Original article

Determination of postmortem interval through histopathological alterations and collagen evaluation in the prostate of Wistar albino rats

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ABSTRACT

Background: Determination of postmortem interval (PMI) has always been a fundamental issue in forensic fields. Therefore, the purpose of this study was to assess usefulness of histopathological changes and collagen degradation in the prostate for estimation of PMI in adult male rats. **Methods:** Forty male albino rats were euthanized using CO2 chamber and then classified randomly into 8 groups; each group consisted of 5 rats. Rats were used for determining postmortem changes at 0, 6, 12, 24, 36, 48, 60 and 72 hs. Sections of prostate were fixed and stained for histopathology and quantitative analysis of collagen by Masson trichrome. Moreover, slices of 5µm mounted on positively charged slides were carried out for collagen III immunohistochemistry. **Results:** The prostate showed normal histology at 0 and 6 hs postmortem (PM). While, at 12 hs, most sections of the gland revealed no abnormalities except for mild focal widening between the acini. Starting from 24 hs, epithelial desquamation was seen in most acini. Seventy-two hours, obvious necrosis of prostatic acini appeared in most of samples. Degradation of collagen started and the spaces between acini became wide at 12 and 24 hs PM. Complete loss of stain reactivity was observed at both 60 and 72 hs. Moreover, a significant decrease in the amount of collagen's stained areas in the prostate started from 12 hs. Positive immunohistochemical reaction of collagen III was detected at 0, 6 and 12 hs PM. However, it was completely lost at 60 and 72 hs. Interestingly, the immuno-reactive area of collagen III was significantly increased at 6 hs, then a remarkable decline in immuno-reactive area was observed at 12 hs. **Conclusion:** Collagen type III proved to be a successful parameter with histopathology in determination of PM interval. However, further studies still needed to confirm the accuracy of these parameters.

Keywords: Prostate, Collagen, Histopathology, Immunohistochemistry**,** Rats

I. INTRODUCTION

Death is described as the cessation of physiological processes that keep the integrity and function of the cells. Almost after death, the body starts to endure an irreversible and progressive sequence of physical and chemical changes (Brooks and Sutton, 2018). The postmortem interval (PMI) is the amount of time that has elapsed since the time of death, and its determination has always been a fundamental issue in the forensic field. However, PMI estimation remains controversial, and it is often not possible to draw any precise conclusions regarding the time of death based on the appearance of a single postmortem change (Di Maio and Dana, 2007; Madea, 2016).

Numerous physico-chemical changes begin to occur in the body in a sequence order after death. In the first hour PM, physical changes like initiation and relapse of rigor mortis, progress of livor mortis and algor mortis can be assessed, whereas additional approaches may be related to the difference in specific chemical substances within various body fluids (Cordeiro et al., 2019).

The estimation of the time of death in spite of its importance remains to be one of the most difficult responsibilities of the forensic investigator. In spite of, huge amount of researches that have been carried out, reliable and accurate methods still needed for estimating the length of PMI, especially as the duration of this interval increases. Histological and immunohistochemical investigations of different tissues after death have become crucial because they might help in the estimation the PMI (Thaik-Oo et al., 2002; Dettmeyer, 2011). Immunohistochemical staining in sweat glands of skin (Cingolani et al., 1994), follicular cells of the thyroid (Wehner et al., 2000; 2001a) or pancreatic cells (Wehner et al., 2001b); were investigated previously. However, collagen considered as an ultimate parameter for determination of PMI because it degraded slowly by the time after death if compared to other functional or structural proteins (Mazzotti et al., 2019). Until now, histochemical studies on degradation of collagen have been done in skeletonized human remains for the determination of PMI (Boaks et al., 2014; Jellinghaus et al., 2018; Jellinghaus et al., 2019). Moreover, immunohistochemistry for collagen proteins in gingival tissue was only done by Mazzotti et al. (2019).

Prostate is considered among the most resistant organs to decomposition and to postmortem (PM) changes, as it may still be recognizable in a partially skeletalized body for a year PM. This make the prostate a good subject for histopathological analysis for PMI estimation (Saukko and knight, 2016). Additionally, collagenous protein is the primary component of the fibromuscular stroma and may play a significant role in the growth of the prostate either physiologically or pathologically (Thompson et al., 1979; Nakada et al., 1982). Moreover, available research of using collagen as a biomarker for PMI estimation in prostatic tissue is still scarce. Therefore, the purpose of this study was to evaluate the usefulness of histopathological changes and collagen degradation in the prostate for estimation of PMI in adult male rats.

II. MATERIALS AND METHODS

2.1 Animals and sample collection

Forty male Wistar albino rats, weighing 185 - 225 g, were fed *ad libitum* and were kept at Laboratory Animal House of Faculty of Veterinary Medicine, Suez Canal University, Egypt. Rats were euthanized using CO_2 chamber and then classified randomly into 8 groups, each group consisted of 5 rats. These rats were used for determining postmortem changes at 0, 6, 12, 24, 36, 48, 60 and 72 hs. The rats were kept at constant temperature (20 ± 2 ºC) throughout the time intervals of the experiment. The procedures of the study were agreed by institutional ethical committee and carried out according to the ethical guidelines for the use of laboratory animal in experiments at the Faculty of Veterinary Medicine, Suez Canal University (Approval No. #2020044).

2.2 Sample processing and histopathology

Prostate sections were fixed in formalin 10% (El-Gomhoria Co., Egypt) in phosphate buffered saline (PBS) and dehydrated in ascending concentrations of ethyl alcohol (70-100%) and embedded in paraffin wax and stained with Hematoxylin and Eosin (H&E) for histopathology (Bancroft et al., 1996).

2.3 Masson's trichrome stain

Sections of 4µm were stained with Masson trichrome and observed under light microscope (Zhang et al., 2015). Quantitative analysis of collagen stained areas by Masson trichrome (μm^2/ 200 μm^2), was performed via Image J program, Japan. Subtracting of noise was performed for three slides per each sample at 40 X magnifications.

2.4 Morphometric study

The reactions of the Masson's trichrome stain that illustrating the steps of collagen degradation at different time intervals were analyzed in five random fields per slide.

2.5 Immunohistochemistry of collagen III

Slices of 5µm mounted on positively charged slides for collagen III immunohistochemistry were carried out on rat's prostate embedded in paraffin. Sections were subjected to de-waxing then 10 min antigen retrieval via autocalving at 120 °C using 10 Mm citrate buffer (pH 6). After washing with PBS, the endogenous peroxidase was clogged via adding 0.3% H_2O_2 in methanol for 15 min duration. Sections were washed with PBS again and blocking was achieved by adding blocking buffer for 30 min at room temperature. Sections were incubated with primary mouse monoclonalanti-collagen III (FH-7A) antibody (Abcam, ab6310, USA) for 2 h at 25 °C. The dilution rate for the primary antibody was 1:200. After incubation, sections were washed 4 times for 4 min each with PBS. The stained prostate sections were visualized using Polyvalent HRP/DAB detection kit (Abcam, ab64264, USA) as recommended by manufacturer's instruction. Quantitative analysis of the immuno-reactive area % (IRA%) was performed (% IRA = IR stained area/Total area × 100) by

an image analyzer (Image J program, Japan) after subtracting background noise according to Elgawish et al. (2015).

2.6 Statistical data analysis

For estimation of collagen in Masson stained and in immuno-reactive areas, one-way ANOVA and Tukey's Multiple Comparison (Graph Pad Prism 5.0 software, San Diego, CA, USA) were applied to find the difference in collagen degradation through the time after death. A probability level of $P \leq 0.05$ was considered significant.

III. RESULTS

3.1 Histopathology

In 0 and 6 hs PM, samples of prostate revealed normal rounded to irregularly branching glands. Each gland was lined by two cell layers, an outer cuboidal layer and an inner columnar mucin-secreting epithelium layer with invagination of papillary projections. Each gland was surrounded by connective tissue and smooth muscle. While, at 12 hs PM, most sections of the gland revealed no abnormalities except for mild focal widening between the acini and intact fibro-muscular septa between the acini. Starting from 24 hs PM, epithelial desquamation of most acini in most cases was seen and inflammatory cells and degeneration started to invade the fibro-muscular stroma. In 36 hs PM, the epithelium of the gland showed degeneration along with mild atrophy of the prostatic acini and infiltrations of the inflammatory cells were observed in the fibro-muscular stroma. Furthermore, epithelial destruction, atrophy and necrosis of prostatic acini, became more obvious and significantly observed at 48 hs PM. The atrophy and necrosis of prostatic acini became more evident and the inflammatory cells had disappeared completely at 60 hs. Seventy-two hours PM, obvious necrosis of prostatic acini appeared in most of the samples (Figure 1).

3.2 Masson's trichrome stain

Positive reaction of the collagen was clear, observed in all sections and arranged in tight manner between the acini in 0 and 6 hs groups. Degradation of collagen started and the spaces between acini became wide at 12 and 24 hs PM. The reaction to the stain became faint and it was restricted around the blood vessels at 36 and 48 hs PM. Complete loss of stain reactivity was observed at both 60 and 72 hs (Figure 2). A significant ($P \leq 0.01$) decrease in the amount of collagen's stained areas in the prostate started from 12 hs (Figure 3).

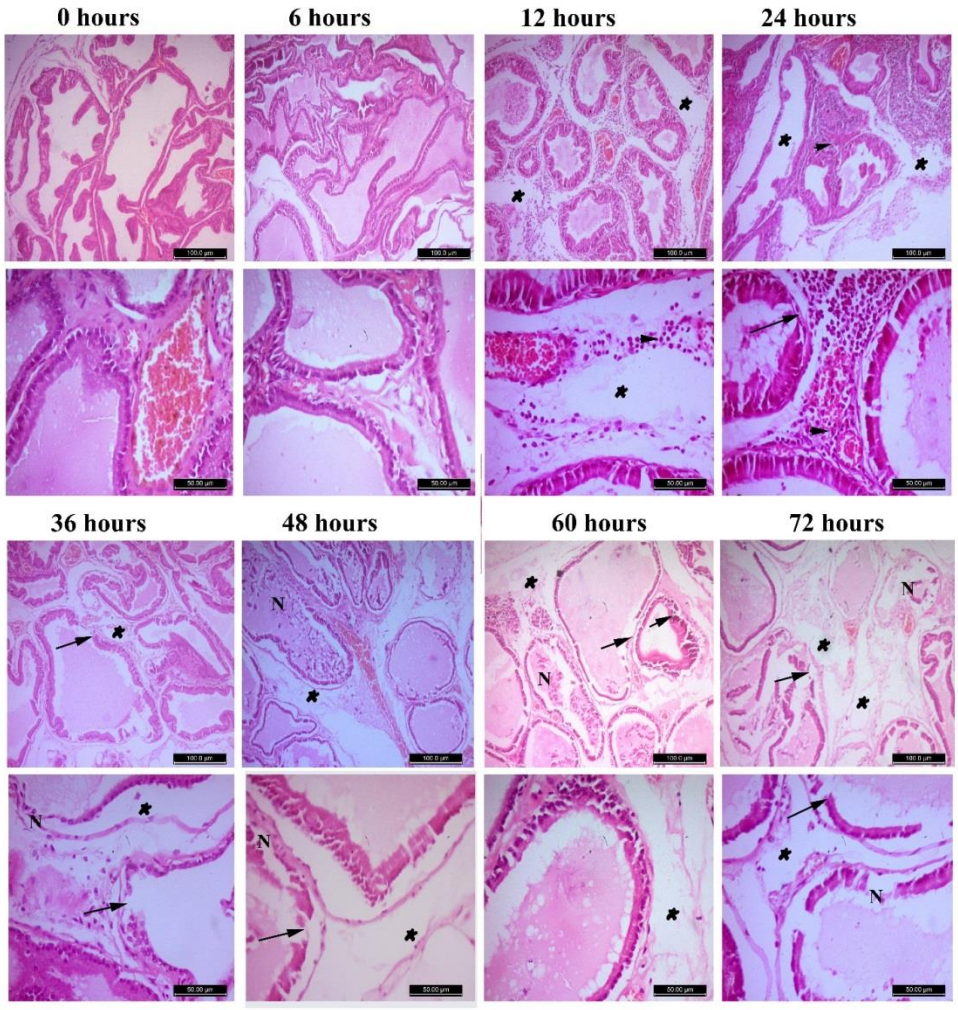

Figure 1: Histopathology of prostate gland of rats showing the different postmortem changes at different time intervals (0-72 hs). Epithelial degeneration (arrows), necrosis (N), inflammatory cells (arrowhead), widening of inter-acinar spaces and degradation of fibro-muscular matrix (asterisk). Sections stained with H&E at 200 X and 400 X.

3.3 Immunohistochemistry

The mean area % of collagen III immunostaining for different studied groups was illustrated and positive immunohistochemical reaction of collagen in most examined sections was detected at 0, 6 and 12 hs PM. However, degradation of collagen and widening of the space between the prostatic acini was observed at 24 and 36 hs PM. The immunohistochemical reaction was nearly absent in most of the sections at 48 hs PM, and completely lost at 60 and 72 hs (Figure 4). The immuno-reactive area of collagen III was remarkably ($P \leq 0.01$) increased at 6 hs PM compared to all time intervals, then a remarkable decline in immuno-reactive area was observed from 12 hs PM. Notably, there was no significant difference in the immuno-reactive areas of collagen III started from 48 and 72 hs (Figure 5).

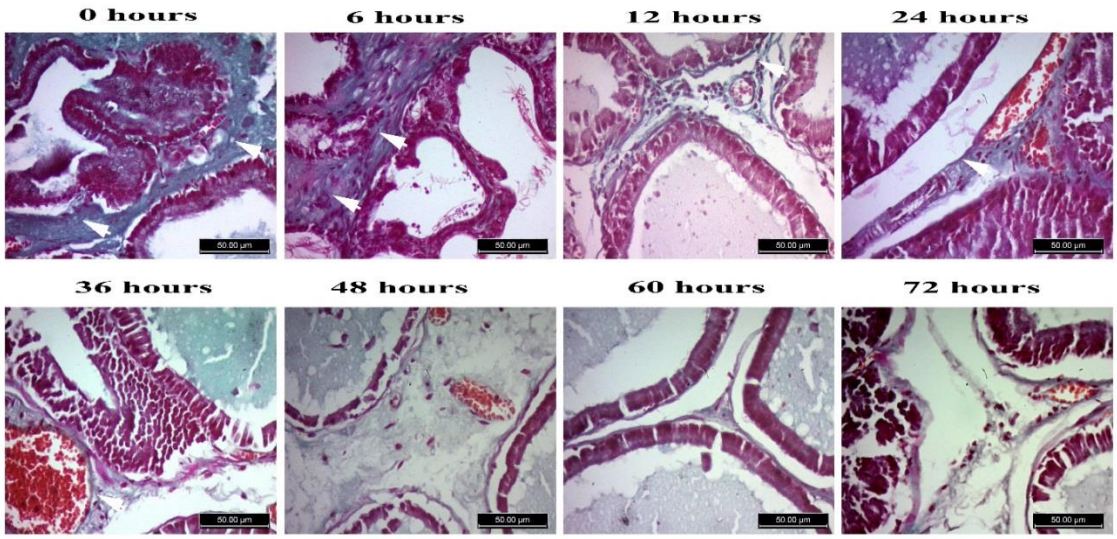

Figure 2: The reactions of the Masson's trichrome stain (400 X) illustrating the steps of collagen degradation at different time intervals. At 0 and 6 hs PM, positive reaction of the collagen was clear. After 12 and 24 hs PM, degradation of collagen started and the spaces between acini became wide. After 36 and 48 hs PM, the reaction of stain became faint. Complete loss of stain reactivity was observed at both 60 and 72 hs PM. Positive stained collagen area was demonstrated by white arrowhead.

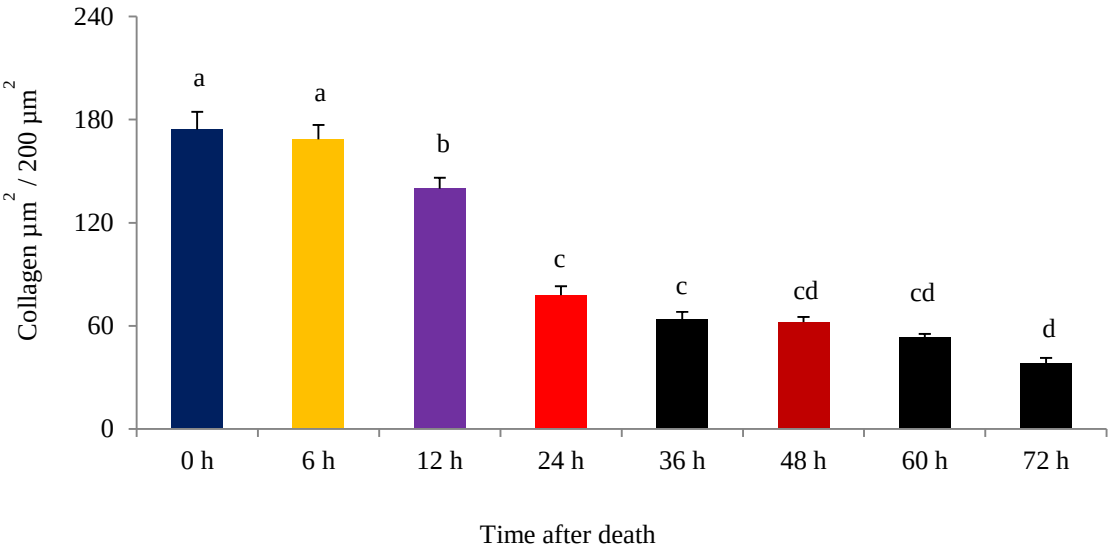

Figure 3: Statistical analysis of Masson's trichrome stained areas demonstrated a significant ($P \leq 0.01$) decrease in the amount of collagen stained areas in the prostate started from 12 hs PM. Different superscripts indicated significances as time interval with a superscript (a) is significantly different with all other groups of different time interval with different superscripts as (b) or (c),..while time interval with two superscript as (cd) was non-significantly different with time interval of (c) or (d) superscript.

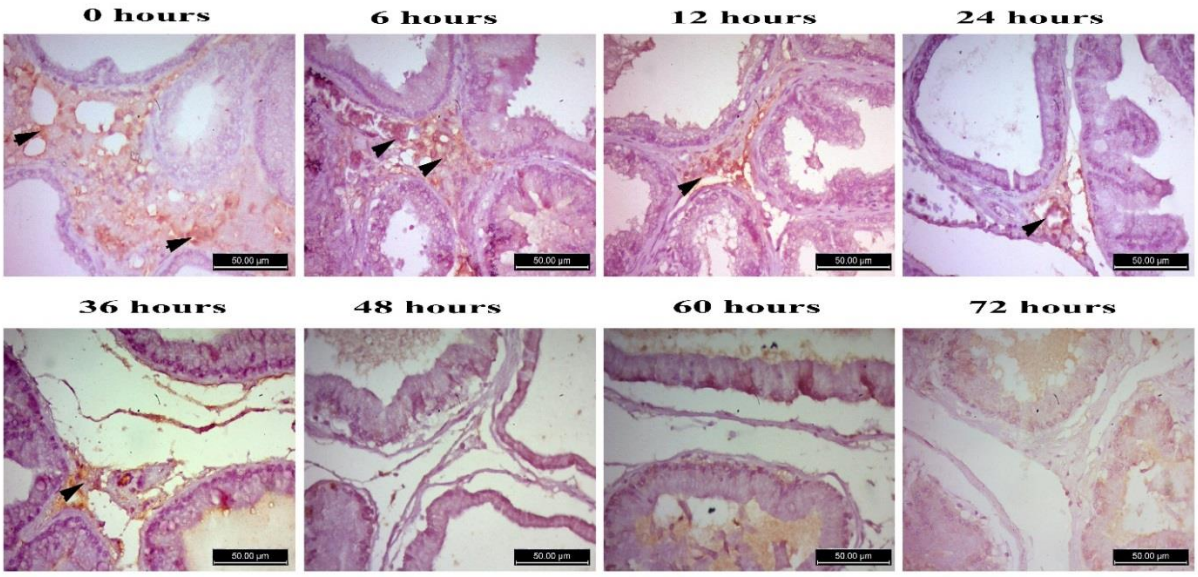

Figure 4: Immunohistochemistry of collagen III in prostate gland of rat showing the postmortem changes of the collagen at different time intervals (0-72 hours). Positive stained collagen III areas appear as brown color (black arrowhead). Immunohistochemical reaction was absent in most of the examined sections at 48 hs PM, while it cannot be observed completely at both 60 and 72 hs PM. Stained sections at 400 X.

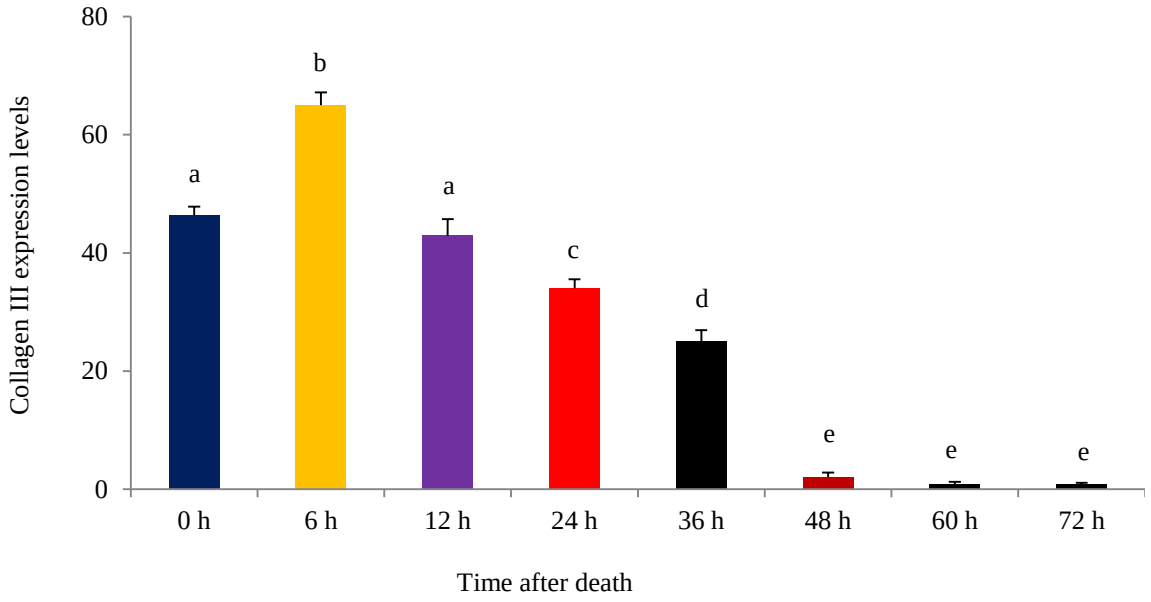

Figure 5: The immuno-reactive area of collagen III of different groups of the study. Different superscripts indicated significances as time interval with a superscript (a) is significantly different with all other groups of different time interval with different superscripts as (b), (c), (d) or (e)

IV. DISCUSSION

Ordinary methods for determination of PMI depend mainly on expected alterations that occur within the body following death (Young et al., 2013). Although these techniques are reliable, there are still several parameters, which make the estimation of PMI of limited value (DiMaio and DiMaio, 2001).

Prostate resists autolysis and putrefactive processes and could be used in determination of PMI, however, there were a scarce information in the literature regarding this issue and the only report in this field was done by Mahmoud et al. (2018).

Light microscopic examination of prostate samples didn't detect any structural abnormality during the first 12 hs PM except for slight changes in the form of for mild focal widening between the acini, and then a series of prostatic changes were observed that include necrosis and atrophy specially at 60 to 72 hs. These results agreed with Mahmoud et al. (2018) who found that there were no detectable histopathological changes in the prostatic tissue during the first 12 hs PM and significant epithelial disruption, inflammatory cells and fatty degeneration began to appear in the prostatic acini after 24 hs. The prostatic acini showed significant atrophy and necrosis 2 days PM. In contrary to the histopathological findings in the current study, Saukko and Knight (2016) reported that prostate of human being may still be recognizable in a partially skeletalized body for a year PM. The rapid degradation rate of the rats' prostate in the present study may be attributed to the smaller size of rats' prostate in comparison to that of human being as well as the difference in the lining epithelium of the rats' prostate which is lined by simple columnar epithelium compared to the more resistant pseudo-stratified and stratified columnar epithelium lining of human prostate (Aaron et al., 2016). In other explanation for the rapid decomposition of the prostate of rats in the current experiment, Matuszewski et al. (2014) stated that the mass of the carcass is the most important endogenous factor that influences the rate of postmortem decomposition as well as the estimation of PMI. They demonstrated that bodies of small masses decomposed more rapidly than those with larger one.

Degradation of collagen started between acini at 12 and 24 hs PM. Significant decrease in the amount of collagen's stained areas in the prostate started from 12 hs PM. The present results were in accordance with Mazzotti et al. (2019) who found a statistically significant reduction of the arrangement of collagen fibers in gingival tissue stained with Masson trichrome that was evident in the short postmortem interval samples (1-3 days) compared to control group.

The histological evaluation in combination with immune-labeling technique provided a comprehensive analysis of the changes and degradation of the collagen in relation to different PMIs. Considering the pattern of collagen type III expression, a progressive reduction in immuno-staining was observed with time, resulting in a significant difference between the average values of the postmortem samples and the controls at the statistical analysis which was in agreement with the

results obtained by Mazzotti et al. (2019). This result could be explained by the postmortem disruption of blood flow leading to a prolonged state of oxygen deficiency and the beginning of the chemical breakdown of proteins (Cingolani et al., 1994; Hostiuc et al., 2017).

V. CONCLUSION

Collagen type III proved to be a successful parameter in association with histopathology in determination of PM interval. However, further studies still needed to confirm the accuracy of these parameters.

VI. REFERENCES

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تحديد الفاصل الزمني بعد الوفاة من خلال التغيرات النسيجية المرضية وتقييم الكولاجين في البروستات لدى الفئران البيضاء

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تحديد فترة ما بعد الوفاة كان دائما قضية اساسية في مجال الطب الشرعي ,لذلك كان الغرض من هذه الدراسة هو تقييم التغيرات النسيجية المرضية و تدهور الكولاجين في البروستاتا لتقدير فترة ما بعد الوفاة في ذكور الجرزان البالغة . تم قتل أربعين جرذا بطريقة رحيمًه عن طريق استخدام غرفة غاز ثاني أكسيد الكربون، ثم تم تقسيمها بشكل عشوائي إلى 8 مجموعات. كل مجموعة تتكون من 5 جرذان. تم استخدام هذه الجرذان لتحديد التغييرات بعد الوفاة خلال الفترات 0 و 6 و 12 و 24 و 36 و 48 و 60 و 72 ساعة. تم تثبيت قطاعات من البروستاتا لعمل قطاعات نسيجية مرضية. تم إجراء التحليل الكمي للمناطق المصبوغة بالماسون تراي كروم و التي تدل على وجود الكولاجين. علاوة علي ذلك تم إجراء شرائح موجبة الشحنة من أجل الكيمياء المناعية للكولاجين III. كشفت غدة البروستات عن تركيب طبيعي في الساعة 0-6 بعد الوفاه، بينما في الساعة 12 لم تظهر معظم أقسام الغدة أي تشوهات باستثناء اتساع بؤري خفيف بين الحويصلات. ابتداء من 24 ساعة شوهد تقشر طلائي في معظم الحويصلات، في الساعة 60 بعد الوفاةً ، كان هناك ضمورا واضحا في حويصلات البروستاتا كما اختفت الخلايا الالتهابية تمامًا في جميع العينات. ظهر نخر لحويصلات البروستاتا في معظم العينات عند الساعة 72 ما بعد الوفاه. في العينات المصبوغة بصبغة ماسون ، لوحظت صبغة واضحة و ايجابية للكولاجين في الساعة 0-6 ما بعد الوفاه. ومع ذلك ، لوحظ فقدان كامل في صبغة الكولاجين في كل من 60 و 72 ساعة بعد الوفاه، مع ملاحظة انخفاض معنوي كبير في كمية المناطق المصبوغة بالكولاجين ابتداء من 12 ساعة بعد الوفاه. غاب التفاعل النسيجي الكيميائي والمناعي للكولاجين III تمامًا عند 60-72 ساعة بعد الوفاه. ومن المثير للاهتمام ، أن الصبغة المناعية النسيجية الكيميائية للكولاجين III زادت بشكل معنوي في الساعة 6 بعد الوفاه ثم انخفضت بشكل ملحوظ بعد 12 ساعة من الوفاه. في الختام ، يمكن استخدام النوع الثالث من الكولاجين البروستاتي كأداة تكميلية ، جنبًا إلى جنب مع الفحص النسيجي المرضي في تقدير فترة بعد الوفاه على الرغم من أن نتائج هذه التجربة تشير إلى احتمالية استخدام الكولاجين لتحديد فترة ما بعد الوفاه في نسيج البروستاتا، الا أنه يجب إجراء دراسات إضافية لضمان دقة هذه الدلالات.