UNIVERSITI PUTRA MALAYSIA

DETERMINATION OF POST-MORTEM INTERVAL (PMI) VIA IMMUNOHISTOCHEMICAL LOCALIZATION AND EXPRESSION OF BIOGENIC AMINE (CADAVERINE)

YEOW MEI JUAN

DETERMINATION OF POST-MORTEM INTERVAL (PMI) VIA IMMUNOHISTOCHEMICAL LOCALIZATION AND EXPRESSION OF BIOGENIC AMINE (CADAVERINE)

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A project paper submitted to the
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In partial fulfilment of the requirement for the
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CERTIFICATION

It hereby certified that I have read this project paper entitled "Determination of Post-mortem Interval (PMI) via Immunohistochemical Localization and Expression of Biogenic Amine (Cadaverine)", by Yeow Mei Juan and in my opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

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(Supervisor)

DEDICATION

This project is dedicated to my beloved family for being my tower of strength;

To all the animals that have suffered injustice;

And

To those who seek justice for them.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, Prof. Dr. Noordin Mohamed Mustapha for giving me this opportunity to conduct this project. It was indeed one of the most unforgettable and interesting experiences throughout my DVM years.

A billion thank you I would like to bid to Dr. Mazlina Mazlan, for lending a warm, helping hand during the most difficult times, and also to the staff members of histopathology lab. All of your guidance and assistance will be remembered.

To my ever-supportive family and White White, thank you for the positive encouragements and support in everything I pursue, especially throughout the FYP period. Not forgetting my high school friends and best friends who are always there for me despite being far apart. Also my college roommate, Ginny Lim for being patient and spontaneously fun throughout these 5 years. A billion thank you to my rotation mates for 3 semesters (Gam Gajah) and my beloved DVM 2012/17 classmates for the physical and moral support whenever I needed the most.

Last but not least, I would like to thank myself for the perseverance, the courage and the undying spirit to strive despite all possible challenges. Never forget to stay humble, positive and firm in everything you pursue. Have faith, and always stay strong!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	percent
°C	degree Celcius
µl	microliter
BSA	bovine serum albumin
cm	centimeter
DPX	dibutyl phthalate xylene
H&E	Hematoxylin and Eosin
IHC	immunohistochemical
kg	kilogramme
mL	mililiter
mM	milimolar
PBS	phosphate buffered solution
PMI	post-mortem interval

ABSTRAK

Abstrak kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus

VPD 4999 - Projek Tahun Akhir

PENGANGGARAN TEMPOH KEMATIAN MELALUI PEWARNAAN IMUNOHISTOKIMIA DAN KEPEKATAN AMINA BIOGENIK (KADAVERINA)

Oleh

Yeow Mei Juan

2017

Penyelia: Prof. Dr. Noordin Mohamed Mustapha

Kekurangan pengetahuan mengenai sela masa pasca kematian (PMI) masih wujud disebalik penyelidikan meluas termasuklah amina biogenik (kadaverina) belum pernah dinilai sebagai petunjuk PMI. Maka, kajian ini bertujuan untuk mencadangkan parameter baru untuk penganggaran PMI melalui imunohistokimia dan taburan kepekatan kadaverina. Sampel otak, hati, otot dan testis yang diperolehi daripada tiga ekor anjing pada jam ke-0, ke-12, ke-18, dan ke-24 pasca eutanasia diawet dalam formalin 10% dan diproses untuk histologi dan imunohistokimia kadaverina (IHC). Sampel pewarnaan H&E juga dijalankan dan didedahkan kepada analisis morfometri. Kepekatan setanding kadaverina didapati pada jam ke-18 dan ke-24 pasca kematian adalah lebih tinggi daripada yang lain ($p < 0.01$). Kepekatan kadaverina hepatik melebihi tisu-tisu lain, kecuali kepada otak ($p<0.01$). Walau bagaimanapun, kesan

bergantungan kepada masa didapati pada semua organ diuji ($p<0.01$). Walau bagaimanapun, kesan bergantungan kepada masa didapati pada semua organ diuji ($p<0.01$). Ukuran morfometri hepatosit, neuron dan tubul seminiferous berbeza secara ketara ($p<0.01$). Kesimpulannya, ternyata bahawa ungkapan kadaverina boleh berfungsi sebagai penganggar PMI. Walau bagaimanapun, kajian yang sama perlu tertakluk kepada amina biogenik yang berbeza dengan kuantifikasi tepat.

Kata kunci: Amina biogenik, imunohistokimia, kadaverina, sela masa pasca kematian

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 – Final Year Project

DETERMINATION OF POST- MORTEM INTERVAL (PMI) VIA IMMUNOHISTOCHEMICAL LOCALIZATION AND EXPRESSION OF BIOGENIC AMINE (CADAVERINE)

by

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2017

Supervised by: Prof. Dr. Noordin Mohamed Mustapha

A dearth of knowledge still exists despite extensive research being conducted on a reliable post mortem interval (PMI) indicator. Biogenic amine has never been attempted as an indicator of PMI and is evaluated in this study based on immunologically expressed cadaverine. Samples of the brain, liver, muscle and testis obtained from three dogs at 0, 12^{th}, 18^{th}, and 24^{th} hour post-euthanasia were immediately fixed in 10% formalin and routinely processed for histology (Haematoxylin and Eosin) and cadaverine immunohistochemistry (IHC). The H&E-stained samples were also subjected to morphometric analysis. Comparable cadaverine expressions found at 18^{th} and 24^{th} hour post-mortem were higher than 0 and 12^{th} hour ($p<0.01$). Hepatic cadaverine expression surpassed other tissues, except

of the brain ($p<0.01$). However, a time-dependent effect was found on all organs tested ($p<0.01$). The morphometry of hepatocytes, neurons and seminiferous tubules were significantly different ($p<0.01$). In conclusion, it appears that cadaverine expression may serve as a PMI indicator. However, similar studies should be subjected to different biogenic amines along with its exact quantification.

Key words: Biogenic amine, cadaverine, immunohistochemistry, post-mortem interval

1.0 INTRODUCTION

The study of the time since death, or the post-mortem interval (PMI) has been one of the most popular fields of research in human forensic medicine. However, in a veterinary context, published data on the estimation of the PMI is scarce, especially studies on companion animals and domestic livestock (Erlandsson and Munro, 2007). Furthermore, most of the data were from finding under temperate conditions which may be incompatible to those found in the tropics. Accurate estimation of the time of death is crucial to the investigation of death via eliminating possible suspects or events, especially in cases of alleged offences related to neglect or abuse of companion animals, deaths of high number of animals, or animal deaths during transportation. Besides, in accordance with the Malaysian Government Act 999 (2009) and Animal Welfare Act (2015), the establishment of a legal framework which primarily aims for animal welfare warrants the need of a veterinary forensic knowledge. Hence, a reliable medico-legal death time estimation would allow evidence-based prosecutions to be carried out.

Despite some improvements which have been made for the past 30 years, according to Swift (2010), the existence of any single, reliable and accurate method in estimating the time since death during the early PMI remains debatable. Munro and Munro (2012) stated two basic approaches to the estimation of time of death: (1) the measurement of change that takes place at a known rate, and (2) the comparison of the occurrence of events known to have taken place at a specific time with the time of death. A list of techniques adopted in veterinary forensic investigations for the

estimation of time of death was stated by Munro and Munro (2012), namely temperature-based methods, post-mortem chemistry, electrical stimulation of muscle and nerves, gross appearance of body (rigor mortis, decomposition, shape, colour and luminosity of the eye etc.), histopathology and electron microscopy, radiology, DNA and RNA analyses, entomology and environmental and associated evidence. However, the lack of validation of many of these methods due to failure in demonstrating quantitative measurement and inclusion of a mathematical description causes the results open to challenge which may lead to ambiguity in legal cases.

Besides, it is worth noting that veterinary pathologists often estimate the time since death based on their experience of gross post-mortem changes such as autolysis, livor mortis, rigor mortis and putrefaction. However, the vast range of species covered and the variety of circumstances in which they are found renders these estimations to be questionable. Similarly, experienced pathologists in the field of human forensic pathology frequently underestimate PMI (James and Knight, 1965).

Forensic entomology can be of considerable value in a veterinary context, although the identification of the types and stages of maggots and beetles requires a prerequisite of knowledge on insect fauna (Anderson and Huitson, 2004). However, it is deemed necessary for veterinary pathologists to acquire appropriate skills and knowledge in the collection of entomological evidence.

On the other hand, various studies on the pattern of temperature drop (algor mortis) in estimating PMI showed important findings. These include the thumb rule of post-mortem rectal temperature fall of 1.5 ^{0}F per hour (Baccino *et al*.., 1996) and the

development of nomogram based on a single rectal measurement (Henssge, 1988), whereby the latter is currently the most widely accepted and practical method on time of death estimation. Body weight, varying degrees of ambience, the effect of wind, surface conductance and irradiation were among the confounding factors which have to be taken into consideration when applying temperature-based methods. A study by Abdulazeez and Noordin (2010) showed the rate of cooling under tropical conditions was less consistent than in temperate climates, also the absence of lag phase in the cooling curve which was derived from temperature measurements of organs in dogs as compared to human (Erlandsson, 2003; Abdulazeez and Noordin, 2010; Okene, 2010).

Thus, careful consideration and attention are required during the application of temperature-based methods in different regions, also during extrapolation between species. Researches on other methods in the determination of post-mortem interval, such as post-mortem chemistry, microscopic and ultrastructural changes and post-mortem radiology, however may serve as means of support and refinement, or as alternatives for further investigation and independent validation which are of relevance to forensic veterinary pathology (Munro and Munro, 2012).

Three main sources in which investigators could derive additional information during the determination of PMI would be evidences i. obtained from the carcass, ii. associated with the environment and iii. anamnestic which is based on ante-mortem movements or daily activities of an individual (Swift, 2010). Studies on methods extolling the vast number of identifiable changes which may occur during the PMI

should therefore being constantly conducted, in order to attribute a temporal value for the time since death.

2.0 LITERATURE REVIEW

The determination of the PMI plays a crucial role in assisting investigators to narrow the 'window of opportunity', therefore excluding specific events and suspects. Despite much time and effort being put forth in researching a variety of methods for determining the time since death, the lack of accuracy and reliability of most methods was of main concern (Henssge, 1988). "The margin of error remains large and unpredictable, even in controlled research conditions, let one for the more variable environment of an actual scene of death" (Knight, 1988). In the veterinary context, estimation of the PMI is particularly complex, due to diverse objectives depending on the category and species of animal involved.

Tamime (2017) showed that biogenic amines are nitrogenous compounds with one or more amine groups, formed mainly by decarboxylation of amino acids or via aldehydes and ketones amination and transamination. Besides, biogenic amines have been found to be of biological importance in vegetable, microbial and animal cells (Santos, 1996). Polyamines, which are the subtypes of biogenic amines, are low molecular weight aliphatic amines that perform essential functions in living organisms (Casero and Pegg, 2009; Igarashi and Kashiwagi, 2010; Pegg, 2009; Wallace *et al*., 2003). Main examples of polyamines would be putrescine, cadaverine, spermidine, and spermine, which are regulated by the activity of the enzyme ornithine decarboxylase (Pegg and McCann, 1982). In mammals, polyamines are involved in various physiological functions such as cell proliferation and apoptosis (Igarashi *et al*., 1975; Marton and Pegg, 1995; Seiler and Raul, 2005), immunity (Seiler and

Atanassov, 1994), and oxidative stress response. In addition, alterations in the production of polyamines and their metabolic enzymes have been associated with numerous pathological and psychiatric conditions (Chen *et al*.., 2010).

In general, the process of decomposition after death occurs by two mechanisms, which are autolysis and putrefaction (Dent *et al*., 2004; Janaway *et al*., 2009). Autolysis can be defined as a process of cellular self-destruction caused by hydrolytic enzymes originally contained within cells (Enwere 2008; Shirley *et al*., 2011), whereas putrefaction is the degradation of tissue via activity of microorganism such as bacteria, fungi and protozoa, from normal biota of the human body especially in the gastrointestinal tract (Dent *et al*., 2004; Paczkowski & Schütz, 2011). Vass (2001) pointed out that, putrefaction often results in the catabolism of tissue into gases, liquids and simple molecules. In addition, compounds including indole, skatole, putrescine, cadaverine and various fatty acids have been detected and are significant decomposition products.

The effect of putrefaction of poultry carcasses found an accumulation of high levels of eight biogenic amines, namely putrescine, cadaverine, tryptamine, phenylethylamine, histamine, tyramine, spermidine and spermine (Tamim and Doer, (2003). Significant increase in the concentration of all amines, excluding spermidine and spermine were observed with increasing putrefaction time. Besides, it is worth stating that during the first 24 to 36 hours of putrefaction the increase was relatively slow, after which high concentration levels were then observed. This statement is supported by Farn and Sims (1987) findings on decomposition of tuna fish, whereby

levels of putrescine, cadaverine and histamine markedly increased after 36 hours of decomposition at 21 ^{0}C. Both aforementioned studies have showed an elevated production of biogenic amines with time during decomposition of carcasses. However, significant differences were noted between them: species of animals, and the environment (study on poultry carcasses was conducted at 30 ^{0}C, 70 to 80% relative humidity) which could affect the rate of decomposition, thereby the concentration and rate of biogenic amines being produced. Besides, both studies were aimed to study the effects of biogenic amines on the quality and freshness of final rendered food product, rather than the determination of post-mortem interval.

On the other hand, a study in the field of human neuropsychopharmacology, particularly on the evidence of altered biogenic amine concentrations in cerebral cortex of suicide completers was conducted. Quantitation of polyamines from post-mortem brain tissue was carried out via high-resolution capillary gas chromatography with mass spectrometry (Chen *et al.*, 2009). The finding was a significant elevation of both putrescine and spermidine levels in brain of suicide completers with major depressive disorder (Chen *et al.*, 2010). According to Gilad and Gilad (2003), and Sohn *et al.* (2002), elevated levels of polyamines particularly putrescine, serves as an important component of the polyamine stress response, which is associated with destructive effects of stress and anxiety in addition to the development of other psychiatric disorders. Besides, only male subjects were used in the study to avoid gender-specific differences in terms of levels of polyamines and their metabolic enzymes, as well as responses to polyamine exposure (Barron *et al.*, 2008; Bastida *et*

al., 2007; Ferioli *et al*., 1999; Gliad *et al*., 2002). Therefore, given the role of putrescine in the polyamine stress response, an elevated putrescine level in brain tissue may be associated with stress and anxiety ante-mortem, besides the effect of microbial activity during putrefaction. However, further studies and validations are warranted for its application in the context of forensic veterinary pathology.

In conclusion, it has been noted that research on the determination of PMI based on production of biogenic amines is currently limited. Based on plausible finding of studies which is the increase in biogenic amines level with time after death, a study on the expression or quantitation of these biogenic amines as a mean of PMI determination is thus warranted. Therefore, this study was conducted with the objectives of:

i. Defining and scoring the extent of biogenic amine (cadaverine) expression over time in different tissues
ii. Identifying the best tissue to be used as a PMI indicator based on immunogically expressed cadaverine

It is hypothesised that biogenic amines would serve as reliable and sensitive indicators in the determination of PMI in dogs which correlate well with the morphometric changes of cells over time.

3.0 MATERIALS AND METHODS

3.1 TISSUE SAMPLING

Three mongrel with a median weight of 14.9 kg were utilized for the study. Following euthanasia with pentobarbital sodium, the carcasses were placed on right lateral recumbency. Necropsy examination including observation of rigor mortis, development of odour, insect succession pattern, ocular changes and alterations to the internal organs and tissues was carried out on each sampling time. Histology samples were collected from four different tissues namely the brain, liver, epaxial muscle and testis from each dog at 0 hour, 12^{th} hour, 18^{th} hour and 24^{th} hour post-euthanasia. Tissue samples of the cerebrum were collected via dorsal craniotomy technique, whereas liver tissue samples were obtained following an incision on the left thoraco-abdominal wall along the costochondral length of the last rib. Scrotal tissue samples were collected from the right testis of all dogs, via a longitudinal incision into the scrotum. Lastly, sampling of epaxial muscle was carried out via open biopsy technique along the left epaxial muscle. All tissues were maintained in a closed environment via sealing of the outermost skin layer after each sampling. The cerebral tissue samples were fixed in 40% neutral buffered formalin whilst the other samples (liver, testis and epaxial muscle) were collected into 10% neutral buffered formalin.

3.2 HEMATOXYLIN-EOSIN STAINING METHOD

Tissues were routinely processed to wax blocks, sectioned serially at 4 µm and stained with haematoxylin and eosin (H&E). The histology slides were dipped in 100% xylene and mounted with coverslips using dibutyl phthalate xylene (DPX), then

left overnight. The slides were viewed under the light microscope (*Motic*, BA410 Trinocular, Hong Kong).

3.3 IMMUNOHISTOCHEMISTRY STAINING METHOD

3.3.1 Deparaffinisation and Rehydration

The paraffinized tissue sections were incubated in oven for 15 minutes at a temperature of between 56 to 60°C, then immersed in xylene for 5 minutes. Rehydration was carried out by subsequently immersing the slides into descending concentration of alcohol solution (100% alcohol, 70% alcohol and 50% alcohol, each concentration for 3 minutes then rinsed with distilled water). The slides were then placed in phosphate buffered saline (PBS) for 15 minutes, followed by blocking with liquid blocker at the peripheral of the tissue sections.

3.3.2 Inactivation of Peroxidase

A 3% hydrogen peroxidase solution was dropped onto the slides before incubation at room temperature for 10 minutes. It was then rinsed off via PBS for 5 minutes.

3.3.3 Antigen Retrieval

The slides were incubated in 10mM of sodium citrate buffer, pH 6.0 and placed in a microwave oven at lowest setting (50 Watt) for 10 minutes to unmask the antigens. They were then allowed to cool at room temperature for at least 20 minutes before placed in PBS for 10 minutes.

3.3.4 Protein Blocking

For non-specific protein blocking, 100 µl of 5% Bovine Serum Albumin (BSA) was dropped onto each slide then incubated in a humidified chamber at room temperature (37°C) for 15 minutes. The solution was then drained off from the slides prior to the immunohistochemical staining.

3.3.5 Immunohistochemistry Staining

The primary antibody used was a rabbit polyclonal antibody against the cadaverine antigen (AbnovaDiamino-pentane polyclonal antibody, PAB0085). It was diluted with Tris-buffered solution (TBS) containing 0.5% of crystalline bovine serum albumin (BSA) to a dilution of 1:2000, then applied to the tissue sections (100 µl per slide) before overnight incubation in a humidified chamber of 4°C. The primary antibody was rinsed off in PBS for 10 minutes. An anti-rabbit secondary antibody diluted with 0.1% TBS to a dilution of 1:50 was prepared and applied to the slides (100 µl per slide). Incubation was carried out in a humidified chamber at room temperature for 30 to 60 minutes, before rinsing off the secondary antibody using PBS.

The antigen-antibody complexes were detected via ABC-peroxidase technique using DAB (3-3'-diaminobenzidine) (Dako, Inc., North America) as chromogen substrate to reveal the immunoreaction. The slides were then immersed in Harris Hematoxylin as a cytoplasmic counterstain for five minutes before rinsed with distilled water for 10 minutes, followed by dehydration of slides in 95% alcohol solution (five minutes, twice) and 100% alcohol solution (five minutes, twice). Similarly as for histology preparation, the slides were dipped in 100% xylene and

mounted with coverslips using DPX, then viewed under the light microscope (Motic, BA410 Trinocular, Hong Kong).

3.4 IMAGE ACQUISITION

Images were captured via the Motic BA410 Trinocular (Motic, Hong Kong) which was equipped with a MoticamPro camera (Motic, Hong Kong). The light and camera settings were controlled using the Motic Images Plus 2.0 (Motic, Hong Kong) software. Images for the analysis of the expression of cadaverine and H&E-stained tissue sections were randomly captured at 200X and 400X respectively, whereas for the analysis of periodic distribution of cadaverine, images were captured at 100X.

3.5 IMMUNOHISTOCHEMISTRY DATA ANALYSIS-EXPRESSION OF CADAVERINE

The immunohistochemistry (IHC) images were analysed using ImageJ software with IHC Profiler plugin (Rasband, 1997). The IHC profiler could be freely downloaded from Sourceforge website (https://sourceforge.net/projects/ihcprofiler/). Guidelines on the use of IHC profiler and the embedment of ImageJ program to the Microsoft Windows operating system can be obtained in the package (Varghese *et al*., 2014). Three types of images were produced, namely the DAB, the hematoxylin and a complimentary image. The DAB image was selected and analysed for the intensity of brown colour, indicating positive immunoreaction of the cadaverine antigen and antibody. A total of 10 images from different sites on each slide were analysed. Lastly, the intensity of brown colouration in the images was quantified and interpreted as the mean expression of cadaverine in each sample.

3.6 STATISTICAL ANALYSIS

Statistical analysis was performed using IBM SPSS version 23 software. The data obtained were normally distributed and of equal homogeneity of variance and only $p<0.05$ were considered significant. Two-way ANOVA, a parametric test was chosen to analyse the mean expression of cadaverine of organs at different time points and between organs, with 0 hour being the pre-mortem time and the 12^{th} hour, 18^{th} hour and 24^{th} hour as the post-mortem time.

The extended data on the morphometric analysis of the cells were neither normally distributed nor equal in homogeneity of variance. Therefore, a non-parametric test (Kruskal-Wallis test) was used to analyse the mean area of hepatocytes, neurones and seminiferous tubules at different time points, followed by comparison between any two organs using Mann-Whitney test. Correlation was also carried out to test between mean cadaverine expression and the mean morphometric measurements of cells over time.

4.0 RESULTS AND DISCUSSION

4.1 MEAN EXPRESSION OF CADAVERINE IN BRAIN, LIVER, MUSCLE AND TESTIS

Table 1 shows the mean expression of cadaverine in the brain, liver, muscle and testis during the experimental period. A two-way ANOVA revealed significant differences between groups over time post mortem and the organs ($F = 27.964$, $p<0.01$ and $F = 21.253$, $p<0.01$, respectively). Comparable expressions of cadaverine found at the 18^{th} and 24^{th} hour were significantly higher than those at 0 and 12^{th} hour. However, cadaverine expression at the 12^{th} hour was significantly higher than that at 0 hour. For the organs, the expression of hepatic cadaverine surpassed ($p<0.05$) those of other tissues, except that of the brain. The brain and testicular cadaverine expressions were significantly ($p<0.05$) higher than that of the muscle.

Table 1. The expression of cadaverine in the brain, liver, muscle and testis during the experimental period (Mean± SE)

	Hours-post mortem			
	0	12	18	24
Brain	21.73^{bcA}± 4.92	46.57^{bcB}± 4.92	46.85^{bcC}± 4.92	62.42^{bcC}± 4.92
Liver	23.66^{cA} ± 4.92	41.26^{cB} ± 4.92	74.91^{cC} ± 4.92	64.85^{cC} ± 4.92
Muscle	12.99^{aA} ± 4.92	18.33^{aB} ± 4.92	35.83^{aC} ± 4.92	30.74^{aC} ± 4.92
Testis	33.46^{bA} ± 4.92	40.74^{bB} ± 4.92	52.67^{bC} ± 4.92	29.90^{bC} ± 4.92

^{abc}Means bearing different superscripts within column are significantly different at $p < 0.05$
^{ABC}Means bearing different superscripts within row are significantly different at $p < 0.05$

The scores of cadaverine expression in different organs were also plotted against time to obtain its function as shown in Figures 1 and 2. The mean expression of cadaverine was

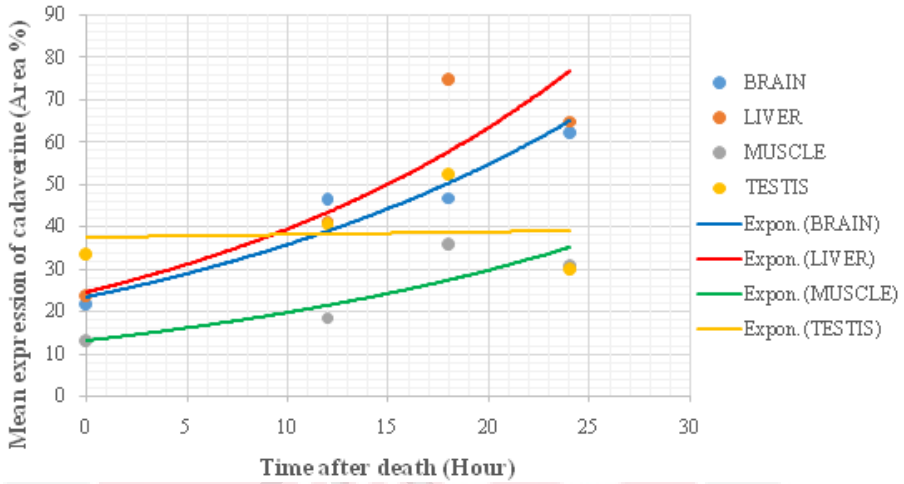

Figure 1. The exponential curve for mean expression of cadaverine in the brain, liver, muscle and testis over 24 hours post-mortem period.

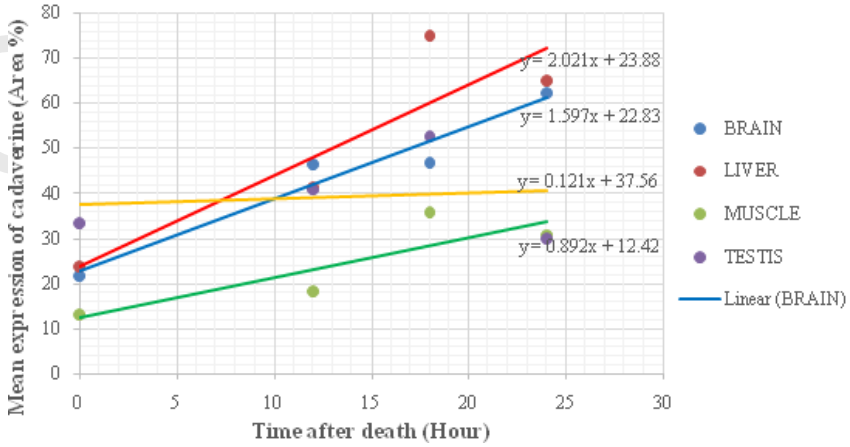

Figure 2. The linear curve for mean expression of cadaverine in the brain, liver, muscle and testis over 24 hours post-mortem period.

lowest in the muscle tissue since the beginning as compared to other organs. This is in agreement with earlier findings (Tomita *et al*..,2004), whereby the onset of decomposition is slowest in the muscle, due to a lack of digestive enzymes. Conversely, in the liver the mean expression was highest due to its storing a high concentration of digestive enzymes (Dix & Graham, 2000). Thus, leading to a faster onset and higher rate of decomposition in the liver. The mean expression in the brain was comparably high due to the likelihood of harbouring more lysine which in turn causes a much higher cadaverine concentration (Dolezalova *et al*.., 1974) following a higher production of its decarboxylation end-product (cadaverine). The mean cadaverine expression in the testis remained stable over time.

Plates 1 to 4 show the photomicrograph of the periodic expression of cadaverine in the brain, liver, muscle and testis in a 24-hour period. Based on the findings, the brain and liver served as potential PMI indicators due to more significant and predictable changes in cadaverine levels with time. However, the muscle tissue which showed a stable cadaverine expression over time represented a poor indicator of PMI. This could be further explained by various properties of the muscle tissue. As aforementioned, the decomposition rate of a tissue could be influenced by its level of digestive enzymes. Therefore, the muscle decomposes at a slower manner due to its low level of digestive enzymes. Besides, the stability of the muscle tissue observed throughout the study could be supported by findings from Maxie (2015), whereby myofilaments have resistance towards autolysis, which leads to a better preserved overall architecture of the muscle tissue post-mortem. Lastly, muscle tissues are much

well-protected thus less contaminated. Thus, a lower bacterial load present in the muscle which leads to a lower proteolysis rate.

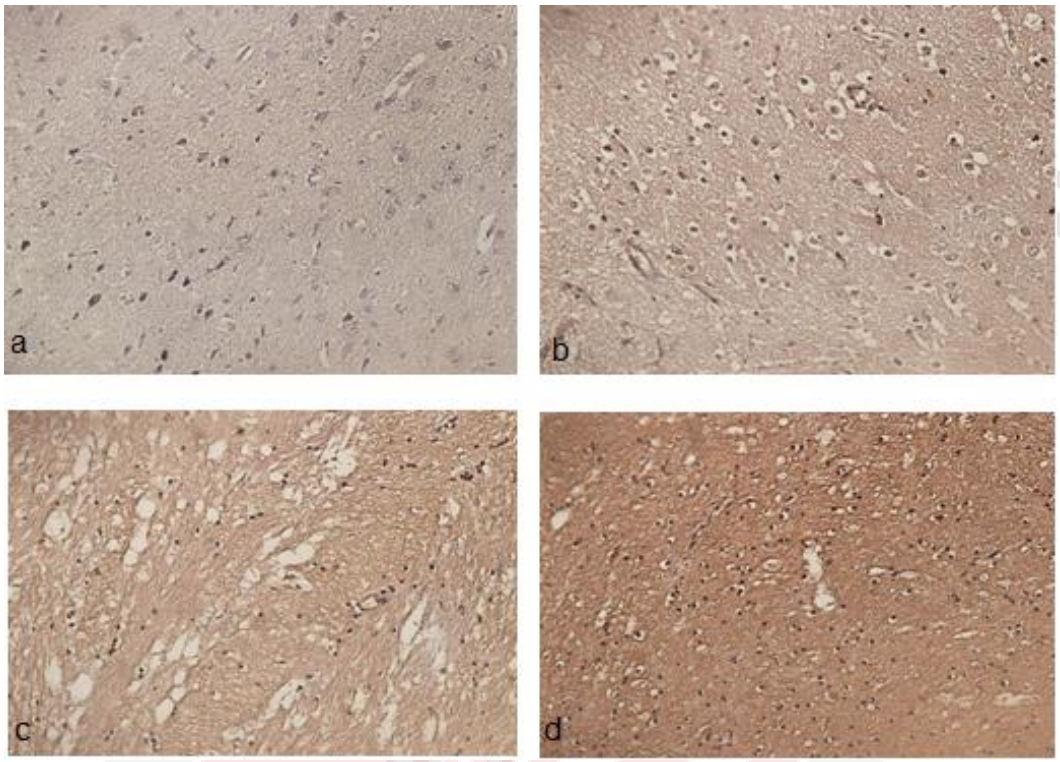

Plate 1. Photomicrograph of periodic expression of cadaverine in the brain at (a) 0 hour, (b) 12 hour (c) 18 hour and (d) 24 hour post-mortem

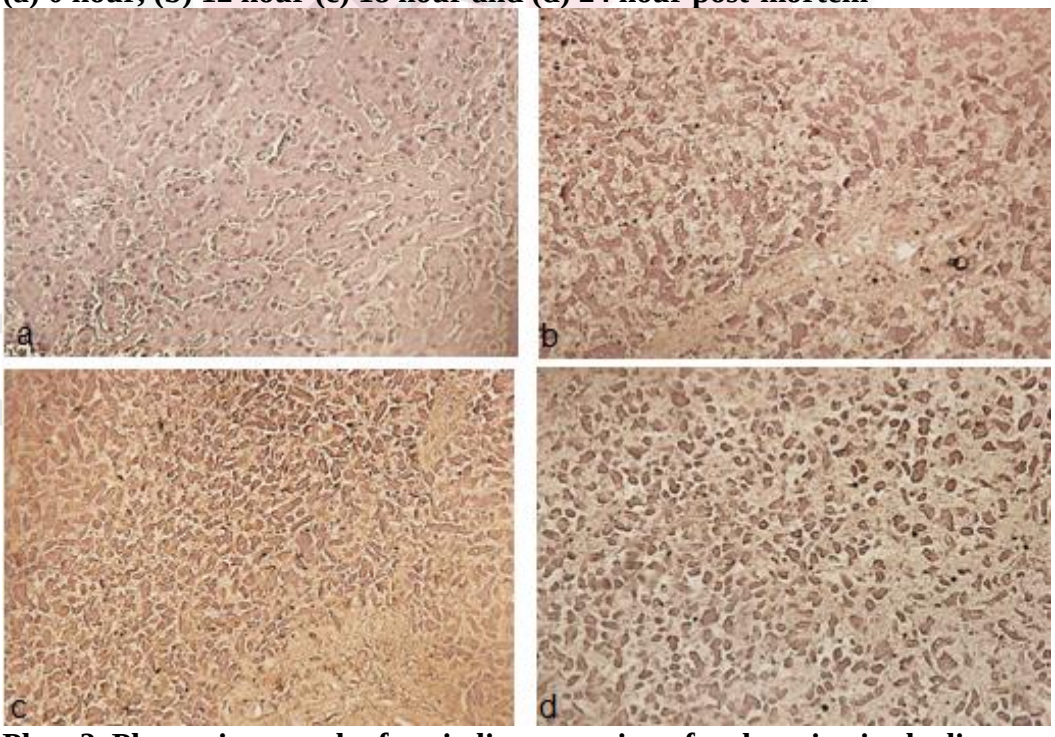

Plate 2. Photomicrograph of periodic expression of cadaverine in the liver at (a) 0 hour, (b) 12 hour (c) 18 hour and (d) 24 hour post-mortem

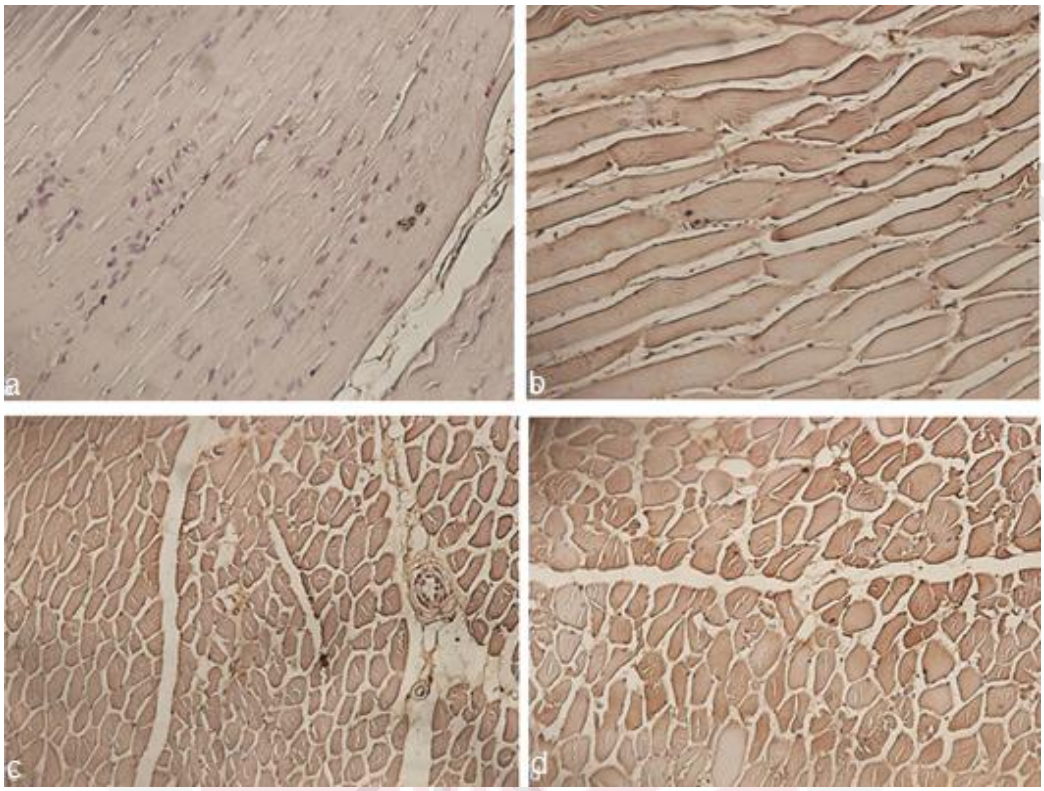

Plate 3. Photomicrograph of periodic expression of cadaverine in the muscle at (a) 0 hour, (b) 12 hour (c) 18 hour and (d) 24 hour post-mortem

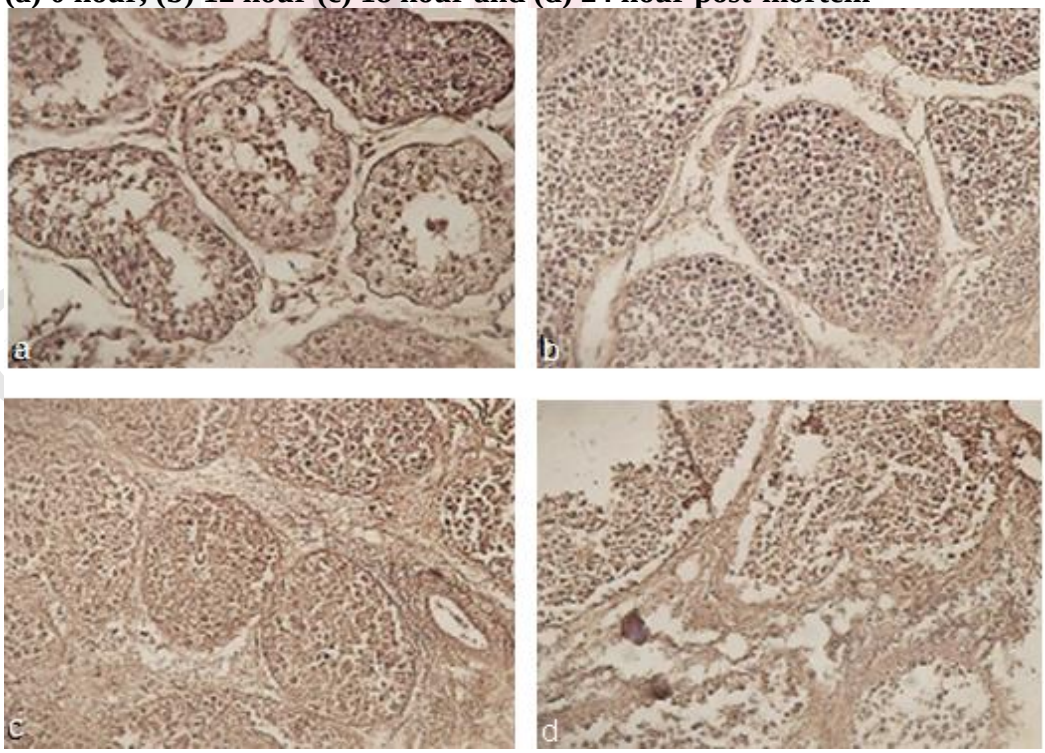

Plate 4. Photomicrograph of periodic expression of cadaverine in the testis at (a) 0 hour, (b) 12 hour (c) 18 hour and (d) 24 hour post-mortem

4.2 MORPHOMETRIC ANALYSIS OF CELLS

The mean morphometric measurements of area of the hepatocytes, neurons and seminiferous tubules at different time points are as shown in Table 2 and Figure 3. The muscle tissue was not included in this analysis due to its unconvincing properties as PMI indicator, as discussed preciously. The area of hepatocytes decreased in an almost linear manner, whereas the neuronal area increased until the 18th hour then decreased significantly.

However, inconsistent findings were observed in the area of seminiferous tubules. Kruskal-Wallis test revealed significant differences between groups in organs ($p < 0.01$). Significant differences were observed between any two organs via Mann-Whitney test ($p < 0.01$). However, the liver proved to be a more reliable and consistent organ to be measured in a 24-hour period, based on its pattern in morphometric measurements.

Table 2. The mean morphometric measurement of area of the hepatocytes, neurons and seminiferous tubules over four time points post-mortem (Mean ± SD)

	Zero hour	**12th hour**	**18th hour**	**24th hour**
Neuronal area	345.73^{a}± 236.73	358.34^{a}± 166.98	460.26^{a}± 173.22	366.05^{a}± 87.85
Hepatocyte area	44087.62^{b} ± 19637.10	28095.88^{b} ± 15878.39	13768.50^{b} ± 5360.92	9100.29^{b}± 2849.29
Seminiferous tubular area	57606.60^{c} ± 44320.43	70760.83^{c} ± 57717.13	53227.48^{c} ± 44318.99	60344.19^{c} ± 56277.28

^{abc}Means bearing different superscripts within column are significantly different at $p < 0.05$

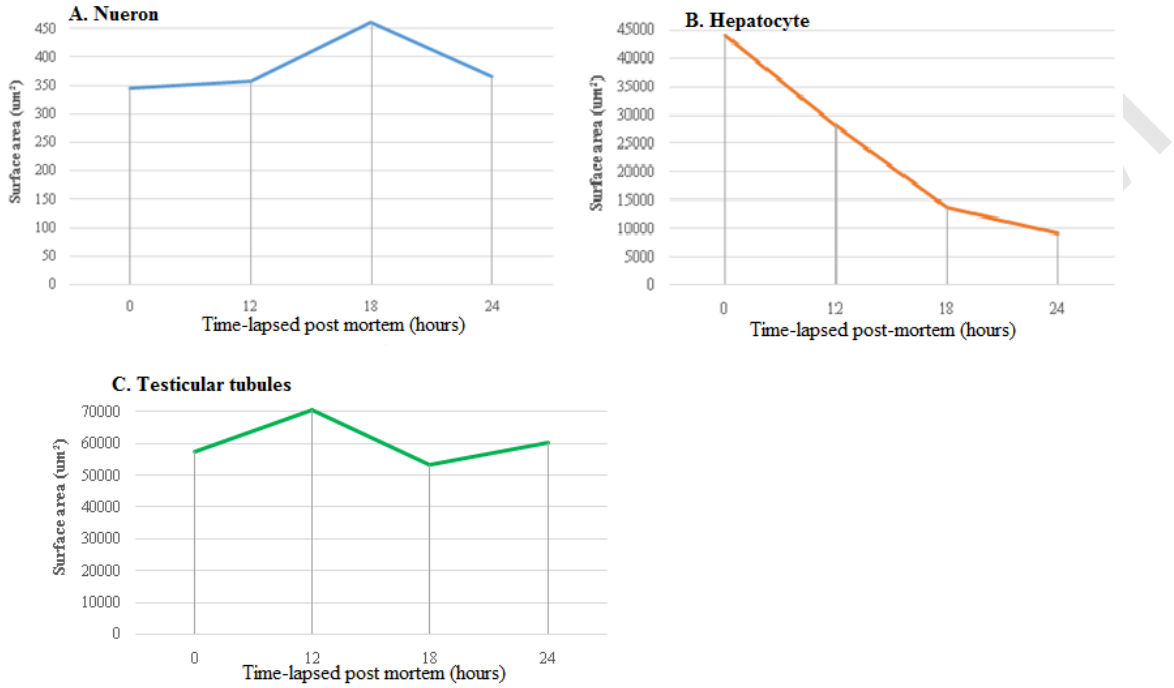

Figure 3. The mean surface area of (A) neurons, (B) liver (C) testicular tubules during the experimental period

4.3 CORRELATION BETWEEN MEAN EXPRESSION OF CADAVERINE AND MORPHOMETRIC ANALYSIS OF THE BRAIN, LIVER AND TESTIS

Correlation between the mean cadaverine expression and morphometric changes of cells was found to be insignificant.

4.4 CORRELATION BETWEEN ARBTRARY TEMPERATURE CHANGES AND MEAN EXPRESSION OF CADAVERINE IN BRAIN AND LIVER

In addition, the mean expressions of cadaverine in the brain and liver were mapped onto temperature changes post-mortem. The expression was found to be

moderately correlated with temperature changes in the liver but not in the brain, as shown in Figure 6.

Table 3. Correlation between temperature changes against mean expression of cadaverine in the brain and liver.

	Mean expression of cadaverine	
Temperature changes	**Brain**	**Liver**
R^2 value	0.111*	0.528*
p-value	0.293	0.007

* Correlations are significant at $p < 0.05$ (one-tailed)

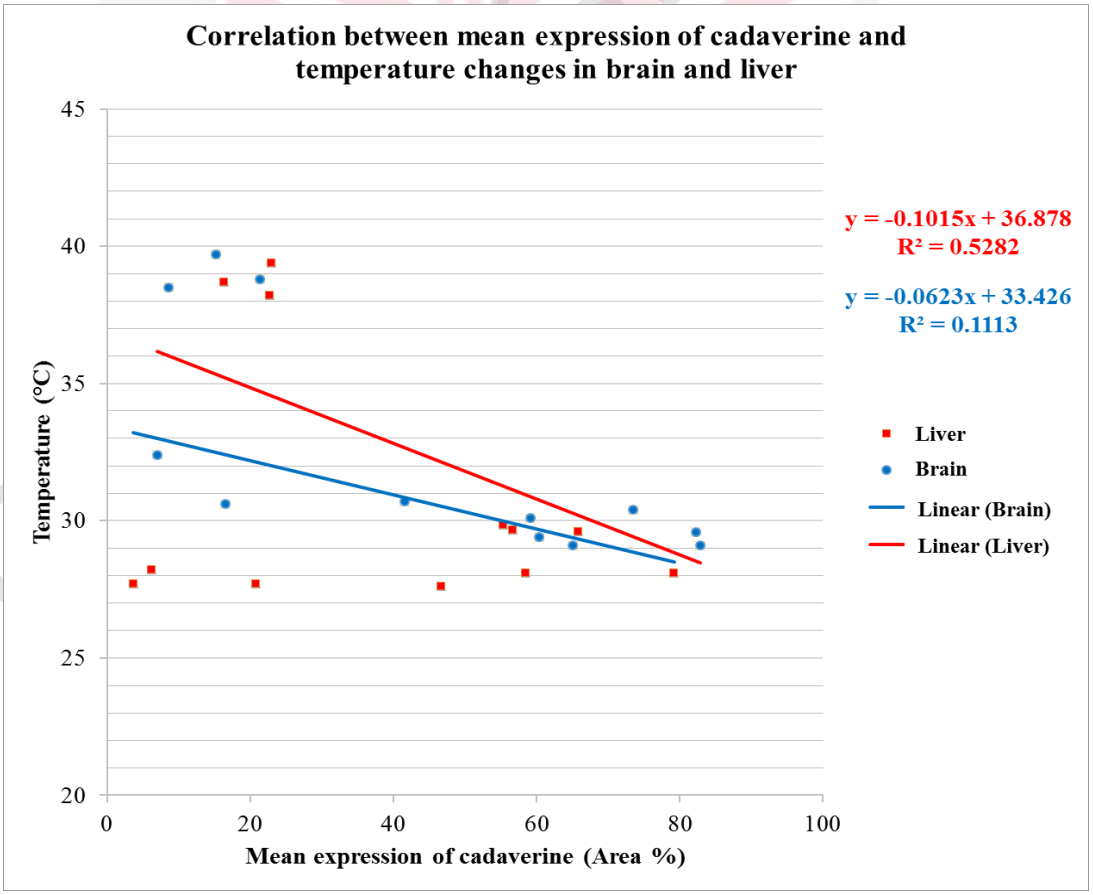

Figure 4. Graph of linear curve of correlation between mean expression of cadaverine and temperature changes in brain and liver at different time points post-mortem.

5.0 CONCLUSION

It could be concluded that expression of cadaverine in tissues can serve as a potential indicator for PMI determination in dogs. Based on findings on the cadaverine expression, the morphometric analysis and correlation with arbitrary temperature changes, the best tissue being identified in this study as a PMI indicator was the liver. However, it still appears that there is no single determinant of a PMI and possibly a combination of several well correlated parameters is the best choice in determining a PMI.

6.0 RECOMMENDATIONS

Similar study should be conducted on the expression of other biogenic amines such as putrescine and spermidine or in pigs. Besides, studies could also be carried out for a longer post-mortem period and in a controlled physical environment such as the temperature and relative humidity which could influence the rate of carcass decomposition.

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