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Cryopreservation of protozoan parasites

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Abstract

Conventional methods for the propagation and preservation of parasites *in vivo* or *in vitro* have some limitations, including the need for labor, initial isolation and loss of strains, bacterial and fungal contamination, and changes in the original biological and metabolic characteristics. All these disadvantages are considerably reduced by cryopreservation. In this study, we examined the effects of various freezing conditions on the survival of several protozoan parasites after cryopreservation. The viability of *Entamoeba histolytica* was improved by seeding ($p<0.05$, χ^2test), while this was not so effective for *Trichomonas vaginalis*. Of six cryoprotectants examined, dimethyl sulfoxide (Me_2SO) and glycerol showed the strongest cryoprotective effects. The optimum conditions for using Me_2SO were a concentration of 10% with no equilibration, and those for glycerol were a concentration of 15% with equilibration for 2 hr. The optimum freezing rate depended on the parasite species. *Trypanosoma brucei gambiense* and *Leishmania amazonensis* were successfully cryopreserved over a wide range of freezing rates, whereas the survival rates of *E. histolytica*, *T. vaginalis*, *Pentatrichomonas hominis*, and *Blastocystis hominis* were remarkably decreased when frozen at improper rates. Unlike the freezing rate, exposure of the protozoans to a rapid thawing method produced better motility for all parasites.

Keywords: cryopreservation; protozoa; parasites; seeding; cryoprotectant; freezing; thawing

Introduction

Conventional methods for the propagation and preservation of parasites *in vivo* or *in vitro* have some limitations including the need for labor, initial isolation and loss of strains, bacterial and fungal contamination, and changes in the original biological and metabolic characteristics [5, 8, 12]. All these disadvantages are considerably reduced by cryopreservation, which has been applied to several species of parasites. Blood protozoa, such as *Trypanosoma* spp. and *Plasmodium* spp., can be frozen easily without cryoprotectants or precise control of the freezing rates [4, 8, 14]. On the contrary, studies on the cryopreservation of helminths have been limited to only a few nematodes. Species that have been successfully cryopreserved are microfilariae of *Wuchereria bancrofti*, *Brugia malayi*, and *Dirofilaria corynodes* [6]; newborn larvae of *Trichinella* spp. [9]; first-stage larvae of *Haemonchus contortus*, *Trichostrongylus colubriformis*, and *Ostertagia circumcincta* [5]; and second-stage larvae of *Toxocara canis* [10].

In general, the concentration and type of the cryoprotectant, and the freezing-and-thawing rates are known as factors affecting the viability after

cryopreservation, yet previous studies have barely investigated these factors. Farri et al. [3] reported that the best survival rate of *Entamoeba histolytica* (10%) was obtained in the presence of 7.5% dimethyl sulfoxide (Me_2SO), with equilibration at 37℃ for 15 min, followed by cooling at 1℃/min. Booth et al. [1] reported that the highest survival rate of *Toxoplasma gondii* was obtained when a *T. gondii* suspension containing 12.5% Me_2SO and 4% BSA was preincubated for 30 min at room temperature, followed by freezing at a rate of 1℃/min and rapid thawing. A similar finding using *Giardia lamblia* was also reported [7].

When a cell suspension is cooled slowly, the suspension is not frozen even at temperatures below its freezing point. This phenomenon, called “super-cooling”, causes rapid temperature elevation thereafter. Whittingham et al. [15] reported that the influence of super-cooling could be minimized by adding ice crystals to the suspension around its freezing point, thus preventing the temperature elevation effect. Uga and Matsumura [12] first introduced this technique for cryopreservation *of Trichomonas vaginalis*, and reported that seeding at –5℃ was effective for improving survival of this protozoan.

Most cryopreservation studies have mentioned good survival rates of parasites, but comparative studies and the phenomena occurring during the freezing process have

not been described. A few papers [7, 9] reported optimal procedures for the cryopreservation of individual parasites, but these reports did not make it clear whether such methods could be applied to other species of parasites. In the present study, we examined the influences of (i) seeding, (ii) type and concentration of the cryoprotectant, (iii) equilibration, and (iv) rates of freezing and thawing, on the survival rates of six different species of protozoan parasites after cryopreservation.

Materials and methods

Parasites

In our study, we used six species of protozoa that have been maintained in our laboratory. These were: *E. histolytica* (origin unknown), *T. vaginalis* (isolated from a patient in 1975), *Pentatrichomonas hominis* (isolated from a patient in 2000), *Blastocystis hominis* (isolated from a rat in 1998), *Leishmania amazonensis* (Ecuador strain), and *Trypanosoma brucei gambiense* (Welcome strain). *E. histolytica* had been maintained using Tanabe-Chiba medium at 25℃, and cultivated for 10-18 days. Numbers of 1×10^5-5×10^5 trophozoites/ml were usually used in our study. Trophozoites of *P. hominis* and *B. hominis* were cultured in the same medium at 37℃ for 2-8 days (1×10^5-4×10^6/ml). *T. vaginalis* was maintained in SYS medium at 37℃, and 2-day-old

cultures with numbers between 1×10^5-1×10^6 trophozoites/ml were used in the study. *L. amazonensis* was cultured in Eagle's minimum essential medium containing 15% fetal calf serum and 1% hemolyzed blood at 25℃. The number of parasites was 1×10^7 promastigotes/ml. The culture medium containing the respective protozoa was used as the basic medium for cryopreservation. *T. brucei gambiense* was maintained using laboratory mice by intraperitoneal injection of 1×10^5 parasites per inoculum. When the parasitemia reached 1×10^7-1×10^9 trypomastigotes/ml, blood was collected from the tail of the experimental animals. The infected blood suspension thus obtained was diluted 100-fold and used as the basic medium.

Cryoprotectants

Six cryoprotectants were used in this study, i.e., Me_2SO (Wako, Osaka, Japan), glycerol (Wako), propanediol (PROH; Wako), polyvinylpyrrolidone (PVP; Wako), horse serum, and egg yolk. The appropriate amount of each cryoprotectant was added to 0.2ml of the basic medium in order to adjust to final concentrations of 3-20% as appropriate for different subcultures. The whole mixture was then incubated for 0.5-12 hr depending on the parasite species at either 25 or 37℃ (equilibration).

Freezing equipment

The freezing equipment used in this study is shown in Fig. 1. Cooling rates were controlled by adding small pieces of dry ice to alcohol in which a cryotube (Sarstedt, Neumbrecht, Germany) was dipped. To cool the alcohol uniformly, it was stirred using a magnetic stirrer. The temperature changes of the alcohol and the whole mixture in the cryotube were recorded to –70℃ with digital thermometers (Yokogawa Instruments, IM2455, Tokyo, Japan).

Freezing and thawing

The whole mixture was cooled to 0℃ at a rate of 1.0℃/min, and to –30℃ at rates of 0.1, 0.3, 1.0, 5.0, or 10.0℃/min. Thereafter, the suspension was cooled to –70℃ at a rate of 2.0℃/min and then kept at –70℃ for at least 20 min. In this study, the pellet freezing method and two-step freezing method were also performed. In the former, 15 μl of the suspension was dropped directly onto dry ice and ice pellets formed immediately (>10.0℃/min). In the latter, a cryotube filled with the suspension was embedded in a styrene forming block and kept at –25℃ for 8 hr and then at –70℃ overnight (<0.1℃ /min). The frozen suspensions were thawed at rates of 2, 7, 35, 70 or 140℃/min. These rates were obtained by keeping the frozen suspension kept: (i) on ice for 30 min (2℃

/min), (ii) in air at room temperature for 10 min (7℃/min), (iii) in air at room temperature for 1 min and in a 37℃ water bath for 1 min (35℃/min), (iv) in a 37℃ water bath for 1 min (70℃/min), and (v) in a 100℃ water bath for 25 sec and then in a 37℃ water bath for 5 sec (140℃/min).

Seeding

Ice crystals made of the respective culture medium or PBS (about 2 mm in diameter) were seeded to the suspension at –4, –6, or –8℃.

Viability assessment

After thawing, the suspension was observed using a light microscope to check the viability and morphology of the protozoa. The viability of *E. histolytica*, *T. vaginalis*, *P. hominis*, *L. amazonensis*, and *T. brucei gambiense* was assessed by the motility. In the case of *B. hominis*, we could not use motility as a criterion. Therefore, trypan blue exclusion (0.5% trypan blue solution mixed with an equal volume of the suspension) was used. It is likely that dead *B. hominis* had disappeared by either autolysis or physical damage.

Results

Seeding

Figure 2 shows the changes in the cooling curves of the medium in a cryotube at a time when alcohol was being cooled at a rate of 1℃/min. When the medium was frozen without seeding, a rapid temperature rise from –17.2±1.9℃ to –6.0±0.6℃ (equivalent to its freezing point) was observed, and then cooled to –20℃ in a few minutes (broken line in Fig. 2). The occurrence of this temperature rise was confirmed by a total of 10 trials for each experiment. Alternatively, when seeding was performed at –4℃, the remarkable temperature rise was not observed (bold line in Fig. 2). To determine the optimum seeding temperature, seeding was done at –4, –6, and –8℃. Temperature elevations of 0.5±0.4℃ and 2.9±0.6℃ were observed when seeding was performed at –6℃ and –8℃, respectively. On the other hand, no obvious elevation was observed when seeding was done at –4℃.

The influence of seeding on the survival rates was examined using two protozoa, *E. histolytica* and *T. vaginalis*. The survival rate of *E. histolytica* increased from 40±13% to 48±6% when seeding was performed at –4℃, and these differences were statistically significant ($p<0.05$, χ^2test). In contrast, the survival rate of *T. vaginalis* with seeding (70±6%) was not significantly different from that without seeding (67±5%).

Cryoprotectants

Table 1 shows the effects of the cryoprotectant type and concentration as well as the effects of equilibration time on the survival rate of *T. vaginalis* after cryopreservation. Of the six cryoprotectants examined, Me_2SO, glycerol, and PROH showed protective activity for *T. vaginalis*. When Me_2SO was used as the cryoprotectant, a maximum survival rate (60%) was achieved at a concentration of 10% without equilibration, and equilibration for more than 30 min decreased the survival rate. On the other hand, when glycerol was used as the cryoprotectant, the survival rate increased sharply as the equilibration time increased. The best survival rate (75%) was obtained when glycerol was used at a concentration of 15% with equilibration for 2 hr. In the case of PROH, the maximum survival rate was obtained at a concentration of 15% without equilibration, but it was lower than the best survival rate with Me_2SO or glycerol.

To clarify the cytotoxicity of the cryoprotectants, *T. vaginalis* was incubated in the presence of 5 or 10% Me_2SO, or 10 or 15% glycerol. The survival rates of *T. vaginalis* in 5% Me_2SO_4, 10% Me_2SO_4, 10% glycerol, and 15% glycerol after 6 hr were 62%, 1%, 96%, and 47%, respectively. With the medium containing 10% glycerol, there was no difference in the survival rate of *T. vaginalis* with regard to the incubation time,

and it showed the lowest level of cytotoxicity.

Freezing and thawing rates

Table 2 shows the effects of various freezing rates on the survival rates of six protozoa. *T. brucei gambiense* showed a survival rate of more than 60% at all freezing rates examined. *L. amazonensis* survived at a rate of about 90% at freezing rates of 0.1-10.0℃/min. The best survival rate of *B. hominis* (82%) was obtained at a freezing rate of 1℃/min. On the contrary, the maximum survival rates of *E. histolytica*, *T. vaginalis*, and *P. hominis* were obviously lower than those of the three protozoa mentioned above. Furthermore, the optimum ranges of the freezing rates were narrow.

Three species of protozoa (*E. histolytica*, *T. vaginalis* and *L. amazonensis*) were thawed using five different thawing rates after cryopreservation (Table 3). The results for all these protozoa showed the same trend. The maximum survival rate was observed when the suspension was thawed at 35 and 70℃/min.

Discussion

Since the first successful cryopreservation of *Plasmodium* spp. in 1939, some

species of parasites have been successfully cryopreserved, including protozoa such as *T. gondii* [1], *E. histolytica* [3], *Trypanosoma cruzi* [4], *G. lamblia* [7], and *T. vaginalis* [12], as well as helminths such as trichostrongylidae [5], filarioidea [6], *Trichinella* spp. [9], and *T. canis* [10]. For example, Weathersby and McCall [14] reported that *Plasmodium gallinaceum* frozen without cryoprotectants was as infective as the nonfrozen organism. Lyman and Marchin [7] reported that 90% of *G. lamblia* survived after cryopreservation. On the other hand, some parasites showed very low survival rates after freezing. In the case of *E. histolytica*, the rate was only 10% [3], and muscle-stage larvae of *Trichinella* spp. after freezing showed motility but were not infective [9]. To obtain higher survival rates, further experiments to determine the optimum conditions for cryoprotectants and freezing-and-thawing rates are needed [2].

Few reports have mentioned the effect of temperature elevation due to super-cooling on the survival rate after freezing. Uga and Matsumura [12] reported that seeding significantly improved the survival rate of *T. vaginalis*, from 60% to 79%. In addition, Trad et al. [11] confirmed that seeding increased the survival rate of human oocytes. However, in the present study, seeding was only effective for *E. histolytica*, but not so effective for *T. vaginalis*. This result conflicted with the report by Uga and Matsumura [12]. The reason for this discrepancy may be differences in the culture

conditions or strains of protozoa used in the different studies.

In our study, six types of cryoprotectants were examined. Among them, Me_2SO and glycerol showed the highest cryoprotective effects. Me_2SO has been used as a cryoprotectant for the cryopreservation of many species of protozoa, and its optimum concentration was previously reported to be around 10%. For example, the maximum survival rate of *E. histolytica* (10%) was obtained when it was cryopreserved in the presence of 7.5% Me_2SO [3]. When 6.5% Me_2SO was used, 90% of *G. lamblia* survived [7]. Furthermore, an optimum Me_2SO concentration of 12.5% was obtained for the cryopreservation of *T. gondii* [1]. Using *T. vaginalis*, the optimum concentration of Me_2SO was 5-10%. These results suggest that regardless of the species of parasite, Me_2SO shows an optimum protective effect at concentrations between 5 to 12.5%.

On the other hand, the usefulness of glycerol as a cryoprotectant is a matter of controversy. For example, when *T. vaginalis* and *Plasmodium chabaudi* were cryopreserved using 10% glycerol, no protective effect was observed for the former [12], but a protectiveness similar to that of Me_2SO was obtained for the latter [8]. In both Me_2SO and glycerol, the mechanism of the protective effect on frozen cells occurs by intracellular permeation. The difference between Me_2SO and glycerol is the speed at which they permeate the cell: glycerol permeates more slowly than Me_2SO. Therefore, it

has been reported that equilibration is necessary when glycerol is used as a cryoprotectant. In our study, equilibration remarkably improved the survival rate of *T. vaginalis*. The optimum duration of equilibration was revealed to be 6 hr for 10% glycerol and 2-3 hr for 15% glycerol. Mutetwa and James [8] reported that the best survival rate of *P. chabaudi* was obtained with equilibration for 15 min in the presence of 10% glycerol.

We also examined the cytotoxicities of Me_2SO and glycerol, since they showed the highest cryoprotective effects. Me_2SO showed a density-dependent cytotoxicity effect. This corresponds with our observation that when Me_2SO was used as a cryoprotectant, the survival rate decreased as the equilibration time increased. The cytotoxicity of glycerol was also confirmed, but it was not as adverse as that of Me_2SO. Therefore, glycerol may be considered an effective cryoprotectant for the cryopreservation of helminths that have never been successfully cryopreserved.

Both rapid and slow freezing methods have been used for the cryopreservation of parasites. In the rapid freezing method, a cryotube containing the parasite is directly immersed into liquid nitrogen (3,600-5,100℃/min). *Babesia rodhaini* [2], *T. cruzi* [4], and *Plasmodium* spp. [8, 14] were successfully cryopreserved by this method. The slow freezing method uses a programming freezer or alcohol bath to control the freezing rate (1-20℃/min), and this method has been applied to protozoa such as *T. gondii* [1], *E.*

histolytica [3], *G. lamblia* [7], and *T. vaginalis* [12]. This method is applicable to other protozoa (e.g. *Plasmodium* spp. and *Trypanosoma* spp.), which can also be cryopreserved by the rapid method.

In the present study, we examined different freezing rates using six species of protozoa. *T. brucei gambiense* and *L. amazonensis* each tolerated a wide range of freezing rates, and thus we considered them to have high levels of freezing tolerance. On the other hand, for the four remaining protozoa, the ranges of optimum freezing rates were narrow, and incorrect freezing rates remarkably decreased their survival rates. Therefore, we classified these protozoa as having low freezing tolerances. From the results obtained, we concluded that the majority of blood protozoa have high freezing tolerances, whereas the majority of intestinal protozoa have low freezing tolerances. Although tolerance to freezing has been reported to be related to cell size and/or the kind of organism [2], we could not clarify how these factors influenced the freezing tolerance. Uga et al. [13] cryopreserved *Angiostrongylus cantonensis* eggs and concluded that these smaller cells may be less affected by freezing. They also found that the optimum cooling rate for 16-cell stage eggs was lower than that for 1-cell stage eggs. This was thought to be related to the water contained in the cytoplasm.

Unlike the case with freezing rates, few studies on thawing rates have been

reported. Of the studies that have been done, most have adopted a rapid thawing method using a water bath at 35-40℃. The few studies on thawing rates have revealed that faster thawing rates produce better infectivity or motility [2, 8, 12]. The same result was obtained in this study, and thus we concluded that rapid thawing (more than 35℃/min) was superior and ensured the best recovery for all parasites. In our system, however, we could not obtain linear thawing curves, although these were the same thawing methods that have been used in other studies [3-7, 9, 10].

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Figure legends

Fig. 1. Freezing equipment.

Fig. 2. Changes in cooling curve due to seeding. Seeding was performed at -4℃.

Fig.1

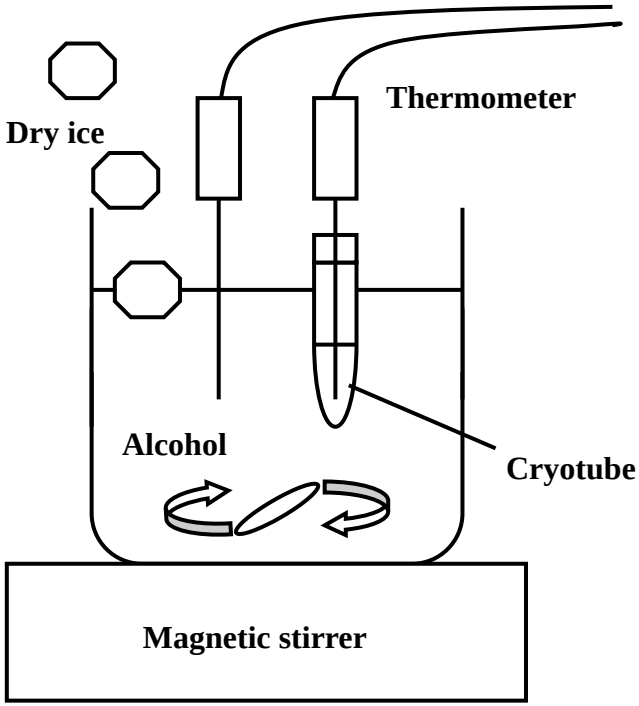

Fig.2

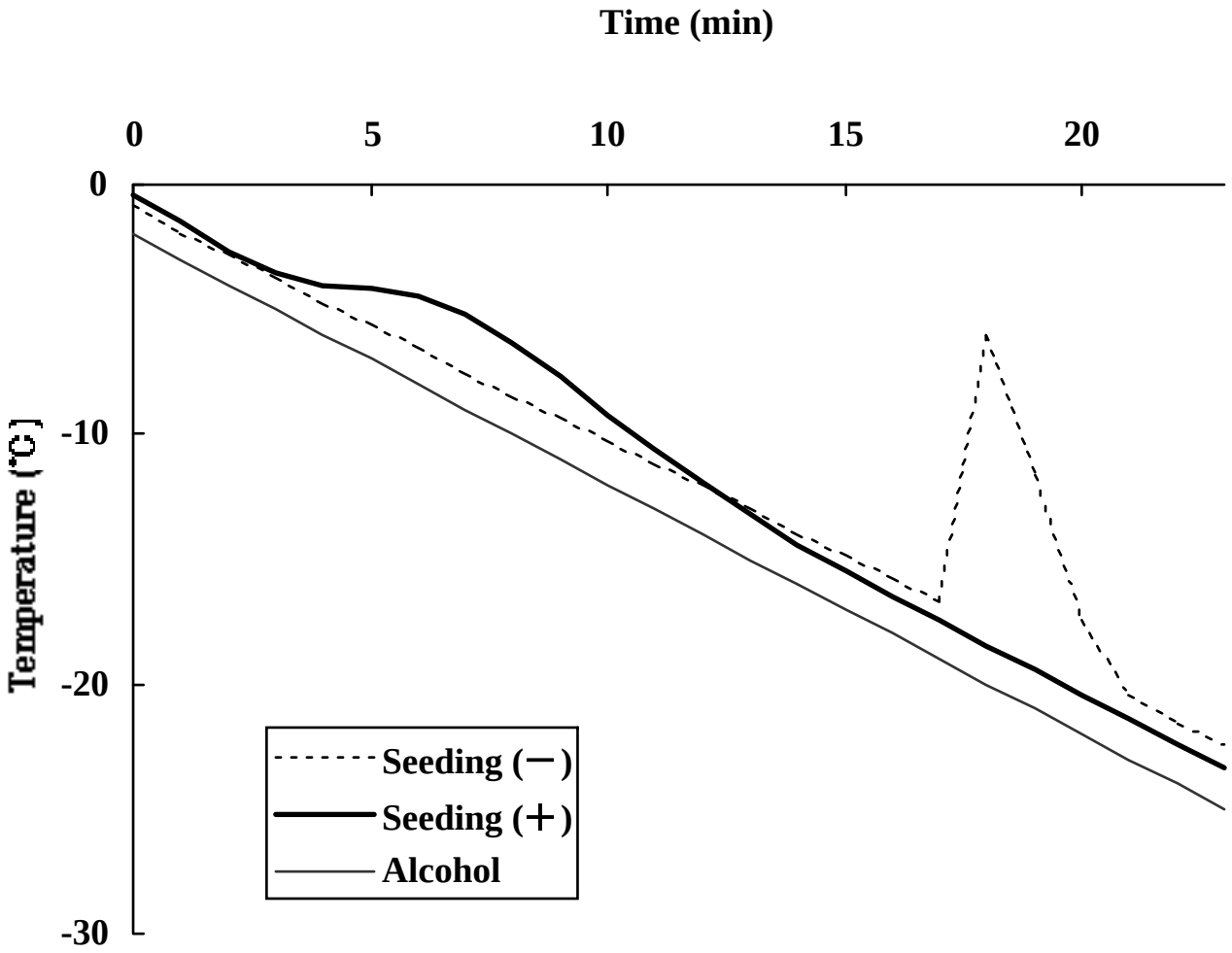
Time (min)
0
5
10
15
20
0
-10
-20
-30
Temperature (℃)
Seeding (－)
Seeding (＋)
Alcohol

Table 1

Effects of type, concentration, and equilibration time of cryoprotectants on survival rates of *Trichomonas vaginalis*

Type of cryoprotectants	Equilibration time (hr)	Concentration of cryoprotectants (%)				
		3	5	10	15	20
Me_2SO	0	24	35	60	15	0
	0.5	36	59	48	0	0
	1	12	29	44	3	0
	2	7	19	26	2	0
	3	9	24	9	0	0
Glycerol	0	0	0	0	2	0
	1	4	6	24	50	13
	2	0	3	52	75	25
	3	4	3	61	74	45
	4	0	14	66	68	32
	6	1	25	73	30	10
	8	9	45	50	22	2
	10	1	11	48	6	0
	12	1	18	46	4	0
PROH	0	0	0	34	48	13
	0.5	0	0	30	45	1
	1	0	1	29	12	0
	2	0	0	6	0	0
	3	0	2	8	0	0
PVP	0	0	0	0	0	0
	3	0	0	0	0	0
Horse serum	0	0	0	0	0	0
	3	0	0	0	0	0
Egg yolk	0	0	0	0	0	0
	3	0	0	0	0	0

Cryoprotectants were added at 37℃. After equilibration, the samples were cooled to -30℃ at 1℃/min, and to -70℃ at 2℃/min. Seeding was performed at -4℃. They were then thawed in a 37℃ water bath for 1 min after being kept at -70℃ for at least 20 min. Each experiment was performed two times.

Table 2

Survival rates of six protozoa after cryopreservation at different freezing rates

Parasites	Freezing rate (℃/min)						
	< 0.1	0.1	0.3	1.0	5.0	10.0	10.0 <
E. histolytica	41	55	47	32	1	0	0
T. vaginalis	6	27	61	61	57	25	0
P. hominis	49	51	55	58	44	7	0
T. brucei gambiense	68	66	85	83	92	94	61
L. amazonensis	11	88	89	94	92	93	6
B. hominis	40	49	70	82	66	36	1

10% Me_2SO was used as a cryoprotectant. The samples were cooled to 0℃ at 1℃/min, and to -30℃ at the different freezing rates. After that, they were cooled to -70℃ at 2℃/min. Seeding was performed at -4℃. They were then thawed in a 37℃ water bath for 1 min after being kept at -70℃ for at least 20 min. Each experiment was performed three times.

Table 3

Survival rates of three protozoa after cryopreservation at different thawing rates

Parasites	Thawing rate (℃/min)				
	2	7	35	70	140
E. histolytica	23	28	34	45	44
T. vaginalis	8	22	63	59	58
L. amazonensis	78	84	94	90	89

10% Me_2SO was used as a cryoprotectant. The samples were cooled to -30℃ at 1℃/min, and to -70℃ at 2℃/min. Seeding was performed at -4℃. They were then kept at -70℃ for at least 20 min, and next were thawed at different rates. Each experiment was performed three times.