UNIVERSITI PUTRA MALAYSIA

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

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

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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 Frankie Lau Pick Ping 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)

DEDICATIONS

This thesis paper is dedicated to the One Almighty God who make anything possible,

To my father and mother,

My grandmother and grandfather,

My brothers and sisters,

Lee Chee Yien

And to all my beloved teachers who guided me through the education pathway**.**

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Prof. Dr. Noordin Mohamed Mustapha, my beloved project supervisor for his time, support, advices, guidance, valuable comments and suggestions throughout the completion of this project. The door of his office was always open whenever I ran into a trouble or had a question about my research. He always make sure I'm enjoying the process of conducting this project.

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Most importantly, none of this could have happened without the support from my loves one, my mother, father, brothers, sisters and Lee Chee Yien. They never failed to provide me positive energy from the beginning.

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LIST OF ABBREVIATIONS

%	percent
°C	degree Celsius
BSA	bovine serum albumin
cm	centimetre
DPX	dibutyl phthalate xylene
H&E	Hematoxylin and Eosin
IBD	interlobular bile ducts
IHC	immunohistochemical
kg	kilogramme
mL	millilitre
mM	milimolar
PBS	phosphate buffered solution
PMI	post-mortem interval
µl	microlitre

ABSTRAK

Abstrak kertas projek yang dikemukakan kepada FakultiPerubatanVeterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999-Projek TahunAkhir

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

Oleh

Frankie Lau Pick Ping

2016

Penyelia: Prof. Dr. Noordin Mohamed Mustapha

Sela masa pascakematian (PMI) adalah tempoh sejak kematian sehingga jasad haiwan atau manusia ditemui. Penentuan PMI amatpenting bagi penyiasat forensic, terutamanya dalam isu forensik yang melibatkan binatang atau manusia. Anggaran PMI yang tepat dapat dijadikan *prima facie* yang kukuh untuk penyiasatan pihak pendakwa. Banyak parameter PMI telah dikaji oleh saintis dari seluruh dunia. Namun, kebanyakan data adalah dari negara-negara barat. Maka, kajian ini bertujuan untuk mencadangkan parameter baru untuk penanggaran PMI melalui perwarnaan imunohistokimia dan kepekatan amina biogenik (kadaverina). Sampel tisu otak, hati dan otot epaksial dari tiga ekor anjing telah diperolehi pada sela 0, 12, 18, dan 24 jam pasca kematian. Sampel telah diproses untuk prosedur perwarnaan imunohistokimia menggunakan antibodi kadaverina dengan histologi H&E . Setiap sampel dianalisis

menggunakan perisian profil immunohistokimia (Image J IHC Profiler) bagi menentukan kepekatan kadaverina pada setiap titik masa pasca kematian. Keputusan menunjukkan perbezaaan keertian pada kepekatan kadaverina antara setiap titik masa pasca kematian bagi otak ($p = 0.000$) dan hati ($p = 0.000$) sahaja. Peningkatan kepekatan kadaverina adalah tererti antara otak dan hati ($p = 0.043$), hati dan otot ($p = 0.008$) dan otot dan otak ($p = 0.009$). Terdapat juga perkaitan yang tererti antara perubahan suhu dengan kepekatan kadaverina pasca kematian pada otak ($r = -0.898$, $N = 12$, $p = 0.000$) dan hati ($r = -0.958$, $n = 12$, $p = 0.000$) tetapi tiada bagi otot. Perubahan histology berdasarkan pewarnaan Hematoxylin-Eosin (H&E) turut berkait rapat dengan perubahan berdasarkan pewarnaan immunohistokimia pada sampel tisu sepanjang tempoh kematian. Kajian lanjut yang serupa perlu dilakukan dengan menggunakan amina biogenik lain seperti putresina dan spermidina. Secara kesimpulan, kadaverina dari otak dan hati boleh dijadikan parameter untuk penggangaran PMI.

Kata kunci: sela masa pasca kematian, patologi, amina biogenik, kadaverina, otak, hati, otot

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 LOCALISATION AND EXPRESSION OF BIOGENIC AMINE (CADAVERINE)

by

Frankie Lau Pick Ping

2016

Supervised by: Prof. Dr. Noordin Mohamed Mustapha

Post- mortem interval (PMI) is the time since death until the moment the dead body of either an animal or human is discovered. The determination of PMI is of crucial importance in a forensic investigation as it would narrow down uncertainties with respect time and justifying a much more valid prima facie. Despite being extensive, most studies on PMI originated from the temperate regions. Thus, this study aimed to establish a parameter to estimate PMI via immunohistochemical localisation and expression of biogenic amine (cadaverine). Brain, liver and epaxial muscle tissue samples from three dogs were obtained at 0, 12, 18, and 24-hour post-mortem. The samples were then processed for cadaverine immunohistochemistry and H&E stained histology. Each sample was scored using an immunohistochemistry profiler software (ImageJ IHC profiler) to assess the expression of cadaverine at each interval. Results

demonstrated significant differences in expression of cadaverine in a time-dependent manner for the brain ($p = 0.000$) and liver ($p = 0.000$) but none in the muscle ($p = 0.817$). These expression was comparable between the brain and liver but was much higher ($p = 0.009$ & 0.008 respectively) than that of the muscle. There was significant correlation between temperature changes against concentration of cadaverine in the brain ($r = -0.898$, $N = 12$, $p = 0.000$) and the liver ($r = -0.958$, $N = 12$, $p = 0.000$) but again none was demonstrated in the muscle. Likewise, the post-mortem changes based on H&E correlated well with those of cadaverine immunohistochemistry throughout the assessment period. Similar study should be subjected to different biogenic amines such as putrescine and spermidine. In conclusion, brain and hepatic cadaverine serves as a potential indicator for the determination of PMI.

Key words: Post- mortem interval, pathology, biogenic amines, cadaverine, brain, liver, muscle

1.0 INTRODUCTION

The importance of post-mortem interval (PMI) is an upcoming parameter in veterinary science especially with regards to medicolegal issue. In accord with the Government Act 999 (2009) and establishment of Animal Welfare Act (2015) and various legal frame to protect animal welfare, warranting the need of a veterinary forensic team. To do so, one of the important question posed in sudden death inquiry is "When did the animal die?" Hence determining an accurate PMI will assist prosecution with strong evidence. The main principle of determination of the time since death is calculation of a measurable date along a time-dependent curve back to the start point (Henssge, 1986).

In general, there are various methods studied to estimate PMI through physical, chemical and biological parameters. Traditional methods since centuries ago are based on detection of phenomena happen near to the death including rigor mortis, livor mortis, and external post-mortem appearance of body. These physical parameters have their limitations due to several environmental and the host factors. There are changes, either destructive or conservative events that may accelerate, slow down or suppress some stages of tissue changes (Estracanholli *et al*., 2011). Biochemical detection is based on electrolyte changes in cerebrospinal fluid, vitreous humour and pericardial fluid (Kawamoto *et al*., 2013; Chandrakanth *et al*., 2013). Detection of temperature changes based on algor mortis, mechanical stimulation of skeletal muscle contraction based on rigor mortis, study of ultrastructure changes of certain organ post-mortem and maggot activity are examples of biological approaches to determine PMI (Mall *et*

al., 2005; Tomita *et al.*, 2004; Warther *et al*., 2012; Anders *et al*., 2013;Villet & Amendt, 2011).

Traditional methods of determining time since death are based on naked eye observations of the gross changes in dead body to provide a rough approximation of post- mortem interval. These various gross changes in the body after death are cooling of the body, rigor mortis, loss of corneal reflex, post-mortem hypostasis, decomposition and other putrefactive changes. (Kushwaha *et al*., 2009).

Most of the parameters established for human work rather than animal use. Munro & Munro (2008) described the particular difficulties for the animal pathologist particularly when they are more likely to be dealing with different species and more environmental factors to be take consider. There are limited studies established for veterinary use. Most literatures on this issue are reported or studied in the temperate (Edges, 1984; Erlandsson & Munro, 2007; Cooper & Cooper, 2007; Merck, 2007; Munro & Munro, 2008; Munro & Munro, 2011; Sinclair *et al*., 2006). Recently three reports were documented from Malaysia (Heng *et al*., 2009; Abdulazeez & Noordin, 2010; Okene, 2010). Determination of PMI used to estimate time since death rather than to determine time of death. Hence most of currently available methods focus on constructing a timeframe since animal death and the events happen post-mortem.

To date there is no definite method to pin-point PMI although various methods were studied. An estimation of PMI cannot be just merely based on a single parameter due to various factors. For example, post-mortem cooling or algor mortis has been established as standard in practice with wide applicability and reliability (Henssge &

Madea, 2007). The principle site used for temperature recording is the rectum although other body sites include brain, skin, axilla and internal organs. If there were circumstances where rectal temperature or any organ temperature cannot be established due to no rectum remaining or organ available for the examination.

Hence, most practical way suggested by Henssge & Madea (2007) estimation of time since death is via integration of different parameters into a single system which enable a much more accurate, reliable and applicable estimation of PMI in all cases. Thus, this study was undertaken to fulfil the following objectives:

i. to determine and score the extent of biogenic amine expression overtime in tissues

ii. to map the scores with previously obtained data on PMI using time and body temperature

It is hypothesised that biogenic amines are reliable and sensitive indicators of PMI in dogs which correlate well against body temperature changes.

2.0 LITERATURE REVIEW

2.1 DECOMPOSITION OF TISSUE AFTER DEATH

The decomposition of a body is a mixed process that varies from cellular autolysis by endogenous chemical destruction to tissue autolysis, by either the release of enzymes or external processes, resulting from the bacteria and fungus in the intestines or from outside (Knight & Saukko, 2004). There are five general stages used to describe the process of decomposition in animals: Fresh, Bloat, Active and Advanced Decay, and Skeletal/Remains (Goff, 2009). These five stages of decomposition are coupled with two stages of chemical decomposition: autolysis and putrefaction. These two stages contribute to the chemical process of decomposition, which breaks down the main components of the body. (Dix & Graham, 1999; Pinheiro, 2006; Goff, 2009)

As soon as cell death, cell membranes become permeable and breakdown with release of cytoplasm containing enzymes. The proteolytic, glycolytic and lipolytic action of ferments leads to autolysis and disintegration of organs which occurs without bacterial influence. Putrefaction is the final stage following death, produced mainly by the action of bacterial enzymes, mostly anaerobic organism derived from the bowel. (Rao, 2013). There are four primary categories of organism involved in decomposition which are bacteria, fungi or moulds, insects and vertebrae scavengers. (Goff, 2009). The main destructive organism is bacteria who produce a large variety of enzymes and these breakdowns the various tissues of the body (Rao, 2013). It begins immediately after death at the cellular level, which is not evident grossly. There is progressive breakdown of soft tissues and the digestion of their proteins, carbohydrates and fats.

The organisms enter the tissues after death mainly from gastrointestinal tract, respiratory tract or external skin wound. They multiply and spread through the blood vessels using proteins and carbohydrates of the blood as culture media. (Rao, 2013)

Combination of autolysis and putrefaction lead to breakdown of large molecules (protein, carbohydrate, fat) into smaller molecules (amino acid, fatty acids etc.). Therefore, as said by Di Maio & Di Maio (2001), decomposition involves autolysis (the destruction of cells and organs by an aseptic chemical process) and putrefaction (the destruction by bacteria and other microorganisms).

2.2 RELEASE OF BIOGENIC AMINES

The body undergoes changes at cellular level long before any changes can be seen with the unaided eye. Autolytic enzymes are released following cellular autolysis and digest the tissues around them. This process of autolysis is different than the process of necrosis which is death of cells and tissues that occurs when the individual is still alive. Tissues rich in digestive enzymes are most readily subjected to autolytic changes. (Dix & Graham, 1999)

Autolysis is readily seen under microscope and may be apparent grossly. The cellular architecture appears to fade away or, in case of tissues rich in digestive enzymes, dissolve. There is no reaction by the body's immune or inflammatory system. If cells are injured or die while the animal is alive, there will be an inflammatory response to the injury. Therefore, death and disintegration of cells and tissues can be associated and apparent microscopically. (Dix & Graham, 1999)

2.2.1 Degenerative changes of liver

In liver, degenerative changes are mainly seen in portal triad, hepatocytes and cytoplasm, nucleus and Kupffer cells. There is mild cloudy swelling in cytoplasm; reduced stainability of erythrocytes; atrophy of hepatocytes and sinusoidal dilatation in subcapsular region; swelling of nuclei and karyopyknosis of endothelial or Kupffer cells in epithelia of the interlobular bile ducts (IBD) after 12 hours in room temperature. By 24-hour post-mortem, there was loss of lobular architecture; degenerated portal triad; karyolysis, degeneration, fragmentation and vacuolation of nuclei of hepatocytes and loss of distinct cellular border of hepatocytes. (Kimura & Abe, 1993; Palmer *et al*., 2005).

2.2.2 Degenerative changes of brain

Neurones are very vulnerable to hypoxia which occur following cardiac arrest or respiratory arrest after death (Busl & Greer, 2010). At early stage, dead neurones become shrunken and eosinophilic with pyknosis and karyorrhexis of the nuclei. Swelling of neurone which accompanied with development of tiny holes (microvacuoles) in neuronal cytoplasm occurred after 12 hours. By 24 hour post-mortem, nuclei of neurone completely degenerated while endothelial cells and astrocytes tend to swell up and become more faded in color (Krafts, 2010).

2.1.3 Degenerative changes of muscle

Studies carried out by Tomita *et al*. (2004) and Penttila & Ahonen (1976) suggested that skeletal muscle showed the greatest delay in post-mortem changes, most probably due to lack of digestive enzyme at that region.

3.0 MATERIALS AND METHOD

3.1 TISSUE SAMPLING

The euthanised mongrel carcasses weighing 12.5 kg to 18 kg were used in this study. They were euthanized with sodium pentobarbitone intravenously. The carcasses were placed on left lateral recumbency on the examination table in the necropsy laboratory. Brain, liver, and epaxial muscle tissue samples were obtained at 0-hour, 12-hour, 18-hour, and 24-hour post-mortem. Tissue sample of the cerebrum were collected using a 7cm hollow metal tube with diameter of 3cm attached to a 10ml syringe. Liver tissue sample were obtained from four different sites through incision caudal to the last right rib. The incision was sutured using simple continuous method with nylon sutures. Epaxial muscle tissue sample were obtained by using open biopsy technique along the right epaxial muscle. Liver and epaxial muscle samples were placed in 10% formalin while brain samples were placed in 40% formalin for fixation.

3.2 HEMATOXYLIN-EOSIN STAINING

Tissues for histopathology were fixed in 10% formalin and then paraffin embedded and cut serially at 4 µm and stained with H&E in the routine manner. The slides were dipped in 100% xylene and mounted with coverslips by using DPX (dibutyl phthalate xylene), left to dry overnight. The slides were viewed by using light microscope (*Motic*, BA410 Trinocular, Hong Kong)

3.3 IMMUNOHISTOCHEMISTRY STAINING METHOD

3.3.1 Deparaffinisation and rehydration

Paraffin sections were incubated in oven (56-60°C) for 15 minutes. For deparaffinisation, paraffin sections slides were immersed in xylene for 5 minutes following by rinsing in distilled water twice subsequently. For rehydration, the slides were immersed in gradually descending strength of alcohol (100% alcohol for 3 minutes followed with rinsing in distilled water twice subsequently; 70% alcohol for 3 minutes followed with rinsing in distilled water; 50% alcohol for 3 minutes followed with rinsing in distilled water). The slides were placed in PBS for 15 minutes and blocked with liquid blocker at peripheral of the tissue sections.

3.3.2 Inactivation of peroxidase

The slides were neutralized for endogenous peroxidases with 100µl 3% hydrogen peroxidase and incubate at room temperature for 10 minutes. The slides were then rinsed in PBS for 5 minutes.

3.3.3 Antigen retrieval

The slides were incubated in 10 mM Sodium Citrate Buffer, pH 7.0, and processed in a microwave oven at lowest setting for two cycles of 10 minutes each to unmask antigens. Slides were then allowed to cool at room temperature for at least 20 minutes before rinsed in PBS for 10 minutes.

3.3.4 Protein blocking

For nonspecific protein blocking, 100µl 5% Bovine Serum Albumin (BSA) was dropped onto each slide and incubated in humidified chamber at room temperature (37°C) for 60 minutes. Slides were then drained off from 5% BSA prior to immunostaining.

3.3.5 Immunohistochemistry staining

Tissue sections were incubated for 60 minutes in a humidified chamber at 37°C with rabbit polyclonal antibody against cadaverine antigen (Abnova, Diamino-pentane polyclonal antibody, PAB0085, dilution 1: 2000). The primary antibody was diluted in Tris-buffered solution (TBS) containing 0.1% crystalline bovine serum albumin (BSA). The antigens-antibodies complex was detected by ABC-peroxidase technique using DAB (3,3'-diaminobenzidine) (Dako, , Inc., North America.) as chromogen substrate to reveal the immunoreaction. The slides were immersed in Harris Hematoxylin as cytoplasm counterstain for 5 minutes before rinsed with distilled water for 10 minutes. The slides were dipped in 100% xylene and mounted with coverslips by using DPX (Dibutyl phthalate xylene) and viewed by using light microscope (Motic, BA410 Trinocular, Hong Kong).

3.4 IMAGE ACQUISITION

Images were captured using the Motic BA410 Trinocular (Motic, Hong Kong) equipped with a MoticamPro camera (Motic, Hong Kong). Light and camera setting

were controlled using the Motic Images Plus 2.0 (Motic, Hong Kong) software. Images for analysing expression of cadaverine and H&E stained sections were captured at 20X objective lenses.. However, images for analysing periodic distribution of cadaverine overtime were captured at 10X objective lenses.

3.5 IMMUNOHISTOCHEMISTRY DATA ANALYSIS – EXPRESSION OF CADAVERINE

The IHC images were analysed using ImageJ software with IHC Profiler plugin (Rasband, 1997). The IHC profiler can be freely downloaded from Sourceforge website (https://sourceforge.net/projects/ihcprofiler/). Guidelines regarding the use of IHC profiler and embedding it to the Microsoft Windows operating system based ImageJ program can be obtained in the package (Varghese *et al*., 2014). These IHC images were subjected to the software lead to production of three images, namely, DAB, hematoxylin and a complimentary image. The DAB image was selected and analysed for the intensity of brown colour which indicate positive immunoreaction of cadaverine and cadaverine antibody. A total of 10 images from different sites on the slide per organ per dog were analysed. The average intensity of brown colouration in the images represent the expression of cadaverine in each sample. Mean expression of cadaverine in the organ was obtained by average of the expression of cadaverine in the organs from three dogs.

3.5 STATISTICAL ANALYSIS

Statistical analysis was performed using IBM SPSS version 20 software (Statistics, 2011). The data obtained were normally distributed and of equal of homogeneity of variance. Differences between groups in each organ were assessed by parametric test (One-way ANOVA) to evaluate variance with zero hour being the control group while 12-hour, 18-hour and 24-hour post-mortem as treatment group. Comparison between different organs were analysed by Post- hoc Tukey test. Correlation of mean expression of cadaverine against temperature changes were analysed by Pearson correlation. Analyses were considered as significant at $p < 0.05$.

RESULTS AND DISCUSSION

4.1 MEAN EXPRESSION OF CADAVERINE IN BRAIN, LIVER AND MUSCLE

Table 1 shows the mean expression of cadaverine across the four time points in the tissues. The ANOVA revealed significant differences in mean expression of cadaverine in brain and liver between the treatment groups ($F(3, 8) = 1177.449$, $p<0.05$ and $F(3, 8) = 105.285$, $p<0.05$ respectively). However, there was no significant difference in muscle between groups ($F(3, 8) = 0.312$, $p=0.817$).

Table 1. The expression of cadaverine across the four time points in the brain, liver and muscle (Mean±SE)

	Zero hour	12-hour	18-hour	24-hour
Brain	$5.72^{aA} \pm 1.99$	$50.9^{a} \pm 0.33$	$83.47^{aA} \pm 0.73$	$88.78^{aA} \pm 0.58$
Liver	$14.57^{aB} \pm 0.43$	$49.27^{a} \pm 2.97$	$56.24^{a} \pm 1.15$	$65.06^{a} \pm 2.87$
Muscle	$55.9^{C} \pm 6.61$	56.43 ± 3.43	54.29 ± 2.31	56.84 ± 2.17

[a]Means with different superscripts within column are significantly different
[ABC]Means with different superscripts within row are significantly different

At zero hour, expression of cadaverine is highest in the muscle, followed by the liver and brain. Cadaverine expression in muscle remain the same throughout the study period. As time advances, the expression of cadaverine in the brain is the highest ($p=0.000$), followed by the liver ($p=0.000$). However, there is no significant different in expression of cadaverine in the muscle from zero hour to 24-hour after death ($p=0.817$).

Table 2 shows the comparison between brain, liver and muscle against mean expression of cadaverine over the 24 hours post-mortem period. Post- hoc Tukey test shows that the expression of cadaverine was comparable between the brain and liver but was much higher than that of the muscle (p= 0.008 & 0.009 respectively).

Table 2. Comparison between brain, liver and muscle against mean expression of cadaverine over the 24 hours post-mortem period (Mean±SE)

Multiple Comparison		**Significant Value**
Liver	Brain	$p < 0.043$
Liver	Muscle	$p < 0.008$
Brain	Muscle	$p < 0.009$

Comparison are significant at $p< 0.05$

Figure 1 and Figure 2 show the exponential and linear curve for mean expression of cadaverine in brain, liver and muscle over the 24 hours post-mortem period. When mapped, the mean expression of cadaverine in these three organs shows a comparable pattern (Figure 1). The mean expression of cadaverine in liver and brain show a significant increase from time zero hour to 24-hour ($p<0.05$ and $p<0.05$ respectively). Studies done by Schmidt-Glenewinkel *et al*. (1977), Dolezalova *et al*. (1974) and Chang (1976) showed that the liver, brain and large intestine are organs with high lysine content. Hence, cadaverine formation are expected to be higher in these organs across the time.

From the exponential curve in Figure 1, the mean expression of cadaverine is the highest in the muscle at zero hour and remain unchanged overtime. The decomposition onset supposed to be slowest in muscle due to lack of digestive enzymes (Tomita *et al*., 2004). This is not consistent with our study that the expression of cadaverine in muscle is highest at zero time when compared to other organs. It is possible that muscle contains other component which can stained positive with IHC although it could not be proven in this preliminary study.

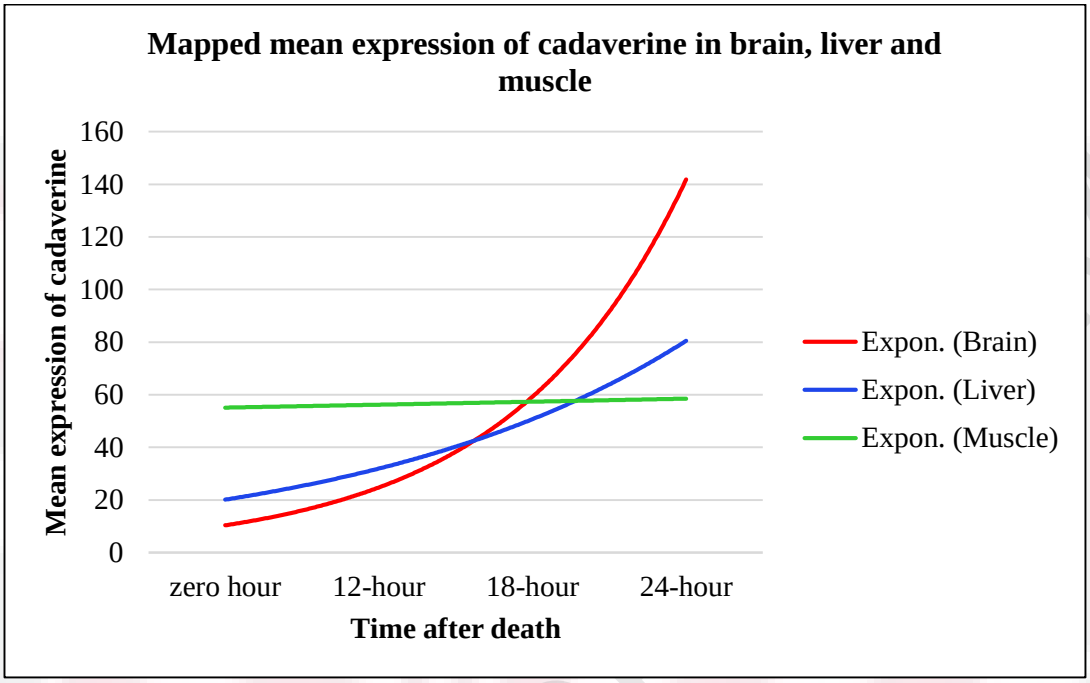


Figure 1. The exponential curve for mean expression of cadaverine in brain, liver and muscle over the 24 hours post-mortem period.

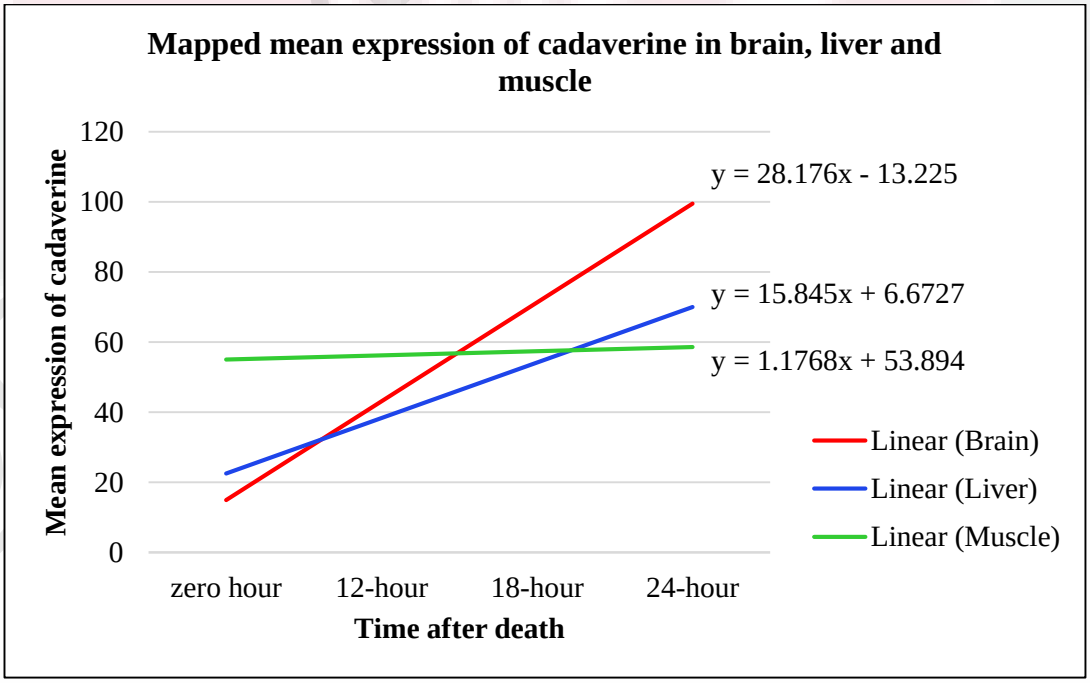


Figure 2. The linear curve for mean expression of cadaverine in brain, liver and muscle over the 24 hours post-mortem period.

From the linear equation in Figure 2, the rate of cadaverine expression in brain is much faster than that of the liver. This is likely to be due to the brain harbouring more lysine compared to other organs (Dolezalova *et al*., 1974) leading to a higher production of cadaverine.

The occurrence of autolysis is quick in tissues which have a high concentration of autolytic enzymes, such as pancreas, liver, and gastric mucosa; moderate in the heart, kidney and brain tissue; slowest in fibroblasts, which are poor in lysozymes and hydrolytic enzymes Scarpelli & Iannaccone, 1990; Penttilä & Ahonen, 1976; Van Cruchten & Van Den Broeck, 2002). This shows that expression of cadaverine over time is dependent on type of tissue that contain high lysine level which is the precursor of cadaverine. It is also depends on how fast the bacteria able to reach the organ and start decarboxylation of lysine into cadaverine. As mentioned earlier, even though liver has higher lysozymes and hydrolytic enzymes, it has lower level of lysine compared to brain. Hence rate of cadaverine expression is higher in the brain. On the same time, lysine can be degraded into other amine through different pathway. Production of cadaverine is mainly catalysed by lysine decarboxylase which produced following decomposition by bacteria. However, there is no comprehensive documentation on the pathway of lysine degradation. Hence, the usage of expression of cadaverine as PMI indicator is reliable only in organs that harbour high lysine content.

4.2 CORRELATION BETWEEN TEMPERATURE CHANGES AGAINST MEAN EXPRESSION OF CADAVERINE AGAINST IN BRAIN AND LIVER

Table 3 and Figure 3 show the correlation between mean expression of cadaverine against the temperature changes in brain and liver as time advances. There was significant correlation between temperature changes against mean expression of cadaverine in the brain ($r = -0.898$, $N = 12$, $p = 0.000$) and the liver ($r = -0.958$, $N = 12$, $p = 0.000$) but none was demonstrated in the muscle.

If new parameters or methods are recommended for PMI estimation, the reliability and accuracy should always be compared to methods developed for the same post-mortem period (Henssge & Madae, 2007). Body organ temperature changes after death (algor mortis) is worldwide accepted parameter to determine PMI. (Mall *et al*., 2005; Rutty *et al*., 2005; Henssge, 1988; Al-Alousi *et al*., 2002; Nelson, 2000; Baccino *et al*., 2007). Study conducted by Abdulazeez & Noordin (2010) and Rachel (2015) were the first in tropical country and demonstrated the reliability of body organ temperature changes as PMI indicator in Malaysia. Hence, data of temperature changes in brain and liver were obtained from study with same study design done by Rachel (2015) and compared with the mean expression of cadaverine.

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

	Mean expression of cadaverine	
Temperature changes	Brain	Liver
	-0.898*	-0.958*

* Correlations are significant at 0.05 level (2 tailed)

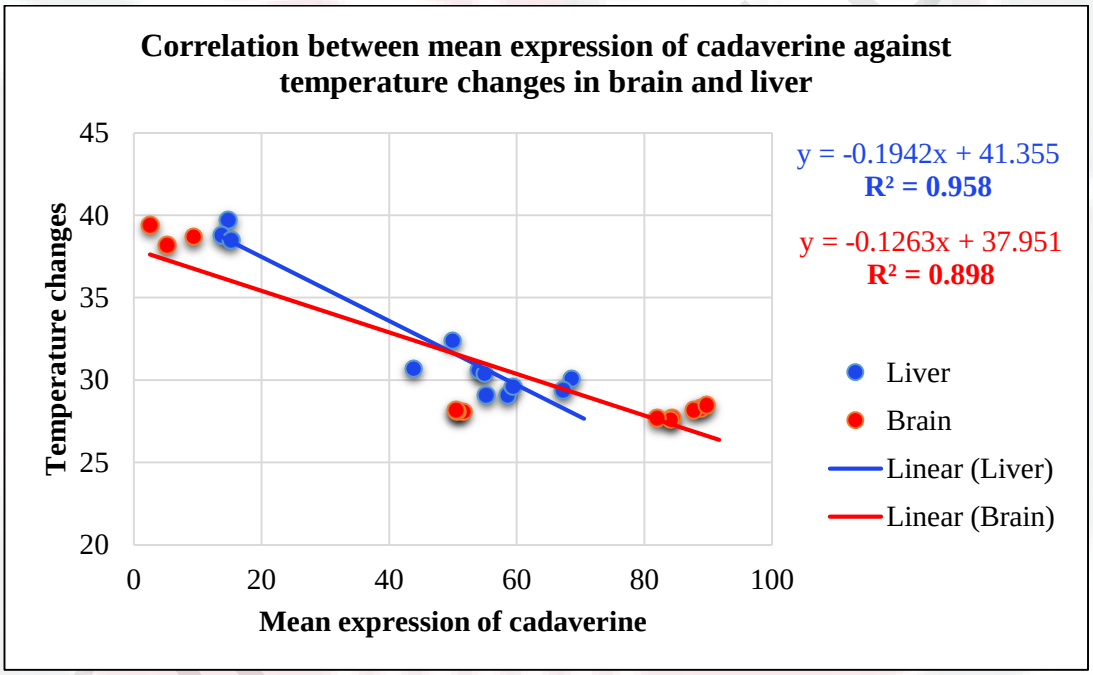


Figure 3. The linear curve for correlation between mean expression of cadaverine against the temperature changes in brain and liver throughout the 24 hours post-mortem. Strong linear correlation.

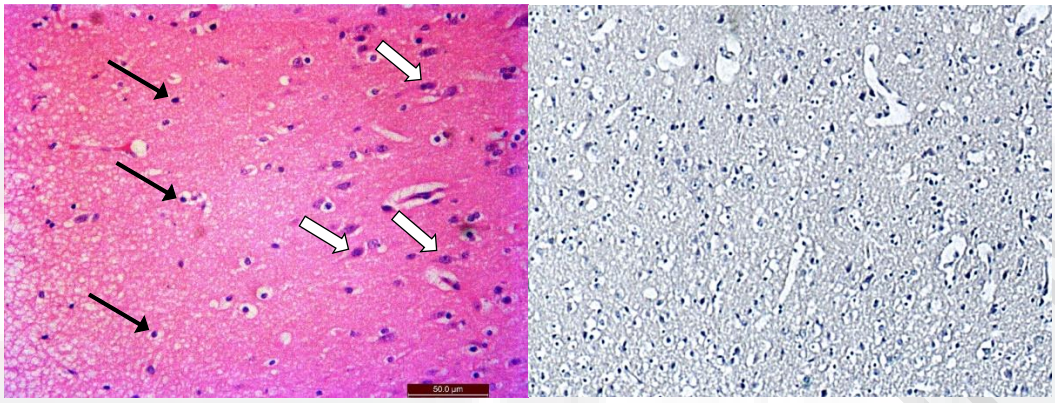
From Table 3 and Figure 3, a strong correlation between temperature changes against mean expression of cadaverine in the brain and liver indicated expression of brain and liver cadaverine is a reliable parameter to determine PMI in dog.

4.3 HISTOLOGICAL SCORES OF H&E STAINING AND IHC STAINING

Plates 1-12 show the photomicrograph of the tissues following H&E and IHC staining. The histological changes in brain, liver and muscle tissue stained with IHC were observed across zero hour, 12-hour, 18-hour, and 24-hour post-mortem. The changes with respect to localization and amount of cadaverine expressed were compared with tissue decomposition changes histologically under H&E staining.

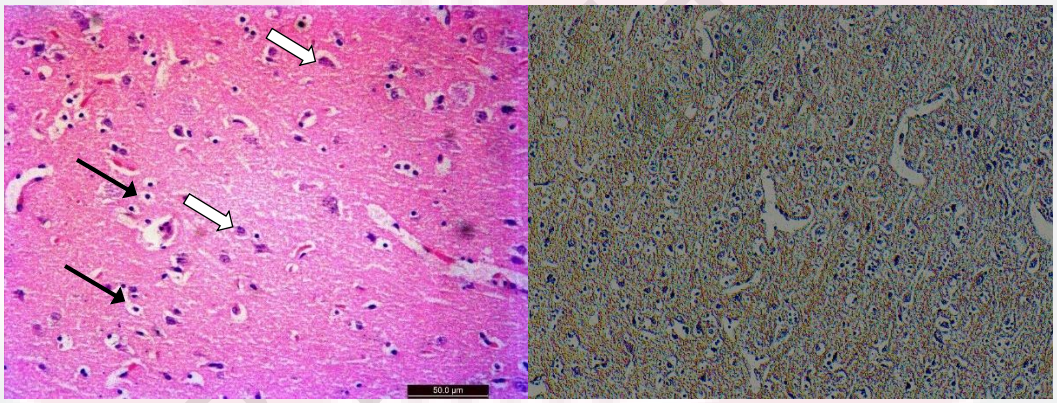
4.3.1 Brain

Plate 1 to 4 show photomicrograph of brain changes between H&E and IHC staining across the 24 hours post-mortem period. At 0-hour post-mortem, the neurons (black arrow) and neuroglia cells (white arrow) were still intact under H&E staining without any positive stain for cadaverine. At 12-hour post-mortem, almost half of the brain parenchyma was stained positively with cadaverine. The nuclei of neurons started to darken and disintegrated as seen under H&E staining. There were no much changes in 18-hour compared to that of the 12-hour post-mortem. By 24 hours post-mortem, the entire parenchyma of brain tissue stained positive with cadaverine. Disappearance of nuclei of neurons and massive vacuolization of neuroglia cells cytoplasm in brain parenchyma marked the terminal stage of brain autolysis. Accumulation of cadaverine in brain parenchyma is consistent with degeneration of neurones and neuroglia cells as observed in the H&E stained tissues.



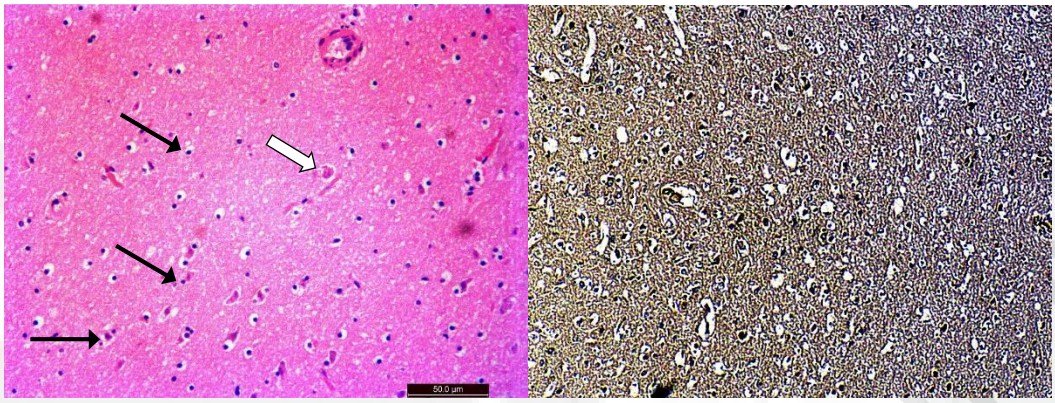
(a) **(b)**

Plate 1. Photomicrograph of brain at zero hour post-mortem under H&E (a) and IHC (b) staining. The white arrows indicate neurons while the black arrows indicate neuroglia cells. There was no positive stain of cadaverine at this point in time.



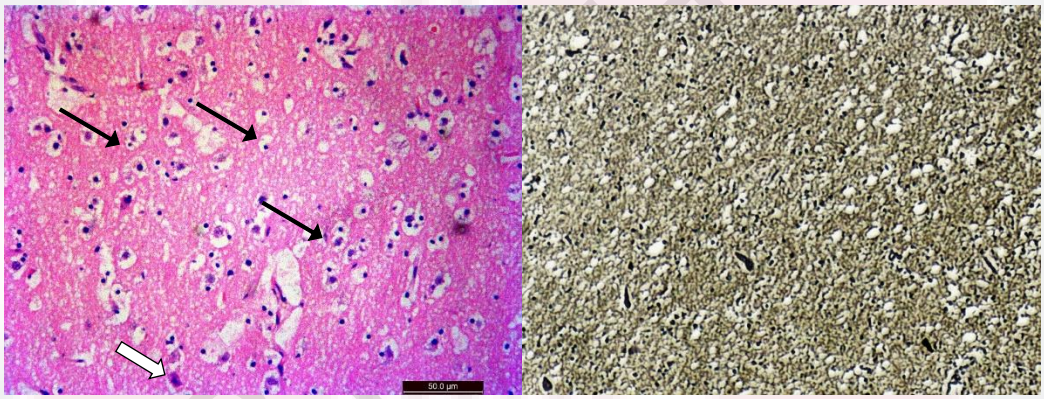
(a) **(b)**

Plate 2. Photomicrograph of brain at 12-hour post-mortem under H&E (a) and IHC (b) staining. The white arrows indicate neurons while the black arrows indicate neuroglia cells.



(a) **(b)**

Plate 3. Photomicrograph of brain at 18-hour post-mortem under H&E (a) and IHC (b) staining. The white arrows indicate neurons while the black arrows indicate neuroglia cells.

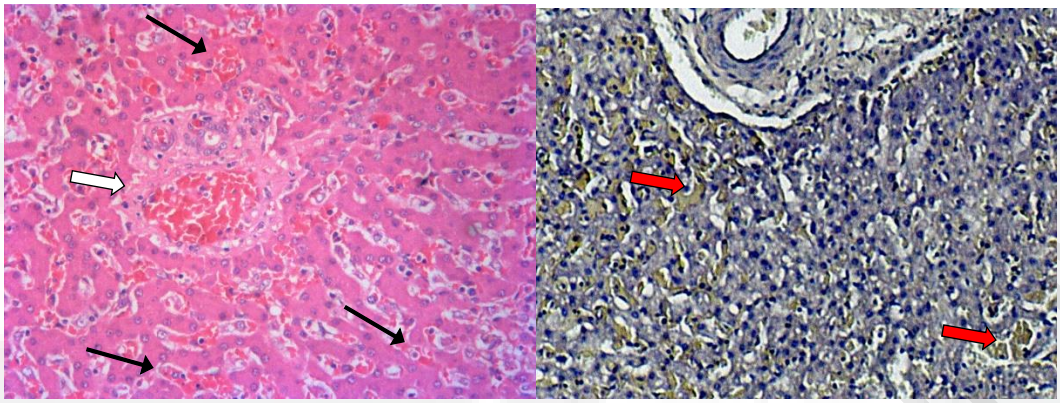


(a) **(b)**

Plate 4. Photomicrograph of brain at 24-hour post-mortem under H&E (a) and IHC (b) staining. The black arrows indicate neuroglia cells. There were no visible nuclei of neurons. The entire brain parenchyma is now stained positive with cadaverine.

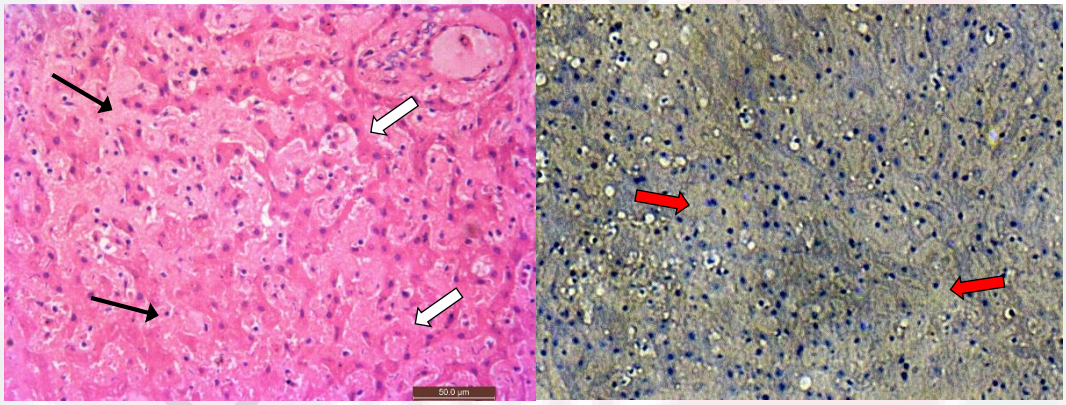
4.3.2 Liver

Plate 1 to 4 show photomicrograph of liver changes between H&E and IHC staining over the 24 hours post-mortem period. At 0-hour post-mortem, liver portal triad architecture remain intact; the hepatocyte remain intact with blood accumulated within the sinusoid under H&E staining. The cadaverine was positive staining in the sinusoid but not in the hepatocyte. At 12-hour post-mortem, the hepatocytes shrunken with accumulation of cellular cast in the sinusoid which stained positive with cadaverine. At 18-hour post-mortem, there was no much changes in localisation and amount of cadaverine expression as compared to 12-hour post-mortem period. However, there were nucleus fragmentation and cytoplasm swelling under H&E staining which are part of autolytic process. By 24 hours post-mortem, the architecture of portal triad had disintegrated. There were clear demarcation between hepatocytes, absence of hepatocyte nucleus and only hepatocytes been stained positively with cadaverine which marks the terminal stage of decomposition in liver.



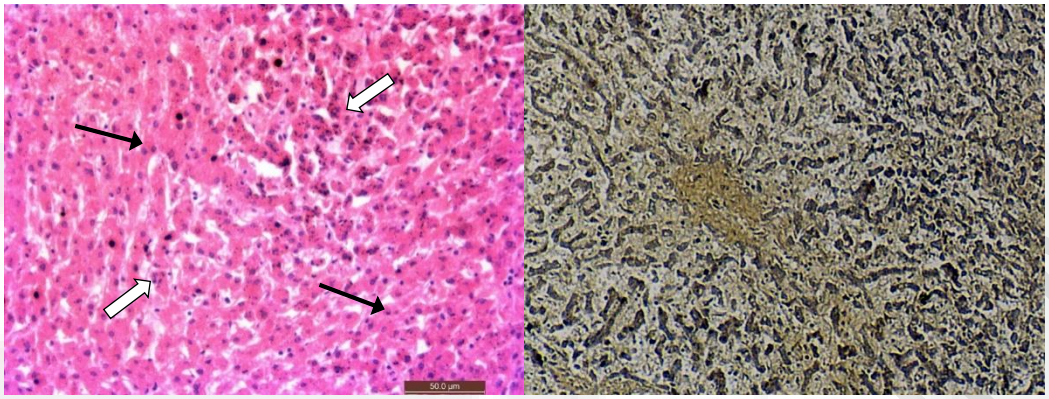
(a) **(b)**

Plate 5. Photomicrograph of liver at zero hour post-mortem under H&E (a) and IHC (b) staining. The white arrow indicates portal triad while the black arrows indicate blood accumulation within hepatic sinusoid. The red arrows show positively stained cadaverine within hepatic sinusoid.



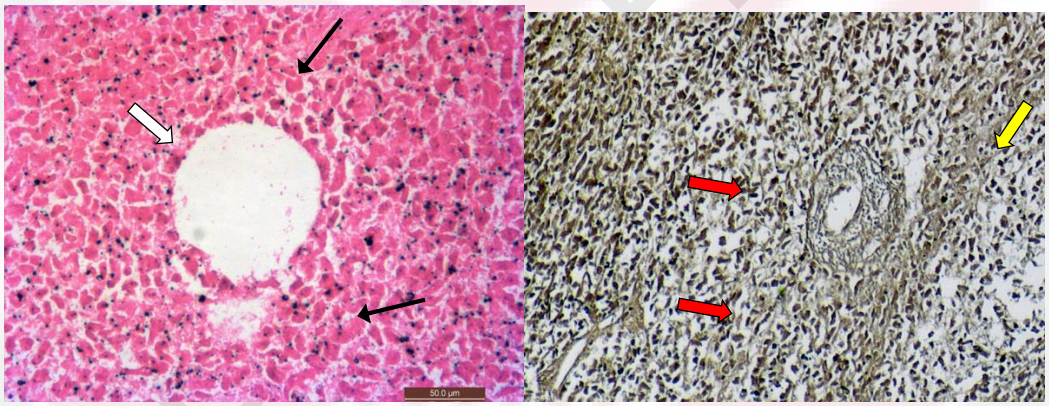
(a) **(b)**

Plate 6. Photomicrograph of liver at 12-hour post-mortem under H&E (a) and IHC (b) staining. Black arrows indicate cellular cast accumulation within hepatic sinusoid while white arrows indicate shrunken hepatocytes. The red arrows show positively stained cadaverine within hepatic sinusoid.



(a) **(b)**

Plate 7. Photomicrograph of liver at 18-hour post-mortem under H&E (a) and IHC (b) staining. White arrows show nucleus fragmentation while black arrows show cytoplasm swelling within hepatocytes. Hepatocytes and sinusoid stained positively with cadaverine.



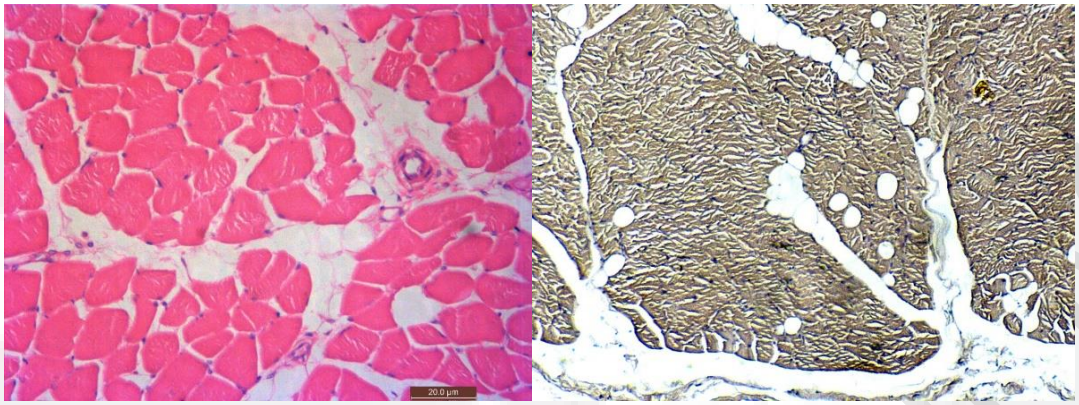
(a) **(b)**

Plate 8. Photomicrograph of liver at 24-hour post-mortem under H&E (a) and IHC (b) staining. White arrow shows disintegrated portal triad while black arrows show well demarcated hepatocytes that loss nuclei. All hepatocytes (red arrows) and partial sinusoid (yellow arrow) were stained positively with cadaverine.

Blood consist of exogenous or ready-made cadaverine by bacteria in the intestine before the animal died (Bardócz, 1995). This could be the reason of sinusoid stained positively with cadaverine at zero hour post-mortem. The cellular cast formed by 12-hour post-mortem are the decomposition product of hepatocytes which consist of cadaverine.

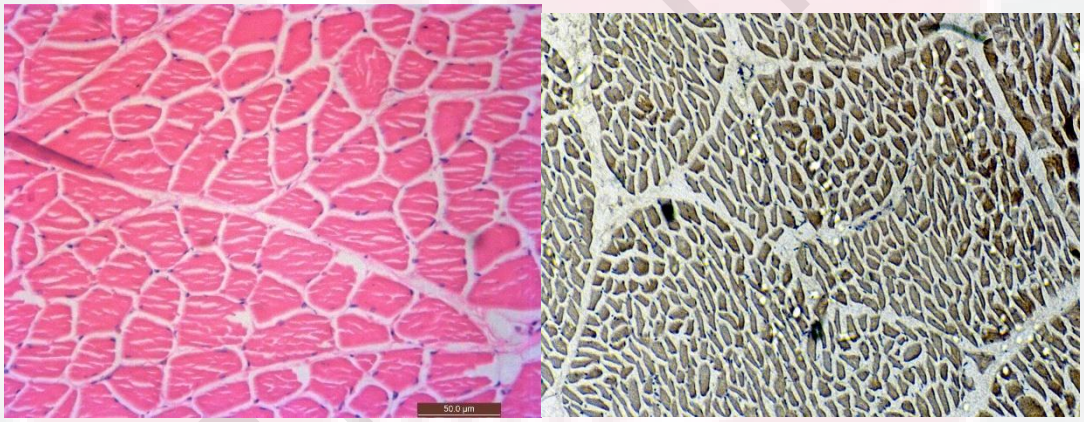
4.3.3 Muscle

In muscle, there was no significant changes over time both in H&E d and IHC stained tissues. The architecture of skeletal muscle remain similar at 0-hour and 24-hour. At zero hour and 24-hour, the whole muscle tissue were stained positively with cadaverine under IHC staining. There is no documentation explaining the reasons of muscle being stained positively with cadaverine despite the muscle undergoes the slowest rate of decomposition among all organs.



(a) **(b)**

Plate 9. Photomicrograph of epaxial muscle at zero hour post-mortem under H&E (a) and IHC (b) staining.



(a) **(b)**

Plate 10. Photomicrograph of epaxial muscle at 24-hour post-mortem under H&E (a) and IHC (b) staining.

4.4 TEMPORAL DISTRIBUTION OF CADAVERINE

4.4.1 Brain

From Plate 11, the distribution of cadaverine in brain was from the surface the parenchyma to the meninges. This suggest that decomposition of brain start from inner medulla layer of brain and extended out to cortex throughout the period of death. Brain cavity (cranial cavity) is an aseptic area where there is no digestive enzyme or bacteria. The only way for bacteria to get in is from the inner layer which consist of major blood vessels and CSF fluid which is the source of bacteria proliferation.

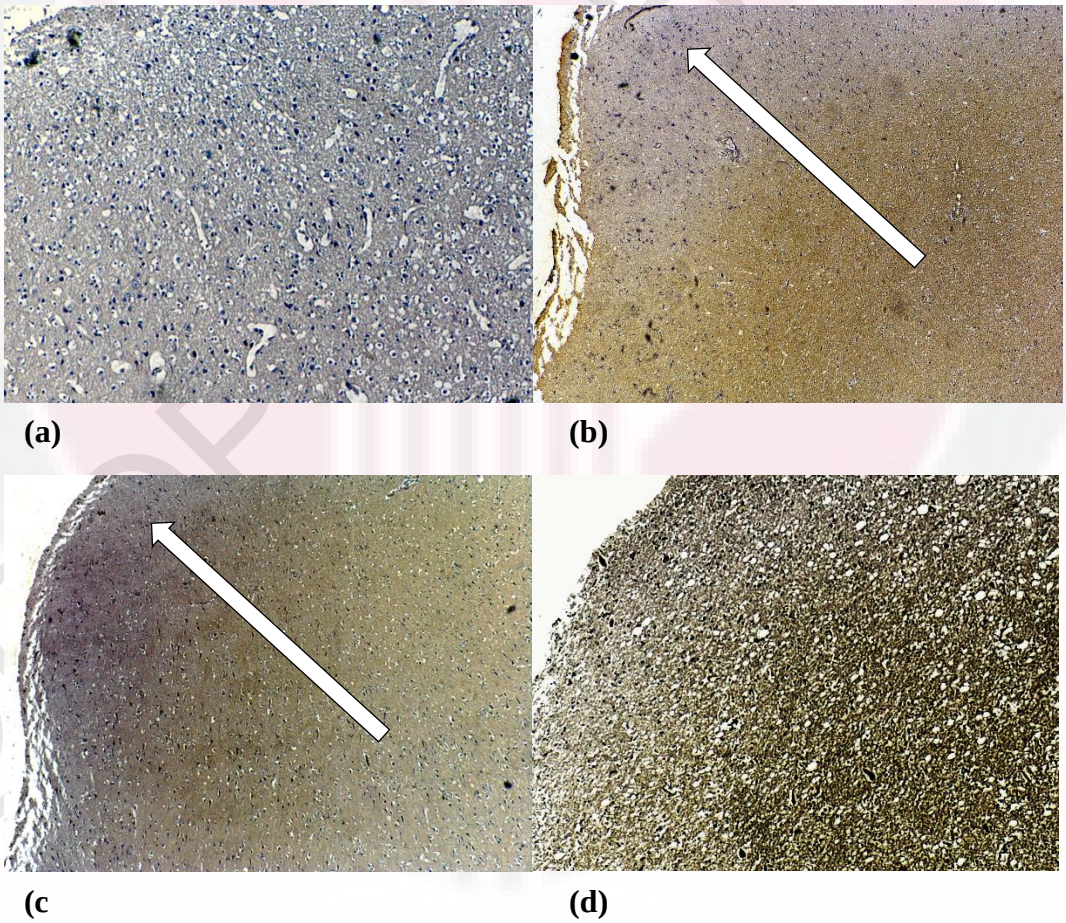


Plate 11. Temporal distribution of cadaverine in brain. (a) zero hour. (b) 12-hour. (c) 18-hour. (d) 24-hour. White arrows indicate direction of cadaverine distribution overtime.

4.4.2 Liver

In liver (Plate 12), cadaverine is expressed first in the sinusoid followed by the sinusoid again and the hepatocytes, then lastly only hepatocytes. As opposed to the brain, the changes is from the capsule to the parenchyma. Presence of cadaverine in the blood at zero hour is probably due to exogenous cadaverine as mentioned earlier as the liver is within abdominal cavity. When an animal dies, autolysis and putrefaction start in intestine and spread quite rapidly and easily to liver owing to the vicinity of this organ to the intestines. Therefore, autolysis or putrefaction of the liver mainly begins or is faster from the capsule to the parenchyma.

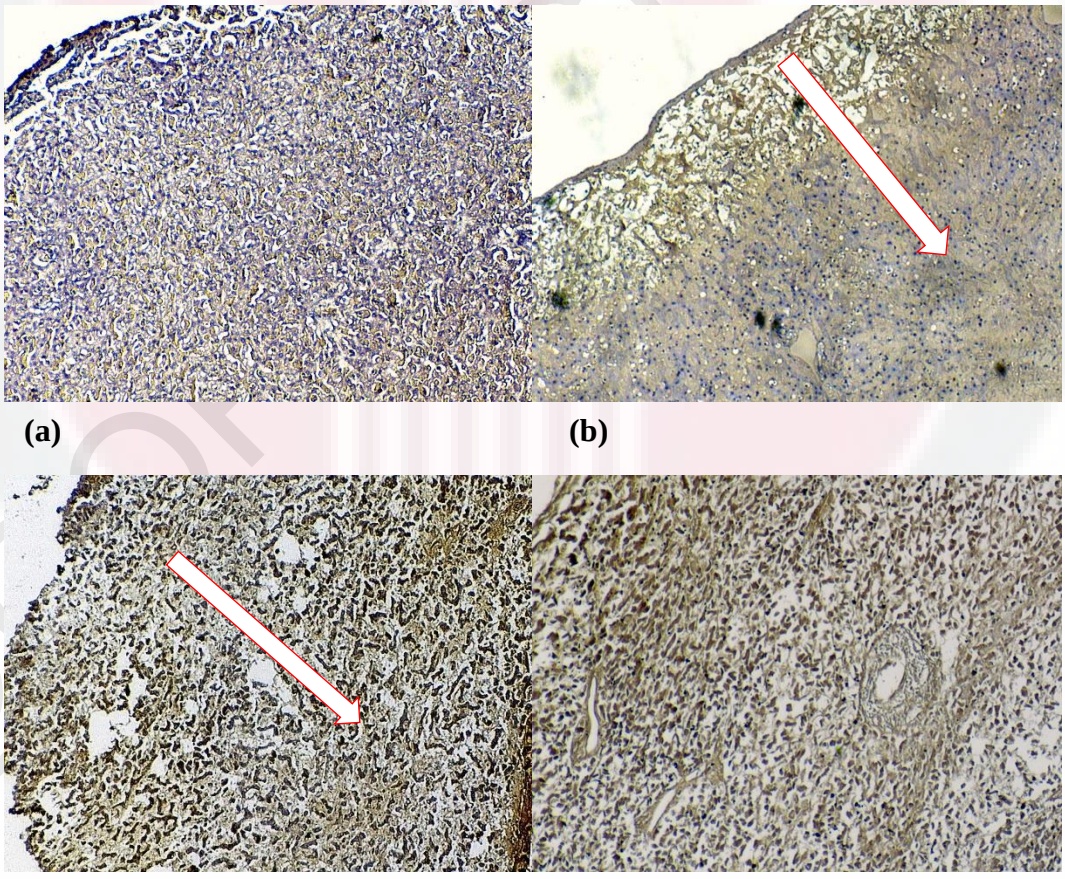


Plate 12. Temporal distribution of cadaverine in liver. (a) zero hour. (b) 12-hour. (c) 18-hour. (d) 24-hour. White arrows indicate direction of cadaverine distribution overtime.

CONCLUSION

Brain and hepatic cadaverine may serve as potential indicators for the determination of PMI. Expression of cadaverine correlate well with temperature and histopathological changes in the brain and liver within a 24 hours after death.

RECCOMENDATIONS

Although a potential estimator nut the usage of cadaverine expression alone cannot provide a reliable PMI. Therefore, similar study should be conducted by using other biogenic amines such as putrescine and spermidine, prolonging the study period and the use of different species of animal. Combination of different biogenic amines may enable a more accurate and reliable PMI. Ultimately, study should be conducted by integrating different PMI parameter into a system of different parameters which furthermore increase the accuracy of PMI.

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