UNIVERSITI PUTRA MALAYSIA

THE EFFECTS OF DIFFERENT CRYOPRESERVATION CONDITIONS ON FELINE PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs) VIABILITY

SITI AISYAH BINTI AZHAR

FPV 2016 34

THE EFFECTS OF DIFFERENT CRYOPRESERVATION CONDITIONS ON FELINE PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs) VIABILITY.

SITI AISYAH BINTI AZHAR

A project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfillment for the requirement of the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia

43400 UPM, Serdang, Selangor Darul Ehsan

Malaysia.

MARCH 2016

It is hereby certified that we have read this project paper entitled “The Effects Of Different Cryopreservation Conditions On Feline Peripheral Blood Mononuclear Cells (PBMCs) Viability”, by Siti Aisyah binti Azhar and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 - Project

DR. FARINA MUSTAFFA KAMAL
DVM (UPM), PhD (UC Davis, USA)
Senior Lecturer,
Faculty of Veterinary Medicine, Universiti Putra Malaysia
(Supervisor)

DR. KHOR KUAN HUA
DVM (UPM), PhD (Queensland, Australia)
Senior Lecturer,
Faculty of Veterinary Medicine, Universiti Putra Malaysia
(Co-Supervisor)

DR. MOHD. HEZMEE MOHD. NOOR
DVM (UPM), PhD (Queensland, Australia)
Senior Lecturer,
Faculty of Veterinary Medicine, Universiti Putra Malaysia
(Co-Supervisor)

DEDICATIONS

"Hakuna matata"

-The Lion King, 1994-

To my dearest family and friends,

thank you for making me the way I am today.

ACKNOWLEDGEMENT

In the name of Allah, the Beneficent, the Merciful. I would like to thank the Almighty God for giving me life and granting my wish in completing this project. Million thanks to my parents, Rohayah binti Abdullah and Azhar bin Awi; my two lovely aunts, Rosnine binti Abdullah and Noraini binti Abdullah; and my younger brother, Muhammad Anwar bin Azhar for loving and raising me with all of their heart. My deepest gratitude to my supervisor, Dr. Farina Mustaffa Kamal and co-supervisors, Dr. Khor Kuan Hua and Dr. Mohd. Hezmee Mohd. Noor, for their guidance throughout the project. I would also like to thank Mr. Mohamad Habid bin Hasan, research assistant who has been sharing his knowledge and willing to help me throughout my final year project. My deepest gratitude to the staffs of University Veterinary Hospital (UVH), especially Dr. Purshyla Manikam, Dr. Hemadevy Manoraj, Dr. Jessie Bay, Mr. Maniam Munusamy, Mr. Mohd. Faizal Hamzah and Mr. Mohd. Azuwan Abu Hassan for being very helpful during my sampling. I would also like to thank Dr. Nor Emeliawati Mohd. Napiah, the private veterinarian for assisting me in my sample collection, and all the cat owners for their willingness in allowing their cats to participate in this research.Million thanks to Dr. Yeap Swee Keong and Dr. Nadia from Faculty of Biotechnology and Biomolecular Science for assisting me in flow cytometry procedure. Thank you to Prof. Mohamed Ariff Omar for assisting me in statistical analysis. Not to forget my dearest friends, Raquel Yong, Nuur Fatin, Nik Fatin, Husna Omar, Husna Atika, Tan Shin Yi, Jong Kwang Yan, Sameerah Hani, and Hakim Narwawi for lending a hand when they are most needed. Last but not least, thank you to all of my classmates, DVM 2016 for being a part of my life.

LIST OF CONTENTS

LIST OF TABLES

Page number:

LIST OF FIGURES

ABBREVIATIONS

%	percentage
^{o}C	degree Celcius
7-AAD	7-Aminoactinomycin
DMSO	Dimethyl sulfoxide
FBS	Fetal Bovine Serum
FCoV	Feline Coronavirus
FeLV	Feline Leukaemia Virus
FIV	Feline Immunodeficiency Virus
FS	Feline Serum
HBSS	Hank's Balanced Salt Solution
K_2EDTA	Potassium Ethylenediamine tetraacetic acid
IACUC	Institutional Animal Care and Use Committee
ID	Identification
mL	millilitre
PBMCs	Peripheral Blood Mononuclear Cells
PBL	Peripheral Blood Lymphocyte
PBS	Phosphate Buffered Saline
PI	Propidium iodide
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
UPM	Universiti Putra Malaysia
UVH	University Veterinary Hospital

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek.

IMPAK BERLAINAN KONDISI PENGAWETANKRIO TERHADAP KEBOLEHHIDUPAN SEL DARAH MONONUKLEAR KUCING

Oleh

SITI AISYAH BINTI AZHAR

2016

Penyelia: Dr. Farina Mustaffa Kamal

Penyelia Bersama: Dr. Khor Kuan Hua

Dr. Mohd. Hezmee Mohd. Noor

Sel darah mononuklear kucing (PBMCs) mengandungi subset sel imuniti, seperti lymfosit dan monosit. Pencirian fungsi imun mereka amat berkait dalam menyediakan maklumat asas mengenai mekanisme dan tindak balas sel. Dalam kajian ini, suhu dan komposisi media pengawetankrio diselidik untuk mengenal pasti kesannya terhadap kebolehhidupan PBMC kucing. Sampel darah telah diperolehi dari 15 ekor kucing sihat dan PBMC kucing diasingkan menggunakan teknik emparan kecerun-tumpatan. PBMC tersebut diawetkrio dalam -196°C untuk 2 minggu dalam tiga media pengawetankrio berlainan kondisi, iaitu 10% DMSO bersama 90% FBS, 10% DMSO bersama 90% serum kucing (FS), kombinasi 10%

DMSO bersama 45% FBS dan 45% FS. Tambahan lagi, dua suhu berlainan (4°C dan suhu bilik) media pengawetankrio juga dinilai. Kebolehhidupan sel dinilai menggunakan teknik pengecualian pewarna Trypan Blue dan sitometri aliran. Hasil kajian dari teknik pengecualian pewarna menunjukkan bahawa penambahan FS memberi pemulihan yang sama terhadap PBMC kucing sebaik sahaja dicairkan berbanding penggunaan FBS. Walaubagaimanapun, hasil kajian dari sitometri aliran menunjukkan pemulihan PBMC kucing yang lebih tinggi apabila FS digunakan berbanding FBS. Tambahan pula, suhu media pengawetankrio yang berbeza tidak memberi perbezaan ketara dalam pemulihan PBMC kucing setelah pengawetankrio. Keseluruhannya, penggunaan FS dalam media pengawetankrio memberi keputusan yang sama terhadap kadar pemulihan PBMC kucing sebaik sahaja dicairkan berbanding penggunaan FBS. Tambahan lagi, FS mengekalkan kebolehhidupan PBMC kucing dalam jangka masa yang lebih panjang. Walaubagaimanapun, peranan mereka dalam titik keputusan akhir penilaian klinikal lain tidak boleh dikecualikan.

Kata kunci: PBMC kucing, pengawetankrio, komposisi media, suhu media, kebolehhidupan sel, sitometri aliran

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 - Project.

THE EFFECTS OF DIFFERENT CRYOPRESERVATION CONDITIONS ON FELINE PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS) VIABILITY.

By

SITI AISYAH BINTI AZHAR

2016

Supervisor: Dr. Farina Mustaffa Kamal

Co-supervisors:Dr. Khor Kuan Hua

Dr. Mohd. Hezmee Mohd. Noor

Peripheral blood mononuclear cells (PBMCs) contain immune cell subsets such as lymphocytes and monocytes. Characterisation of their immune functions are pertinent in providing fundamental information on its mechanism and response. In this study, the temperature and composition of cryopreservation media were examined to determine the effects on feline PBMCs viability. Blood samples were obtained from 15 healthy cats and feline PBMCs were isolated using density gradient centrifugation technique. The PBMCs were stored in -196°C for 2 weeks in cryopreservation media of three different conditions; namely, 10% DMSO with 90% FBS, 10% DMSO with 90% feline serum (FS) and the combination of 10% DMSO with 45% FBS and 45% FS. In addition, two different temperatures (4°C and room

temperature) of cryopreservation media were assessed. The cell viability was measured by using trypan blue dye exclusion technique and viability staining using flow cytometry. The result from dye exclusion technique showed that the addition of FS provide a similar recovery of feline PBMCs immediately upon thawing as compared to the utilization of FBS. However, the result from flow cytometric analysis revealed a higher PBMCs viability when using FS as compared to FBS. In addition, different cryopreservation medium temperature did not provide significant recovery of feline PBMCs after cryopreservation.Overall, the use of FS in cryopreservation media has provided comparable feline PBMCs recovery compared to FBS immediately upon thawing. In addition FS may play a role in maintaining feline PBMCs viability. However, their role in the clinical endpoints of other assays could not be excluded.

Key words: Feline PBMCs, cryopreservation, medium composition, medium temperature, cell viability, flow cytometry

1.0 INTRODUCTION

Peripheral blood mononuclear cells (PBMCs) contain immune cell subsets, namely lymphocytes, monocytes, and macrophages. Characterisation of their immune functions are pertinent in providing fundamental information on its mechanism and response. Feline PBMCs are important in research works, such as isolation of feline parvovirus (Miyazawa *et al.*, 1999), productive infection of feline immunodeficiency virus (FIV) *vif* gene in feline PBMCs and monocyte-derived macrophages (Lockridge *et al.*, 1999), and expression of CXCR4 on feline PBMCs in FIV infection (Willet *et al.*, 2003). Often, analyses could not be performed immediately due to various issues such as logistics, unavailability of materials, or simply time constraint. Therefore, cryopreservation is performed whereby low temperature is used to preserve different types of cells for long term by using liquid nitrogen. Low temperature storage (-196°C) and slow cooling rate (1°C per minute) are generally recommended for optimal cells recovery. In addition, using cryoprotectant such as dimethyl sulfoxide (DMSO) can prevent the formation of ice crystals which can lyse the cells during thawing. The freezing medium is usually supplemented by higher concentration of serum such as fetal bovine serum (FBS). Although certain aspects in cryopreservation have been studied in human PBMCs, none has been done in feline PBMCs.In this study, the temperature and composition of cryopreservation media were examined using dye exclusion technique and cell viability staining by flow cytometry to determine the effects on feline PBMCs viability.

The objectives of this study were:

1) To identify the best cryopreservation conditions in order to get the maximum feline PBMCs recovery upon thawing.
2) To determine the effect of compositions and temperatures of cryopreservation media on feline PBMCs viability.

The hypotheses for this study were:

1) The addition of feline serum in cryopreservation media provides higher feline PBMCs recovery.
2) Low temperature (4°C) of cryopreservation media is less toxic to feline PBMCs.

7.0 REFERENCES

Alipoor F. J., Sadighi Gilani M. A., Eftekhari-Yazdi P., Daneshzadeh M.T., Khakzad H., (2010). The effect of FBS concentration on cryopreservation of isolated spermatogonial cells from neonatal mouse by MACS method. *Journal of Iranian Anatomical Sciences, 7*, 121-31.

Brunner, D., Frank, J., Appl, H., Schöffl, H., Pfaller, W., & Gstraunthaler, G. (2010). Serum-free cell culture: the serum-free media interactive online database. *Altex*, *27*(1), 53.

Freshney, R. I. (2005). Cryopreservation. *Culture of Animal Cells*: a manual of basic technique. New York: Wiley-Liss Inc, pp 321-324.

Freshney, R. I. (2010). Specialized cells. *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications, Sixth Edition*, pp 383-432.

Gao, D., & Critser, J. K. (2000). Mechanisms of cryoinjury in living cells.*ILAR journal*, *41*(4), 187-196.

Golab, K., Leveson-Gower, D., Wang, X. J., Grzanka, J., Marek-Trzonkowska, N., Krzystyniak, A., Millis, J. M., Trzonkowski, P., & Witkowski, P. (2013). Challenges in cryopreservation of regulatory T cells (Tregs) for clinical therapeutic applications. *International immunopharmacology*, *16*(3), 371-375.

Kleeberger, C. A., Lyles, R. H., Margolick, J. B., Rinaldo, C. R., Phair, J. P., & Giorgi, J. V. (1999). Viability and recovery of peripheral blood mononuclear cells cryopreserved for up to 12 years in a multicenter study. *Clinical and diagnostic laboratory immunology*, *6*(1), 14-19.

Kreher, C. R., Dittrich, M. T., Guerkov, R., Boehm, B. O., & Tary-Lehmann, M. (2003). CD4+ and CD8+ cells in cryopreserved human PBMC maintain full functionality in cytokine ELISPOT assays. *Journal of immunological methods*, *278*(1), 79-93.

Litvan, G. G. (1972). Mechanism of cryoinjury in biological systems.*Cryobiology*, *9*(3), 182-191.

Lockridge, K. M., Himathongkham, S., Sawai, E. T., Chienand, M., & Sparger, E. E. (1999). The feline immunodeficiency virus vif gene is required for productive infection of feline peripheral blood mononuclear cells and monocyte-derived macrophages. *Virology*, *261*(1), 25-30.

Makino, M., & Baba, M. (1997). A cryopreservation method of human peripheral blood mononuclear cells for efficient production of dendritic cells.*Scandinavian journal of immunology*, *45*(6), 618-622.

Martinello, T., Bronzini, I., Maccatrozzo, L., Iacopetti, I., Sampaolesi, M., Mascarello, F., Patruno, M. (2010) Tissue Engineering Part C: Methods. 16(4): 771-781

Mazur, P. (1984). Freezing of living cells: mechanisms and implications.*American Journal of Physiology-Cell Physiology*, *247*(3), C125-C142.

Miyazawa, T., Ikeda, Y., Nakamura, K., Naito, R., Mochizuki, M., Tohya, Y., Vu, D., Mikami, T., Takahashi, E. (1999). Isolation of Feline Parvovirus from Peripheral Blood Mononuclear Cells of cats in northern Vietnam. *Microbiology and immunology*, 43 (6), 609-612.

Nakamura, Y., Asahi, S., Nakaya, T., Bahmani, M. K., Saitoh, S., Yasui, K., Mayama, H., Hagiwara, K., Ishihara, C., Ikuta, K. (1996). Demonstration of Borna Disease Virus RNA in Peripheral Blood Mononuclear Cells Derived from Domestic Cats in Japan. Journal of Clinical Microbiology, 34 (1), 188–191.

Nazarpour, R., Zabihi, E., Alijanpour, E., Abedian, Z., Mehdizadeh, H., & Rahimi, F. (2012). Optimization of human peripheral blood mononuclear cells (PBMCs) cryopreservation. *International journal of molecular and cellular medicine*, *1*(2), 88.

Owen, R. E., Sinclair, E., Emu, B., Heitman, J. W., Hirschkorn, D. F., Epling, C. L., Tan, Q. X., Custer, B., Harris, J. M., Jacobson, M. A., McCune, J. M., Martin, J. N., Hecht, F. M., Deeks, S. G., & Norris, P. J. (2007). Loss of T cell responses following long-term cryopreservation. *Journal of immunological methods*, *326*(1), 93-115.

Pegg, D. E. (2015). *Cryopreservation and Freeze-Drying Protocols: Principles of Cryopreservation.* New York: Springer New York. pp 3-19.

Riss, T., O'Brien, M., & Moravec, R. (2003). Choosing the right cell-based assay for your research. *Cell notes*,*6*(1), 6.

Riss, T. L., Moravec, R. A., Niles, A. L., Benink, H. A., Worzella, T. J., & Minor, L. (2004). *Cell viability assays*. United States of America: National Center for Biotechnology Information.

Sattui, S., de la Flor, C., Sanchez, C., Lewis, D., Lopez, G., Rizo-Patrón, E., Clinton White Jr. A., & Montes, M. (2012). Cryopreservation modulates the detection of regulatory T cell markers. *Cytometry Part B: Clinical Cytometry*, *82*(1), 54-58.

Simione, F.P. (2009). Thermo Scientific Nalgene and Nunc Cryopreservation Guide. Thermo Fisher Scientific Inc. pp1-8

Strober, W. (2001). Trypan blue exclusion test of cell viability. *Current protocols in immunology*, A3-B.

Studdert, V.P., Gay, C.C., Blood, D.C. (2012). *Saunders Comprehensive Veterinary Dictionary (4th Edition).* United States of America: Elsevier Saunders. pp 281.

Ulmer, A. J., Scholz, W., Ernst, M., Brandt, E., &Flad, H. D. (1984). Isolation and subfractionation of human peripheral blood mononuclear cells (PBMC) by density gradient centrifugation on Percoll. *Immunobiology*,*166*(3), 238-250.

Wang, T., Xu, Z., Jiang, W., & Ma, A. (2006). Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell. *International journal of cardiology*, *109*(1), 74-81.

Wewetzer, K., & Dilmaghani, K. (2001). Exposure to dimethyl sulfoxide at 37 C prior to freezing significantly improves the recovery of cryopreserved hybridoma cells. *Cryobiology*, *43*(3), 288-292.

Willett, B. J., Cannon, C. A., & Hosie, M. J. (2003). Expression of CXCR4 on feline peripheral blood mononuclear cells: effect of feline immunodeficiency virus infection. *Journal of virology*, *77*(1), 709-712.