

## Cryopreservation of lymphocytes for immunological studies in horses<sup>1</sup>

Renata F. Siqueira<sup>2\*</sup> and Wilson Roberto Fernandes<sup>2</sup>

**ABSTRACT.-** Siqueira R.F. & Fernandes W.R. 2018. **Cryopreservation of lymphocytes for immunological studies in horses.** *Pesquisa Veterinária Brasileira* 38(11):2019-2022. Departamento de Clínica Médica, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Dr. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-270, Brazil. E-mail: [refarinelli@yahoo.com.br](mailto:refarinelli@yahoo.com.br)

The use of frozen cells allows studies on diseases and other immunological assays, since it facilitates the logistics of collecting and transporting, including laboratories located in different cities or other countries. The objectives of this study were to verify if the storage in the refrigerator after collection at different times changes the viability of total leukocytes after months of freezing and the ratio of CD4/CD8 is affected by the freezing process. Venous blood of 15 healthy horses was used and the experiment was divided into 2 stages. In the first, the viability of the leukocytes before and after freezing was verified, as well as different storage times in the refrigerator (fresh blood, stored for 24 and 48 hours) before the freezing process. In the second part, the immunophenotyping of the T lymphocytes was performed, in order to observe if after thawing the relationship between LT CD4 and LT CD8 undergoes change. There was no difference between the amounts of viable leukocytes from frozen fresh blood compared to fresh blood before freezing, nor difference between the viability of blood left in the refrigerator (4°C) for 24 hours and fresh blood and fresh frozen blood. There was a decrease in viability of frozen leukocytes after 48 hours left in the freezer for other samples; however, the recovery was 10<sup>7</sup>x cells. Regarding the immunophenotyping of CD2CD4+ and CD2CD8+ double-labeled T lymphocytes in the blood stored in the refrigerator for 24 hours before freezing, no difference was observed between before and after 6 months of freezing. It is concluded that cryopreservation of equine total leukocytes is possible and, although there was a difference between freezing times, even in the less viable sample, sufficient numbers of cells were recovered for other immunological assays.

INDEX TERMS: Cryopreservation, lymphocytes, immunology, horses, immunophenotyping, mononuclear cells.

### RESUMO.- [Criopreservação de linfócitos para estudos imunológicos em equinos.]

A utilização de células congeladas possibilita estudos sobre doenças e outros ensaios imunológicos, pois facilita a logística de coleta e transporte, inclusive para laboratórios localizados em cidades diferentes ou outros países. Os objetivos desse estudo foram verificar se o armazenamento sobre refrigeração em diferentes tempos e a criopreservação alteram a viabilidade de leucócitos totais e se a relação entre LT CD4/CD8 é afetada pelo processo de congelamento. Utilizou-se sangue venoso de 15 cavalos hígidos e o experimento foi dividido

em 2 etapas. Na primeira foi analisado se houve alteração na viabilidade dos leucócitos provenientes de amostras de sangue armazenadas em diferentes tempos em geladeira antes e depois de 6 meses de congelamento a -80°C. Na segunda parte, realizou-se a imunofenotipagem dos linfócitos T, com a finalidade de observar se após o descongelamento a relação entre LT CD4 e LT CD8 sofre alteração. Não houve diferença entre a quantidade de leucócitos viáveis da amostra de sangue fresco descongelado em relação ao sangue fresco antes do congelamento, nem diferença entre a viabilidade do sangue deixado em congelador (4°C) por 24 horas e do sangue fresco. Houve uma diminuição da viabilidade dos leucócitos, após o descongelamento de 6 meses (-80°C), das amostras de sangue deixado em geladeira por 48 horas antes do congelamento em relação às outras amostras, porém, a recuperação foi de células x10<sup>7</sup>. Quanto à imunofenotipagem de linfócitos T com dupla marcação CD2CD4+ e CD2CD8+, no sangue armazenado

<sup>1</sup> Received on January 20, 2018.

Accepted for publication on February 7, 2018.

<sup>2</sup> Departamento de Clínica Médica, Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade de São Paulo (USP), Av. Dr. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-270, Brazil.

\*Corresponding author: [refarinelli@yahoo.com.br](mailto:refarinelli@yahoo.com.br)

em geladeira por 24 horas antes do congelamento, e não foi observada diferença entre antes ou depois de 6 meses de congelamento. Conclui-se que a criopreservação de leucócitos totais de equinos é possível e, embora tenha havido diferença entre os tempos de congelamento, mesmo na amostra menos viável, houve recuperação de uma quantidade de células suficientes para outros ensaios imunológicos.

**TERMOS DE INDEXAÇÃO:** Criopreservação, linfócitos, imunologia, equinos, imunofenotipagem, células mononucleares.

## INTRODUCTION

The use of frozen blood cells, mainly mononuclear leukocytes, is a routine procedure very well established in human laboratories for both diagnostic and therapeutic purposes (Sputtek et al. 2007). Cryopreservation allows late assays of both cellular function and phenotyping, besides centralizing in a single laboratory samples collected in several places, thus minimizing possible interlaboratory variability (Aziz et al. 2013).

Cell freezing and thawing processes can cause cell death or irreversible damage due to water crystallization and organelle modifications and DNA damage. Lymphocytes and monocytes are more resistant to such damage because they have fewer granules. However, the use of solutions with cryoprotective substances such as dimethylsulfoxide (DMSO), fetal bovine serum, RPMI can minimize damages if the freezing and thawing time is respected (Svedentsov et al. 2007).

Cryopreservation of human mononuclear leukocytes maintains the ability to express histocompatibility antigens and cellular immunity parameters such as lymphocyte proliferation in response to mitogens, cytokine expression and surface antigens, and NK cell cytotoxicity (Truax et al. 1990). CD4 and CD8 T lymphocytes are crucial components of the antiviral immune response, since limiting virus replication and monitoring the function of these cells is prerequisite for the development of antiviral therapies and vaccines (Kutscher et al. 2013).

Although the use of frozen cells in Veterinary Medicine is not as routine as in Medicine there are numerous possibilities of application in the study of vaccines, therapies and in the knowledge of all stages of development of some viral diseases of animals that are in great distances of the laboratories, facilitating logistics. In horses, in the acute phase of Equine Infectious Anemia the immune response is mediated by the major histocompatibility complex (MHC) and cytotoxic CD8 T lymphocytes (Craig & Montelaro 2013). In West Nile virus infection the CD8 T lymphocyte also plays an important role in the control of infection through perforins and interleukins such as tumor necrosis factor (TNF) and interferon gamma (IFN $\gamma$ ), but the importance of LT CD4 (Netland & Bevan 2013). Equine Viral Arteritis virus has tropism by monocytes and CD3 LT, especially by CD4 (Go et al. 2010). Immunity of neonates is not fully understood and the mechanism of passive transfer by colostrum in several mammalian species suggests that the maternal lymphocyte plays key roles in the development of the immune system of foals (Perkins et al. 2014).

The use of frozen cells would allow more studies on these diseases and other tests, as it facilitates collection and transport logistics, including for laboratories located in different cities or other countries.

The objectives of this study were to verify if refrigeration storage at different times and cryopreservation alter the viability of total leukocytes and whether the relationship between CD4/CD8 LT is affected by the freezing process.

## MATERIALS AND METHODS

This study was approved by the CEUA (Ethics Committee for the Use of Animals) of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo, under the protocol number 2606/2012.

Fifteen healthy, vaccinated and vermifugated horses, aged between 3 and 5 years, weighing between 400 and 450 kg, were fed with commercial concentrate and Coast-cross hay.

The experiment was divided into two stages. In the first one, it was verified if different storage times of the blood at 4°C alter the viability of the leukocytes before and after the freezing at -80°C. For this, nine heparinized tubes of peripheral blood of the jugular vein of each horse were collected. Three samples from each animal were immediately processed and frozen (fresh blood). Three samples were stored for 24 hours at 4°C and only then were processed and frozen (24h blood). The last three samples were stored for 48 hours at 4°C and then processed and frozen (48h blood).

Both fresh and blood samples stored in the refrigerator were centrifuged at 800g for 10 minutes, the leukocyte bud removed and deposited in PBS (phosphate buffered saline) and the erythrocytes lysed three times. Total leukocytes were stained with Trypan blue and counted. Then suspended in heat-inactivated fetal bovine serum solution (FCS, Invitrogen, Karlsruhe, Germany) and 10% DMSO (dimethylsulfoxide, Sigma-Aldrich, Munich, Germany) and gradually frozen at -80°C. Six months then all samples were thawed and the leukocytes counted.

In the second part, the immunophenotyping of the T lymphocytes was performed, in order to observe whether after the thawing the relationship between LT CD4 and LTCD8 changes. Immunophenotyping of the leukocytes before freezing the blood stored in the refrigerator was carried out 24 hours before the freezing and after 6 months frozen in a freezer -80°C. Leukocytes were thawed rapidly in a water bath at 37°C, centrifuged at 800g for 5 minutes, resuspended in 1ml of PBS, pH 7.4, and counted. 1x10<sup>6</sup> cells were deposited in 1 ml PBS in each tube. For each sample, 3 tubes were used, the first as white, the second for monoclonal anti-CD2 mice (IgG2a anti-horse CD2, Serotec MCA1278) and CD4 (IgG1 mouse anti-horse CD4, Serotec MCA1078) tube for monoclonal anti-CD2 and CD8 (anti-horse CD8 mouse IgG1, Serotec MCA 2385) monoclonal antibodies. After a half-hour incubation at room temperature and second wash, goat anti-rat IgG RPE (Serotec 303009) and Goat anti-mouse IgG FITC (Serotec 103002) secondary antibodies were labeled, another half-hour incubation in the dark and last wash. The labeled cells were resuspended in 1ml of PBS and subjected to analysis on the flow cytometer (BD FACS Calibur, United States) for identification and enumeration of lymphocyte subpopulations.

The data read by flow cytometry were analyzed by a computer program (Flow Jo software), which provided the histogram, and the table with the number of cells identified by immunophenotyping.

The data were analyzed using SPSS statistical software v.9.0. The behavior of the studied variables, both in the comparison between viability before and after freezing, and in the comparison between lymphocyte subpopulations, was shown to be non-parametric. Therefore, the Wilcoxon test was paired with 5% significance (P<0,05).

## RESULTS AND DISCUSSION

The cryopreserved human mononuclear leukocytes are separated by the Ficoll-Hypaque separation gradient where only the target cells of the study are obtained. Because it is a more laborious and time consuming technique, it was decided to work with total leukocytes obtained from the leukocyte bud after centrifugation, as suggested by Truax et al. (1990), where a single person can process larger numbers of samples.

Initially, we tested whether the permanence of whole blood in the refrigerator at different times (24 and 48 hours) alter the viability of total leukocytes compared to fresh blood, or which has been processed immediately after harvest. The results are shown in Table 1.

There was no difference between the amounts of viable leukocytes from fresh frozen blood relative to fresh blood before freezing, which suggests that the freezing process used was adequate. There was also no difference between the viability of blood left in the refrigerator (4°C) for 24 hours and fresh blood and fresh frozen blood. There was a decrease in the viability of the frozen leukocytes after 48 hours left in the freezer relative to the other samples, however, the recovery was of  $\times 10^7$  cells, which means that even if a significant decrease occurred, a sufficient amount of cells were recovered for the accomplishment of some tests. According to Sputtek et al. (2007), if the purpose of the freezing is laboratory assays, quantities of  $0.5 \times 10^6$  are sufficient, especially if 5 to 10% DMSO is used as a cytoprotectant. Pereira-Cunha et al. (2014) worked with human umbilical cord mesenchymal stem cells and other mononuclear cells stored at room temperature for 96 hours prior to freezing and found that despite the decrease in the number of live cells there was still sufficient T and B lymphocyte viability to perform assays.

In the second part of this study the immunophenotyping of CD2CD4+ and CD2CD8+ double-labeled T lymphocytes was performed in the blood stored in the refrigerator for 24 hours before freezing, and no difference was observed between before and after 6 months of freezing, as illustrated by Table 2.

Weinberg et al. (2009) performed the immunophenotyping and cell proliferation of peripheral blood mononuclear cells

from 104 human volunteers (49 seropositive for HIV and 55 healthy) using blood samples frozen at -70°C and in liquid nitrogen and concluded that fresh and frozen blood samples of the two forms, present a good correlation, since they did not find difference in the amount of CD4 and CD8 LT before and after freezing and between fresh and frozen blood. Other researchers (Reimann et al. 2000, Alam et al. 2012, Aziz et al. 2013) also analyzed the difference in the number of the same cells after a long freezing period (6 months to 6 years) and found no alteration, as in the present study. Duthie et al. (2002) evaluated antioxidant status, DNA damage and the ability to repair fresh and frozen human lymphocytes for more than seven months. They concluded that the thawing process does not cause DNA damage and that the antioxidant capacity is also preserved.

Kutscher et al. (2013) report that rest of a few hours or all night after lymphocyte thawing at 37°C is a common practice in laboratories that work with human blood and aims to increase the functional activity of these cells. The same authors tested the effect of overnight rest on in vitro stimulation, cytokine production and the antiviral capacity of CD4 and CD8 T lymphocytes. They concluded that although there was a decrease in cell viability after rest, there was a significant increase in the antiviral capacity of both cells. CD8 T lymphocytes increased expression of IFN $\gamma$ , IL-2, TNF $\alpha$  and MIP 1 $\beta$ . The findings of this study and others cited by these authors, who use the rest of the cells, reinforce the idea of this present work that it is possible to use frozen cells and to use the blood stored for 24 hours in many immunological tests in Equine Veterinary Medicine also, where transporting the blood to the laboratory can be time consuming.

## CONCLUSIONS

Cryopreservation of total leukocytes from equines is possible, and although there was a difference between freezing times even in the less viable sample, sufficient numbers of cells were recovered for other immunological assays.

Blood may be stored for up to 24 hours in a refrigerator prior to freezing for immunological assays using mononuclear cells, as up to that time there has been no change in cell viability or the relationship between CD4/CD8 T lymphocytes.

Further studies should be carried out in order to understand the action of storage time on the activity of these cells.

## REFERENCES

- Alam I., Goldeck D., Larbi A. & Pawelec G. 2012. Flow cytometric lymphocyte subset analysis using material from frozen whole blood. *J. Immunoassay Immunochem.* 33(2):128-139. <<http://dx.doi.org/10.1080/15321819.2011.604370>> <PMid:22471603>
- Aziz N., Margolick J.B., Detels R., Rinaldo C., Phair J., Jamieson B.D. & Butch A. 2013. Value of quality assessment program in optimizing cryopreservation of peripheral blood mononuclear cell in a multicenter study. *Clinic. Vac. Immunol.* 20(4):590-595. <<http://dx.doi.org/10.1128/0014-8179-2013-000693-12>> <PMid:23408528>
- Craig J.K. & Montelaro R.C. 2013. Lessons in AIDS vaccine development learned from studies of equine infectious, anemia virus infection and immunity. *Virus* 5(12):2963-2976. <<http://dx.doi.org/10.3390/v5122963>> <PMid:24316675>
- Duthie S.J., Pirie L., Jenkinson A.M. & Narayanan S. 2002. Cryopreserved versus freshly isolated lymphocytes in human biomonitoring: endogenous and induced DNA damage, antioxidant status and repair capability.

**Table 1. Median values, first and third quartiles of the variables fresh blood, fresh frozen blood, 24 hours frozen blood and 48 hours frozen blood**

	Fresh blood	Fresh frozen blood	24 hour frozen blood	48 hour frozen blood
Median	7.250 <sup>A</sup>	7.225 <sup>A</sup>	7.388 <sup>A</sup>	3.850 <sup>B</sup>
(1 and 3 Quartiles)	(5.950-15.70)	(5.819-9.282)	(3.850-8.560)	(2.863-7.388)

Different letters on the same line represents difference between variables (P<0.05).

**Table 2. Median values, first and third quartiles of the LT CD4 / CD8 ratio pre and post freezing**

	CD4 / CD8 Pre Freezing	CD4 / CD8 Post Freezing
n	15	15
Median	0.9664	0.8803
(1 and 3 Quartiles)	(0.9009 - 0.9906)	(0.9420 - 0.8395)
P	0.1055	

- Mutagenesis 17(3):211-214. <<http://dx.doi.org/10.1093/mutage/17.3.211>> <PMid:11971991>
- Go Y.Y., Zhang J., Timoney P.J., Cook R.F., Horohov D.W. & Balasuriya U.B.R. 2010. Complex interactions between the major and minor envelope proteins of equine arteritis virus determine its tropism for equine CD3 T lymphocytes and CD14 monocytes. *J. Virol.* 84(10):4898-4911. <<http://dx.doi.org/10.1128/JVI.02743-09>> <PMid:20219931>
- Kutscher S., Dembek C., Deckert S., Russo C., Korber N., Bogner J.R., Geisler F., Umgelter A., Neuenhahn M., Albrecht J., Cosma A., Protzer U. & Bauer T. 2013. Overnight resting of PBMC changes functional signatures of antigen specific T-cell responses: impact for immune monitoring with clinical trials. *Plos One* 8(10):1-13. <<http://dx.doi.org/10.1371/journal.pone.0076215>> <PMid:24146841>
- Netland J. & Bevan M.J. 2013. CD8 and CD4 T cells in West Nile Virus immunity and pathogenesis. *Viruses* 5(10):2573-2584. <<http://dx.doi.org/10.3390/v5102573>> <PMid:24153060>
- Pereira-Cunha F.G., Duarte A.S.S., Reis-Alves S.C., Olalla Saad S.T., Metzke K., Lorand-Metze I. & Luzo Â.C.M. 2014. Umbilical cord blood CD34+ stem cells and other mononuclear cell subtypes processed up to 96 h from collection and stored at room temperature maintain a satisfactory functionality for cell therapy. *Vox Sanguinis* 108(1):72-81. <<http://dx.doi.org/10.1111/vox.12199>> <PMid:25333825>
- Perkins G.A., Goodman L.B., Wimer C., Freer H., Babasyan S. & Wagner B. 2014. Maternal T-lymphocytes in equine colostrum express a primarily inflammatory phenotype. *Vet. Immunol. Immunopathol.* 161(3-4):141-150. <<http://dx.doi.org/10.1016/j.vetimm.2014.07.009>> <PMid:25174977>
- Reimann K.A., Chernoff M., Wilkening C.L., Nickerson C.E. & Landay A.L. 2000. Preservation of lymphocyte immunophenotype and proliferative responses in cryopreserved peripheral blood mononuclear cells from human immunodeficiency virus type-1 infected donors: implication for multicenter clinical trials. *Clin. Diagn. Lab. Immunol.* 7(3):352-359. <PMid:10799445>
- Sputtek A., Kühnl P. & Rowe A.W. 2007. Cryopreservation of erythrocytes, thrombocytes and lymphocytes. *Transfus. Med. Hemother.* 34(4):262-267. <<http://dx.doi.org/10.1159/000104136>>
- Svedentsov E.P., Tumanova T.V., Zaytseva O.O., Solomina O.N. & Utemov S.V. 2007. Preservation of leukocytes in conditions of cryoanabiosis. *Cell Preserv. Tech.* 5(2):144-150. <<http://dx.doi.org/10.1089/cpt.2007.0508>>
- Truax R.E., Powell M.D., Montelaro R.C., Issel C.J. & Newman M.J. 1990. Cryopreservation of equine mononuclear cells for immunological studies. *Vet. Immunol. Immunopathol.* 25(2):139-153. <[http://dx.doi.org/10.1016/0165-2427\(90\)90031-M](http://dx.doi.org/10.1016/0165-2427(90)90031-M)> <PMid:2378055>
- Weinberg A., Song L.Y., Wilkening C., Sevin A., Blais B., Louzao R., Stein D., Defechereux P., Durand D., Reidel E., Raftery N., Jesser R., Brown B., Keller M.F., McFarland E. & Fenton T. 2009. Optimization and limitations of use of cryopreserved peripheral blood mononuclear cells for functional and phenotypic T-cell characterization. *Clin. Vac. Immunol.* 16(8):1176-1186. <<http://dx.doi.org/10.1128/CVI.00342-08>> <PMID: 19515870>