Cryopreservation of *Plasmodium* Sporozoites for Whole Organism Vaccination Against Malaria

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Abstract

Effective cryopreservation methods for *Plasmodium* sporozoites are extremely difficult to develop, and their success is highly dependent on various factors, such as vial geometry and the composition of cryopreserving solutions, including the cryoprotectants (CPAs) employed. We proposed to optimize this methodology by testing a wide combination of CPAs and sporozoite freezing conditions in order to identify the optimal freezing formulation for cryopreservation of *Plasmodium* sporozoites. Our results established glass vial as presenting the most appropriate geometry for sporozoite cryopreservation and showed that a rationally selected blend of CPAs leads to a significant retention of ~50% of *P. berghei* viability after cryopreservation and thawing. In parallel, we also identified freezing mixtures that very effectively cryopreserve sporozoites of the human-infective *P. falciparum* parasite. Further *in vivo* tests have aimed at understanding the impact of injecting several freezing compositions *in vivo*, employing mouse models. These results have the potential to significantly advance the current capacity to cryopreserve *Plasmodium* sporozoites, establishing an essential tool for the development of new anti-malarial interventions.

Introduction

Malaria remains a significant public health threat, responsible for an estimated 445 thousand yearly deaths worldwide and placing at risk approximately half of the world's population[1]. This disease is caused by apicomplexan parasites of the *Plasmodium* genus that infect mammalian hosts through the bite of an infected female *Anopheles* mosquito vector. There are five species of *Plasmodium* parasites that can cause malaria in humans, with *P. falciparum* and *P. vivax* being responsible for the highest mortality and morbidity worldwide[2]. The life cycle of mammalian-infective *Plasmodium* parasites comprises 3 main stages: the obligatory liver stage, the symptomatic erythrocytic stage and the transmission stage in the mosquito vector. The cycle starts with the bite of an infected *Anopheles* mosquito and concomitant injection of sporozoites on the mammalian host's dermis[3]. A small number of the injected sporozoites will reach the host's vasculature and enter the blood stream, where they travel to the liver[3]. Sporozoites bind to liver cells through the action of circumsporozoite (CS) protein[4], and subsequently invade hepatocytes with the formation of a parasitophorous vacuole (PV). Inside the PV, the parasites initiate their multiplication and differentiation into merozoites[5]. Upon completion of this developmental process, merosomes, merozoites-filled vesicles, exit the liver cells, lyse and released merozoites infect erythrocytes[6]. Inside erythrocytes, merozoites replicate and are released into the blood stream by lysing the infected erythrocyte and initiating another cycle of erythrocytic infection. During this phase, some merozoites differentiate into gametocytes that may be ingested by a mosquito when it bites an infected host[7]. In the mosquito midgut, gametocytes are fused and form a zygote that will originate a mobile form termed ookinete. Ookinetes traverse the midgut wall and eventually develop into oocysts. Thousands of sporozoites mature inside the oocysts, and subsequently infect the mosquito's salivary glands[8].

Given its obligatory and asymptomatic nature, the liver stage of *Plasmodium* infection is considered an ideal target for vaccination, blocking parasite development as soon it enters in the human body and preventing replication. Until this moment, several subunit vaccine candidates have been proposed, among which RTS,S/AS01 is the most advanced candidate, but whose efficacy is very limited[9]. Alternatively, whole-organism approaches such as radiation attenuated sporozoites (RAS), genetically attenuated parasites (GAP) and chloroquine chemoprophylaxis with sporozoites (CPS) have shown to be very efficacious, and currently constitute the most promising malaria vaccine candidates[10]. However, they also pose safety concerns that arise from the fact that they are based on the use of human-infective *P. falciparum* sporozoites[11,12]. The iMM's Prudêncio lab is currently developing a whole-organism pre-erythrocytic vaccine inspired by the old smallpox vaccine concept. The concept is employs rodent *P. berghei* parasites, which are non-pathogenic to humans, genetically modified to express *P. falciparum* immunogens, leading to an immune response free of safety risks. In this context, a vaccine such as the one being developed at iMM, would constitute an alternative to current whole sporozoite-based vaccine candidates. Nonetheless, the efficiency of all whole-organism malaria vaccines is highly dependent on the number of live cryopreserved sporozoites that remain able to reach the liver after thawing, and elicit an immune response[10,13]. Current cryopreservation methods lead to losses of around 90% of sporozoite viability[13]. Thus, a more effective method for sporozoite cryopreservation is urgently required, that will increase sporozoite survival after cryopreservation, thereby enhancing the efficiency of whole-organism malaria vaccines and facilitating vaccine production and storage[14,15].

Effective cryopreservation methods are extremely difficult to develop for most cells, as is the case of *Plasmodium* sporozoites[16]. During the freeze-thaw processes, cells undergo multiple stresses, including mechanical damage on the cell's outer surface due to the formation of ice crystals, increase in osmotic pressure due to chemical and residual unfrozen water between ice crystals, and the formation of intracellular ice crystals[17,18,19]. Cell type, ice growth direction, vial geometry, cooling and thawing rates are among the most important factors that affect cryopreservation. An ideal cryopreservation methodology relies on the optimization of these factors towards achieving a vitreous state, a process alternative to cryopreservation where there is no ice formation[20,21]. Importantly, the composition of the freezing mixture employed, which normally includes cryoprotective agents (CPAs), has a significant impact on the effectiveness of cryopreservation and can directly lead to a vitreous state[22]. CPAs are chemical compounds that remain in the extracellular environment, (non-penetrating) or even penetrate (penetrating CPAs) cells, preventing the damages caused by the freeze-thaw process [21,22].

As result of the collaboration between iMM's Prudêncio lab and the IST startup Smartfreez Lda, a new strategy for cryopreservation of sporozoites is being developed that uses a patented unidirectional freezing technology, which has been shown to provide better results than conventional freezing by controlling ice formation[23]. Other parameters that influence cryopreservation have been also optimized by the Prudêncio lab and an appropriate cooling and thawing rate has already been determined.

The aim of this thesis was to further optimize this cryopreservation methodology, by evaluating the impact of several freezing conditions on sporozoite viability, towards formulating an ideal cryopreservation mixture capable of maximizing sporozoite survival. Furthermore, we also aimed to provide a comprehensive analysis on the function of each CPA, individually or in combination, in protecting sporozoites during the cryopreservation process.

Materials and Methods

Parasites

Luciferase-expressing *P. berghei* ANKA sporozoites were obtained from the dissection of the salivary glands of infected female *Anopheles stephensi* (*A. stephensi*) mosquitoes, bred at Instituto de Medicina Molecular (iMM) (Lisbon, Portugal), prior to being employed on *in vitro* and *in vivo* essays. *P. falciparum* NF54c sporozoites were obtained from the salivary glands of infected *A. stephensi* mosquitoes provided by Radboud UMC in Nijmegen (Netherlands).

Briefly, infected mosquitoes were chilled at -20ºC for 5 min and washed with 70% ethanol before dissection. Mosquito salivary glands were removed by manual dissection and collected in sterile incomplete RPMI1640 (Gibco) supplemented with 1:300 fungizone 0.25 µg/ml (Gibco), 1% penicillin 5 U/ ml (Gibco), 1% streptomycin 5 µg/ml (Gibco), 1:1000 gentamycin 50 mg/ml (Gibco), at day 19-21 post blood meal. Salivary glands collected were then homogenized by using a pestle and the free sporozoites were counted in a Bürker-Türk counting chamber on a phase-contrast microscope.

CPAs & Vials

All CPAs tested during these experiments were purchased from VWR Chemicals or Sigma (**Supplementary tables**). The vials geometries tested acquired from several manufactures (**Supplementary tables**).

Freeze and Thawing Process

Sporozoites were frozen employing a steel platform designed by SmartFreez Lda. which led to a unidirectional freezing (from the bottom to the top). The steel platform was incubated in dry ice for 30 min prior to use. Sporozoites resuspended in incomplete RPMI1640 were maintained on ice after their isolation from salivary glands and subsequently mixed and chilled with the respective cryopreservant solutions. At each VG2 (n.a) were added $80x10^3$ sporozoites in v(iii) of cryopreservative solution which was immediately frozen. An ice thin layer on the bottom of the vial was induced (nucleation) and the vials were readily placed on a y mm polystyrene foam plate over the steel platform so that a freezing rate of approximately XºC/ min was achieved.

Heat transfer during the freezing was promoted by adding absolute ethanol (VWR Chemicals) to eliminate the air layer between the bottom of the vial and the source of heat. The thawing process was performed at 37ºC, as rapidly as possible. The thawed sporozoites were resuspended in RPMI1640 (supplemented as described above) to dilute cryoprotectants before infection.

Huh7 and HC-04 cell Maintenance

Human hepatoma cells (Huh7 cells) were cultured in RPMI culture medium supplemented with 10% FBS (Gibco), 1% of non-essential amino acids (Gibco), 1% of penicillin 5 U/ml and 1% streptomycin 5 µg/ml, 1% glutamine 2mM (Gibco), and 1% Hepes at pH 7 (Gibco). The cells were seeded in 96-well plate ($10x10^3$ cells/ well) and incubated at 5% CO2 and 37ºC, one day before infection with sporozoites.

Hepatocytoma HC-04 cells 1fg were grown in culture flasks in DMEM F12 (Gibco) medium supplemented with 10% FBS, 1% of non-essential amino acids, 1% of penicillin 5 U/ml and 1% streptomycin 5 µg/ml, 1% glutamine 2 mM, incubated in 5% CO2 at 37ºC. For experimental use cells were seeded in 24-well plates with or without glass coverslips, ($100x10^3$/ well) one day prior to infection with sporozoites.

Characterization of *P. berghei* Hepatic Infection

In order to evaluate hepatic infection, Huh7 cells were seeded in 96-well plates ($10x10^3$ cells/ well) and infected next day with $10x10^3$ firefly luciferase-expressing *Plasmodium berghei* line obtained from the salivary glands of infected *A. stephensis* mosquitoes. The addition of sporozoites was followed by centrifugation at 3000 rpm, for 5 min. The viability of huh7 cells was analyzed 46h after sporozoite addition through AlamarBlue assay (Invitrogen™) following the manufacture´s protocol. Parasite load was measured by luminescence following addition of the luciferin substrate (Biotium) 48 h after infection, as previously described[24].

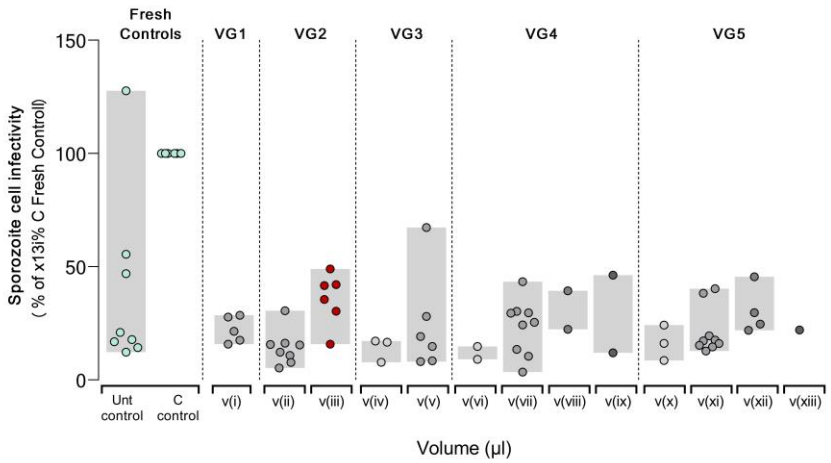


Figure 1 - Performance of different vial geometries for *Plasmodium* sporozoites cryopreservation: (i) Fresh controls; (ii) VG1; (iii) VG2; (iv) VG3, (v) VG4, (Vi) VG5

Characterization of *P. falciparum* Hepatic Invasion

In the conditions described above, HC-O4 cells were seeded in a 24-well plate (100x10^3 cells/ well) with or without glass coverslips, 24 h prior to addition of *Plasmodium* sporozoites. At 0 h, 100x10^3 sporozoites were added to each well previously filled with 800 µl of RPMI supplemented (as described above). Following addition of sporozoites, the plate was centrifuged at 3000 rpm, for 5 min. After 3h of incubation at 37ºC and 5% of CO_2, the wells with glass coverslips were rinsed with 1xPBS and fixed for 10 min with 4% of paraformaldehyde to be used for immunofluorescence microscopy

Immunofluorescence Microscopy Imaging of *P. falciparum* Infected HC-04 Cells

Following fixation with 4% of paraformaldehyde for 10 min, coverslips containing HC-04 cells were blocked with a solution of 1% D (VWR Chemicals) in 1xPBS, for 30 min at room temperature (RT). Incubation with an anti-*P. falciparum* CS protein primary antibody (mAb 2A10 1:300 diluted in 1% BSA/ 1xPBS) at RT for 1 h to stain extracellular parasites was followed by two washing steps and incubation with a secondary antibody goat anti-mouse Alexa Fluor® 546 (1:300 diluted in 1% BSA/ 1xPBS) for 1h at RT. Ice-cold methanol was added and incubated for 15 min at RT in order to induce cell permeabilization and then washed-out by 2 rinse steps with 1x PBS. After cell permeabilization and rinse, an additional blocking step with 1% BSA/ 1xPBS was performed prior 1 h incubation with the anti-CS protein *P. falciparum* primary antibody for extracellular staining. Incubation of 1 h at RT with a secondary antibody goat anti-mouse Alexa Fluor® 488 (1:300 diluted in 1% BSA/ 1xPBS) and Hoechst 33342 (1:900 diluted in 1% FCS/ 1xPBS) was followed by 2 washes with 1xPBS after the second blocking. Samples were mounted using Flourmount G (Southern Biothec) and images were acquired on a Zeiss Axiovert 200M Widefield Fluorescence microscope.

Mice

In vivo experiments were performed using C57BL/6 and Balb/c mice that were purchased from Charles River and housed in IMM's specific pathogen-free rodent facility. All animal experiments were performed in strict compliance to the guidelines of our institution´s animal ethics committee and the Federation of European Laboratory Animal Science Associations (FELASA)).

Liver Infection

Mice were inoculated by injection of infected luciferase-expressing *P. berghei* ANKA sporozoites. For each inoculation, sporozoites were resuspended either in incomplete RPMI1640 (controls without CPA) or in freezing mixtures (with CPA) and inoculated by retro orbital intravenous injection (3x10^4 sporozoites per mice).

Assessment of Liver Infection Load Infection by Bioluminescence

Parasite liver loads in live mice after infection were quantified by real time *in vivo* bioluminescence imaging as previously described[25,26,24,27]. The bellies of C57BL/6 mice were shaved prior to imaging in order to minimize the absorption of light by the highly pigmented fur. At 44 h post-infection, animals were anesthetized using the isoflurane (Zoetis), and D-luciferin (Biotium) dissolved in PBS, was injected subcutaneously. Within 3 to 5 min after injection of D-luciferin, *Plasmodium* liver stages were visualized and liver loads quantified by measuring luciferase activity of parasites in whole bodies of mice through IVIS Lumina II Imaging System (Perkin Elmer Life Sciences).

Quantitative analysis of bioluminescence of whole bodies was performed by measuring the luminescence signal intensity using the region of interest (ROI) settings of the Living Image® 3.0 software. The ROI was set to measure either the abdominal area at the location of the liver for whole body imaging. ROI measurements are expressed in total flux of photons.

Statistical Analysis

All data were tested for normality with the Shapiro-Wilk test prior to analysis. The statistical difference between groups was determined using the 1-way analysis of variance for data following a normal distribution and the Kruskal-Wallis for data not following a normal distribution. A Dunett multiple comparison test was used to identify the statistical differences between groups. All statistical analyses were performed using the GraphPad Prism 7.0 software (GraphPad,San Diego, CA, USA).

Results

Evaluation of Different Cryogenic Vials

Vial geometry is known to be a key parameter for cryopreservation of biological material[28]. We evaluated 5 commercially available cryogenic vials made of different

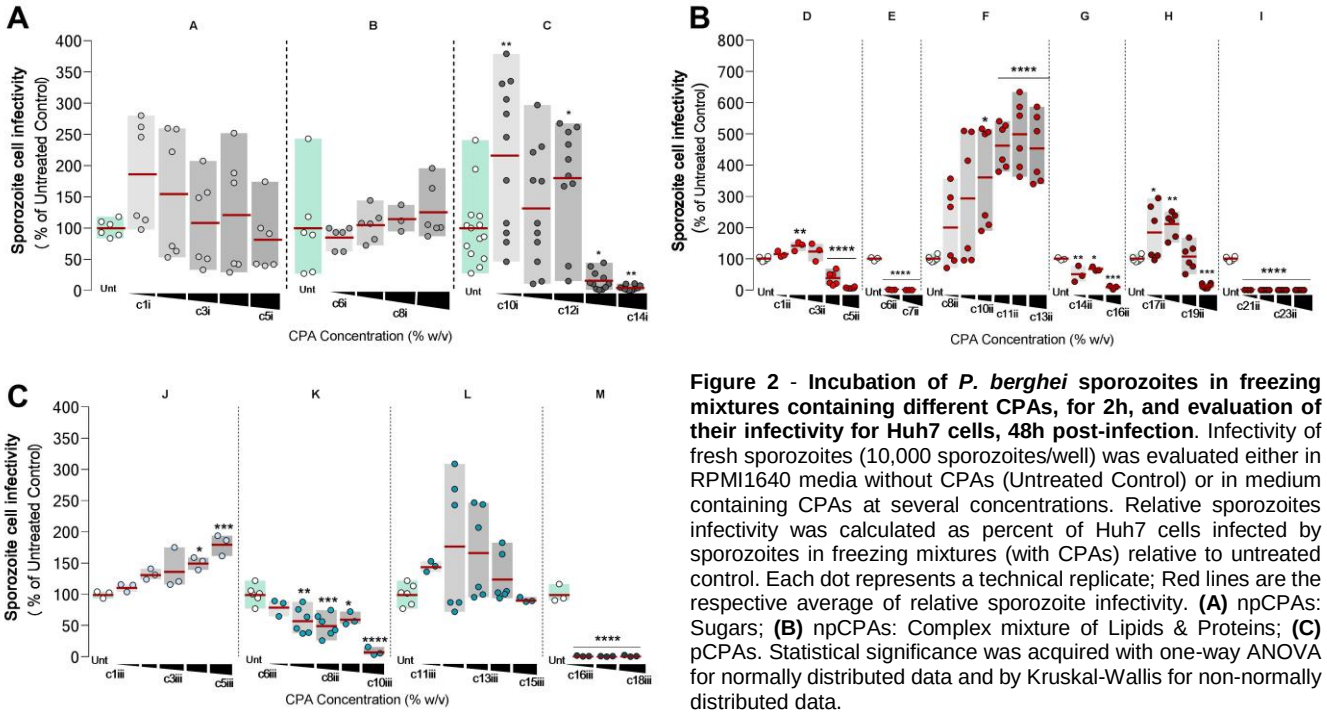


Figure 2 - Incubation of *P. berghei* sporozoites in freezing mixtures containing different CPAs, for 2h, and evaluation of their infectivity for Huh7 cells, 48h post-infection. Infectivity of fresh sporozoites (10,000 sporozoites/well) was evaluated either in RPMI1640 media without CPAs (Untreated Control) or in medium containing CPAs at several concentrations. Relative sporozoites infectivity was calculated as percent of Huh7 cells infected by sporozoites in freezing mixtures (with CPAs) relative to untreated control. Each dot represents a technical replicate; Red lines are the respective average of relative sporozoite infectivity. **(A)** npCPAs: Sugars; **(B)** npCPAs: Complex mixture of Lipids & Proteins; **(C)** pCPAs. Statistical significance was acquired with one-way ANOVA for normally distributed data and by Kruskal-Wallis for non-normally distributed data.

materials and with different physical-thermodynamic features in terms of their performance for sporozoite cryopreservation. Our results confirmed that cryopreservation in VG2 vial using v(iii) of freezing mixture (x13i% C in RPMI) retains sporozoite survival rates above 30% and is the best option among the various vial geometries evaluated. Regarding the freezing mixture volume, our results indicate that decreasing of total volume below 400 µl has a negative impact on sporozoite survival during cryopreservation (**Figure 1**). We hypothesize that lower volumes can results in faster freezing rates, which leads to a decrease in sporozoite survival rates. Based on these observations, we have selected VG2 and v(iii) of total volume of freezing mixture as the most appropriate conditions for the subsequent studies.

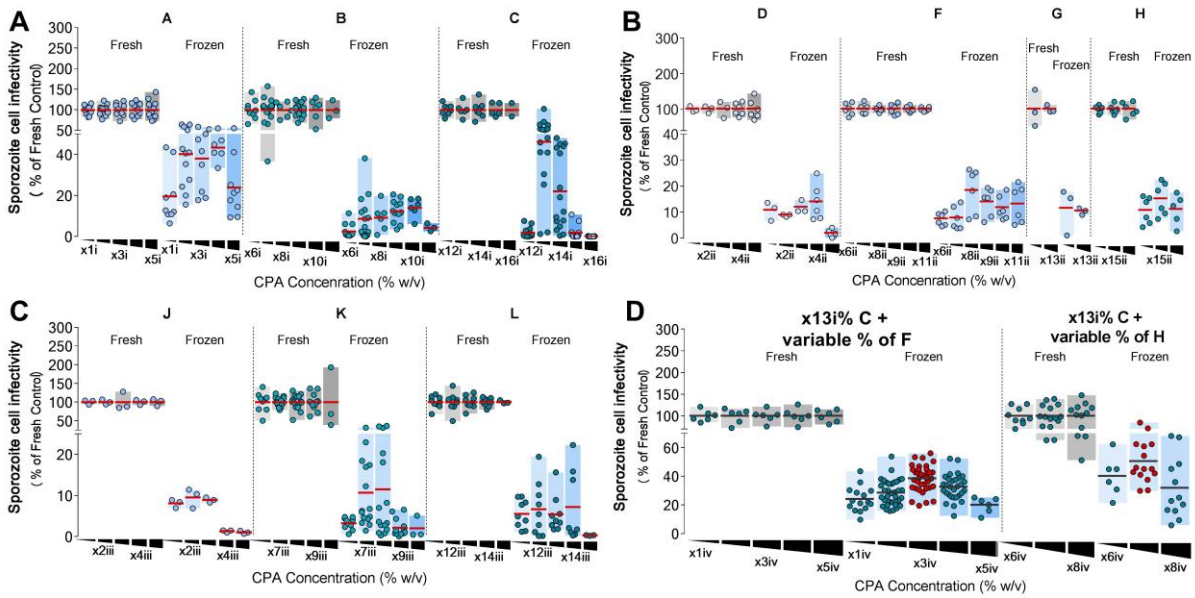
In-House *Plasmodium* Sporozoites Freezing Mixture Formulation

Having determined the most appropriate type of vial geometry and the ideal volume of the freezing mixture, and employing some parameters previously determined by the Prudêncio lab, such as an optimal cooling rate of approximately XºC/min and unidirectional freezing technology, the next step was to evaluate various formulations of freezing mixtures for sporozoite cryopreservation. We aimed for a comprehensive analysis of the effect of adding a variety of well-established chemical compounds with known cryoprotectant qualities (CPAs: cryprotectant agents) to the freezing mixture used for sporozoite cryopreservation (Hubalek, 2003)[22]. We selected a list of CPAs that can be divided into 3 different categories regarding their function, mechanism of action and molecular features: Non-penetrating sugars; Non-penetrating complex mixtures of lipids & proteins; Penetrating CPAs (pCPAs).

Effect of CPA on the Infectivity of Fresh Sporozoites

Although the addition of CPAs to freezing mixtures can have an important role in protecting cells against the deleterious effects of freezing, under specific conditions and concentrations, CPAs can also have a toxic effect that should be considered. Therefore, we conducted a detailed study to determine the effect of selective CPAs addition to mixtures used for sporozoite suspension, in the absence of cryopreservation. To that end, fresh sporozoites were either incubated on ice in a mixture including the CPA concentration under evaluation, or without CPA (untreated control) for 1h, and their relative infectivity was subsequently assessed. We started by evaluating the effect of adding different sugars at various concentrations in the sporozoite suspension. Our results demonstrate that there is no significant decrease in viability associated to A and B in all concentrations tested (**Figure 2 (A)**). The presence of C at concentrations higher than c12i% led to a considerable decrease in sporozoite viability, indicating a potential toxicity profile for this CPA.

We then extended our evaluation to another category of non-penetrating CPAs (npCPAs), which includes complex mixtures of Lipids & Proteins. Our results indicate that the addition of I, E, and very high concentrations of H, D and G resulted in an average decrease of over 60% in sporozoite infectivity, relative to untreated controls (**Figure 2 (B)**). These CPAs were therefore excluded from subsequent studies. The next category of CPAs evaluated was that of Penetrating-CPAs, which included compounds well known for their cryoprotective features[29,30,22]. However, some are also known to have a high toxicity profile for some types of cells, which impairs their widespread use. Of the pCPAs assessed, both K and M resulted in reduced sporozoite survival. However, while a drastic reduction in survival could only be observed at concentrations K higher that c8iii%, all the tested concentrations of M presented a high toxicity profile, which excluded its further inclusion in sporozoite freezing mixtures (**Figure 2 (C)**).



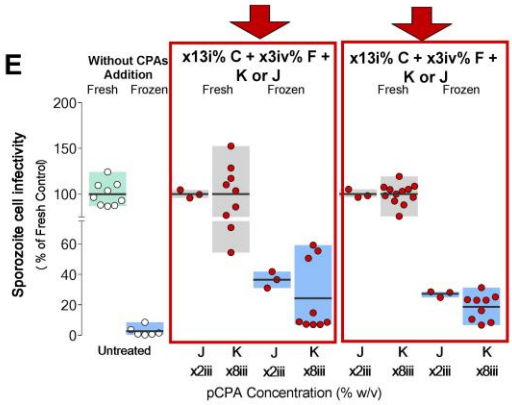


Figure 3 - **Cryopreservation of *P. berghei* sporozoites in freezing mixtures containing specific CPAs or combination of CPAs, and evaluation of their infectivity for Huh7 cells, 48h post-infection.** Infectivity of sporozoites (10,000 sporozoites/well) was evaluated after cryopreservation, with respective CPA at several concentrations. Relative sporozoite infectivity was determined as percent of Huh7 cells infected by cryopreserved sporozoites relative to fresh control in the same freezing mixture. Each dot represents an individual technical replicate; Red lines are the respective average of relative sporozoite infectivity. **(A)** npCPAs: Sugars; **(B)** npCPAs: Complex mixture of Lipids & Proteins; **(C)** pCPAs. **(D)** Freezing mixtures containing x13i% C and variable concentrations of H or F; **(E)** Cryopreservation of *P. berghei* sporozoites in freezing mixtures supplemented with x13i% C, x7iv% H or x3iv% F and x2ii% J or x8iii% K

Performance of CPAs Included in Freezing Mixtures of *Plasmodium* Sporozoites

Following the evaluation of the impact of exposing sporozoites to various CPA-supplemented mixtures, we sought to analyze the protective effect of selected mixtures against the hostile conditions of sporozoite freezing-thawing processes. To this end, we compared the infectivity of cryopreserved sporozoites suspended in different freezing mixtures with that of fresh sporozoites exposed to the same freezing mixtures. The cryopreservation process employed was based on unidirectional freezing, a technology developed in collaboration with our partners, SmartFreez, which has been proven to provide better survival results than conventional freezing through the effective control of ice formation[23]. A single CPA was employed per freezing mixture so that any effects could be directly correlated to a single parameter. We started by evaluating the performance of 3 sugars, A, B, C. As shown in **Figure 3 (A)**, the addition of x13i% of C to the freezing mixture provided high protection for sporozoites, retaining approximately 46% of sporozoite survival after cryopreservation and thawing. A provided similar protection at a wider range of concentrations (from x2i% to x4i%), showing that it is well tolerated by sporozoites. We next assessed the cryoprotection provided by complex mixtures of lipids and proteins, for which sporozoite survival is generally inferior, to what was observed upon sugar addition to the sporozoite suspension. This is particularly the case of x13ii% G and x5ii% D, which already had shown to have a negative impact on sporozoite survival (**Figure 2 (B)**), a profile that was maintained in terms of cryoprotection (**Figure 3 (B)**). Within this category of CPAs, F and H appear to provide the highest protection among the CPAs evaluated. Lastly, we assessed the effect of adding pCPAs., K, J and L, to the freezing mixtures. **Figure 3 (C)** shows that the introduction of very high concentrations of pCPAs leads to a reduction of sporozoite survival, whereas x8iii% and x2iii% K and J, respectively, led to the highest sporozoite survival. Although pCPAs conferred lower protection than all other CPAs, a potential combination of CPAs from different categories, including pCPAs, has been described as enhancing the overall protective effect[22,31].

Combined Effect of Different CPAs in the Same Freezing Mixture

Potentially, a combination of CPAs from different categories, acting in a variety of manners, may boost the protective capacity of a freezing mixture and improve sporozoite survival after cryopreservation. Based on that assumption, we started by selecting x13i% C, the best performing CPA among the Non-Penetrating Sugars tested, and added several concentrations of F or H, which yielded the most promising results among complex mixtures of Lipids & Proteins, to the freezing mixture. Using a freezing mixture containing x13i% C and x3iv% F we obtained a very consistent result of approximately 40% of sporozoite survival. When x7iv% H and x13i% C were employed

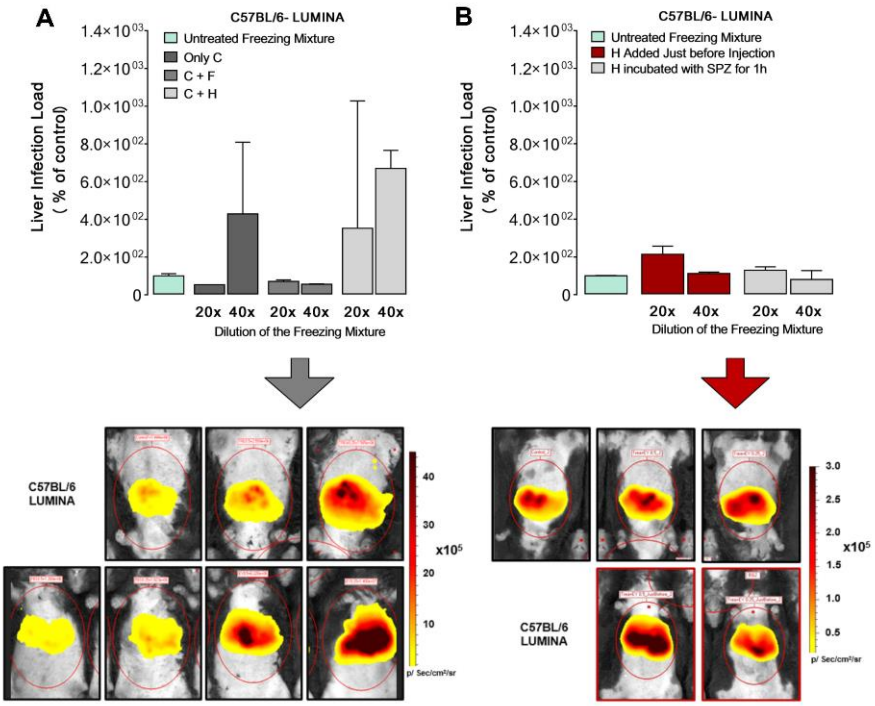


Figure 3 - **Effect of injecting different sporozoite freezing mixtures in mice.** The sporozoites were incubated in 3 freezing mixtures: x13i%C; x13i%C and x3iv%F; or x13i%C and x7iv%H. Before mice injection, each freezing mixture was diluted 4 or 5 times. **(A)** Effect of injecting sporozoite freezing mixtures in C57BL/6 assessed by RT-Imaging. Images: **(upper – left to right)** Untreated Control; z1i%C; z1i %C; **(Lower – left to right)** z1i%C and z1ii%F; z1ii%C and z2ii%F; z1i%C and z1iii%H; z1i%C and z2iii%H. **(B)** Difference of injecting sporozoites incubated for 1 h in the freezing mixture containing C and H or injecting sporozoites without previous incubation; Images: **(upper – left to right)** Untreated Control; z1i%C and z1iii%H; z2i%C and z2iii %H; **(lower – left to right)** z1i%C and z1iii%H; without incubation z2i%C and z2iii %H without incubation.

Plasmodium sporozoite survival after cryopreservation reached 50,4% (**Figure 3 (D)**). This excellent result represents a substantial increase in comparison to the current standard in sporozoite cryopreservation. We next hypothesized that survival rates might be further increased by supplementing the mixture with pCPAs, which are able to penetrate cells and may therefore complement the actions of the other CPAs present. Thus, we supplemented the freezing mixture containing x13i% C and x7iv% H, with K or J, which yielded the most promising results among the pCPAs tested. However, the introduction of pCPA in the mixture did not lead to further improvements, as shown in **Figure 3 (E)**.

Analysis of the Impact of Selected Freezing Mixtures in *in vivo* Mouse Models

Having established that several freezing mixtures are able to provide a substantial protective effect on sporozoites during freezing-thawing cycles, warranting high sporozoite infectivity after cryopreservation, we decided to evaluate selected mixtures using *in vivo* models of sporozoite infection. Specifically, we employed a freezing mixture supplemented with only x13i% C; a freezing mixture with x13i% C and x7iv% H and, a freezing mixture supplemented with x13i% and x3iv% F. We started by analyzing the effect of the selected freezing mixtures on mouse hepatic infections by *Plasmodium*, *in vivo*, in the absence of cryopreservation. To that end, we compared the infectivity of fresh sporozoites injected in the presence of each freezing mixture with that of fresh sporozoites in a freezing mixture without any CPA. Since we observed that an increase in the concentration of injected C led to an enhanced liver infection load (**data not shown**), the concentration of CPA to inject in mice was reduced by diluting each freezing mixture 20 and 40 times so that the percentage of C injected was z1i% and z2i%, respectively. At these dilutions, similar infection loads were observed in the presence and in the absence of C (**Figure 4 (A)**). However, the injection of freezing mixtures containing H enhanced the parasite's liver load in C57Bl6 mice compared with sporozoites injected with the remaining freezing mixtures or in the absence of CPAs, as shown in **Figure 4 (A)**.

In order to understand the reason behind the increase in liver parasite load observed when freezing mixtures containing H are injected, we performed a study where sporozoites were either resuspended in a freezing mixture supplemented with H either one hour prior to injection or only at the moment of the injection. Potentially, this would allow us to determine whether the boosted effect of supplementing the freezing mixtures with H rely in providing a more effective medium for maintaining sporozoites viability after their isolation. The results in **Figure 4 (B)** indicate that there is no significant difference between previously incubating sporozoites in freezing mixtures with H compared to adding this medium to sporozoites only at the moment of injection, suggesting that H acts mostly after being injected in mice.

Invasion Capacity of Cryopreserved *P. falciparum* Sporozoites in Selected Freezing Mixtures

We then sought to evaluate the efficiency of selected freezing mixtures on the cryopreservation of human-infective *P. falciparum* sporozoites. To this end, we compared the invasion rate of HC-04 cells by cryopreserved and fresh *P. falciparum* sporozoites. This method relies on quantifying the number of sporozoites inside and outside cells, as well as the total number

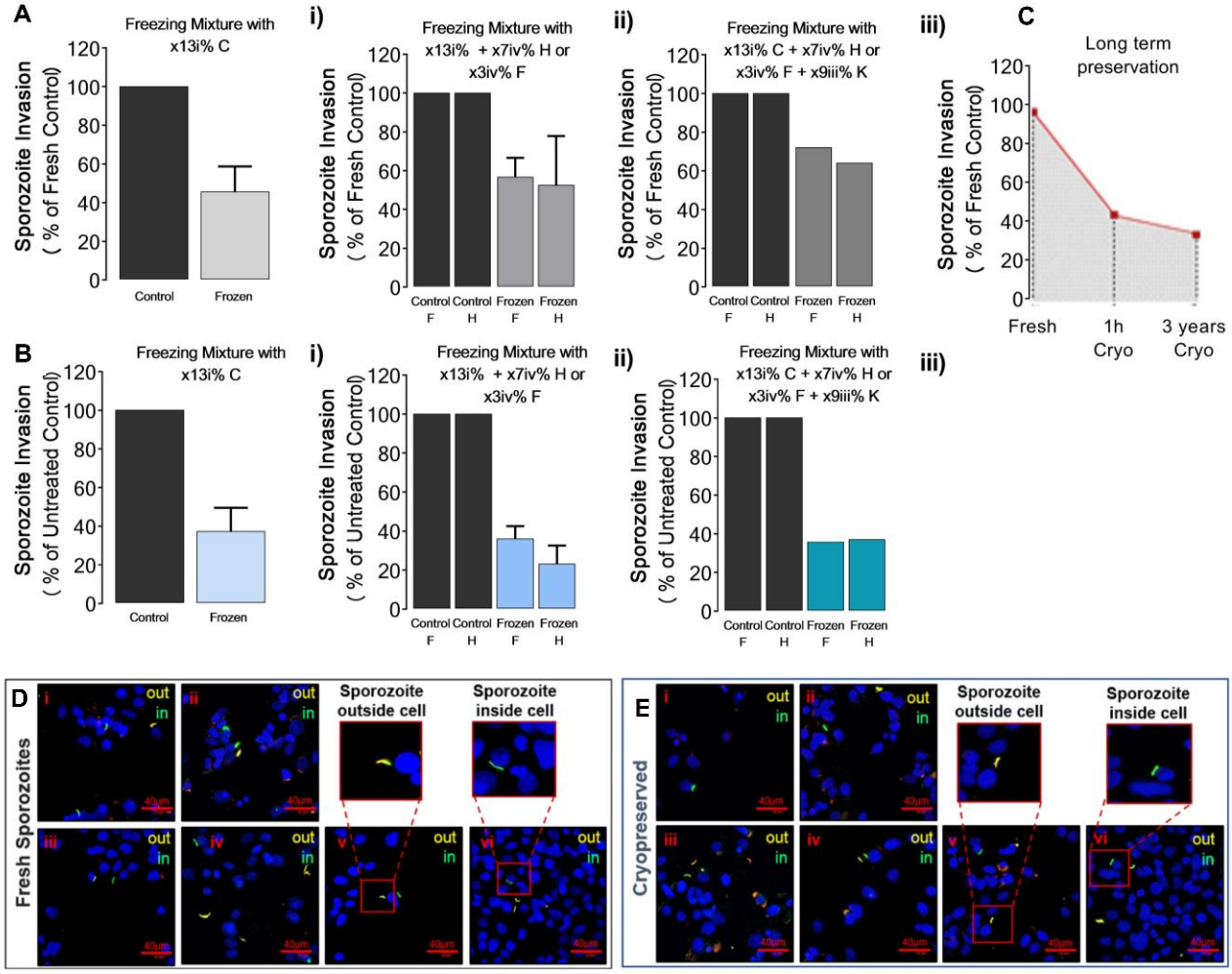


Figure 5 - **Invasion capacity of fresh and freezing mixture cryopreserved P. falciparum sporozoites in HC-04 cells. 100,000 sporozoites, either cryopreserved or not (Fresh Controls), were added per well. (A)** Cryopreserved sporozoite invasion rate compared with fresh sporozoites exposed to the same freezing mixture: **i)** only x13i% C; **ii)** x13i% C and x7iv% H or x3iv% F; **iii)** with further introduction of x9iii% J. **(B)** Cryopreserved sporozoite invasion rate compared to an untreated fresh control in the same freezing conditions of **(A); (C)** Sporozoite invasion capacity after 3 years of cryopreservation; **(D)** and **(E)** Representative images acquired of fresh and cryopreserved sporozoites in each condition, respectively: **i)** x13i% C + x3iv% F+ x9iii% K; **ii)** x13i% C + x7iv% H + x9iii% K; **iii)** x13i% C; iv) In **(D)** is the untreated control and in **(E)** is x13i%C cryopreserved 3 years ago; **v)** x13i% C + x7iv% H ; **vi)** x13i% C + x3iv% F.

of cells (**Figure 5 (D)** and **(E)**). As expected, the percentage of invasion in the controls was higher than in the cryopreserved sporozoites, indicating that the cryopreservation process affects the sporozoites' cell invasion capacity. Nevertheless, 45% of the *P. falciparum* sporozoite invasion ability was retained when the parasites were cryopreserved using freezing mixture containing only 10% C, compared with non-cryopreserved controls (**Figure 5 (Ai)**). Furthermore, when we introduced x3iv% F or x7iv% H in x13i% C freezing mixture, the invasion capacity of cryopreserved sporozoites was increased to approximately 55% (**Figure 5 (Aii)**). Strikingly, addition of the pCPAs, K, to the freezing mixtures containing x13i% C and x3iv% F or x7iv% H led to the retainment of approximately 70% of the HC-04 invasion capacity of cryopreserved *P. falciparum* sporozoites relative to fresh sporozoites incubated in the same freezing mixture. However, this last result needs to be repeated to confirm its reproducibility. Additional normalization between sporozoites cryopreserved in the selected freezing mixtures and fresh sporozoites incubated in RPMI (without any CPA) was also performed (**Figure 5 (B)**). This normalization revealed a slight decrease which may result of fresh sporozoites are in medium without any CPA. Another evaluation was based on comparing the invasion capacity of *P. falciparum* storage during 3 years after cryopreservation with those of fresh sporozoites and of sporozoites cryopreserved for one hour (**Figure 5 (C)**). This long-term assessment showed that there is no substantial loss of invasion capacity during the storage period. Further tests are required to better understand the protection provided by these freezing mixtures in *P. falciparum* and to increase the consistency of our results.

Discussion

An effective sporozoite cryopreservation process would constitute a significant scientific advance towards the development of new antimalarial strategies[32]. To be effective, a cryopreservation process for *Plasmodium* sporozoites should preserve adequate sporozoite infectivity, maintaining the parasites' ability to complete their liver stage development and produce infective blood-stage parasites. Sanaria is developing a malaria vaccine, PfSPZ, which involved the establishment of a process of sporozoite isolation and cryopreservation under good manufacturing practice conditions[30,13,31]. Other recent studies have also focused on evaluating different cryogenic solutions for resuspension of *Plasmodium* sporozoites for cryopreservation[14,32,33,34]. A retainment of 24% viability of *P.*

berghei cryopreserved sporozoites was observed when a commercially available cryosolution (CryoStor CS2) was employed[35,]. In 2018, Singh applied this previously established cryopreservation protocol[36,35] to evaluate the efficiency of a genetically attenuated *P. berghei* vaccine[34]. Nevertheless, the efficacy reported by either the Singh *et al.* study[34] or by Sanaria's PfSPZ vaccine has been compromised mostly by the requirement of high numbers of cryopreserved sporozoites per subject to achieve protective efficacy[37,15,34]. We were able to identify cryopreservation conditions that reproducibly retain 50% of *P. berghei* sporozoite viability (**Figure 3 (D)**), which represents a 36% increase in viability relative to that observed for sporozoites cryopreserved in CryoStor CS2[33]. Existing reports show that Sanaria's cryopreservation process leads to approximately, 7.4[13] and 6.4[38] -fold losses in sporozoite infectivity in mice and humans, respectively. Using the cryopreservation method described in this work, cryopreserved *P. berghei* sporozoites exhibited only a 2-fold reduction in compared with fresh ones, a clear improvement over the current gold standard.

Several freezing conditions were optimized to achieve the results described above. Our assessment identified VG2 as most appropriate to cryopreserve v(iii) of sporozoite suspension (**Figure 1**). Glass has an higher thermal conductivity than plastic materials[39], which may help explain the success of our cryopreservation process. Additionally, we speculate that volumes below v(iii) freeze faster, which may impact the optimal cooling rate for sporozoites and may explain the drastic decrease in sporozoite survival observed when a v(ii) suspension is used.

Besides vial geometry, composition of the solution used to resuspend sporozoites during freezing is an essential parameter[22]. The formulation of cryopreservation freezing mixtures normally relies upon 3 main components: a basal carrier solution, several types of CPAs[19,40]. A carrier solution is a component of freezing mixtures that normally holds the rest of the components in suspension. It contains a pH buffer, osmotic agents (balanced salt solution) and sometimes apoptosis inhibitors that provides basic support for cells at near-freezing temperatures[40,41]. RPMI1640 was employed as the carrier solution for sporozoite suspension following their extraction from mosquitoes since it presents a near isotonic salts concentration preventing sporozoite shrinking or swelling. This carrier solution can be further supplemented with several types of CPAs at different concentrations, towards improving the solution's protective capacity and potentially inducing vitrification under specific freezing conditions[42].

Sporozoites are very susceptible to freezing mixtures containing high concentrations of specific CPAs, being totally intolerant to M, E and I (**Figure 2**). The presence of these CPAs in sporozoite suspensions showed a toxicity profile[43,44,45,40]. Usually, cryopreservation solutions are not physiological solutions, since the high concentrations of CPAs increases the hypertonicity of the solution[40]. Osmotic and biochemical toxicities are two independent mechanisms of damage that can occur during introduction, incubation, and removal of a cryopreservation solution[40]. Conversely, sporozoites exposed to some npCPAs exhibited high levels of infection (**Figure 2 (B)**). It has been shown that albumin, present in some of these CPAs, may enhance sporozoite motility *in vitro* and potentially increase their infection capacity[46].

Our results identified non-penetrating sugars, particularly C and A, as the class of CPA preserving the highest sporozoite infectivity upon cryopreservation and providing the highest protection during freezing and thawing. C is an important component of different freeze-drying protocols because of its essential features in recovering biological materials upon thawing[19]. Interestingly, C is the most abundant sugar in the hemolymph of *Anopheles* mosquitoes, which suggests that it is likely an energy source for *Plasmodium* parasites[47]. For that reason, we hypothesize that its addition to sporozoite suspension contributes to the recovery of the fitness of post-thawed sporozoites. Additional mechanisms of CPA action have been proposed for C, such as stabilizing cell membranes, reducing alterations in membrane morphology and stability during freezing[48].

H and F are examples of npCPAs of lipids and proteins that are also essential for preserving the cell membrane integrity and maintaining the physiological viscosity during cryopreservation. H is a very common component of sperm freezing extenders, where low density lipoproteins, LDL, are believed to be the main responsible for H success during cryopreservation[49]. The major functions associated to this molecule are its interaction with cell membranes either to stabilize phospholipidic layer, replace the damaged phospholipids or binding to cell membrane proteins, leading to the efflux of phospholipids and cholesterol[50]. F is another complex mixture of proteins and lipids widely employed in cryopreservation of several types of cells and organisms[22]. Indeed, one of the first studies involving sporozoite cryopreservation of *Plasmodium* parasites used a solution containing blood serum or plasma, whose composition is similar to that of F[51]. A solution containing similar components was recently described for *P. vivax* cryopreservation, but only provided a very low protection[32]. pCPAs are able to cross cell membranes, which reduces the intracellular ice formation and thus prevents cell lysis[43]. However, under certain conditions (temperature, cell type, cooling rate, target, etc.), its intracellular presence may also trigger a toxic response for cells[43]. The molecular weight of water is 18 Da, while that of pCPAs is, on average, 70 Da[18]. This difference in molecular weights leads to water moving more rapidly than pCPA into and out of the cell, resulting in significant changes in cell[40]. However, some of these pCPAs are only toxic at a specific temperature and after a given period of time, which is the case of J[22,43].

CPAs can interact with each other in mixtures, or with crucial cell molecules, thereby producing effects other than those that would occur with individual CPAs[40]. In a freezing mixture one of the components might have a dominant role or they may combine to produce additive or synergistic effect[40]. Our results confirmed this assumption with the inclusion of two different types of npCPAs, H and C, in the sporozoite suspension (**Figure 3 (D)**). This result suggests that the protection of the cell membrane provided in the extracellular environment by H (and also F) and C is essential during cryopreservation for maintaining sporozoite viability and infectivity. Nonetheless, contrary to what was observed in several studies in other microorganisms[19,52], the introduction of pCPA in the freezing mixture led to a decrease in sporozoite survival (**Figure 3 (E)**). This might be a consequence

of the osmolarity of solution. The osmolarity of a physiological solution is 270–300mOsm. A freezing mixture like the one tested in this work contains 3 different types of CPAs and should result in a very high osmolarity that leads to detrimental effects on cells. **Figure 3 (E)** also shows the importance of having CPAs in sporozoite freezing mixtures, since sporozoites cryopreserved only in RPMI1640 were unable to survival during cryopreservation.

Additional validation of the present cryopreservation process in *in vivo* models is required. CPAs such as H, F or C contain several factors and proteins that may behave as foreign molecules in the mice, triggering an immune response that can influence infection, *in vivo*. Our results showed that injection of H, even at very low concentrations, leads to an increase in the parasite liver load, in mice. A possible explanation could be the improved ability of solutions containing H (in comparison with RPMI without any CPA) to better preserve sporozoites when they are isolated from mosquito's salivary glands. However, that hypothesis was excluded by the results in **Figure 3 (B)**, which showed that there is no significant difference between incubating or not sporozoites in H freezing mixture prior injection.

Finally, it is essential to translate the achievements made with the *P. berghei* model to the human-infective *P. falciparum* parasite. Singh *et al.* described a reduction of approximately 5-3-fold in cryopreserved *P. falciparum*[34] whereas we obtained only a slight reduction of 1.4-2-fold relative to fresh sporozoites (**Figure 5 (Aiii)**). Despite these excellent preliminary results, it is crucial to understand whether this parasite is able to complete its development after invasion. The damages resulting from cryopreservation may have a cumulative effect, allowing a normal invasion but blocking the subsequent parasite development due to injuries in parasite replication machinery. Long-term preservation in liquid nitrogen for a period of 3 years after freezing showed only a slightly decrease comparatively with sporozoites cryopreserved only for 1h (**Figure 5 (C)**). This validates that at -196ºC sporozoites are being well preserved with almost total absent of metabolic activity.

This work contributes significantly to the goal of developing an ideal method for cryopreservation of *Plasmodium* sporozoites. Importantly, this method for *Plasmodium* sporozoite cryopreservation employs inexpensive, commercially available CPAs, with no need for specialized equipment. It is expected that most malaria research laboratories currently employing infected mosquitoes can adopt the procedures developed in this study. Furthermore, we speculate that this reproducible cryopreservation method has the potential to contribute to the development of novel anti-malarial strategies and impact the development and evaluation of whole-sporozoite malaria vaccine candidates.

Future Perspectives

Despite the objectives accomplished during this thesis, there is still ample room for further improvements. We propose to continue the optimization of the freezing mixture, taking into account its osmolarity and thus including other CPAs as well as different procedures of addition of the freezing mixture to the sporozoite suspension. Additional *P. falciparum* cryopreservation experiments are also required to evaluate the reproducibility of our results. Another aspect that remains to be optimized is the incubation time required to maximize the mechanism of protection of different CPAs, since this can vary with the molecular features of CPAs. The thawing process also remains largely unexplored. As such, it would be interesting to study different thawing rates at different temperatures. Additionally, other vial geometries can be evaluated, and the possibility of designing and printing dedicated vials can also be explored. Once the cryopreservation process has been optimized using an *in vitro* method to assess sporozoite viability, *in vivo* evaluation of the capacity of cryopreserved sporozoites to reach and develop in mouse livers should be evaluated. Finally, in collaboration with SmartFreez we are developing an automated prototype system that will allow us streamline the process and to test various cooling rates, for optimal recovery of parasite viability after cryopreservation

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