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In vitro evaluation of chemotaxis, vitality, and generation of reactive oxygen species of fresh or frozen equine leukocytes used in the treatment of endometritis

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Abstract

The present study was conducted to determine the chemotactic effects of different leukocyte diluents, dextrose concentrations associated with or without recombinant human interleukin-8 (rhIL-8), lysed leukocyte concentration, and blood plasma source and concentration (homologous vs. heterologous) on fresh equine leukocyte vitality *in vitro* in addition to testing the effects of two post-thaw extenders (phosphate buffered saline - PBS - or saline) on vitality and generation of reactive oxygen species (ROS) by frozen leukocytes. These experiments were conducted as part of the development of a cryopreservation protocol for equine leukocytes for use in the treatment of endometritis. Assays were performed using acryl chemotactic chambers incubated at 37 ^{o}C. After incubation, leukocytes were counted by flow cytometry. The percent of migrating cells did not differ for different concentrations of dextrose. There was no interaction between leukocyte diluents and dextrose concentrations. An interaction between different plasma types and plasma concentrations was observed. The chemotactic effect of lysed leukocytes was directly related to their concentration. The relative amount of live leukocytes after thawing was greater when incubated in PBS than in saline. In conclusion, dextrose was not a strong chemo-attractant for equine leukocytes while homologous plasma, at concentrations of 10, 30, and 60%, was found to be a strong chemo-attractant. Lysed leukocytes and homologous plasma (10, 30, and 60%) had similar chemotactic effects. Cells diluted in PBS and saline had similar vitality and ROS generation in the post-thaw tests when incubated for 15 minutes.

Keywords: equine, leukocyte, freezing, vitality, chemotaxis.

Introduction

Bacterial infection of the genital tract of the mare has been recognized as an important cause of infertility (Hughes and Loy, 1975). The ability to maintain a uterine environment compatible with embryonic and fetal life is essential for reproductive efficiency in equids. However, the uterine environment is easily disturbed by an inflammatory process following bacterial invasion (Asbury and Lyle, 1993), which can occur during breeding, genital examination, and parturition (Ricketts and Mackintosh, 1987). Mares that eliminate contamination easily are resistant to endometritis. Conversely, mares that fail to eliminate contaminants develop persistent uterine inflammation and are susceptible to endometritis (Hughes and Loy, 1975; Kenney *et al*., 1975). Mares susceptible to post-breeding endometritis are also prone to develop persistent bacterial endometritis (Troedsson *et al*., 1997)

Current treatment protocols for bacterial endometritis involve the rapid removal of contaminants and inflammatory debris from the uterine lumen by uterine lavage or pharmacological induction of myometrial contractions. Uterine infusions of fresh homologous plasma with leukocytes in mares experimentally infected with *Streptococcus equi* resulted in a more rapid elimination of bacteria than untreated mares or those treated with infusions of homologous blood plasma or ecbolic drugs (Castilho, 1994; Mattos *et al*., 1999a; c). Plasma with leukocytes was obtained adding a 6% dextrose solution to heparinized blood, modifying the separation gradient and maintaining the leukocytes in the plasma. These results were attributed to increased uterine phagocytic activity due to the infused phagocytes and opsonizing factors present in homologous plasma. However, a chemotactic effect might have influenced these results. The release of by-products from lysed leukocytes or the dextrose solution is suggested to be involved.

Due to some practical limitations in the manipulation of fresh leukocytes, a method for cryopreservation of leukocytes was developed by Castilho *et al*. (1997) and provided *in vitro* viable and functionally competent neutrophils after thawing in phosphate buffered saline (PBS) for therapeutic use in mares susceptible to endometritis. The use of cryopreserved leukocytes for the treatment of mares with endometritis has not yet been reported.

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Materials and Methods

Animals

Seven, clinically healthy, non-pregnant crossbred mares between 4 and 10 years of age, with a good body condition score and weighing 450- 650 kg, were used. Mares were kept in open stalls and fed mixed grass hay and concentrate twice daily.

General procedures

Separation of leukocytes

Blood from each mare was collected into a heparinized container. Erythrocyte lysis and washing were performed to separate leukocytes. Erythrocyte lysis was conducted by adding distilled water at 5 ^{o}C to whole blood (2:1 v/v) followed by the addition of PBS (19.1 g/L; Dulbecco Instant, Fa. Biochrom, Berlin, Germany) to the diluted blood solution (2:1 v/v) 10 seconds later. The blood diluted in PBS was then centrifuged (220 x *g*; Cryofuge 8000- Heraeus-Christ GmbH, D-33660, Osterode, Germany) at 4 ^{o}C for 12 minutes. The supernatant containing erythrocyte debris was discarded, and the pellet was resuspended with PBS (2:1 v/v). This procedure was repeated two to three times until a cell suspension containing only leukocytes was obtained. Cells were counted using a Bürker counting chamber (K. Hecht, Sondheim, Germany). In the absence of erythrocytes, leukocytes were counted and prepared for immediate use by resuspension in PBS or R3F [medium composed of RPMI 1640 medium (Fa. Biochrom, Berlin, Germany) + Hepes 15 nmol/ml + L-Glutamin 2 nmol/L + $NaHCO_3$ 18 mmol/L] with 3% inactivated fetal calf serum and without antibiotics.

Cryopreservation of equine leukocytes

For cryopreservation, cells were resuspended in heterologous equine plasma with 5% DMSO to a final concentration of 200 x 10^6 leukocytes/ml. Immediately after the final dilution, leukocyte samples were placed into macrotubes (Minitüb GmbH, Hauptstrasse 41 - 84184 – Tiefenbach, Germany) and frozen at the following cooling rates: +4 ^{o}C to -70 ^{o}C = 1 ^{o}C/min; and to -70 ^{o}C to -140 ^{o}C = 10 ^{o}C/min; using a semen freezing machine (Gefrierautomat Minidigicool - Fa. IMV- L'Aigle, France). At -140 ^{o}C macrotubes were plunged into the liquid nitrogen (Castilho *et al*., 1997).

Chemotactic assays

Assays were conducted using acryl chemotactic chambers (Cytogen - Dieselstrasse 7 - 61239 - Ober-Morläw, Germany). The chambers consisted of two compartments, a lower and an upper one, and each one consisted of 10 corresponding wells. A silicon membrane separated the compartments allowing communication between them. Compartments were also separated by a polycarbonate filtering membrane with 3 μm pores (Nucleopore Track-Etch membrane - Corning, Corning, NY, USA) through which cells could migrate. The test solution for chemotactic activity was poured into the lower wells (415 μl capacity). The fresh leukocyte suspension for chemotactic testing was poured into the upper wells (200 μl capacity). The chambers were incubated at 37 ^{o}C (Heraeus-Christ GmbH, Hanau, Germany) with 5% CO_2 for two hours.

Measurement of leukocyte vitality

After incubation, the content of corresponding upper and lower wells was stirred, and 100 μl of each mixture were transferred to the cytometry tubes (FACScan® Tubes, Becton Dickinson Labware Europe, 38241 Meylan Cedex- France), and 200 μl of propidium iodide (100 μl/ml, Calbiochem Novabiochem GmbH, Liztweg 1, Bad Soden, Taunus, Germany) were added to each tube. The tubes were then analyzed by flow cytometry (Castilho, 1998; FACScan® - Fa. Becton-Dickinson, Heidelberg, Germany) to count the number of live migrating and non-migrating cells. Three thousand cells were analyzed from each sample. The percentage of migrating cells was calculated from the number of live cells in the lower well divided by the total number of cells originally added to the upper well. Propidium iodide penetrates cell membrane lesions and binds to DNA. Live and dead cells were distinguished by the emission of white or red fluorescence (FL2 or FL3), respectively.

Measurement of the ROS generation

After thawing, cells were incubated for 15 minutes at 37 ^{o}C in a humidified atmosphere with 5% CO_2 and were stimulated thereafter by the addition of 300 nmol/L PMA (Phorbol-12-myristic-13-acetate-Sigma, Deisenhofen, Germany) and gently stirred. Cells were then re-incubated for 15 minutes for PMN activation. After re-incubation, 5 μl of dihydrorhodamin-123 (DHR-123 -Mobitec, Göttingen, Germany), a substance retained by PMN, were added. Myeloperoxidasis, a product of the oxygen metabolism, acted in catalyzing the oxidation of DHR-123, turning it into the fluorescent Rodamin-123. Neutrophil green fluorescence intensity was used to evaluate production of ROS. Two thousand cells from each sample were analyzed by flow cytometry.

Experiment 1: Influence of different concentrations of dextrose associated or not to rhIL 8 on chemotaxis of fresh equine leukocytes.

Leukocytes were separated as mentioned above. Cell vitality in this experiment, measured by flow cytometry, was greater than 95%. A 2 x 4 x 2

factorial design was used. Main factors were leukocyte diluents (PBS or R3F), dextrose concentration (0, 1, 2, or 6%), and rhIL-8 concentration (0 µl or 100 µl). A 200µl solution containing either 0, 1, 2, or 6% dextrose (Müller's Mühle Traubenzucker, MN GmbH, 45801 Gelsenkirchen, Germany), with or without 100 µl rhIL-8 (Bio-Concept GmbH- Umkirch, Germany), was placed in each lower well. The remaining space was filled with Percoll. This procedure was conducted twice and 16 wells were filled. The 16 upper wells were filled with 200 µl of a solution containing 2 x 10^6 leukocytes, diluted in either PBS (8 wells) or R3F (8 wells).

The generalized linear model (GLM) procedure of SAS was used for statistical analysis (SAS for Windows, Version 6.12). The model included the main effects of donor, diluent, dextrose concentration, and rhIL-8 concentration and their interactions. The dependent variable was leukocyte migration. Differences among means were considered significant at $P < 0.05$.

Experiment 2: Influence of different concentrations of homologous and heterologous blood plasma on chemotaxis of fresh equine leukocytes

Leukocytes of two non-pregnant mares were used in this experiment. Main factors studied were plasma type (homologous or heterologous) and plasma concentration (3, 5, 30, 60, or 90%). Separation of leukocytes was done as described in previous methods (stated above). The vitality of leukocytes used was greater than 95% (range = 95-98%). To evaluate chemotaxis, 200 µl of solutions containing 3, 10, 30, 60, or 90% of homologous or heterologous plasma diluted in distilled water were poured into each lower well. The remaining volume was filled with Percoll. Positive controls were included for each plasma type consisting of 3% plasma, 100 µl rhIL-8 (25ng/mL), and Percoll as a diluent. All samples and controls were run in duplicates. Each upper well was filled with 2 x 10^6 leukocytes diluted in 200 µl PBS.

Quadratic regression was used for statistical analysis (Minitab for Windows, Version 11.1). The model included the main effects of plasma source (homologous vs. heterologous) and plasma concentration and their interactions. The dependent variable was leukocyte migration. Differences among means were considered significant at $P < 0.05$.

Experiment 3: Influence of the number of lysed leukocytes on the migration of fresh equine leukocytes

Leukocytes of two non-pregnant mares were used in this experiment. Separation of leukocytes was done as described in previous methods. Vitality of leukocytes was greater than 95% (range = 96-99%). Leukocyte lysis was achieved by freezing cells at –100 ^oC without using a cryoprotectant. After 24 hours, samples were thawed at 37 ^oC and resuspended in PBS to the desired concentration.

To evaluate chemotaxis, lower wells were filled with 300 µl of a PBS solution containing 0.4 x 10^6, 2.0 x 10^6, or 10.0 x 10^6 lysed leukocytes. An additional well was filled with 300 µl rhIL-8 (25 ng/ml) and served as a positive control. Percoll was used to fill the volume of the wells. Samples were run in duplicate. Two hundred microliters of PBS containing 1.2 x 10^6 leukocytes were placed into the upper wells, and chemotaxis was assayed as described in previous methods.

The generalized linear model procedure of SAS was used for statistical analysis (SAS for Windows, Version 6.12). The model included the main effect of concentration of lysed leukocytes, and the dependent variable was leukocyte migration. Differences between means were considered significant at $P < 0.05$.

Experiment 4: Influence of two different post-thaw media (PBS or saline) and four leukocyte dilutions on vitality and ROS generation in cryopreserved equine leukocytes.

Leukocytes from all seven mares were pooled and frozen in macrotubes as described in previous methods. Samples from six different batches were randomly thawed at 37 ^oC for 1 minute in a water bath. After thawing, the cell suspension (200 x 10^6 leukocytes/ml) was diluted in either PBS or saline using the following dilutions: 1:50, 1:25, 1:12.5, or 1:6.25.

The samples were incubated in a microtiter plate with a round bottom (Nunc, Wiesbaden Germany). To assess leukocyte vitality, half of the samples were incubated at 37 ^oC with 5% CO_2 for 15 minutes, and the remaining samples were incubated for 120 minutes. To assess ROS production, incubation lasted for 15 minutes.

Analysis of variance was used for statistical analysis (Minitab for Windows, Version 11.1). The model included the main effects of dilution medium and leukocyte dilution. The two dependent variables were leukocyte vitality and ROS production. Differences were considered significant at $P < 0.05$.

Results

Experiment 1

The effect of donor did not influence leukocyte migration. Leukocytes diluted with R3F showed greater ($P < 0.01$) migration than leukocytes diluted in PBS (Fig. 1A). Interactions between medium and rhIL-8 and between medium and dextrose concentration were not significant, thus indicating that varying concentrations of rhIL-8 and dextrose did not change the effect of medium. The presence of rhIL-8 increased migration of leukocytes ($P < 0.01$, Fig. 1B). Similarly, different

dextrose concentrations influenced the number of leukocytes found in the lower compartment of the chamber ($P < 0.02$). There was no interaction between the presence of rhIL-8 and dextrose concentration that affected the migration of leukocytes.

Experiment 2

The influence of different sources of plasma (homologous vs. heterologous) and plasma concentration on migration of equine leukocytes and the influence of rhIL-8 with 3% of the two kinds of plasma are shown in Fig. 2. Source of plasma did not affect migration of leukocytes (Fig. 2A). However, different concentrations of plasma considerably influenced ($P < 0.05$) leukocyte migration, and an interaction between the two types of plasma and different concentrations ($P < 0.05$) on leukocyte migration was found. Using quadratic regression analysis, a high correlation ($R^2 = 0.75$) between plasma concentration and leukocyte migration was found. Figure 2B shows that the presence of rhIL-8 increased the migration of leukocytes ($P < 0.01$). Source of plasma (homologous vs. heterologous) at a 3% concentration did not affect migration of leukocytes in the presence of rhIL-8.

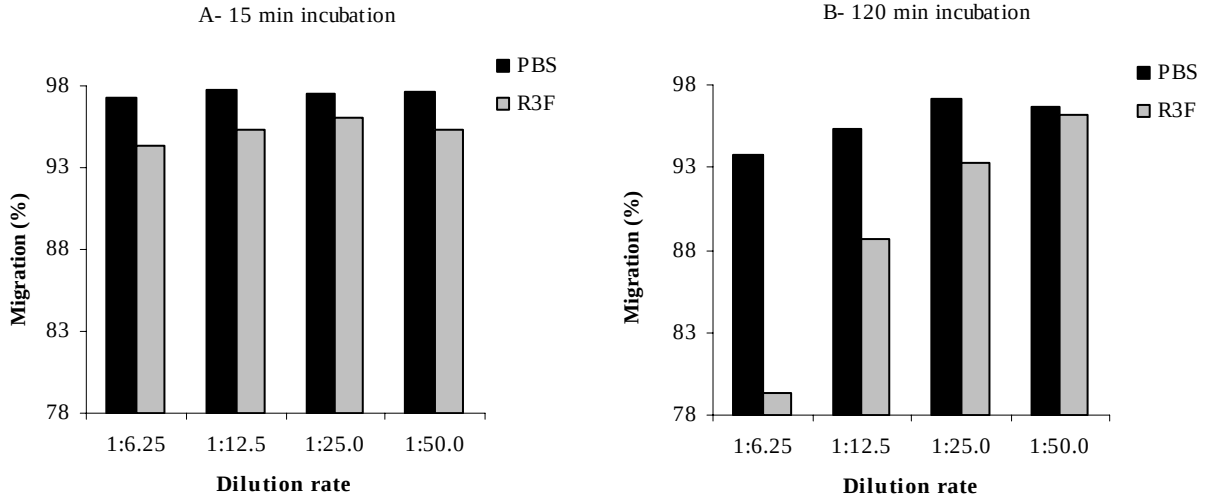

Figure 1. Percent of leukocytes migrating at different dextrose concentrations ($P < 0.01$) diluted in either PBS or R3F cell culture medium (A) or with or without rhIL-8 (B; $P = 0.02$).

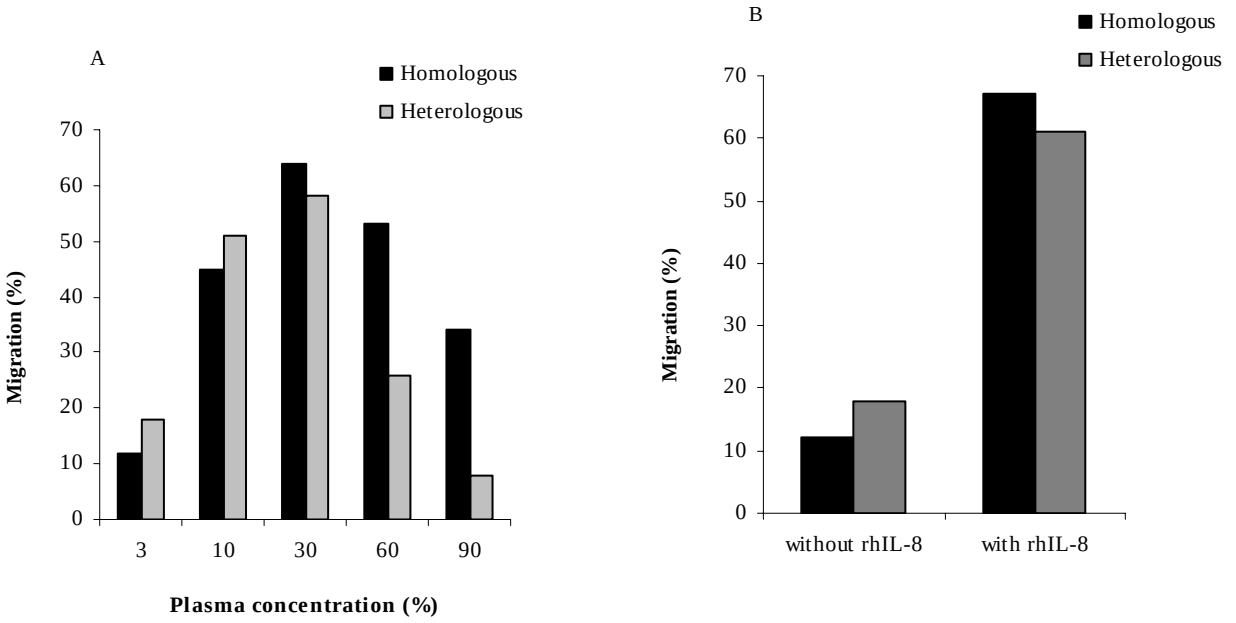

Figure 2. Percent of leukocyte migration at different concentrations (3, 10, 30, 60, and 90%) of homologous or heterologous plasma (A, $P > 0.39$), or in the presence or absence of rhIL-8 (B; $P < 0.01$) with 3% homologous or heterologous plasma ($P = 0.94$).

Experiment 3

Concentration of lysed leukocytes affected leukocyte migration ($P < 0.01$, Fig. 3). A strong correlation between concentration of lysed leukocytes and migration was observed ($R^2 = 0.96$). When 10×10^6 lysed leukocytes were used, the leukocyte migration was similar to that of rhIL-8 treatment and greater than that of the 2.0×10^6 ($P < 0.01$) and 0.4×10^6 ($P < 0.01$) concentration of lysed leukocytes. It was also observed that the concentration of 2×10^6 lysed leukocytes caused a greater migration ($P < 0.05$) of cells than the concentration of 0.4×10^6 leukocytes.

Experiment 4

The percentage of live cells was greater when leukocytes were resuspended using PBS than with saline ($P < 0.05$, Fig. 4). In addition, percentage of live leukocytes increased ($P < 0.01$) with increasing dilution rates. Incubation period did not affect the number of live leukocytes (Fig. 4A and 4B). No interaction was found between the period of incubation and medium. However, there was an interaction between period of incubation and dilution rate ($P < 0.01$) and between the medium used and dilution rate ($P < 0.05$).

Production of ROS was not affected by post thawing extenders, but it was affected by the dilution rate ($P < 0.01$, Fig. 5). There was no interaction between extenders and leukocyte concentration. A strong correlation between leukocyte dilution and formation of ROS was found for leukocytes diluted in both saline ($R^2 = 0.86$) and PBS ($R^2 = 0.88$).

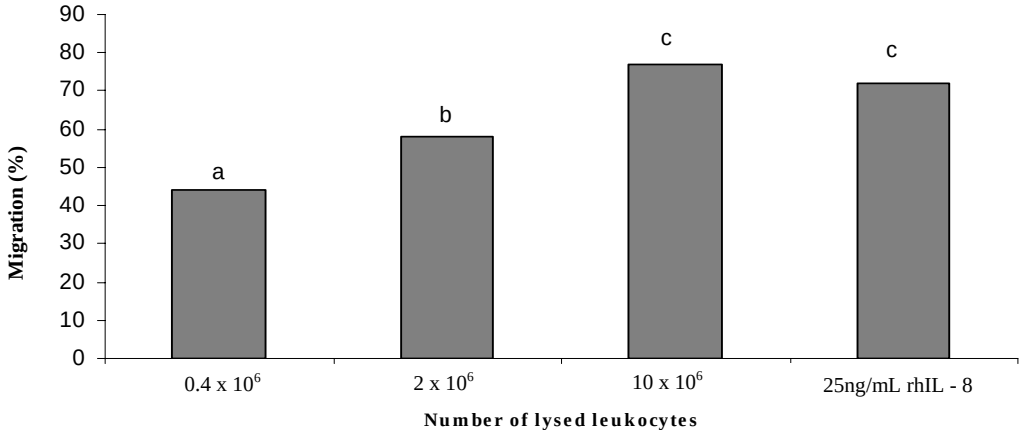

Figure 3. The effect of supplementation with different numbers of lysed leukocytes or 25 ng/ml rhIL-8 on the migration of equine leukocytes. Different letters indicate significant differences ($P < 0.04$).

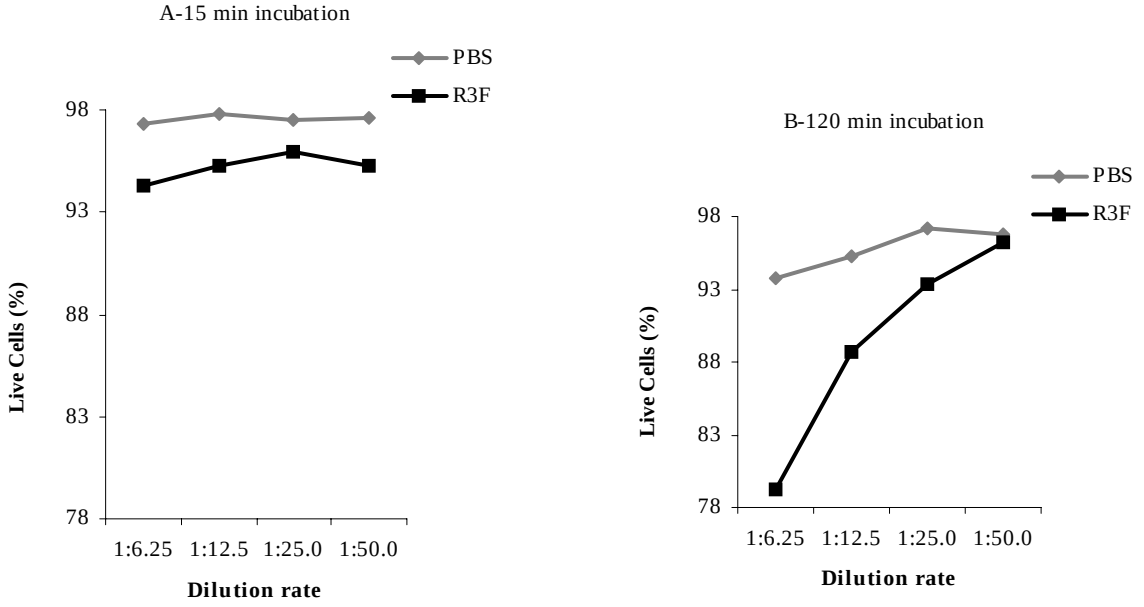

Figure 4. Vitality of leukocytes thawed and resuspended in PBS or in saline after a 15-minute (A) or 120-minute (B) incubation at different leukocyte dilution rates. PBS vs. saline, $P = 0.01$; dilution rate vs. vitality, $P < 0.01$; incubation period vs. vitality, $P = 0.79$.

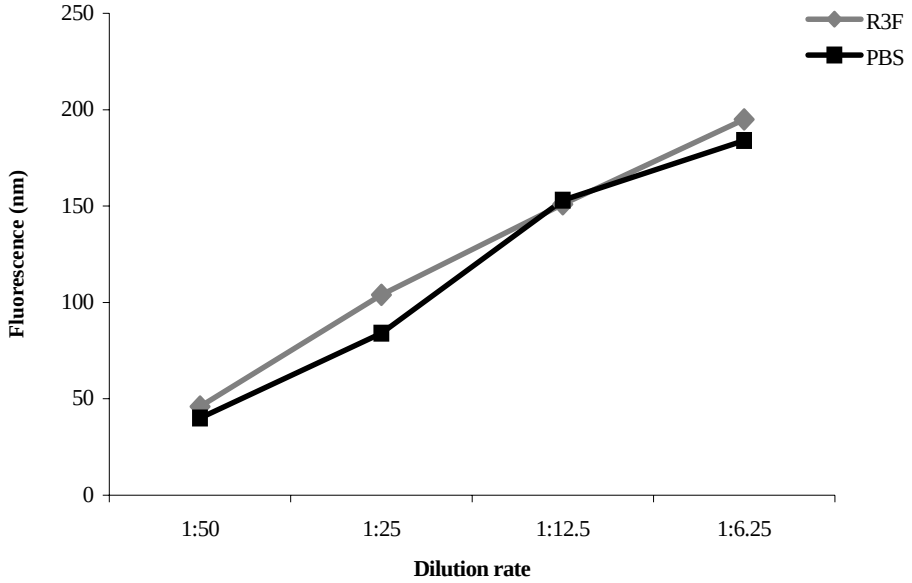

Figure 5. Generation of reactive oxygen species (expressed by fluorescence) by thawed leukocytes stimulated with 300 nMol PMA and incubated for 15 minutes in saline or PBS at different leukocyte dilution rates. PBS vs. saline, P = 0.92; dilution rate vs. fluorescence, P < 0.01).

Discussion

In Experiment 1, the percentage of migrating leukocytes (51%) using only rhIL-8 as a chemo-attractant was lower than the 75% observed by Zerbe *et al*. (2003b) and the 72% found in Experiment 3. In both studies, the same model of chemotactic chamber and the same rhIL-8 concentration were used. The concentration of 25 ng/ml rhIL-8 is considered to have the best chemotactic effect on equine leukocytes (Zerbe *et al*., 2003a). However, in the study of Zerbe *et al*. (2003b) and in Experiment 3, a volume of 300 µl rhIL-8 was used, totaling 7.5 µg per well. In Experiment 1, a volume of 100 µl rhIL-8 per well was used, totaling 2.5 µg per well. The lower migration observed in Experiment 1 could be related to the lower amount of rhIL-8 used. In this regard, RHIL-8 concentration should not be the only variable considered in the evaluation of chemotaxis.

To separate leukocytes from whole blood, Zerbe *et al*. (2003b) used erythrocyte lysis and washing, and then allowed leukocytes to migrate through a 55-78% Percoll gradient. After centrifugation at 100 x *g* at 20 ^{o}C for 25 minutes, neutrophils were washed twice with PBS (220 x *g*, 4 ^{o}C, for 8 minutes) and cells were resuspended only in R3F. Using this method, only live neutrophils (95% vitality) were obtained. In the present study, the separation method used provided a mixed leukocyte population with 95% vitality. In spite of the different separation techniques employed in both experiments, vitality rate was similar and was probably not responsible for the lower migration rate observed in the present study.

Zerbe *et al*. (2003b) used a fluorescence microscope for counting cells, while in the current study, flow cytometry was used. This could also explain the higher percentage of leukocyte migration (29%) without rhIL-8 and dextrose, when compared to the 8% observed by Zerbe *et al*. (2003b).

Dextrose used to separate homologous plasma containing fresh leukocytes could exert a chemotactic effect that may be responsible for the shorter time to eliminate bacteria (Castilho, 1994; Mattos *et al*., 1999a). According to the studies of Mattos *et al.* (1997; 1999b), the use of dextrose for leukocyte separation could also explain the greater pregnancy rates in barren mares infused with plasma containing fresh leukocytes after breeding. In that case, 6% dextrose was used in leukocyte separation and resulted in about 2% dextrose in the supernatant (results not shown). In the present study, dextrose concentrations of 0, 1, 2, or 6% were used in conjunction with or without rhIL-8 (Zerbe *et al*., 1996; 2003a; b). Increasing dextrose concentrations did not increase equine leukocyte chemotaxis when rhIL-8 was not added to the solution. This demonstrates the lack of a chemotactic effect of dextrose. Therefore, the bacterial elimination observed in previous reports (Castilho, 1994; Mattos *et al*., 1999a; c), as well as the better pregnancy rates obtained (Mattos *et al.*, 1997; 1999b), cannot be explained by the chemotactic effect of dextrose but probably by lower leukocyte apoptosis and enhanced phagocytic activity caused by the use of dextrose as a gradient of separation. The use of a 6% dextrose solution to separate leukocytes also causes platelets to move into the plasma fraction. Zalavary *et al*. (1996) found that phagocytosis and respiratory bursts

triggered in neutrophils by immunoglobulin G (IgG)-opsonized yeast particles were potentiated by platelets. Andonegui *et al*. (1997) showed that when neutrophils are cultured in the presence of platelets, the occurrence of apoptosis is reduced in comparison to a control in which the incubation was performed without platelets.

A greater migration occurred when 1% dextrose with rhIL-8 was used. Migration decreased as dextrose concentration increased. However, there was a significant difference only when 6% dextrose with rhIL-8 was used compared with the other concentrations. This is not in agreement with the findings of Oldenborg and Sehlin (1999), who evaluated human neutrophil chemotaxis towards the peptide N-formyl-metionil-fenilalanin. The authors observed that the chemotactic effects of the peptide were regulated by glucose, which in a concentration of 5 mM and 15 mM glucose, induced activity of non-locomoting cells and improved neutrophil adhesion to the substrate.

In Experiment 2, plasma source (homologous vs. heterologous) did not affect chemotaxis. However, plasma concentration influenced leukocyte migration, and the interaction between the two kinds of plasma and concentration affected leukocyte migration. These results demonstrate a chemotactic effect with 10 and 30% heterologous plasma as well as with 10, 30, and 60% homologous plasma. Previous experiments (Castilho, 1994; Mattos *et al*., 1997; 1999a; b; c) used a final plasma concentration of about 60% with leukocytes. Moreover, according to Mattos *et al*. (1997; 1999a), infusion with homologous plasma resulted in a shorter time to eliminate bacteria and higher pregnancy rates when compared to untreated controls. However, that treatment was less effective than treatment with homologous plasma and fresh leukocytes, which demonstrates a possible chemotactic and opsonizing effect from plasma, even without leukocytes. The present results concerning plasma chemotaxis confirm those of Mattos *et al*. (1997; 1999a). These authors explained that the therapeutic action of plasma containing leukocytes was due to a possible synergistic effect of both components of this treatment: first, plasma infusion provides opsonizing factors such as complements and immunoglobulins to the uterine lumen; and second, fresh blood leukocytes improve phagocytosis.

Results obtained in Experiment 3 demonstrate that lysed leukocytes have a chemotactic effect. When 2 x 10^6 lysed leukocytes were used, leukocyte attraction rate was similar to that of 30% homologous and heterologous plasma treatments. Also, a concentration of 10 x 10^6 lysed leukocytes had an effect similar to that of treatment with 25 ng/ml rhIL-8.

The use of PBS as a diluent resulted in greater post-thaw leukocyte vitality than the use of saline. The difference between both diluents increased proportionally to dilution rate and after a 120-minute incubation. In the experiments of Castilho *et al*. (1997) and Zerbe *et al*. (2001), only PBS was used to resuspend thawed cells in a dilution of 1:12.5. The cell separation method, the extender, and the cryoprotectant used were the same as those used in the current study. Vitality and ROS generation data by Castilho *et al*. (1997) and Zerbe *et al*. (2001) were collected after a 6-hour incubation and are similar to data from the current experiment. These authors compared fresh and cryopreserved leukocytes and found that vitality of frozen-thawed leukocytes was similar to that of fresh leukocytes, but frozen-thawed leukocytes generated more ROS than fresh leukocytes. This might be due to stimulation of ROS production during freezing, cryopreservation, or the thawing process, as described for cryopreserved spermatozoa (Weitze and Petzold, 1992).

Although leukocytes showed greater vitality in PBS than in saline, vitality in saline was similar to that of PBS after a 15-minute incubation and dropped below 90% after 2 hours of incubation in both cases. Both extenders exerted a similar effect on the generation of ROS after a 15-minute incubation. Therefore, saline could be used as a post-thaw extender for a quick procedure (thaw, re-suspension and uterine infusion).

The present study showed that dextrose does not have a strong chemotactic effect on equine leukocytes. Among the plasma concentrations used, 10, 30, and 60% homologous plasma and 10 and 30% heterologous plasma showed greater chemotactic effects. Lysed leukocytes, in a concentration of 2 x 10^6 cells/ml, showed a chemotactic effect similar to that of using 10, 30, and 60% homologous plasma. At a concentration of 10 x 10^6 cells/ml, lysed leukocytes were able to attract leukocytes at the same rate as that of treatment with rhIL-8. Cells resuspended either in PBS or saline showed similar vitality and generation of ROS in the post-thaw tests when incubated for 15 minutes. These findings should contribute to the development of a treatment protocol for endometritis in mares based on fresh or cryopreserved leukocytes used in uterine infusions.

References

Andonegui G, Trevani AS, López DH, Raiden S, Giordano M, Geffner JR. 1997. Inhibition of human neutrophil apoptosis by platelets. *J Immunol*, 158:3372-3377.

Asbury AC, Lyle SK. 1993. Infectious causes of infertility. *In*: Mckinnon AO, Voss JL. (Eds.) *Equine reproduction*. Philadelphia: Lea & Febiger. pp.381-391.

Castilho LFF. 1994. *Treatments of endometritis in the mare with homologous plasma containing neutrophils* [in Portuguese]. Porto Alegre, Brazil: Federal University of Rio Grande do Sul. MSc. Dissertation.

Castilho LFF. 1998. *Untersuchung zur Separation, Gefrierkonservierung und Applikation von zirkulierenden polymorphkernigen neutrophilen Granuloziten zur Behandlung der Gebärmutterschleimhautentzündung*

beim Pferd. Hannover, Germany: Tierärztliche Hochschule Hannover. Thesis.

Castilho LFF, Zerbe H, Rabe U, Leibold W, Klug E. 1997. Isolierung und Gefrierkonservierung von immunokompetenten polymorphkernigen neutrophilen Granulozyten zur Endometritisbehandlung beim Pferd. *Pferdeheilkunde*, 13:437-444.

Hughes JP, Loy RG. 1975. The relation of infection to infertility in the mare and stallion. *Equine Vet J*, 7:155-159.

Kenney RM, Bergman RV, Cooper WL, Morse GW. 1975. Minimal contamination techniques for breeding mares: techniques and preliminary findings. *Proc Am Assoc Equine Pract*, 21:237-336.

Mattos RC, Malschitzky E, Mattos R. 1997. Effects of different postbreeding treatments on fertility of Thoroughbred mares. *Pferdeheilkunde*, 13:512-515.

Mattos RC, Castilho LFF, Malschitzky E, Mattos R, Gregory RM. 1999a. Treatment of endometritis of experimentally infected mares: I- effect of lavage, plasma and leukocytes on uterine bacterial clearance. *Arq Fac Vet UFRGS*, 27:49-60.

Mattos RC, Meirelles LS, Malschitzky E, Castilho LFF, Neves AP, Mattos ALG, Vieira MJ, Keller A, Hött AK, Gregory RM. 1999b. Oxytocin, plasma containing leukocytes or combination of both as treatment of postbreeding endometritis in the horse. *Pferdeheilkunde*, 15:584-587.

Mattos RC, Neves AP, Gregory RM, Gomes MJS, Mattos R, Meirelles LS, Lagares, MA, Keller, A, Wald VB, Vieira MJ, Hött AK. 1999c. Treatment of endometritis of experimentally infected mares: II-effect of ecbolic drugs and plasma enriched with leukocytes on uterine bacterial clearance. *Arq Fac Vet UFRGS*, 27:61-72.

Oldenborg PA, Sehlin J. 1999. The glucose concentration modulates N-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe)-stimulated chemokinesis in normal human neutrophils. *Bioscience Reprod*, 19:511-523.

Ricketts SW, Mackintosh ME. 1987. Role of anaerobic bacteria in equine endometritis. *J Reprod Fertil Suppl*, 35:343-351

Troedsson MHT, Ibrahim NM, King VL, Foster DN, Crabo BG. 1997. Post-breeding endometritis: the role of seminal components. *Pferdeheilkunde*, 13:545.

Weitze CF, Petzold R. 1992. Preservation of semen. *J Reprod Sci*, 28:229-235.

Zalavary S, Grenegard M, Stendahl O, Bengtsson T. 1996. Platelets enhance FC(gamma) receptor mediated phagocytosis and respiratory burst in neutrophils: the role of purigenic modulation and actin polymerization. *J Leukoc Biol*, 60:58-68.

Zerbe H, Engelke F, Leibold W, Schoon H-A, Klug E. 2001. Immunophenotypical and functional properties of equine uterine neutrophils of mares with or without uterine degenerative changes. *Pferdeheilkunde*, 17:650-652.

Zerbe H, Schuhberth H-J, Hoedemaker M, Grunert E, Leibold W. 1996. A new model system for endometritis: basic concept and characterization of phenotypic and functional properties of bovine uterine neutrophils. *Theriogenology*, 46:1339-1356.

Zerbe H, Schuberth H-J, Engelke F, Frank J, Klug E, Leibold W. 2003a. Development and comparison of in vivo and in vitro models for endometritis in cows and mares. *Theriogenology*, 60:209-223.

Zerbe H, Castilho LFF, Engelke F, Mattos RC, Schuberth H-J, Klug E, Leibold W. 2003b. Isolation and cryopreservation of functionally competent equine leukocytes. *J Vet Med*, 50:179-184.