

## Determination of Time of Death Based on Basic Histological Stain and Immunostain Changes

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### ABSTRAK

*Penganggaran masa kematian telah dikaji dengan meluas semenjak 30 tahun. Antara pelbagai parameter yang telah dikaji ialah suhu badan mayat, biokimia rigor mortis, perubahan putrefaktif dan entomologi. Walaupun demikian, kesemua kaedah ini amat dipengaruhi oleh faktor luaran seperti perubahan suhu persekitaran dan aktiviti yang telah dilakukan sebelum mati. Untuk memperbaiki permasalahan ini, kami memerhatikan kepada mekanisme semasa proses kematian berlaku. Setakat ini pelbagai kajian telah mendapati bahawa pada permulaan proses kematian, jantung dan perfusi kepada sel akan berhenti. Ini akan menyebabkan sel memulakan proses kematian. Kematian sel ini akan berlaku melalui salah satu daripada dua mekanisme kematian iaitu apoptosis ataupun nekrosis. Sewaktu apoptosis, sel akan mengucis beberapa jujukan protein. Berdasarkan kefahaman ini maka satu kajian telah dijalankan untuk menentukan sama ada nisbah keluasan apoptosis: nekrosis dan petanda apoptosis p53 dan Bcl-2 boleh digunakan sebagai petanda sela masa kematian yang baik (postmortem interval, PMI). Pensampelan telah melibatkan 100 kulit mayat manusia segar dengan PMI yang diketahui dengan tepat dan kesemuanya telah diperolehi daripada unit forensik Hospital Kuala Lumpur (UFHKL). Nisbah keluasan apoptosis: nekrosis ditentukan dengan menggunakan pewarnaan hematoksilin dan eosin manakala petanda apoptosis p53 dan Bcl-2 pula digunakan dengan menggunakan kit apoptosis. Sesemua pewarnaan kemudiannya diindeks dan diplotkan melawan PMI yang diperolehi daripada UFHKL. Keputusan menunjukkan bahawa tidak terdapat korelasi yang signifikan bagi nisbah apoptosis: nekrosis lawan PMI ( $p = 0.144$ ). Korelasi bagi indeks apoptosis iaitu p53 dan bcl-2 juga didapati signifikan apabila diplot lawan PMI ( $p < 0.000$  untuk kedua-duanya). Secara kesimpulan, kami mencadangkan bahawa p53 dan bcl-2 perlu dikaji dengan lebih lanjut ia berkemungkinan besar boleh menjadi satu alat yang baik dalam pengukuran PMI.*

*Kata kunci: sela masa kematian, apoptosis, nekrosis, p53, protein bcl-2.*

### ABSTRACT

*Establishing time of death has been extensively studied for the last 30 years. Parameters that have been studied included body temperature, biochemistry of rigor mortis, putrefactive changes and entomology. Despite an extensive study in these parameters it was found that all of the parameters were very much dependent on external factors like changes in surrounding temperature and activities done prior to death. To solve this problem, we decided to monitor the mechanism that occurs during death. Until now, various researches have found that during the early stage of death, heart and perfusion to the cells will stop. This will cause the cells to start the death process. The death of the cell will occur either through apoptosis or necrosis. During apoptosis the cells will switch on and off a few proteins in a sequence. Based on this understanding, a study was conducted to determine if area ratio of apoptosis: necrosis and apoptotic p53 and Bcl-2 markers can be used as a reliable postmortem interval marker (PMI). Sampling of the study had involved 100 dead human skins with a known PMI. All samples were obtained from forensic unit of Hospital Kuala Lumpur (UFHKL). Ratio of apoptosis: necrosis areas were determined using hematoxylin and eosin staining while apoptosis p53 and Bcl-2 markers were done using an apoptosis kit. All staining were then indexed and plotted against PMI data obtained from UFHKL. Results indicated that there were no significant correlations between ratio of apoptosis: necrosis area against PMI ( $p = 0.144$ ). Whereas for both apoptotic markers p53 and Bcl-2 PMI had shown a significant correlation ( $p < 0.000$  for both results). In conclusion, we suggest that p53 and Bcl-2 parameters should be studied further since it is very likely that it could be a good indicator for PMI.*

*Key words: postmortem interval, apoptosis, necrosis, p53, bcl-2 protein.*

## INTRODUCTION

Establishing the time of death has been a real and huge complicating task in forensic field for years (Kahana et al. 1999). The role of body temperature, rigor mortis biochemistry putrefactive changes and insect succession have all been extensively studied (Vanezis & Trujillo 1996). However, these findings concentrated more on the aspects of macroscopic changes rather than microscopic changes. Thus a high margin of error was created and which eventually lead to an inaccurate postmortem interval (PMI) result (Al-Alousi et al. 2001).

Previously, a similar study was done on histological changes (Khairul 2003). Results from the study indicated an increased in apoptotic area but decreased in necrotic areas in the early stage of PMI. As PMI progressed, the apoptotic areas were found to decrease and the necrotic area increased. This indicated that histological changes could be used as an indicator of PMI. However, the applicability of the study to human was in question since it utilised an animal model rather than a human model.

It is now established that generation of reactive oxygen species (ROS) through lipid peroxidation (LPx) can cause cell death either by apoptosis or necrosis, two distinct cell death pathways (Higuchi & Yoshimoto 2002). Necrosis occurs usually in response to severe injury and is characterised morphologically by cytoplasmic and mitochondrial swelling, plasma membrane rupturing and release of cellular contents into cellular space (Proskuryakov et al. 2003). Apoptosis by contrast, is a tightly regulated form of cell death and is characterised by morphological and biochemical changes (Kiechle & Zhang 2002). These include mitochondrial depolarization and alterations in phospholipids asymmetry, chromatin condensation, nuclear fragmentation, membrane blabbing, cell shrinkage and the formation of membrane bound vesicles termed apoptotic-body (Yoon & Gores 2002). This process can increase the number of cells going to apoptosis. However, the severity of apoptosis in cells can also be increased or decreased by the presence of apoptotic inducers such as p53 marker and apoptotic inhibitor such as Bcl family protein (e.g: bcl-2) (Wang et al. 1999). Based on this understanding, a study was conducted to determine if ratio of apoptosis: necrosis area and apoptotic p53 and Bcl-2 markers can be used as a reliable postmortem interval marker (PMI).

## MATERIALS AND METHODS

A total of one hundred fresh dead human skin obtained from the lower ventral part of the abdomen skin (diameter: 1 cm x 1 cm x 1.5 cm) were collected. All samples were sourced from the Forensic Unit of General Hospital of Kuala Lumpur (FUHKL). Collection and selection was all under the supervision its Director, Dr. Abdul Halim Haji Mansar. Selection of the dead human skin was based on known PMI, race of either Indian or Chinese, storage time in the freezer within 2 hrs after pronounced death and the cause of death must not be due to drugs or anything that would accelerate the decomposition process faster than normal. All tissues were then processed for sectioning and stained using basic stain – H&E based on a method by (Khairul 2003). Apoptotic cells were recognised as cells with intact nuclear membrane, cytoplasmic shrinkage and nuclear fragmentation while necrotic cells were loss of membrane integrity, swelling of organelles and irregular clumping of chromatin. Immunostaining was done using apoptosis kit (Novo Castra Laboratories, UK). This kit was used to identify areas of tissues that expressed p53 and bcl-2 proteins. Tissue areas that expressed p53 were seen as intense nuclear staining in slides while those with bcl-2 had a cytoplasmic staining (Takes et al. 2001). All three staining were then indexed based on method by (Langlois et al. 2000). All the data obtained from each sample was then plotted against their PMI. A linear regression analysis was done for the ratio apoptotic: necrosis vs. log PMI and log Bcl-2 vs. 1/log PMI, while a cubic curve estimation regression was done for the 1/ln p53 vs. PMI. All statistical analysis was done using SigmaStat (USA: Jandel Scientific Software). Significant level were set at  $p < 0.05$ .

## RESULTS AND DISCUSSION

Fig 1 shows that apoptosis area decreases as the tissue decomposes. Regression analysis on the data obtained indicated that the downward trend is not significant ( $p = 0.144$ ) with PMI data. Its regression

square value was found to be at only 0.374. Fig 2 and 3 shows the downward trend of Bcl-2 and p53 expression in decomposing tissue respectively. A linear regression analysis indicated that the reduction seen in Bcl-2 was significant ( $p < 0.000$ ) when correlated with their PMI data. An upward trend was seen in the p53 expression. The trend was found to be significant ( $p < 0.0000$ ) against their PMI. Their regression square value of Bcl-2 and p53 was found to be at 0.938 and 0.993 respectively.

Based on the results it clearly indicates that data on the ratio area of apoptosis: necrosis is an unsuitable indicator for PMI (Figure 1). In general the current trend of the tissue seemed to lead to an increase area of necrosis compared to apoptosis as the tissue decomposed. Various mechanisms could have played a role in what was observed and this included the pro-inflammatory and DNA repair mechanisms (Jamison et al. 2004).

When brain death occurs, pro-inflammatory agents like tumor necrosis factor-alpha (TNF-alpha), interleukin (IL)-1beta, and IL-6 are released in large quantities (Contreras et al. 2003). The release of these chemicals will then lead to increase cell death by necrosis. Apart from the release of pro-inflammatory factors, absence of perfusion would also result in DNA damage. This would then activate an enzyme called poly (ADP-ribose) polymerase (PARP-1) which would then cause a significant decrease in NAD(+), massive adenosine triphosphate (ATP) depletion and cell death by necrosis rather than by apoptosis (O'Valle et al. 2004). Despite the two mechanisms it can be concluded that necrosis predominates cell death as tissue decomposes.

Both expressions of p53 and Bcl-2 were found to change in relation to PMI. In general it is clear that apoptosis was functioning during the tissue decomposition. It is also very likely that since ischemia is occurring on the skin tissue due to an absence of perfusion, cells were very likely undergoing anaerobic metabolism (Rosenfeldt et al. 1998). The utilisation of anaerobic to produce ATP will result in high production of reactive oxygen species (ROS). These will then result in very short-lived inflammatory and endothelial cell-cell interactions and calcium overload, resulting in enhanced release of pro-apoptotic genes from mitochondria (Zhao 2004). ROS will also activate mitogen-activated protein kinases, stimulate nuclear factor-kappaB and promote synthesis of tumour necrosis factor-alpha, another pathway to activate apoptosis.

Further analysis on the apoptosis mechanism has shown that as human tissue decomposes expression of p53 increases dramatically (Figure 2) while Bcl-2 decreases slowly (Figure 3). The extensive expression of p53 in this study confirms studies conducted by (Leker et al. 2004) in which they had also found that expression of p53 was closely linked with cell death due to ischemia. It is felt that the slow reduction of Bcl-2 expression was closely linked to two major factors, which are the resistance of the cell to undergo apoptosis and the destruction of surface cells proteins due to high amount of circulation free radicals. Although there was significant reduction of bcl-2 as the tissue decomposed we felt that the cells were actually trying to increase bcl-2 expression and so inhibiting the apoptosis process (Cao et al. 2001; 2002). The slow reduction seen in Figure 2 could likely be due to the interaction of free radicals on the bcl-2 proteins resulting in a much lower population of intact bcl-2. Since the apoptotic kit is only capable of identifying intact bcl-2, true amount of bcl-2 was actually under reported.

Despite the surprising result from bcl-2 it is very clear that bcl-2 and p53 could be used as a marker to determine PMI. Despite encouraging results, further studies must be done to extend the PMI beyond 18 hrs.

## CONCLUSION

In conclusion, we suggest that p53 and Bcl-2 parameters should be studied further since it is very likely that it could be a good indicator for PMI.

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## REFERENCES

- Al-Alousi, L. M., Anderson, R. A., Worster, D. M. & Land, D. V. 2001. Multiple-probe thermography for estimating the postmortem interval: II. Practical versions of the Triple-Exponential Formulae (TEF) for estimating the time of death in the field. *J. Forensic Sci.* 46 (2): 323-327.

- Cao, Y. J., Shibata, T. & Rainov, N. G. 2001. Hypoxia-inducible transgene expression in differentiated human NT2N neurons-a cell culture model for gene therapy of postischemic neuronal loss. *Gene Ther.* 8 (17): 1357-1362.
- Cao, Y. J., Shibata, T. & Rainov, N. G. 2002. Liposome-mediated transfer of the bcl-2 gene results in neuroprotection after in vivo transient focal cerebral ischemia in an animal model. *Gene Ther.* 9 (6): 415-419.
- Contreras, J. L., Eckstein, C., Smyth, C. A., Sellers, M. T., Vilatoba, M., Bilbao, G., Rahemtulla, F. G., Young, C. J., Thompson, J. A., Chaudry, I. H. & Eckhoff, D. E. 2003. Brain death significantly reduces isolated pancreatic islet yields and functionality in vitro and in vivo after transplantation in rats. *Diabetes.* 52 (12): 2935-2942.
- Higuchi, Y. & Yoshimoto, T. 2002. Arachidonic acid converts the glutathione depletion-induced apoptosis to necrosis by promoting lipid peroxidation and reducing caspase-3 activity in rat glioma cells. *Arch. Biochem. Biophys.* 400 (1): 133-140.
- Jamison, J. M., Gilloteaux, J., Nassiri, M. R., Venugopal, M., Neal, D. R. & Summers, J. L. 2004. Cell cycle arrest and autophagy in a human bladder carcinoma cell line following Vitamin C and Vitamin K3 treatment. *Biochem Pharmacol.* 67 (2): 337-351.
- Kahana, T., Almog, J., Levy, J. & Shmeltzer, E. 1999. Marine taphonomy: adipocere formation in a series of bodies recovered from a single shipwreck. *J. Forensic Sci.* 44 (5): 897-901.
- Khairul, O. 2003. Penentuan lesi antemortem dan postmortem berdasarkan petanda peroksidasi lipid dan pelbagai logam surih. Doktor Falsafah. Universiti Kebangsaan Malaysia.
- Kiechle, F. L. & Zhang, X. 2002. Apoptosis: biochemical aspects and clinical implications. *Clinica Chimica Acta.* 326 (1-2): 27-45.
- Langlois, N. E. I., Eremin, O. & Heys, S. D. 2000. Apoptosis and prognosis in cancer: Rationale and relevance (atas talian). [http://www.resed.ac.uk/journal/vol45\\_4/4540002.htm](http://www.resed.ac.uk/journal/vol45_4/4540002.htm) (3 September 2001 2000)
- Leker, R. R., Aharonowiz, M., Greig, N. H. & Ovardia, H. 2004. The role of p53-induced apoptosis in cerebral ischemia: effects of the p53 inhibitor pifithrin alpha. *Exp Neurol.* 187 (2): 478-486.
- O'Valle, F., Benitez, M. C., Gomez-Morales, M., Bravo, J., Osuna, A., del Moral, R. M., Martin-Oliva, D., Oliver, F. J. & del Moral, R. G. 2004. Correlation of morphological findings with functional reserve in the aging donor: role of the poly (ADP-ribose) polymerase. *Transplant Proc.* 36 (3): 733-735.
- Proskuryakov, S. Y., Konoplyannikov, A. G. & Gabai, V. L. 2003. Necrosis: a specific form of programmed cell death? *Exp Cell Res.* 283 (1): 1-16.
- Rosenfeldt, F. L., Richards, S. M., Lin, Z., Pepe, S. & Conyers, R. A. 1998. Mechanism of cardioprotective effect of orotic acid. *Cardiovasc Drugs Ther.* 12 Suppl 2: 159-170.
- Takes, R. P., Baatenburg de Jong, R. J., Wijffels, K., Schuurin, E., Litvinov, S. V., Hermans, J. & van Krieken, J. H. J. M. 2001. Expression of genetic markers in lymph node metastases compared with their primary tumours in head and neck cancer. *J Pathol.* 194: 298-302.
- Vanezis, P. & Trujillo, O. 1996. Evaluation of hypostasis using a colorimeter measuring system and its application to assessment of the post-mortem interval (time of death). *For. Sci. Int.* 78 (1): 19-28.
- Wang, N., Verna, L., Hardy, S., Zhu, Y., Ma, K. S., Birrer, M. J. & Stemerman, M. B. 1999. C-Jun triggers apoptosis in human vascular endothelial cells. *Circ Res.* 85 (5): 387-393.
- Yoon, J. H. & Gores, G. J. 2002. Death receptor-mediated apoptosis and the liver. *J Hepatol.* 37 (3): 400-410.
- Zhao, Z. Q. 2004. Oxidative stress-elicited myocardial apoptosis during reperfusion. *Curr Opin Pharmacol.* 4 (2): 159-165.

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