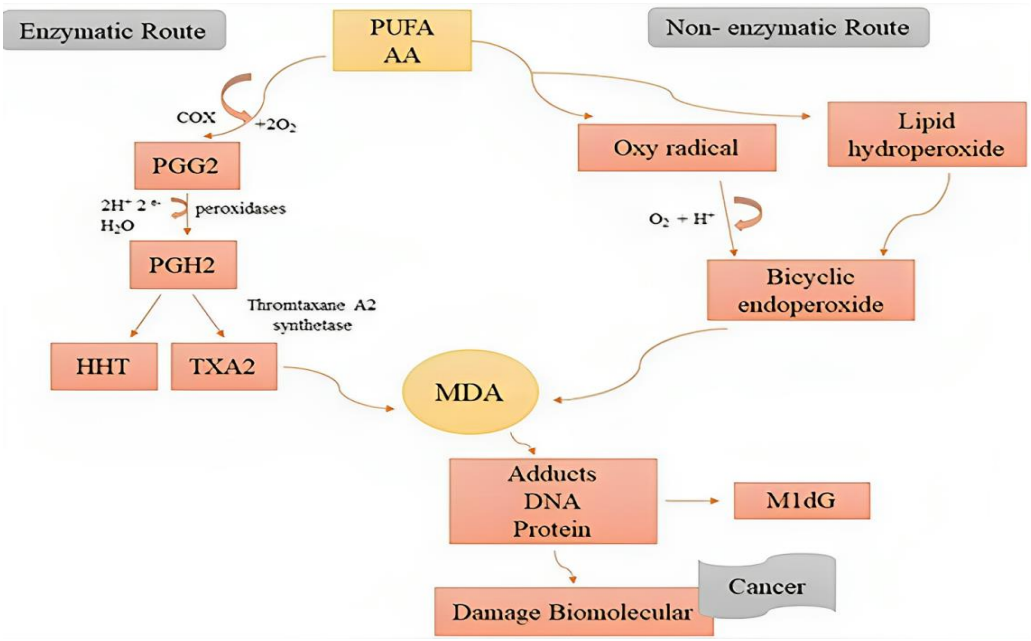

Fig.1: MDA formation by enzymatic and nonenzymatic pathways **(32)**

Under physiological conditions, MDA is not a highly reactive compound, increasing its reactivity at lower pH. At strong acidic conditions, MDA can react with amino acids such as glycine, leucine, valine, and the guanidino group of arginine, to yield different adducts **(33).**

Malondialdehyde (MDA) causes structural changes that mediate its oxidation, such as fragmentation, modification, and aggregation, especially in DNA and protein. The excessive binding of these reactive aldehydes to cellular proteins alters membrane permeability and electrolyte balance. Degradation of proteins leads to progressive degradation of the biological system mediated by oxidative stress **(34).**

Malondialdehyde (MDA) is considered a good marker of oxidative stress, being able to cause damage and form adducts which can become mutagenic, and with irreparable alterations **(35).**

To cope with the oxidative stress, animal and human cells have developed the antioxidant defense system. Under physiological conditions, there are three classes of antioxidant defense systems in the human body to remove excess ROS and avoid oxidative damage, including antioxidant substances, antioxidant enzymes, and repair enzymes **(36, 37).**

The level of reactive species in the cellular system may be reduced by antioxidants either by restricting the expression or activities of free radical-producing enzymes such as xanthine oxidase (XO) and NAD(P)H oxidase, or by enhancing the expression and activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GSR) which protect the body and its tissues from ROS/RNS-induced cellular damage **(21, 38).**

- **Antioxidants:**
  - **Catalase (CAT):**

Gebicka and Krych-Madej (39) mentioned that catalases are heme enzymes which catalyze decomposition of hydrogen peroxide to water and molecular oxygen. They are important members of the antioxidant defense system of cells of almost all aerobic organisms. Role of catalase under physiological conditions and some pathological states is presented in (**Fig.2).**

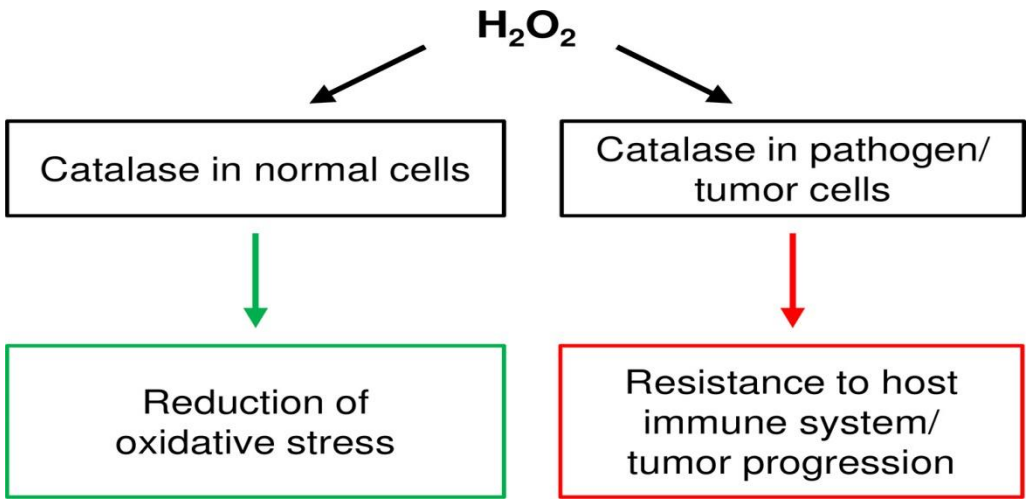

Fig.2: Role of catalase under physiological conditions and some pathological states **(39)**

Catalase (CAT) protects cells by detoxification of the generated H_2O_2 and plays an important role in the acquisition of tolerance to oxidative stress as an adaptive response. CAT can maintain the concentration of O_2 either for repeated rounds of chemical reduction or for direct interaction with the toxin. Furthermore, inhibition of CAT activity results in enhanced cytotoxicity and increased ROS, indicating an important role of CAT in maintaining the oxidative balance **(40).**

- **Glutathione (GSH):**

Glutathione (GSH) is a major antioxidant that maintains the homeostasis of redox states in cells and plays important roles in maintaining the physiological functions of all cells in vivo. The thiol (sulfhydryl, SH) residues play an important role in maintaining the redox state homeostasis intracellularly. In mammals, cysteine (Cys) and methionine (Met) are particularly important as thiol-containing amino acids, but GSH is the most abundant thiol-containing substance (derived from a non-protein) in all kinds of cells **(41).**

It protects cells from damage caused by lipid peroxides, reactive oxygen and nitrogen species, and xenobiotics. Recent studies have highlighted the importance of GSH in key signal transduction reactions as a controller of cell differentiation, proliferation, apoptosis, ferroptosis and immune function **(42).**

The balance between ROS and GSH is essential for the maintenance of normal cellular functions. The imbalance between ROS and GSH can result in oxidative stress that can promote or advance various pathophysiological conditions and may lead to cell death. The sharp decrease of intracellular GSH content would also cause stress to the endoplasmic reticulum, production of a large amount of ROS, lipid peroxidation, and the loss of mitochondrial membrane potential **(43, 44)**.

Under physiological conditions, the oxidant/antioxidant defense system in the human body is in a state of continuous equilibrium. Equilibrium in aerobic metabolism (in Living tissue) is characterized by the formation of free radicals and their removal by means of antioxidant systems. Increase in oxidant levels and a decrease in antioxidant levels are observed in any damaged tissues. After death, it cannot be assumed that oxidant/antioxidant balance be adequately controlled by the body, so changes in oxidant/antioxidant parameters are evaluated as biochemical disturbances Which play important role in determining the early post-mortem intervals **(45,46).**

- **Postmortem histological changes (Histothanatology)**

Other contemporary approaches of PMI measurement include histological and immunohistochemical examinations of degenerative alterations in interior organs which are important because they can aid in the estimation of the PMI **(47).**

Histothanatology is the systematic standard histology for the main organs that should be used in routine forensic autopsies **(48).**

The spectrum of histological, enzyme histochemical, immunohistochemical, and other microscopy investigations can often yield diagnoses crucial to establishing cause of death, chronology of disease processes, wound vitality, intoxication, and postmortem intervals of varying length. However, autolysis and putrefaction are limiting factors that need to be considered in forensic practice and also in histopathological diagnosis **(49).**

Following death many physico-chemical changes begin to take place in the body in an orderly manner and continue until the body eventually decomposes. Similarly, ongoing cellular changes also occur after death that depends on the time interval post-mortem and the circumstances

of the death. These cellular changes can be a useful criterion and marker for estimating the post-mortem interval **(50).**

Degradation of tissues after death results from three basic mechanisms: enzymatic autolysis, oxidation, and microbial growth. Chemical and biological changes occur shortly after death when endogenous enzymes are released, due to loss of cellular integrity and breakdown of cell membranes **(51).**

Decomposition may vary from individual to individual, from environment to environment and even from one part of body to another part of same individual. Process of autolysis is temperature dependent and occurs earlier in some tissue which has high level of hydrolytic enzymes such as the pancreas whereas delayed in fibrous tissue such as uterus or skeletal muscles which have a smaller number of hydrolytic enzymes and few lysosomes **(52).**

Typically, epithelial cells die earlier than connective tissue, cartilage tissue and bones. Nevertheless, macroscopic, and histological investigations, in particular of the heart and lung, are valuable even several years postmortem **(49).**

Death of cells and tissues takes place usually one to two hours after the stoppage of the vital functions & this is termed as molecular death. Autolysis of cells with digestive enzymes starts and residual ATP in cells is also degraded. This cumulative cell degradation makes disintegration of cell membrane possible; cell cytoskeleton is deployed and all nuclear DNA, RNA and proteins are digested in the biochemical environment of degrading cell **(53).**

The mechanisms and histological features of autolysis are the same as for necrosis since the cause is anoxia and absence of life support. It may be quite difficult to distinguish in a histological section, autolysis from necrosis and the only difference is the absence of an inflammatory reaction **(54).**

The autolytic process involves a change in size, shape, electron density, cell structure localization, and typically causes a progressive loss of highly ordered cell structure **(55).**

Cells can be recognized as dead with the light microscope only after they have undergone a sequence of morphological changes **(56).**

Changes that occur in the nucleus after death are pyknosis, karyolysis, and karyorrhexis. Pyknosis is the shrinkage of nucleus due to the loss of water from the nucleus to the cytoplasm. If the nuclear substance is soluble in the cytoplasm, it results in karyolysis and if it is insoluble, it results in karyorrhexis. The enzymes responsible for autolysis disintegrate the intracellular organelles very quickly, and this gives the cytoplasm a homogenous look, which becomes intensively eosinophilic, resulting in a loss of cell details and tissue architecture **(54).**

Microscope diagnostics are influenced by several factors, including **(49).**

- Physical status at the time of death (age, constitution, preexisting diseases, such as fever, sepsis, or injuries)
- Type and duration of the position or storage of the body after death, especially weather conditions (temperature, humidity, etc.)
- Type of burial (with or without coffin, type of wood used to make the coffin, prior embalming, or surface disinfection)
- Location of cemetery, depth of grave, soil temperature, soil properties
- Length of time since burial, cadaver fauna, and cadaver flora

After death, the absence of blood flow brings about tissue anoxia and promotes the process of apoptosis or programmed cell death **(57).**

Apoptosis is a highly regulated type of cell death that promotes the removal of damaged and dead cells from healthy tissues. As such, this form of programmed cell death is an important biological mechanism that plays a key role in normal growth as well as in normal tissue homeostasis **(58).**

The mechanism of apoptosis mainly consists of two core pathways involved in inducing apoptosis: intrinsic pathway and extrinsic pathway. The intrinsic pathway is a mitochondrial-mediated pathway **(Fig.3),** and extrinsic pathway refers to death receptor (DR) mediated pathway **(59).**

Intrinsic apoptosis is triggered by DNA damage, excessive reactive oxygen species (ROS), hypoxia, or cellular/metabolic stress. In contrast, extrinsic apoptosis is initiated by the so-called 'death ligands,' such as Fas ligand (FasL or CD95L), TRAIL (TNF-related apoptosis-inducing ligand), and tumor necrosis factors (TNFs) **(60).**

The key to the regulation and execution of intrinsic apoptosis is the B cell lymphoma2 (BCL-2) family of proteins, which includes anti-apoptotic proteins (i.e., Bcl-2) and pro-apoptotic proteins (i.e., Bax). These proteins are key factors regulating mitochondrial outer membrane permeability and apoptosis. The careful modulation of the balance between these two groups of BCL-2 proteins can largely determine cell fate decisions between life and death **(44, 61).**

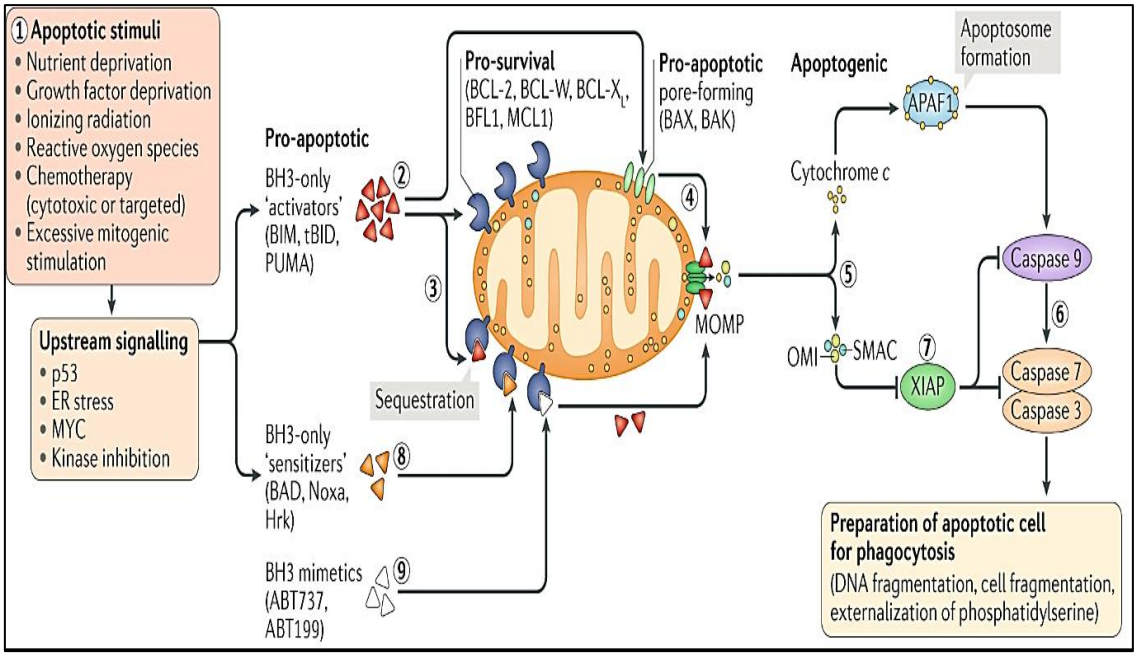

Fig.3: The mitochondrial apoptosis pathway **(61)**

The production of ROS and the depletion of GSH also result in an increase in the ratio of Bax/Bcl-2, and the ratio of Bax/Bcl-2 is suggested as a primary event to determine the

susceptibility to the mitochondrial pathway of apoptosis. Bax translocation to mitochondria and insertion into the membrane may induce cytochrome c release **(62).** ROS can also exhibit an inhibitory effect on apoptotic protein Bcl-2 **(63).**

- **Molecular techniques**

With the advent of the field of molecular biology, the estimation of PMI is proposed to be executed by evaluating the degradation pattern of the biological markers (DNA, RNA, and Proteins) **(64).**

A precise evaluation of PMI, in fact, would require a parameter that changes constantly and proportionally from the time of death with a linear process. This definition seems to fit well in postmortem degradation of nucleic acids. The Deoxyribonucleic acid (DNA) molecule has been considered as a possible parameter for the PMI estimation, considering that it is one of the most stable components of cells, its content is similar among different individuals and different cell types within the same species and its denaturation, in biological samples, begins immediately post-mortem and continues at a constant rate **(45, 65).**

Once an organism dies, the mechanisms of cell division cease in the cells. So, the DNA repair is impeded; endogenous nuclease activity and hydrolytic attacks cause DNA degradation over time to smaller fragments. As the PMI increases, chromatin degrades until there is no DNA of high molecular weight left. Consequently, the degradation of DNA has been increasing with the time extension since death **(66).**

The amount of DNA was detected in various organs like heart, liver, kidney and spleen after death and DNA degradation of heart could be a hallmark for early PMI estimation as rate of DNA degradation in first 6 h after death had a linear correlation with postmortem interval **(67).**

Both qualitative and quantitative estimation of nuclear DNA fragmentation have been studied: from basic UV spectrometry, through dye staining and image analysis technique (IAT), to single-cell gel electrophoresis (SCGE), known as the “comet assay”, and, in the last few decades, DNA amplification methods, such as Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) **(65).**

Over the past few decades, the comet assay, or single-cell gel electrophoresis (SCGE) has become one of the standard methods for assessing DNA damage, with applications in genotoxicity testing, human biomonitoring, and molecular epidemiology, ecogenotoxicology, as well as fundamental research in DNA damage and repair **(68).**

Deoxyribonucleic acid (DNA) has a significant role in biological identification of a person that is considered a final stage in criminal investigation. Deterioration of biological samples may be considered a popular problem in forensic science that negatively influences interpretation of the result in the investigation procedures. In this case, comet assay technique may have an important role and can be used as a screening method and prophylactic tool **(66).**

Comet assay is a fast precise multipurpose and more cost-efficient technique that is particularly can be used for the estimation of DNA fragmentation qualitatively and quantitatively. This technique is mainly sensitive and specific in detection both double & single- stranded DNA breaks and excision repair points in single cells **(Fig.4)**. Also has demonstrated flexibility to use both proliferating and non-proliferating cells. Ii is inexpensive, simple to use, and takes only a short time to research **(69, 70).**

The best way to describe DNA break frequencies is suggested to be expressed as a percentage of tail DNA because the damage done by the comet may be seen clearly. However, many scholars still favor the usage of tail moments. In fact, assay circumstances have a similar impact on the two descriptors **(71).**

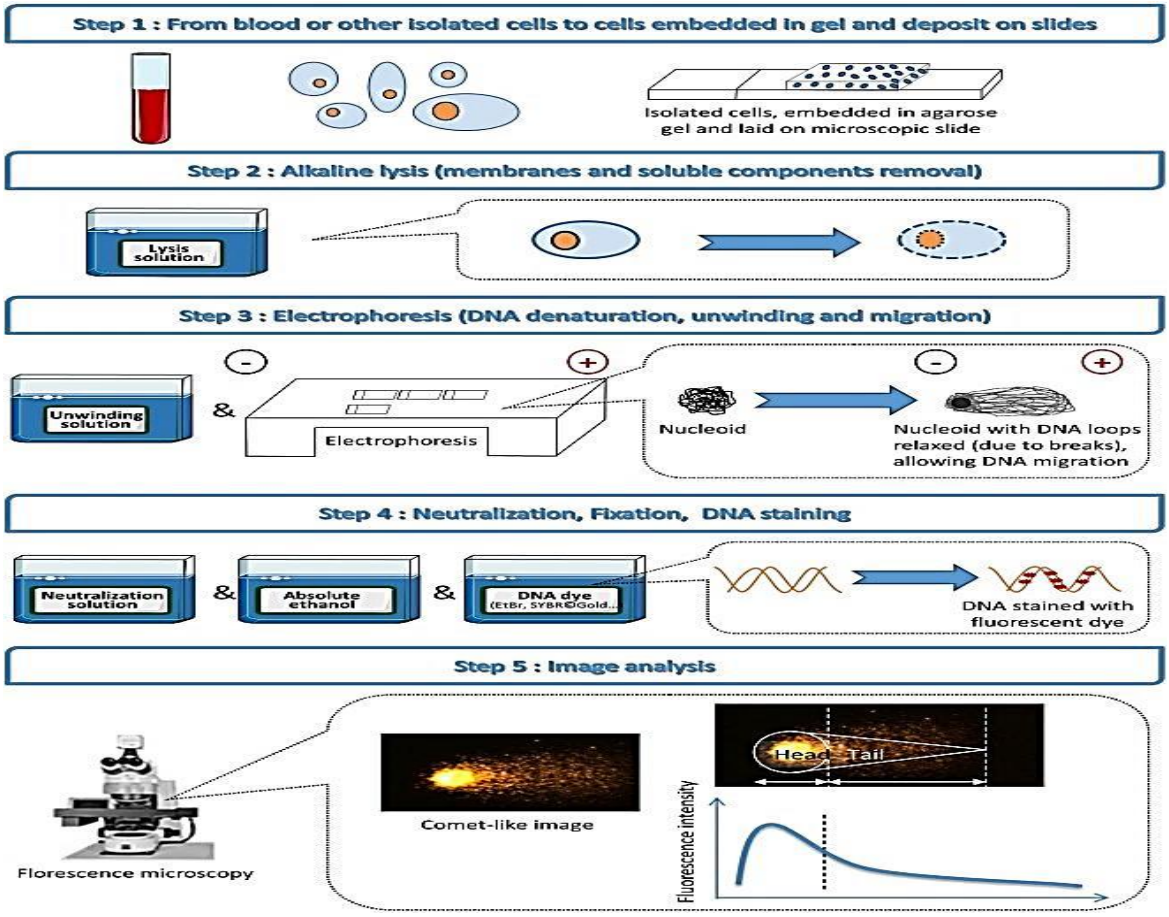

Fig.4: Scheme of the comet assay **(72).**

Over the years, a large number of RNA species have been examined for the measurement of PMI, including messenger RNA (mRNA), ribosomal RNA (rRNA), and microRNA (miRNA). Currently, a wide range of tests is available; the real-time quantitative polymerase chain reaction (qRT-PCR) is the method of choice due to its high sensitivity **(73).**

After death, RNA is degraded by human ribonucleases, bacteria, or environmental contamination. Therefore, its degradation depends not only on time, but also on other main factors, as the cause of death and environmental conditions **(74).**

A major characteristic of total RNA is its prompt beginning of decay after death; the general belief among scientists is that this decay is too rapid to make RNA of any use for forensic science. However, researchers have found that RNA is not so unstable **(75).**

Other than DNA and RNA, Protein is also a basic cellular component of organisms which is found in all tissues and organs. When life ceases, cellular proteins degrade under the influence of proteolytic enzymes, and other than this decomposition accelerated by microbial enzymes. Over the course of time, protein degradation shows evidence as a promising tool in forensic PMI estimation **(64).**

When assessing the body of a deceased individual for postmortem interval analysis, the body itself is both the evidence and the method used for assessment. Thus, a combination of both numerous types of evidence and numerous methods of estimation should be jointly evaluated in order to provide a more accurate time since death determination **(1).**

A mix of conventional, biochemical, and histopathological approaches is required for accurate PMI estimation **(47).**

In reality, many deaths occur outside of these "ideal" settings, and additional confounding variables may be present (eg, layered clothing, obesity, fever). Efforts to improve the precision and reliability of estimating PMI are ongoing, including the use of artificial intelligence in PMI estimation **(76).**

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