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A comparative study in direct cryopreservative efficacy between Triladly® and EDTA saline glucose 10% glycerol cryopreservative media for human and non-human infective trypanosomes

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ABSTRACT

The efficacy of Triladyl®, a commercial cryomedium for bull semen, in the cryopreservation of both human and animal infective trypanosomes as compared to EDTA Saline Glucose (ESG) 10% glycerol was evaluated in the current study. Cryopreserved *Trypanosoma brucei rhodesiense*, *T. evansi*, *T. b. brucei* and *T. congolense* were first propagated in irradiated mice. At the peak of parasitemia, parasites were harvested by cardiac puncture and 10^6,10^5,$10^4$$10^3$,$10^2$ and 10 dilutions made using whole blood bled from clean mice. These dilutions were divided into two equal portions of 0.5 ml each and cryopreserved in both ESG 10% glycerol and neat Triladly®. The procedure was also repeated with *T. congolense* and *T. vivax* species of trypanosomes directly isolated from naturally infected cattle. After 1 month of cryopreservation, 0.4 ml each portion of this dilution was injected intraperitonially into irradiated Swiss white mice. Results on pre-patent period (ppp) and progression of parasitemia showed no difference in the recovery of samples cryopreserved using the 2 media. However, mice injected with *T. b. brucei* cryopreserved in the 2 media showed highly significantly ($p < 0.01$ by *t*-test) lower ppp when compared to the other species of trypanosomes which had no significant difference. However, the ppp in mice injected with trypanosomes cryopreserved in ESG 10% glycerol was significantly lower ($p < 0.05$ by *t*-test) when compared to those cryopreserved in Triladyl®. The interaction between media and species was highly significant indicating therefore that the difference in cryopreservation between the two media varies from one species of trypanosome to the other. The interaction between dose and species was also highly significant ($p < 0.01$ by *t*-test) implying therefore that the effect of the inoculum dose varied from one species to the other leading to the conclusion therefore that although Triladyl® appears as good a cryopreservative medium as ESG 10% glycerol, the choice will be determined by the species of trypanosome.

Key Words: *Trypanosoma*, cryopreservation

INTRODUCTION

Trypanosomes, the causative agent of African trypanosomiasis, are isolated from tsetse flies, domestic animals, wild animals and humans in the field for research and diagnostic purposes. Currently, there are 3 methods of isolating bloodstream trypanosomes from patients, and each has limitations. These methods are: (1) inoculation of infected blood into susceptible rodent, but is limited by non-susceptibility of rodents to some species of trypanosomes (Duke *et al*., 1989; Aerts *et al*., 1992; Matovu *et al*., 2001), (2) inoculation of bloodstream forms into *in vitro* culture, but is limited by risk of contamination of the culture (Aerts *et al*., 1992) and (3) cryopreservation of blood or cerebrospinal fluid (CSF) (Dar *et al*., 1972). Infected host may carry mixed infection and every isolation technique is likely to select the best growing

parts of trypanosome population leading therefore to uncontrolled cloning due to the competitions of various populations during the course of multiple passages in the laboratory animals. In addition, some species such as *T. vivax* will not grow in laboratory rodents and can therefore only be preserved by direct cryopreservation (Dar *et al*., 1972). Cryopreservation of infected samples in liquid nitrogen and subsequent propagation in laboratories works as an ideal method for isolation of trypanosomes. Most of the cryopreservation methods were developed about 50 years ago (Polge and Sotlys, 1957; Cunningham *et al*., 1963) and later improved for field application by Dar *et al*. (1972). However, reduced recovery or complete loss of trypanosomes, especially *T .b. gambiense*, has been reported with cryopreservation method (Burri and Keiser, 2001; Matovu *et al*., 2001). To preserve all parasite populations, it is important to prepare direct cryostabilates in liquid nitrogen as soon as possible without prior propagation in the laboratory animals. This is however limited by the normally low parasitemia in the host that could lead to poor recovery (Duke *et al*., 1989; Aerts *et al*., 1992; Matovu *et al*., 2001). Thus the advantage of cryopreservation over passage in animals is that it avoids modifications of isolates which may affect morphology, infectivity, antigenicity and resistance (Weinman and McAllister, 1947). Direct cryopreservation will also cut down on cost and logistics of taking laboratory animals to the field for the purpose of isolation.

The efficacy of the isolation medium employed determines the recovery of the cryopreserved trypanosomes. EDTA Saline glucose (EGS) in 10% glycerol has been used in the cryopreservation of trypanosomes, but there is need to develop other cryopreservation media which can improve the recoveries of trypanosomes. Triladyl®, a medium normally used for preservation of bull semen, has been reported to be effective in cryopreserving *T. b. gambiense* isolates with superior recovery rate than the standard 10% glycerol (Maina *et al*., 2006). It is not known whether this media can have similar recovery rates for other human and livestock infective trypanosomes species. The aim of this study was therefore to investigate the efficacy of Triladyl® in the cryopreservation of different species of trypanosomes as compared to ESG 10% glycerol.

MATERIALS AND METHODS

Mice

Adult irradiated Swiss White mice weighing between 30 - 33 g used in this study were obtained from International Livestock Research Institute (ILRI). The irradiated group received 600 rads for 6 minutes. They were maintained in mice cages which had wood shavings as beddings. The mice were fed commercial mice pellets (Mice Pellets®, Unga Limited, Kenya) and water was provided *ad libitum*.

Media used in cryopreservation

Triladyl® from Minitüb GmbH (Tiefenbach, Germany) was obtained courtesy of the Swiss Tropical Institute, Basel, Switzerland. It contains 8% glycerol, 20% egg york, Tris buffer, citric acid, fructose, and antibiotics (tylosin 5 mg/L, spectinomycin 30 mg/L, and linomysin 15 mg/L in distilled water). ESG 10% glycerol was prepared using the following formulation: EDTA disodium salt 2.0 g, sodium chloride 8.0 g, potassium dihydrogenhydrogen phosphate 0.30 g, glucose 2 g. These were dissolved in 950 ml distilled water, pH adjusted to 8.0 and then topped to 1 liter and 20% glycerol (v/v) solution prepared.

Trypanosomes used in the study

The trypanosomes *T. b. brucei* KETRI 2710, *T. b. rhodesiense* KETRI 3738, *T. evansi* KETRI 2439 and *T. congolense* KETRI 2267 were randomly selected from KARI-TRC trypanosome bank and propagated in irradiated Swiss White mice. At the peak of parasitemia, the mice were euthanized with concentrated carbon dioxide, bled from heart and the parasitemia quantified using improved neubauer

counting chamber. Dilutions of 10^6,10^5,10^4,10^3 trypanosomes per milliliter were made and each of this dilution was divided into 2 equal portions in sealed ampoules, of which one was cryopreserved in Triladly® and the other in ESG 10% glycerol. The samples were then suspended in liquid nitrogen vapor (-60°C to -80°C) for 2 hours followed by permanent storage in liquid nitrogen at -196°C.

Field *T. congolense* and *T. vivax* isolates were isolated from cattle in Narok District, Kenya after detection of trypanosomes in capillary blood buffy-coat (Paris *et al*., 1982). Infected blood from the jugular vein was then collected in heparinised vacutainer tubes at a parasitemia concentration of 1 trypanosome in 20 microscopical fields. The collected blood was divided into 2 equal portions. One portion was mixed with Triladly® and the other with ESG 10% glycerol in the ratio of 1:1, suspended in liquid nitrogen vapor (-60°C to -80°C) for 2 hours followed by permanent storage in liquid nitrogen at -196°C.

Studies in mice

The trypanosomes referred above were cryopreserved for a period of 1 month. They were then thawed at room temperature and each sample injected intraperitonially (i.p.) as described by Kagira *et al*. (2007) and Gichuki and Brun (1999) into 2 irradiated mice with an inoculum of 0.4 ml. They were monitored daily for trypanosome prepatent period and infection progression. Blood (20 µl) from mouse tail snip method was examined daily for parasites and parasitemia levels assessed using the matching technique of Herbert and Lumsden (1976) for the *T. brucei* group. For *T. congolense*, parasitemia were estimated as described by Paris *et al*. (1982). The pre-patent period (PPP), *i.e.* the time between infection and appearance of trypanosomes in the blood, and parasitemia progression for each mouse was determined and recorded. The animals were monitored for 30 days post infection after which they were euthanized while those that died within the monitoring period were recorded.

Statistical analysis

Data was entered in MS Excel worksheets and statistically analyzed using Genstat 5 Release 3.2 statistical package. Descriptive statistics were presented as tables and graphs. Log linear regression was used to model the data and test effects of the various factors.

RESULTS

Mice inoculated with different trypanosomes levels cryopreserved in the 2 media and which were obtained from the trypanosome bank became parasitaemic, except those injected with *T. evansi* and. *T. congolense* at a dilution of 10 trypanosomes / ml which did not become parasitaemic. Mice injected with *T. b. brucei* cryopreserved in the 2 media showed highly significantly ($p < 0.001$) lower ppp when compared to the other species of trypanosomes which had no significant difference. The prepatent period for the *T. b. brucei* was in the range of 2 - 3 days, *T. b. rhodesiense* in the range of 4 - 5 days, *T. evansi* 4 days while *T. congolense* was in the range of 4 - 6 days. However, the average ppp in mice injected with trypanosomes cryopreserved in ESG 10% glycerol was significantly lower $p < 0.05$ when compared to those cryopreserved in Triladyl®. Media versus species interaction was highly significant at $p < 0.01$ *e.g.* though *T. evansi* had the highest ppp, with ESG 10% glycerol, ppp for the other species were nevertheless lower in relation to ppp in mice injected with trypanosomes cryopreserved in Triladyl® (Fig. 1).

The ppp interaction between media and species is highly significant indicating that the effect of media on ppp varies from one species to the other for example, in *T. b. brucei*, Triladyl® is better while in *T. evansi*, ESG 10% is better.

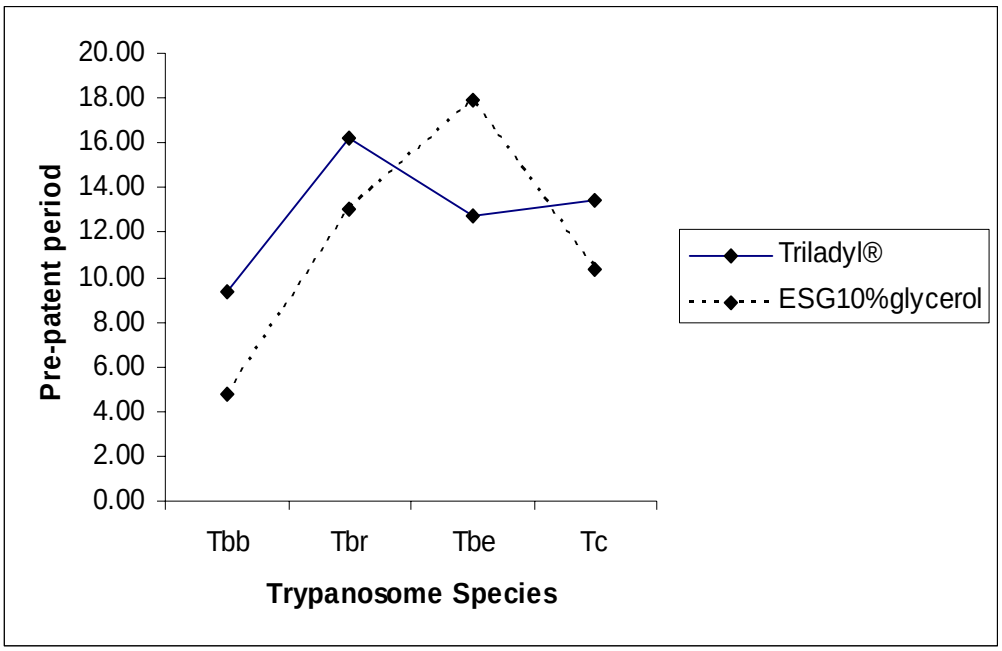

Fig. 1. Average pre-patent period (ppp) of different species of trypanosomes cryopreserved in Triladyl® and ESG 10% glycerol across all the concentrations

Survival in days post onset of parasitemia

Survival was highly significantly different ($p < 0.001$) between the species cryopreserved in the 2 media with the highest and lowest survival observed in *T. b. rhodesiense* and *T. b. brucei* respectively (Fig 2). The survival interactions between media and species and between dose and species were also highly significant irrespective of media used (Fig 3).

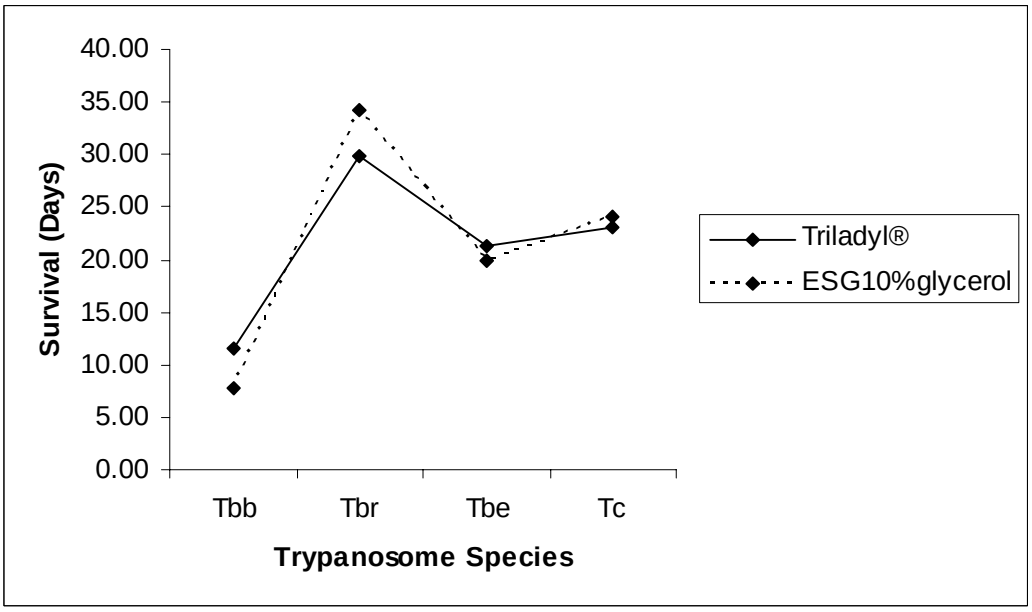

Figure 2: Survival of infected mice across all the inoculum concentrations

No significant survival difference was observed in mice infected with trypanosomes cryopreserved in the 2 media across all the inoculum dosages tested.

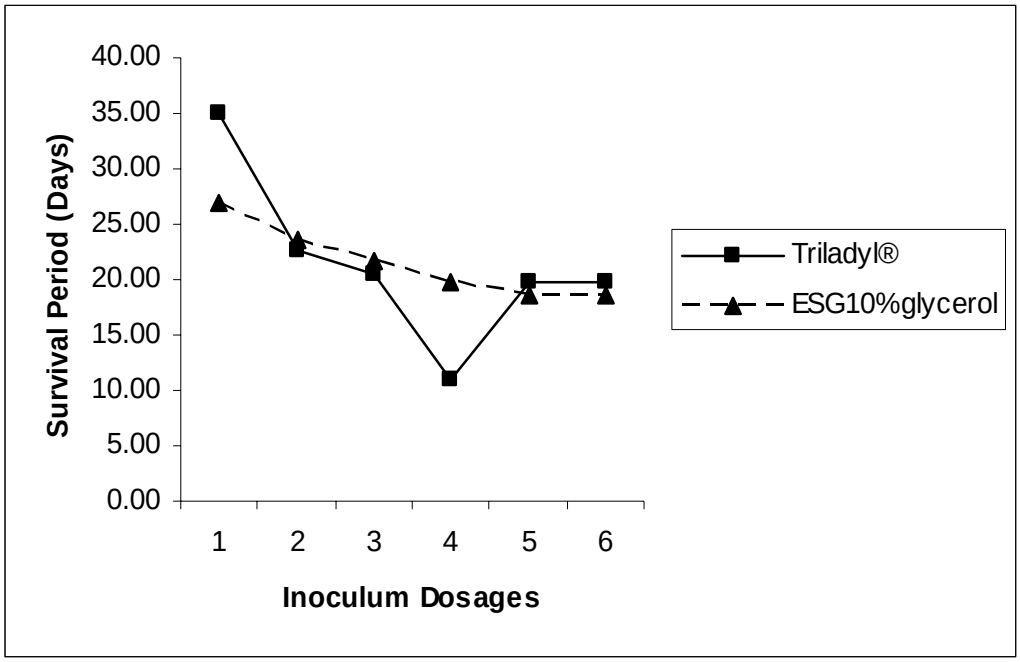

Figure 3: Survival of infected mice in relation to concentration and species

The ppp for the higher inoculum dosages (10^4 - 10^6 trypanosomes / ml) in both media was relatively shorter when compared to lower inoculum dosages (10^2 - 10^3 trypanosomes / ml). In addition, no recovery of cryopreserved *T. congolense* parasite at a dilution of 10 tryps / ml was observed. However, there was recovery of parasites cryopreserved in both media at 1 x 10^2 trypanosomes / ml.

Parasitemia development in mice injected with *T. congolense* and *T. vivax* field isolates

There were no trypanosomes recovered from field samples cryo-preserved using either of the 2 media.

Parasitemia progression in mice injected with trypanosomes from trypanosome bank

There was no significant different in the progression of parasitemia after the onset in mice injected with trypanosomes cryopreserved in the 2 media. The highest parasitemia recorded across all the dosages for both *T. b brucei* and *T. evansi* was 1 x 10^9 trypanosomes while in *T. b rhodesiense* infected mice the highest parasitemia was in the range of 2.5 x 10^8 and 5.7 x 10^8 trypanosomes / ml. However, in *T .congolense* infected mice, the highest parasitemia recorded across all its dosages was 2.5 x 10^8 trypanosomes / ml.

DISCUSSION

The viability and infectivity of different species of trypanosomes parasites cryopreserved in Triladyl® as shown in this study demonstrates this as a good cryopreservative medium. The ability therefore for Triladyl® cryopreserved parasites to grow in mice as well as its ability to preserve low human and animal trypanosome parasites makes it a good alternative cryopreservation medium for these parasites. Previously, the *in vivo* testing of the viability using cryopreserved ESG trypanosomes showed reduced viability or complete loss of parasites especially *T. b. gambiense* (Matovu *et al*., 2001) while Triladyl® was shown to be

a good cryopreservative for *T. b. gambiense* which is known to maintain low parasitemia in the infected host (Maina *et al*., 2006). The ability therefore of Triladyl® to cryopreserve animal trypanosomes shows that it can be used as an alternative cryopreservative medium. Indeed, results from this study have showed that there was no marked difference in the ppp, parasitemia progression, as well as peak parasitemia in mice infected with trypanosomes cryopreserved in the 2 media. This indicates that Triladyl® has no adverse effects on the parasite that would interfere with the intrinsic virulence of the cryopreserved trypanosomes. The observation that ppp for the higher inoculum dosages was relatively shorter when compared to lower inoculum dosages was in agreement with results from previous studies by Maina *et al*. (2006).

The observed good efficacy of Triladyl® has been related to the presence of egg yolk (20%) which acts as cryoprotectant protecting the lipoproteins in the cell membrane during freezing and thawing (Maina *et al*., 2006). A further advantage of Triladyl® is that it is a ready-to-use commercial product, in contrast to ESG which required purchase and constitution of several reagents. Its use as a cryo-preservative will therefore cut down sample processing time and labour-intensive procedures encountered when using the conventional method. However, its long-term efficacy, availability and affordability will need evaluation.

There was no significant difference in the parasitemia progression in mice infected with trypanosomes received from the trypanosome bank. However, *T. b. brucei* was observed to have a significantly shorter ppp and survival period in relation to the other species. From this observation, it can be postulated that the *T. b. brucei* used in this study was of a higher virulence. Previous studies on the relationship between parasitemia and survival, demonstrated that the higher the parasitemia, the greater the degree of pathogenicity in cattle and likelihood of death in mice (Cross, 1975). In another study by Turner *et al*., (1995) it was observed that stocks that grow faster have higher parasitemia and greater virulence.

In this study, recovery of the animal infective trypanosomes isolated directly in the field using the 2 media was not successful. This can be attributed to several factors such as low level of parasitemia in the jugular blood. Previous work has shown that there are more trypanosomes in the peripheral blood than in the jugular blood regardless of the species of trypanosome species (Gitatha, 1967; Krampitz and Cunningham, 1966). Other workers have shown that ear vein blood samples are more likely to test positive for trypanosomes and that diurnal fluctuations of parasitemia status in the same individual are common (Greig *et al*., 1979). In the field, the duration between trypanosome scoring and collection of isolates is normally lengthy possibly leading to the above mentioned limitations in direct cryopreservation. One way of overcoming this predicament would be concentrating trypanosomes prior to preparation of the cryopreserved stabilates (Brun *et al*., 2001) or by mouse inoculation for those species that susceptible to laboratory rodents. In conclusion therefore, this study has shown that Triladyl® is as good as ESG 10% glycerol in the cryopreservation of different species of trypanosomes, but none of the two is good across the species and the choice of media will depend on the species.

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