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CRYOPRESERVATION OF *Trichomonas gallinae* TROPHOZOITES

(*Criopreservação de trofozoítos de Trichomonas gallinae)*

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ABSTRACT

This study aimed at evaluating the viability of *T. gallinae* isolates with the use of cryoprotectants – DMSO, ethylene glycol (EG), glycerol (GL) and propylene glycol (PG) in a freezer, in nitrogen and in an ultrafreezer, 120 days. Cryopreservation with GL, the freezing process was only viable in an ultrafreezer (20%). The use of DMSO led to viable trophozoites (40%) when freezing took place in an ultrafreezer and in nitrogen. Freezing was viable when both cryoprotectants EG (90%) and PG (80%) were used in an ultrafreezer and in nitrogen.

Key words: Trichomoniasis, cryoprotectants, freezer, ultrafreezer, nitrogen.

RESUMO

Este estudo teve como objetivo avaliar a viabilidade de isolados de *T. gallinae* com o uso de crioprotetores - DMSO, etileno glicol (EG), glicerol (GL) e propileno glicol (PG) em freezer, nitrogênio e ultrafreezer, por 120 dias. Criopreservação com GL, o processo de congelamento só foi viável em um ultrafreezer (20%). O uso de DMSO levou a trofozoítos viáveis (40%) quando o congelamento ocorreu em um ultrafreezer e em nitrogênio. O congelamento foi viável quando ambos os crioprotetores, EG (90%) e PG (80%), foram utilizados em um ultrafreezer e em nitrogênio.

Palavras-Chave: Tricomoníase, crioprotetores, freezer, ultrafreezer, nitrogênio.

INTRODUCTION

The parasite, *Trichomonas gallinae*, is a cosmopolitan eukaryotic organism which has been described in many countries, except in the Antarctic and in Greenland. It has been known that it occurs in both animals in captivity and wild ones and that its distribution is closely related to *Columba livia*, one of its most important hosts (BUNBURY *et al*., 2007; FORRESTER e FOSTER, 2009).

These methods consists in keeping materials at low temperatures (from -20 °C to -80 °C in freezers) and ultralow ones (from -150 °C to -196 °C in containers with liquid nitrogen). Among cryoprotectants, glycerol (GL), dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol (PG) have stood out since they are organic solvents which have are capable of penetrating cells (RALL *et al*., 1984).

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In order to preserve *T. gallinae* isolates viable for a long period of time in a laboratory, this study evaluated the viability of *T. gallinae* isolates after cryopreservation processes with DMSO, EG, GL and PG in a freezer (-20 ºC), in nitrogen(-196 ºC) and in an ultrafreezer (-96 ºC), for 120 days.

MATERIAL AND METHODS

Trichomonas gallinae

This study used samples of *T. gallinae* which were isolated from naturally infected common pigeons (*C. livia*). It was approved by the Animal Ethics Committee at the Universidade Federal de Pelotas, located in Pelotas/RS, Brazil, on may 7th, 2018 (nº. 23110.012860/2018-81) and by SISBIO on February 2nd, 2018 (nº. 61235-1). Samples were collected with the use of oral swabs and placed on trypticase-yeast extract-maltose (TYM) medium Diamond (1957).

Viability of trophozoites

In order to carry out the test, cultures that had 95% viability and density of $1x10^5$ trophozoites/mL were used in supplemented TYM medium. Trophozoites were obtained by centrifugation at 1500 rpm for 10 minutes.

Cryopreservation

Cryoprotectants DMSO, EG, PG and GL were used at 10% concentration (CASTRO *et al*., 2011; DUMAS, 1974; FERREIRA *et al*., 2015; LUMSDEN *et al*., 1966). The experiment was conducted in 10 cryotubes and transparent sterile pallets (0.5 ml) for every cryoprotectant. Aliquots of trophozoites and cryoprotectants were placed in cryotubes, which were kept in a freezer (-20 ºC) and in an ultrafreezer (-80 ºC). In nitrogen freezing, cryogenic tubes and pallets were used in four different protocols: (A) Direct freezing in nitrogen in pallets, (B) Previous freezing in liquid vapor at -45 ºC for 10 minutes and, then, in nitrogen in cryotubes, (C) Freezing in a freezer at -20 ºC for 24 h and, then, in liquid nitrogen at -196 ºC, in cryotubes, and (D) Slow freezing at -4 ºC for 5 minutes, followed by freezing at -45 ºC for 45 minutes and cryopreservation in liquid nitrogen at -196 ºC in cryotubes.

Readings were carried out on the 2th, 15th, 30th, 60th and 120th days when cryotubes (n=2) and pallets were removed from all freezing methods (freezer, ultrafreezer and nitrogen) at determined periods of time.

The first evaluation of freezing viability (after 48 h) was conducted after thawing in warm water (25 ºC) of two cryotubes/pallets of every cryoprotectant (n=48).

In order to evaluate trophozoite viability throughout the experiment, 10 µL of the content of every cryotube was placed on slides, which were observed by an optical microscope (40x magnification) after thawing with the use of Trypan Blue (0.4%) dye exclusion (1:1) to verify protozoan motility.

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After this process, a 100mL aliquot was replicated in TYM broth at 37 ºC in aerobiosis for 48h so that viability could be observed by trophozoite multiplication at the four different reading periods.

Statistical analyze

Statistical analysis was performed by univariate analysis of variance (ANOVA), followed by the Tukey Test (GraphPad Prism 8.0 Software).

RESULTS AND DICUSSION

Abreu and Tutunji (2004) stated that preservation processes to be used for microorganisms have been routine in the development of researches. Some species of parasites, such as protozoa and helminths, have been successfully cryopreserved (LEVINE e MARQUARDT, 1955; LEVINE *et al*.,1962; WEATHERSBY e McCALL,1981). In this study, recovery of *T. gallinae* samples 120 days after freezing at –196 ^{0}C shows that long storage periods may be feasible at low temperatures.

In the study reported by this paper, several methodologies were tested to evaluate the efficiency of cryoprotectants on the flagellated protozoan *T. gallinae*, so as to preserve cultures that enable stocks of isolates or strains to be formed and used experimentally in different situations (GIRÃO *et al*., 2004). Common freezing is based on preserving agents at relatively low temperatures, from -4 to -20°C. It is one of the simplest and cheapest maintenance methods, besides being safe to store several microorganisms (TORTORA *et al*., 2012). In this study, this methodology was not effective, it may be due to the fact that the process breaks cell membranes of trophozoites. Intracellular ice crystals, which often break cell structures mechanically, are believed to form in cell fast freezing processes (LAW e MERYMAN, 1991).

Slow freezing is the technique which uses low concentrations of cryoprotectants and whose temperature drop is gradual, besides, cells undergo previous cooling before being frozen (DEL VALLE, 2008). Rôlo *et al*. (2018) found significantly lower results in vitrification than in slow freezing. It corroborates results of this study, which did not reach positive results, by comparison with fast freezing. Ultrafast freezing and use of cryoprotectants, followed by abrupt temperature drop, are believed to cause direct passage from the liquid state to an amorphous one, with no formation of ice crystals (GONÇALVES *et al*., 2008). The challenge cells have in the freezing process is to be capable of standing alteration – due to the passage through intermediate ranges of temperature in both freezing and thawing –, rather than resisting storage temperature at -196 °C (COSTA e FERREIRA, 1991; MAZUR, 1984; OLIVEIRA *et al*., 2010).

In general, cryoprotectant agents act inside the cell by replacing water partially and connecting to hydrogen of molecules of intracellular water (JAIN e PAULSON, 2006). It may have occurred in this study when EG and PG prevented intracellular crystals from forming, damaging structures and destroying membranes of *T. gallinae* trophozoites. However, efficiency of cryoprotectants may vary, depending on the structure (cell or

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tissue) (FULLER e PAYNTER,2004), exposure time before the cryopreservation process itself and their concentrations.

EG it has been widely used as an intracellular cryoprotectant agent (NEWTON *et al*., 1998). Therefore, in this study, EG was more efficient than the other cryoprotectants under investigation. There may be smaller barriers in the membranes of trophozoites which, associated with the low molecular weight of the cryoprotectant, favor higher penetration of these substances into cells (SNOECK *et al*., 2007).

The evaluation of EG by microscopic exam, which was carried out on the 2nd, 15th, 30th, 60th and 120th days after thawing, showed a large number of viable forms with motility and trophozoite growth after sample cultivation. It suggests that microorganisms kept viable (70%) in periods of time under investigation in a freezer up to the 30th day and in the other periods of time (10%). Concerning freezing in an ultrafreezer (90%) and in protocol A (80%) in nitrogen (-196 ºC), there were no significant differences among different periods of time (Fig. 01).

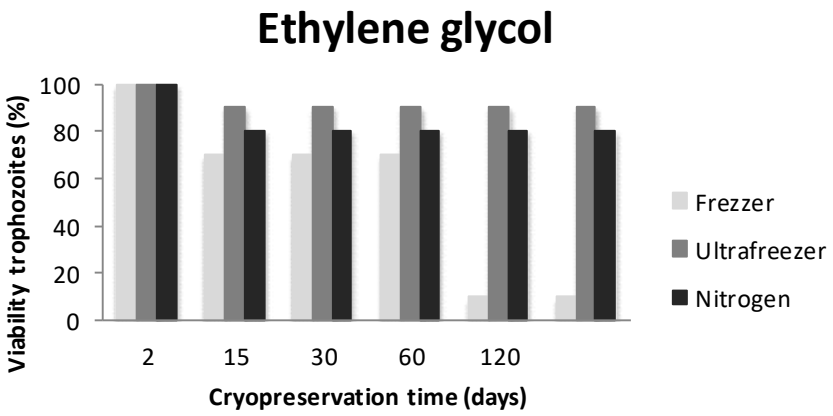

Figure 01: Cryopreservation of *Trichomonas gallinae* trophozoites under cryoprotectants ethylene glycol.

Obs.: The evaluations were carried out at different times (0-120 days).

Cryopreservation in PG reached results which were similar to the ones provided by EG in ultrafreezer (80%) and in protocol A (70%) in nitrogen (-196 ºC). The freezing process in a freezer reached different results. Reading on the 2nd day showed 30% of viable forms, this result kept the same after 48 h. Reading on the 15th day also showed that trophozoites reached the same results that were found on the 2nd day. On the 30th day, when cryotubes were thawed and observed microscopically, they had few viable forms (10%). Finally, readings on the 60th and 120th days showed that the number of viable trophozoites decreased significantly (1%) (Fig. 02).

Tests of DMSO provided 40% of viable forms and good motility in readings carried out on the 2nd and 15th days. Readings on the 30th, 60th and 120th days showed that the number of trophozoites was lower and that motility was low (10%) in ultrafreezer. In protocol A at different periods of time, cultures had 40% viability and motility.

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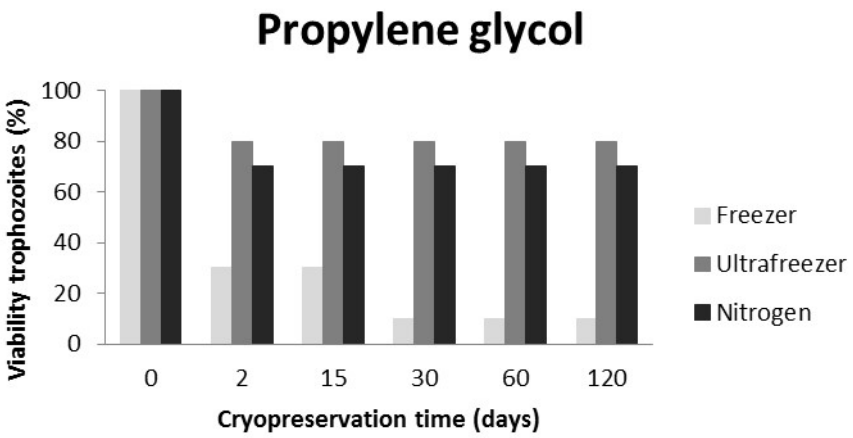

Figure 02: Cryopreservation of *Trichomonas gallinae* trophozoites under cryoprotectants propylene glycol.

Obs.: The evaluations were carried out at different times (0-120 days).

When DMSO was used for freezing in a freezer, readings on the 2nd and 15th days showed 20% of viable forms in the microscope. Readings on the 30th, 60th and 120th days showed that the number of trophozoites was lower and that there was low motility (1%). (Fig. 03).

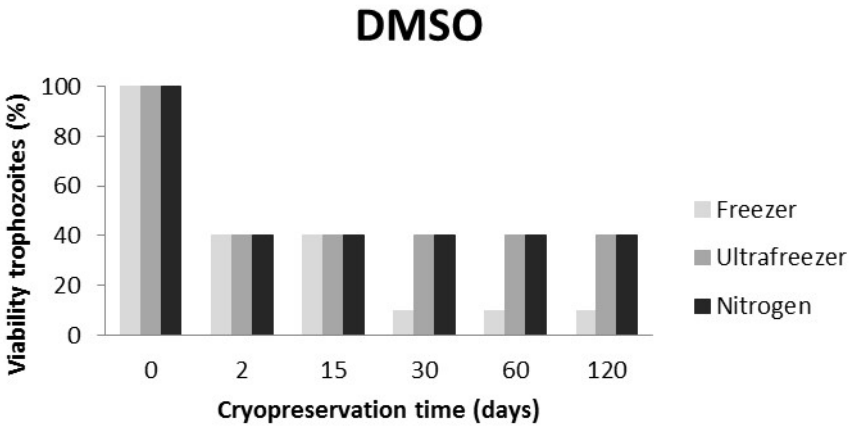

Figure 03: Cryopreservation of *Trichomonas gallinae* trophozoites under cryoprotectants dimethyl sulfoxide.

Obs.: The evaluations were carried out at different times (0-120 days).

Cryoprotectant DMSO features activity has been highlighted and widely used in cryopreservation (CAMPERO, 1989; MATSUO, 2007; MC ENTERGART, 1954; YUAN e XUE, 2010). In this study, DMSO was effective in cryopreservation of trophozoites at different periods of time, mainly in freezing in an ultrafreezer and in nitrogen. Its main activity derives from the fact that it is capable of penetrating cells fast as a penetrating agent, thus, decreasing ice crystal formation in cryopreservation processes (STEDMAN, 2003; THIRUMALA *et al*., 2006).

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Uga and Matsumura (1979) cryopreserved *Trichomonas vaginalis* in liquid nitrogen, with the use of both GL and DMSO, which showed 60% trophozoite viability in DMSO. These results differ from the ones found by Matsuo (2007) in an experiment with fast freezing of *Trichomonas vaginalis* sedimentation, which used DMSO, EG, PG and GL. DMSO exhibited maximum protective effect at 20%. In the study reported by this paper, both EG and PG showed high efficacy in different types of freezing and at distinct reading times.

Regarding GL, readings on the 2nd, 15th and 30th days exhibited viable forms with motility (50%). Readings on the 60th and 120th days showed few viable trophozoites with low motility, when freezing was carried out in an ultrafreezer (20%) and in protocol A (20%) in liquid nitrogen. In a freezer, viability was also 20% on the 2nd day, but on the 15th, 30th, 60th and 120th days, the number of viable forms decreased considerably (1%) (Fig. 04).

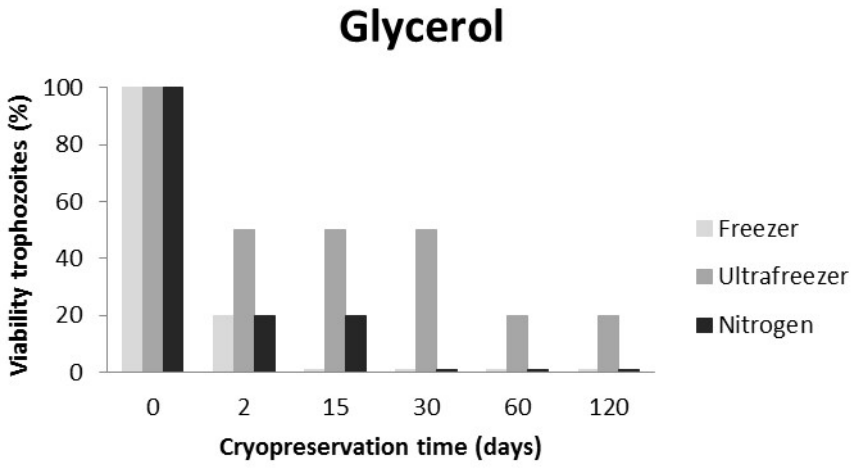

Figure 04: Cryopreservation of *Trichomonas gallinae* trophozoites under cryoprotectants glycerol.

Obs.: The evaluations were carried out at different times (0-120 days).

When GL was used as a cryoprotectant to preserve *T. gallinae* trophozoites, they reached positive results in an ultrafreezer at the periods of time under investigation. These findings corroborate results of the study carried out by Stander-Breedt *et al*. (2004), who tested the effect of GL and DMSO on the preservation of lion (*Panthera leo*) semen. In their study, there was no significant difference between both cryoprotectants under study, it differs from results found by the study reported by this paper. DMSO was more significant in trophozoite preservation at different periods of time and types of freezing than GL. The literature has reported that DMSO better cryopreserves other species of microorganisms, such as protozoa *Toxoplasma gondii* (DUMAS,1974), *Leishmania tropica* (CALOW e FARRANT, 1973) and *Babesia* spp. (HENTRICH e BOSE, 1993; HUBALEK, 2003)

A study of *Tritrichomonas foetus* carried out by Campero (1989) differs from the study reported by this paper when results are compared to protocols B, C and D, since it shows that trophozoites were successfully preserved in liquid nitrogen at -196°C with 10% DMSO and from 65 to 85% of parasites was recovered.

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Concerning freezing in nitrogen, in protocol A with the use of pallets, readings on the 2nd and 120th days showed 40% viability and motility when DMSO was used, while 20% viability and motility was found on the 30th day –and 1% after this period – when GL was used. PG (70%) and EG (80%) were found to be effective in cryopreservation because trophozoites exhibited excellent motility.

McEntegart (1954) cryopreserved four species of *Trichomonas – T. foetus*, *T. vaginalis*, *T. hominis* and *T. gallinae* – with GL and found that fast freezing led to high destruction of trophozoites when the protocol of slow cooling was used. Lumsden *et al*. (1966) also reported that the protocol of fast freezing, with DMSO, GL or polyvinylpyrrolidone 10% as a cryoprotectant, led to protozoan degradation, thus corroborating results found by this study, in which cryoprotectants under fast cryopreservation in cryotubes (protocol B) exhibited neither trophozoite viability nor motility and caused parasite destruction.

Freezing in nitrogen showed very few viable forms in these phases in protocols B, C and D. It should undergo a differentiated freezing process so that trophozoites do not rupture. A study of *T. foetus* carried out by Campero (1989) differs from the study reported by this paper, since the latter successfully preserved trophozoites in liquid nitrogen at -196 °C with 10% DMSO and recovered from 65 to 85%. Diamond (1957) reported the recovery of *T. foetus* after storage at -170 ºC in liquid nitrogen vapor for 1,013 days.

In protocol B, with cryotubes, none of the four cryoprotectants (DMSO, EG, PG and GL) was successful in cryopreservation of flagellated trophozoites, since they had very few viable forms (1%). The same took place in protocols C and D, with cryotubes.

CONCLUSIONS

Both cryoprotectants EG and PG were more effective in cryopreservation of *T. galline* trophozoites throughout freezing in a freezer, in an ultrafreezer and in nitrogen. DMSO and GL exhibited few viable forms after thawing in different types of freezing.

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