Manuscript Title: COMPARATIVE HISTOTAPHONOMY FOR ASSESSING DECAY RATES OF THE SKELETAL MUSCLE, KIDNEY AND TESTES OF SWINE MODEL.

Abstract

Taphonomy, the study of postmortem processes relating to organic remains, provides important data regarding the timeline for decomposition. This study focused on the histological decomposition (histotaphonomy) of the skeletal muscle, kidney, and testes in swine models was conducted to bring refinement in methodologies in estimating the post-mortem interval. This study comprised of sixteen pigs subjected to controlled cervical blunt trauma, followed by periodic sampling of tissues and its histological studies over a five-day post-mortem timeline. Tissues were stained by Hematoxylin and Eosin (H&E), Masson's Trichrome (MT), and Periodic Acid Schiff (PAS) for microstructural changes, collagen deposition, and glycogen depletion, respectively. Results showed that the decomposition rate differed among tissues. Testes had the most rapid decomposition, marked by structural disorganization and rapid glycogen depletion. The kidneys presented a progressive degradation of glomerular and tubular structures, with visible loss of collagen in the advanced stages of decomposition. Skeletal muscle was resilient; it had fewer histological changes in the early stages of PMI, but later stages indicated extensive myocyte degeneration and depletion of connective tissue. A strong correlation between PMI and histological changes was established in this study, with the rate of decomposition influenced by characteristics specific to each tissue type. These findings emphasize the importance of histotaphonomy in forensic investigations, providing a basis for PMI estimation based on micro-anatomical and biochemical changes. Specialized staining techniques enhance the precision of PMI estimations and help forensic anthropologists in death investigations, human identification, and biological profiling.

Keywords: ***Histotaphonomy, Postmortem Interval, Taphonomy, Soft Tissues. Swine Model.***

Introduction

Taphonomy as a field of science consists of the sum of investigations that have expanded understanding of the 'biosphere to lithosphere' transition for all types of organic remains (Anna *et al.*,2018).

The body starts to break down practically soon after death, first by autolysis and then through bacterial putrefaction, along with concomitant apparent changes like rigor mortis and livor mortis, according to post-mortem interval (PMI) assessment. Even though there are many variables that can cause these processes to vary, a general succession of significant changes gradually emerges. Broadly speaking, the body experiences color changes, varying degrees of muscular rigidity, distention with free gas, the creation of purge fluid, epidermal sliding, odor production, soft tissue deterioration, and ultimately bone disintegration (Brooks, 2016).

Some researchers have noticed predictable changes in microanatomy or ultrastructural alterations in cellular architecture, even though most investigations of changes in anatomy during the postmortem period have concentrated on gross anatomy. To properly interpret histopathologic data, the pathologist must, however, be acutely aware of the vital relevance of efficient fixation and the variations among different fixatives. Histologically, the tissues typically show signs of gas bubbles, saprophytic bacteria, loss of cellular stain absorption, and loss of tissue architecture 1–3 days later (Brooks, 2016).

Chemical investigations of bodily fluids are perhaps among the oldest laboratory-based techniques for measuring postmortem interval (PMI). These methods have been used for decades of research, but an appropriate analyte and a perfect sample substrate have not yet been found. The chemical approaches that have been studied up to this point have been proven to be inaccurate, unreliable, and unsuitable for usage in the field, despite a considerable number of studies conducted on a variety of sample types. As a result, while these techniques are still being studied, they are solely of academic interest and are not frequently employed in contemporary research (Brooks, 2016).

Skeletal muscle is one of the most dynamic and plastic tissues of the human body. In humans, skeletal muscle comprises approximately 40% of total body weight, contains 50–75% of all body

proteins, and accounts for 30–50% of whole-body protein turnover. Muscle is mainly composed of water (75%), protein (20%), and other substances including inorganic salts, minerals, fat, and carbohydrates (5%). In general, muscle mass depends on the balance between protein synthesis and degradation and both processes are sensitive to factors such as nutritional status, hormonal balance, physical activity/exercise, and injury or disease, among others.

The importance of the several protein compartments—structural, contractile, and regulatory—to mobility, ability to exercise, functioning, and health has drawn much scientific interest. Skeletal muscle plays a vital role in many body processes. From a mechanical perspective, skeletal muscle's primary job is to transform chemical energy into mechanical energy to produce force and power, maintain posture, and create movement. This activity enables people to engage in social and professional contexts, preserves or improves health, and promotes functional independence (Frontera & Julien, 2015). While microanatomy has been sparsely studied in the assessment of time since death, given its significance in the calculation of postmortem interval, several studies have employed the gross anatomy of both humans and *sus scrofa* in the estimation of postmortem interval (PMI) (PMI). As a result, the goal of this research is to estimate the time of death using the sus scrofa model's microanatomy of the skeletal muscle (Frontera & Julien, 2015).

Materials and Methods

Ethical Approval

An approval was gotten prior to the commencement of this research from the ethics and research committees of the Faculty of Basic Medical Sciences, Research and Ethics Committee through a letter with an approval number UNICROSS/FBMSREC/2023/003

Study Location

This study was carried out in an indoor setting at the Department of Anatomy and Forensic Anthropology Research Facility (DAFARF) specially designed for Forensic Taphonomy studies at the Faculty of Basic Medical Sciences, University of Cross River State, Okuku Campus.

Experimental Design

This study cohorts included sixteen (16) male pigs (*Sus Scrofa domestica*). The experiment animals were sacrificed using cervical blunt trauma typical of clandestine mode of death. Immediately death was confirmed, the sixteen animals were simultaneously eviscerated and vital organs of interest were taken for periodic collection of soft tissues (skeletal muscle, kidney, and testes) for histological analysis of both basic and special stains.

From the design, this research lasted for five days duration. First sets of tissue were collected at the point of death and tagged as control with zero postmortem interval, followed by two hours (2hrs) set of tissues, then six hours (6hrs) after death and final ten hours (10hrs) post death to round up day activities at the research site, to observe the onset of early autolytic postmortem changes like pallor mortis, algor mortis, rigor mortis and livor mortis in the tissues microscopically. Every piece of tissue sliced, was quickly fixed in bouin's fluid and after six hours transferred to 70% alcohol for further processing.

By day two, the research was visited three times daily, morning afternoon and evening for five days period that saw complete decomposition of all the understudied tissues.

Three tissues (kidney, skeletal muscle, and testes) were evaluated histologically for quantification and classification of decay rates and timeline. The outcome of the three results

from basic Hematoxylin and Eosin (H&E), Masson Trichrome (MT) and Periodic Acid Schiff (PAS) for the evaluation of micro-structural alterations, collagen level assessment as well glycogen degradation, as some of the most abundant compounds in the body.

Histological Procedure

After the routine tissue processing procedures (fixation to mounting) Paraffin sectioned tissues (heart, lung, kidney, liver, skin, skeletal muscle, spleen, and testes) on slides were deparaffinised in xylene and hydrated in descending grades of alcohol to distilled water (Bardale *et al.*, 2021). Tissues were stained in haematoxylin for 3-5 minutes, washed in running tap water for five minutes and differentiated in 1% acid alcohol (Bardale *et al.*, 2021). Tissues sections were washed in tap water until they turned blued. The sections were counter-stained in 1% eosin solution for a minute and hydrated in ascending grades of alcohol, cleared in xylene, and mounted using dibutylphthalate xylene (DPX).

Masson's Trichrome Staining Technique

Sectioned tissues on slides were deparaffined in xylene and hydrated in descending grades of alcohol to distilled water. The tissues were sained in Weigert iron haematoxylin working solution for 10 minutes; rinsed in running warm water for 10 minutes, and in distilled water. The tissues were then stained in Biebrich scarlet acid fuchsin solution for 15 minutes and washed in distilled water. Differentiation was done in phosphomolybdic-phosphotungstic acid solution for 10 minutes and stained in aniline blue for 10 minutes. The tissues were rinsed in distilled water and further differentiate in 1% acetic acid solution for 2 minutes, washed in distilled water, dehydrated in ascending grades of alcohol, cleared in xylene, and mounted using DPX (IHC, 2023).

Periodic Acid Schiff Technique

Sectioned tissues on slides were deparaffined and washed in distilled water.Then the tissuesplaced in 0.5% periodic acid solution for 5 minutes. It oxidizes the tissue.After that, the tissues were rinsed properly in distilled water.Then, covered with Schiff's reagent for 5-15 minutes which turns into light pink.After that, washed the stain for 5 minutes using lukewarm water, which turns it into dark pink.Then counterstain the tissue using Mayer's Hematoxylin for

1minute. After that, the tissues were washed running tap water for 5 minutes and rinse using distilled water.Finally, the slides were dehydrated, coverslip and mounted ising synthetic mounting media (IHC World, 2023).

Results

Histology of the Kidney stained with H&E

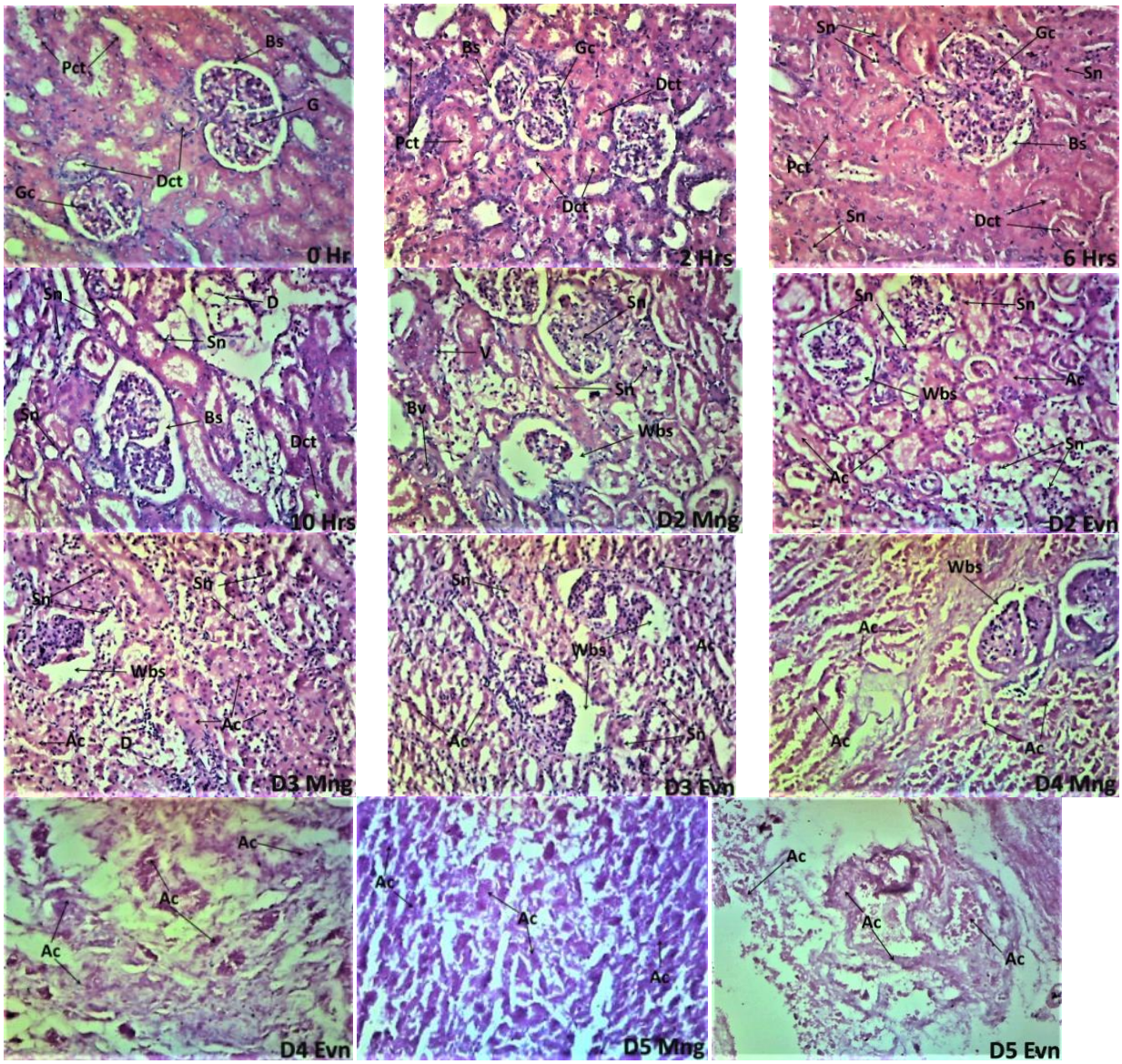

Figure 1: Photomicrographs of the kidney showing the progressive deterioration of renal tissue architecture from normal to severely altered states over exposure time, showing features like

glomerular shrinkage, widening of Bowman's space, cellular anucleation, and tissue disorientation (H&E x100).

Histology of the Kidney stained with Masson Trichrome

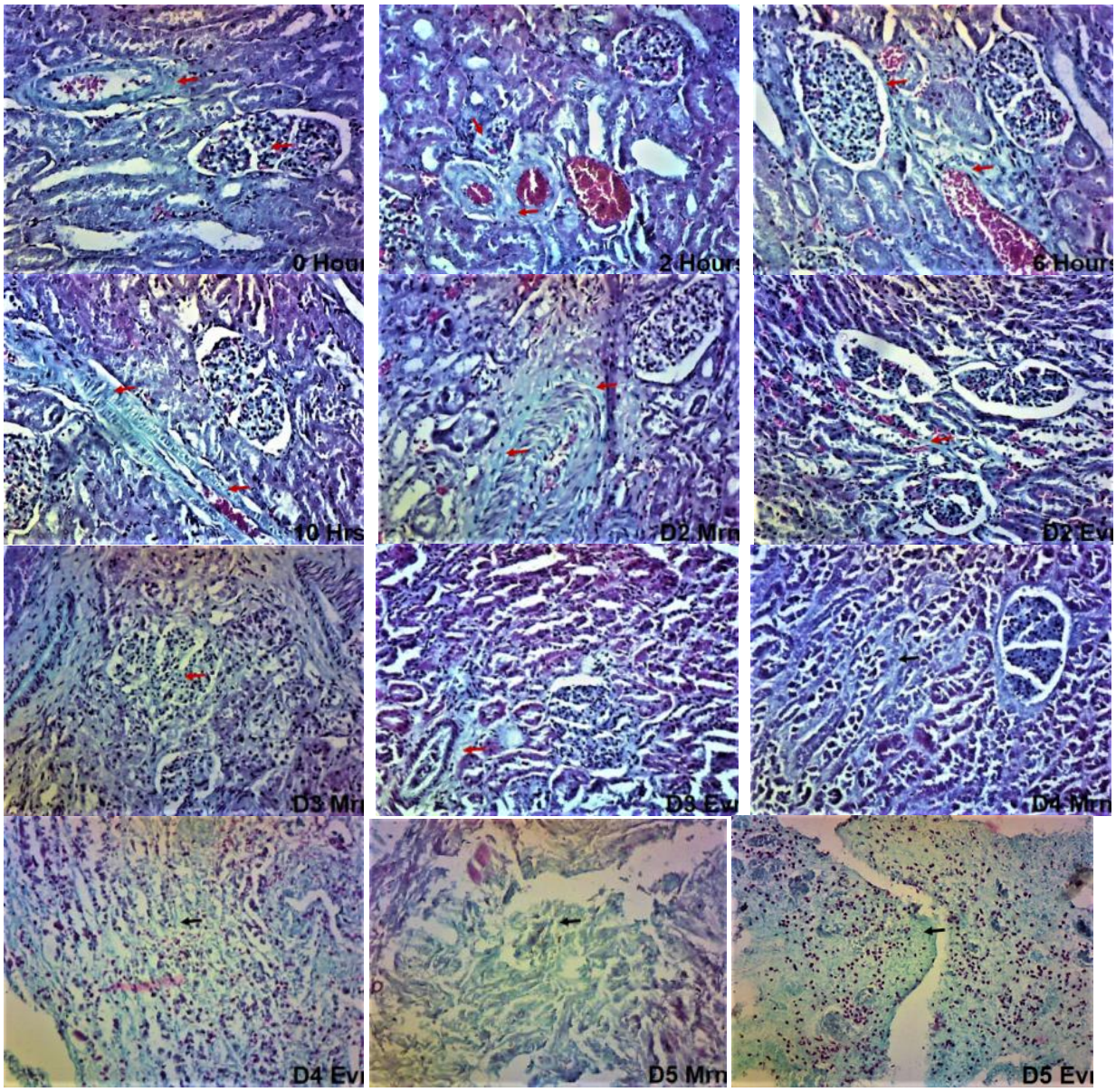

Figure 2: Photomicrographs of the kidney showing the progressive alteration of connective tissue expression in kidney sections over exposure time. Initially, there is normal presentation with wide intense collagen deposits, followed by a gradual reduction in collagen visibility and the emergence of fibrous deposits, indicating progressive tissue alteration and fibrosis (MT x100).

Histology of the Kidney stained with PAS.

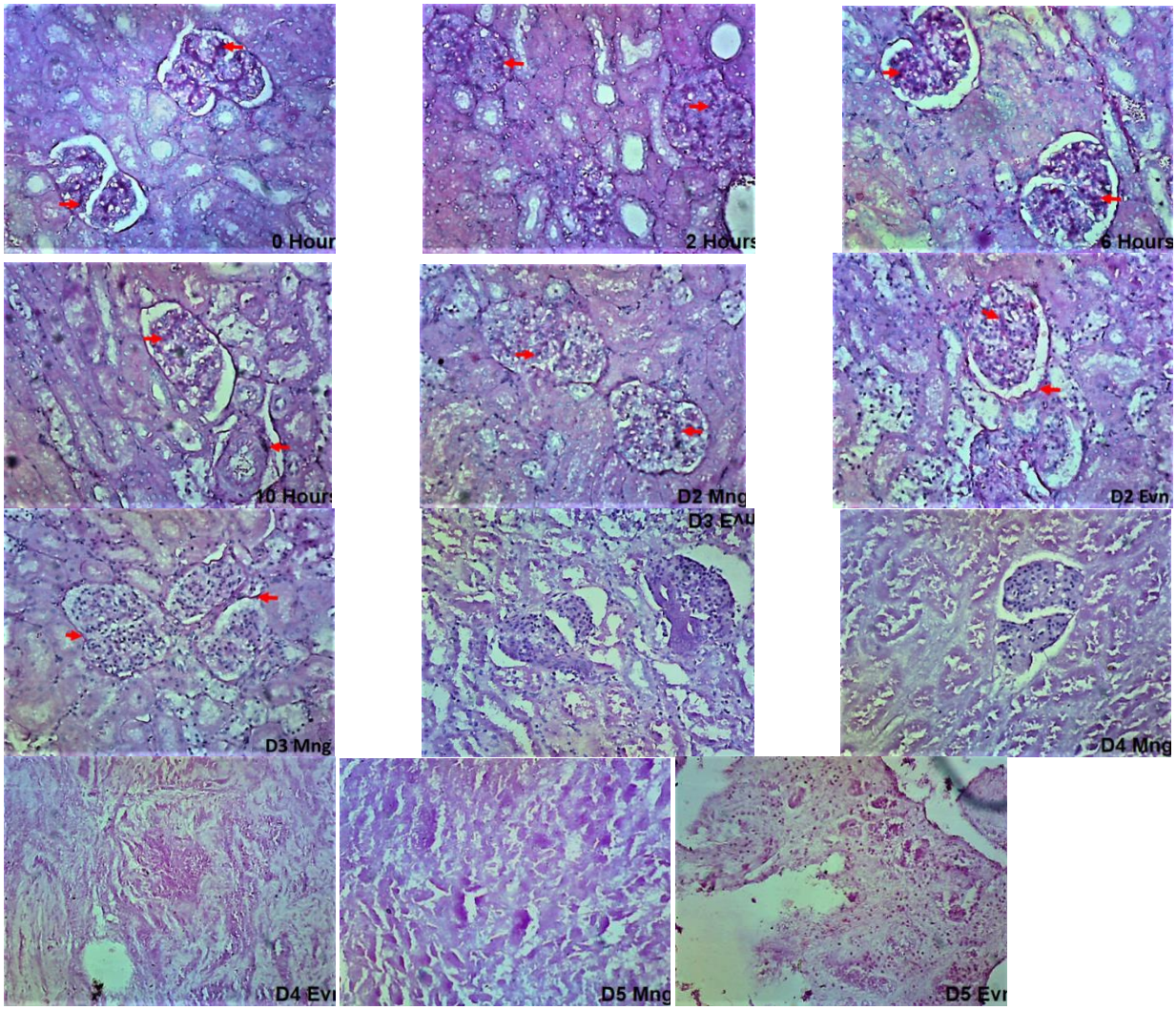

Figure 3: Photomicrographs of the kidney shoeing progressive reduction in glycogen deposits in kidney sections over exposure time. Initial sections show glycogen presence with distinct staining in glomeruli and tubules. Over time, glycogen deposits decrease, accompanied by atrophy of excretory tissue, with staining becoming less prominent, reflecting tissue degradation and altered metabolic activity (PAS x 100).

Histology of the Skeletal Muscle Stained with H&E

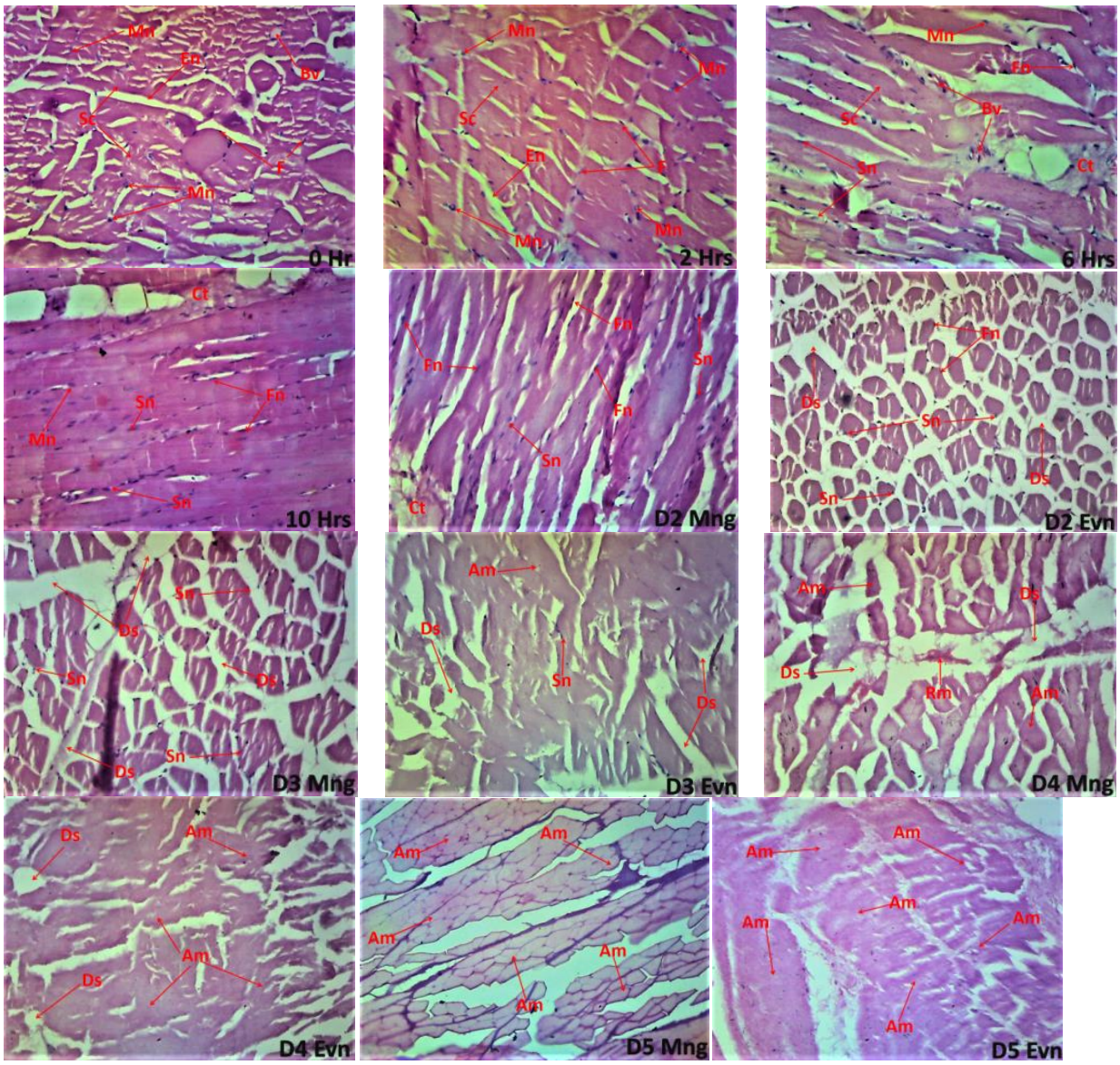

Figure 4: Photomicrographs of the skeletal muscles revealing a progressive distortion of skeletal muscle micro-architecture over exposure time. Early sections show normal features, including intact myocyte nuclei, fibrocytes, and sarcoplasm. As exposure progresses, distortion becomes evident with shrinking nuclei, connective tissue depletion, and degeneration spaces (H&E x100).

Histology of Skeletal Muscle stained with Masson Trichrome

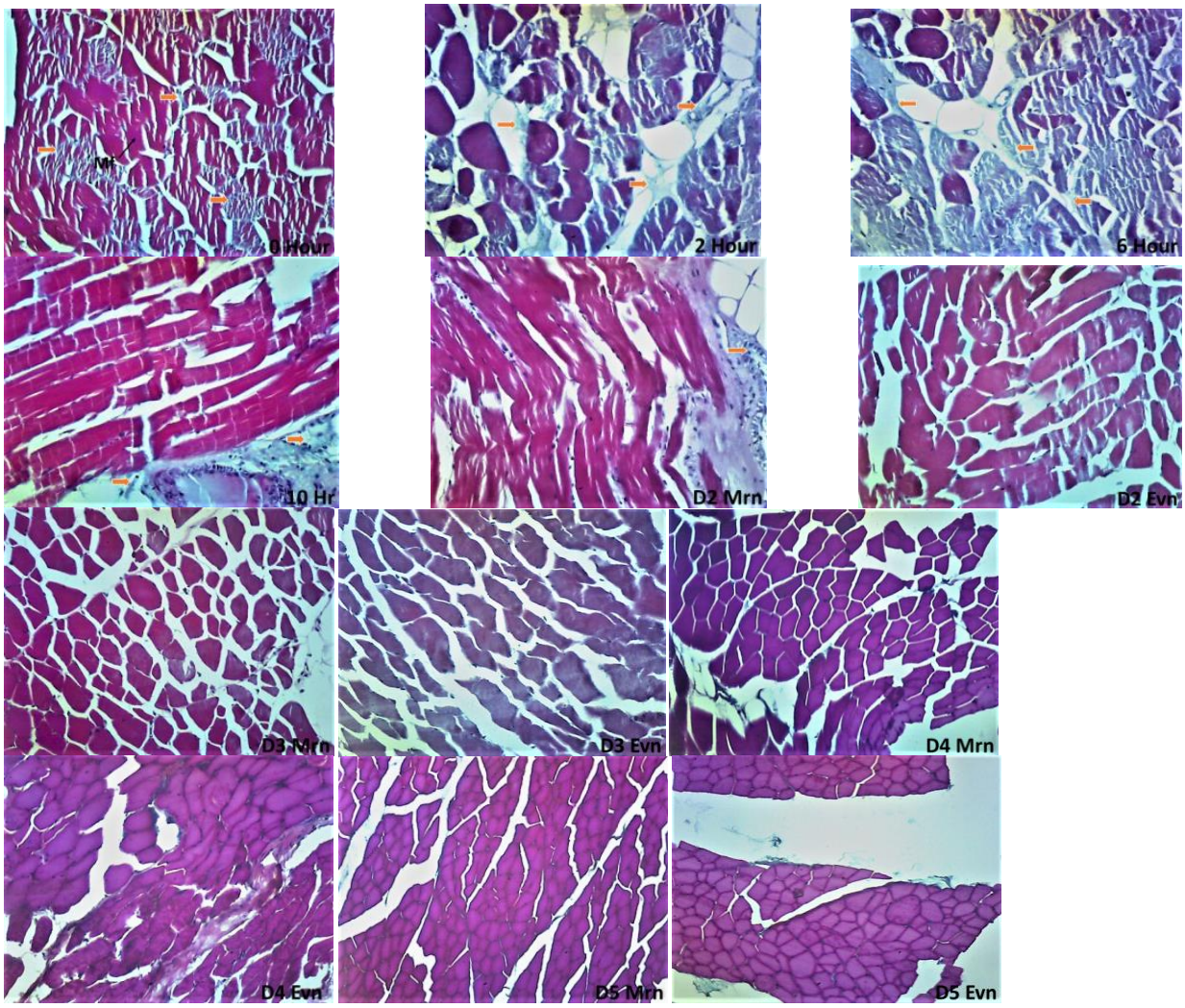

Figure 5: Photomicrographs of the skeletal muscles showing progressive degeneration of connective tissue within skeletal muscle fibers over time. Initial sections display normal connective tissue presence with collagen and fibrous tissue deposits. As exposure duration increases, connective tissue becomes distorted, culminating in the complete absence of connective tissue and visible collagen deposits in later samples (MT x100).

Histology of the Testes stained with Masson Trichrome

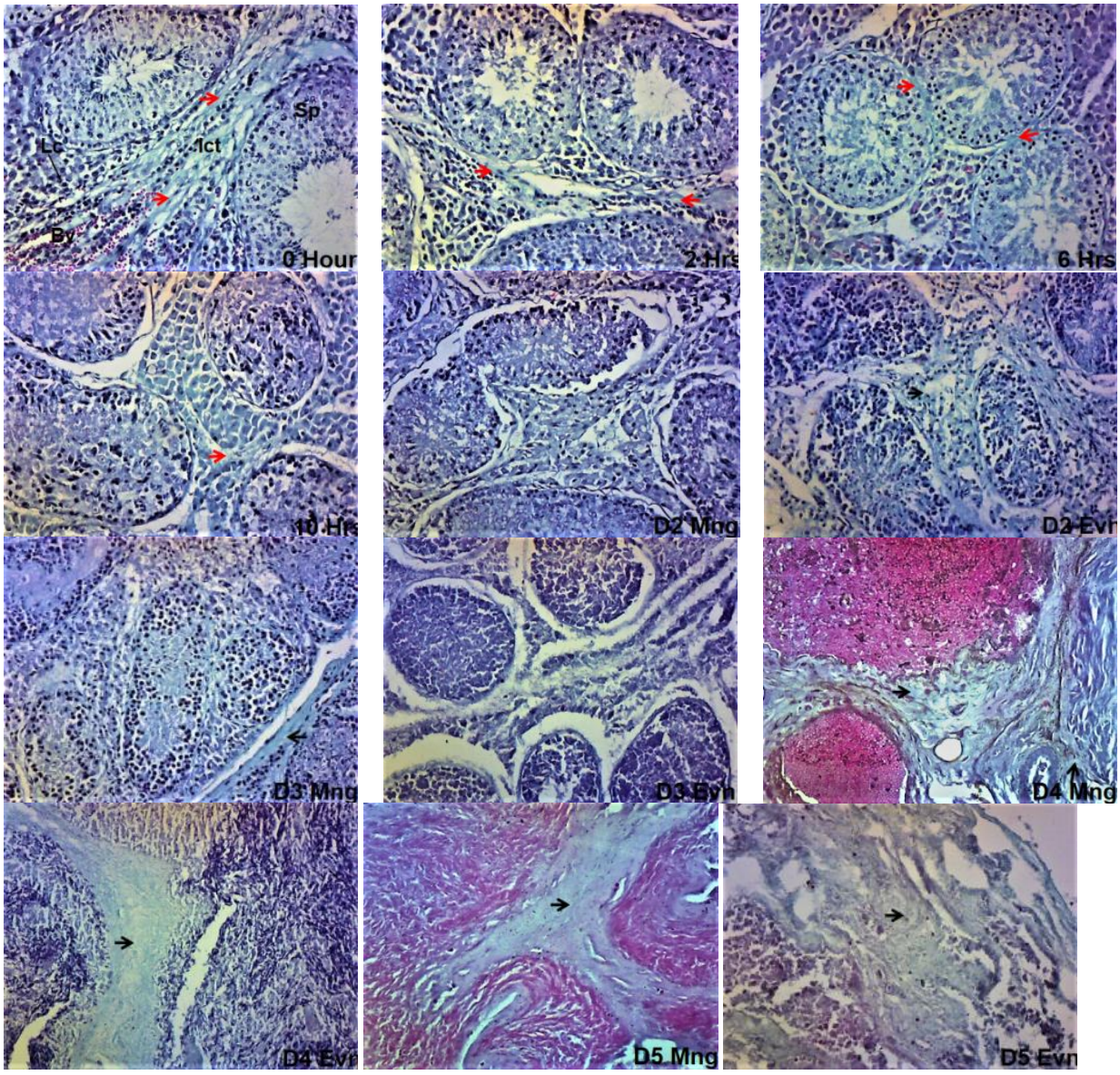

Figure 5: Photomicrographs of the testes showing progressive changes in the testicular tissue over 5 days. Initial sections reveal normal connective tissue structure with intense collagen deposits, identifiable spermatids, Leydig cells, blood vessels, and interstitial connective tissues. As exposure time increases, collagen deposits diminish, and fibrous deposits become more prominent. By the later stages, testicular tissue shows significant structural degradation, with no visible collagen and increased fibrous deposits, indicating progressive connective tissue alteration (MT x100).

Histology of the Testis stained with PAS

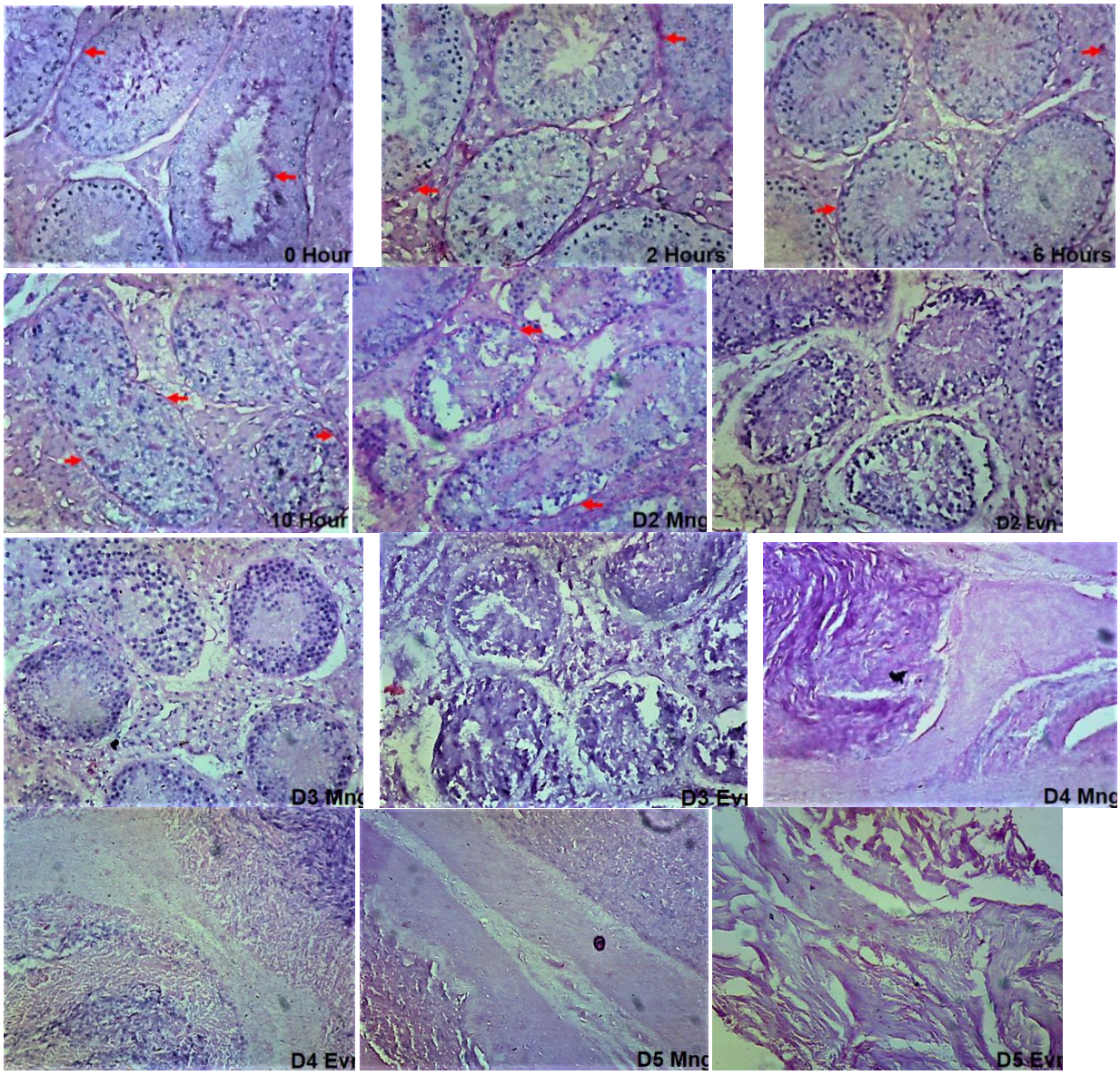

Figure 6: Photomicrographs of the testes showing progressive changes in glycogen deposition within the testicular tissue over varying exposure durations. Initial sections show prominent glycogen deposits in the reproductive tissue, with clear staining in spermatogenic and basement cells. As exposure time increases, atrophying tissue becomes evident, with a marked reduction in glycogen deposits. By later stages, the testicular tissue displays significant atrophy, with minimal to no glycogen visible (PAS x100).

Discussion

The interpretation of the histological decomposition timelines is an important step for forensic scientists that help to expose more knowledge on how to decipher the stages of death using the histological micrograph and why it follows a particular pattern. Histo-taphonomic examination provides a comprehensive view not only of disease diagnosis but also in forensic practice, examination of routinely stained Hematoxylin and Eosin stains slides preserves the underlying tissue architecture, providing an important evaluation in death investigation (El-Nahass *et al*, 2017), and even those stained with special stains such as Masson trichrome and as well as periodic acid Schiff for assessment of the presence of collagen and glycogen respectively in decomposing tissues.

This study evaluated the successive stages of decay from the various periodic soft tissues from various parts of the body (Skin, Heart, lungs, Kidney, testes, Liver, Spleen and skeletal), which are commonly body fragments seen in most crimes that involves death. This component of the study focused on histo-taphonomy that entails microscopic decomposition science. The tissues of interest of interest were subjected to routine histological tissue processing method and stained with basic H&E, and two special stains (Masson trichrome and PAS).

The histology of all the organs studied presented in figure 4.17 to 4.394, showed normal microanatomy for the control groups which are the respectively tissues gotten at the point of death. The subject tissues gotten at 2hours after death, 6hours and 10hours respectively for the first day also showed similar pattern of autolytic changes, but the decay rate from bloating stage to advanced stage of decay showed contrasting decay rates and pattern between the organs. It was observed from the result of hematoxylin and Eosin across the various tissues that some organs decompose faster than others and the pattern of decay is different between the studied tissues.

The findings of Campbell and Maani, (2021) on the skeletal muscle of porcine models and that of Papakonstantinou*et al.,* (2020) on different body soft tissues of humans, conforms to the results of the current study that observed differential decay rates on different tissues.

The histological findings from Fronczek *et al.,* (2014) investigated the use of histology in forensic autopsy are similar. Is a histological test usually required to identify the cause of death? claimed that different body organs degenerate at varying rates and patterns. Evaluation of the aorta's gross and histological alterations after death: Bardale et al. conducted a preliminary investigation in 2021.Their study's findings showed a distinct difference between gross and histological postmortem changes and their application to postmortem prediction. Bardale et al. (2021) also concluded that while intrinsic activities may affect the rate of decay in one organ differently depending on the tissue supporting the findings of the current study, two organs may die at comparable or the same times.

Histology of the Kidney

By emphasizing the glomerular structures, proximal and distal convoluted tubules, and other vital elements of the renal matrix, the H&E staining made it possible to see the general histological features of the kidney tissue. The photomicrographs showed an intact and well-preserved micro-architecture with no notable modifications during the early decay (0–2 hours). But when the PMI increased, observable changes were apparent. After six hours of PMI, there were noticeable changes to the renal micro-architecture, including distal proximal convoluted tubules and shrinking cells. More severe consequences became visible as the PMI approached 10 hours; these included widely displayed decreasing ductal cells and degenerating tubules, which indicated the beginning of alterations connected to decomposition.

Day 2 decay stages demonstrated a series of changes, with severe consequences such anucleated cells, ruptured Bowman's gaps, and other notable deformations becoming more noticeable. These photomicrographs show how these alterations persisted until Day 3. By Day 4, the changes had become quite drastic, resulting in the total disruption of the tissue architecture and the pervasiveness of anucleated cells. A related study by Ito *et al*., (1991), shown that postmortem alterations in the loop of Henle's distal tubules and thick ascending limb were significantly postponed at 0°C, with very slight alterations being observed even 72 hours after death. An intriguing distinction from the results at room temperature was that the postmortem changes in the proximal tubules occurred more quickly at 0°C than in the distal tubules. Masson's Trichrome (MT) staining brought attention to the renal excretory tissue's

connective tissue components. In the preliminary stages of PMI, the staining showed normal expressions of connective tissue (0–2 hours). Deposits of collagen indicated that the structure was stable. As the PMI increased, collagen deposition stayed constant and strong, suggesting the preservation of connective tissue components.

In the later phases of decay, the MT staining showed a consistent decrease in collagen deposition, indicating a modified presentation of connective tissue within the excretory tissue. On Days 4 and 5, the collagen fibers were less evident, and patches of fibrous deposits were seen. These findings demonstrated that the connective tissue experienced notable degradation and changes as the decomposition process advanced. Similar research was done by Kumar et al. (2002), who noted notable changes in tubular and glomerular structures that were characterized by post-mortem tissue integrity loss and cellular degeneration. These results clarified the chronological course of these alterations and highlighted the vital function of each stain in enabling an extensive micro-structural evaluation of post-mortem kidneys.

PAS staining was used on the sample tissue to look for glycogen buildup. Early PMI stages' photomicrographs showed glycogen buildup in the tubules and glomeruli. As the PMI lengthened, the expression of glycogen stores did decrease. The atrophied excretory tissue showed no evidence of glycogen storage by Days 4 and 5, indicating that these biological components had been exhausted.

In all, when the kidney was tested after death at distinct stages of decomposition, a series of changes were observed. The kidney maintained its normal micro-architecture over the first 1-2 hours, showing no discernible changes. But when the postmortem duration grew to 6–10 hours, mild to severe effects, like glomerular architectural changes and the presence of shrinking ductal cells, became noticeable. The effects then intensified on Day 2, with the kidney micro-architecture severely altered and exhibiting extensive anucleated cells and ruptured Bowman's gaps. As time went on, Day 4-5 significantly disrupted the kidney's micro-architecture, resulting in considerable disarray and loss of structural integrity.

Histology of the Skeletal Muscle

The skeletal muscle histological results at 0 and 2 hours show a transverse section of the muscle that is normal, with the myocyte nuclei (Mn) (Sc), endomysium (En), sarcoplasm, and blood veins (Bv) clearly visible. A parallel study by Li *et al*., (2017) for Estimating the Postmortem Interval in Rat Muscle Samples produced comparable results, showing that the muscle samples were stained with H&E to measure the PMIs. From a PMI of 0 to 144 hours, the muscle cell fibers showed progressive changes, including gradual expansion, disintegration, and breaking. Transverse striations became less noticeable as the PMI increased. The nucleus exhibited autolysis-related alterations.

However, in both the 6- and 10-hour periods, there was a little deformation and shrinkage of the nuclei (Sn) and sarcoplasm. Day 2 (morning and afternoon) revealed a moderate deformation of the skeletal muscle micro-architecture with decreasing nuclei and fibrocyte nuclei (Fn). Furthermore, on days two (afternoon) and three (morning and afternoon), there was a significant loss of anucleated myocytes (Am), shrinking nuclei, and deformation. Muscle cell degeneration and serve deformation are seen in skeletal muscle.

Additionally, the results indicate a moderate to severe loss of muscle mass without a recognizable cell border. The skeletal muscle micro-architecture is severely distorted from day three (afternoon) today five (5), exhibiting decreasing nuclei, anucleated myocytes, and degeneration spaces. Related results were seen in a separate study on whether skeletal muscle alterations could offer a trustworthy technique for determining the amount of time since death conducted by Mostafa et al. in 2021: Pittner *et al*., (2020) demonstrated that intra- and intermuscular variations of postmortem protein degradation for postmortem interval (PMI) estimation show the strengths and current limitations of protein degradation-based postmortem interval (PMI) estimation. A histological, biochemical, and DNA study revealed that a significant gradual histopathological alteration of muscle structure started at 48 hpm and progressed thereafter.

Histology of the Testes

Staining with H& E showed microstructural changes in the testes. The initial changes observed in the early postmortem period (2hrs to 10hrs PMI) were shrinking of seminiferous tubules and degeneration of Leydig cells at 10hrs PMI(fresh stage). These spread progressively and became more apparent with increasing length of postmortem interval so that at day five (102Hrs PMI, advanced decay stage), they were total disorientation and distortions in histo architecture with wide presence of anucleated cells and degenerated testicular interstitium.

Similarly, staining with PAS showed presence of glycogen deposit within the reproductive tissue with stained areas of the interstitial connective tissue and basement cells. In the early postmortem period(2hrs to 10hrs PMI, fresh stage). At day 2(18hrs PMI, bloat stage), atrophying tissue was observed and less glycogen deposit by afternoon (24hrs PMI, bloat stage). However, they were no significant changes observed until day 4 morning where the tissues became completely Atrophied; these changes remained same till end of day 5(102hrs PMI, advanced decay stage).

Also, staining with Masson Trichrome showed a normal connective tissue deposit within the reproductive with wide intense collagen deposit at 2hrs and 6hrs PMI However, at 10hrs PMI, all in fresh stage of decay, there was reduction of collagen deposit. At day 2 morning(18hrs PMI, bloat stage), no obvious collagen deposit observed and by evening fibrous deposit observed, which became increased by day 3 morning (42hrs PMI, active decay). No obvious difference from day 3(42hrs PMI) morning was further observed till the end of day 5(102hrs PMI, advanced decay).

The findings of this study showed that they were a progressive decline of the microstructure of testis attributed to autolysis as stated by Henssge *et al.,* (2007) which they agree from their findings that it can be to deduce postmortem interval. This assertion also agrees with the findings of the research conducted by Wei *et al*., (2020) on the histological alterations in human skin 32 days after death and their potential forensic importance. They concluded that the postmortem changes in a tissue's microstructure can be used to estimate PMI.

Conclusion

The histological tissues also showed variations in the pattern and rate of tissue degradation across the studied soft tissues. The histotaphonomy outcome recorded a strong correlation postmortem interval and decay rates based on microstructural alterations, collagen, and glycogen depletion. The knowledge of the decomposition science matrix associated with modes of death and depositional environments and differential histological changes provided in this data will help any forensic investigator to avoid drawing hasty conclusions when determining postmortem interval from different tissues regardless of the stage of decay. Any forensic anthropologist tasked with the responsibility of human individualization, catastrophic victims identification, and biological profiling will find this information useful to predict time of death.

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