Cellular Physiology and Biochemistry

Cell Physiol Biochem 2021;55:206-221
DOI: 10.33594/000000360
Published online: 30 April 2021
Accepted: 23 March 2021







**Original Paper**

# Disaccahrides-Based Cryo-Formulant Effect on Modulating Phospho/Mitochondrial Lipids and Biological Profiles of Human Leukaemia Cells

Marc-Sebastian F. Straka[a] Noha Abdullah Al-Otaibi[a,b] Philip D. Whitfield[c] Mary K. Doherty[c] Bruno F. E. Matarèse[a,d,e] Nigel K. H. Slater[a] Hassan Rahmoune[a]

[a]Department of Chemical Engineering & Biotechnology, University of Cambridge, Cambridge, UK, [b]King Abdulaziz City for Science and Technology Kingdom of Saudi Arabia, Riyadh, Saudi Arabia, [c]Lipidomics Research Facility, University of the Highlands and Islands, Inverness, UK, [d]Department of Physics, University of Cambridge, Cavendish Laboratory, Cambridge, UK, [e]Department of Haematology, University of Cambridge, Cavendish Laboratory, Cambridge, UK

**Key Words**
Cryopreservation • Dimethylsulfoxide • Nigerose • Salidroside • Cardiolipins

**Abstract**
***Background/Aims:*** The use of novel cryo-additive agents to increase cell viability post-cryopreservation is paramount to improve future cell based-therapy treatments. We aimed to establish the Human Leukemia (HL-60) cells lipidomic and biological patterns when cryo-preserved in DMSO alone and with 300 µM Nigerose (Nig), 200 µM Salidroside (Sal) or a combination of Nig (150 µM) and Sal (100 µM). ***Methods:*** HL-60 cells were pre-incubated with Nig/Sal prior, during and post cryopreservation, and subjected to global lipidomic analysis. Malondialdeyhde (MDA), released lactate dehydrogenase (LDH) and reactive oxygen scavenger (ROS) measurements were also carried out to evaluate levels of lipid peroxidation and cytotoxicity. ***Results:*** Cryopreserving HL-60 cells in DMSO with Nig and Sal provided optimal protection against unsaturated fatty acid oxidation. Post-thaw, cellular phospholipids and mitochondrial cardiolipins were increased by Nig/Sal as the ratio of unsaturated to saturated fatty acids 2.08 +/- 0.03 and 0.95 +/- 0.09 folds respectively in comparison to cells cryopreserved in DMSO alone (0.49 +/- 0.05 and 0.86 +/- 0.10 folds). HL-60 lipid peroxidation levels in the presence of DMSO + Nig and Sal combined were significantly reduced relative to pre-cryopreservation levels (10.91 +/- 2.13 nmole) compared to DMSO (17.1 +/- 3.96 nmole). DMSO + Nig/Sal combined also significantly reduced cell cytotoxicity post-thaw (0.0128 +/- 0.00182 mU/mL) in comparison to DMSO (0.0164 +/- 0.00126 mU/mL). The combination of Nig/Sal also reduced significantly ROS levels to the levels of prior cryopreservation of HL-60.

Hassan Rahmoune
University of Cambridge, Chemical Engineering & Biotechnology
Philippa Fawcett Drive, Cambridge CB3 0AS (UK)
E-Mail hr288@cam.ac.uk

***Conclusion:*** Overall, the establishment of the cryopreserved HL-60 cells lipidomic and the corresponding biological profiles showed an improved cryo-formulation in the presence of DMSO with the Nig/Sal combination by protecting the, mitochondrial inner membrane, unsaturated fatty acid components (i. e. Cardiolipins) and total phospholipids.



## Introduction

Cryopreservation is the process of storing cells, tissue or organs at very low temperatures over a prolonged period with the intent of future recovery. DMSO used as a classical cryo-protective agent (CPA) is known to interact with phospholipid membranes and thereby provide cryo-protective effects for cellular membranes [1]. The cellular and organelle membrane roles in maintaining cell integrity exchange [2] and lipid biological/biophysical homestasis are critical for maintaining cell viability post cellular challenges [3]. It is also well established that the loss of membrane integrity, functionality and especially fluidity, is associated with oxidation of unsaturated fatty acids in the phospholipid bilayer, leading to cell death [4]. Moreover, oxidation of cellular polyunsaturated fatty acids (PUFAs) inhibits cell proliferation and induces apoptosis in the THP1 cell line [5]. Cryopreservation might not have to affect the overall fatty acid composition but the proportion of polyunsaturated fatty acids is generally reduced post-freeze/thaw suggesting the possibility of lipid peroxidation [6]. Hence, the composition of the lipidome profiles defines the nature of response to a cryo-challenge and phosphatidylcholine seems to provide cryo-resistance properties, as one study showed this was associated with higher quality of spermatozoa [7]. A recent proteomic study conducted by our group also showed that DNA replication, transcription and cellular repair were also compromised in Human Leukaemia (HL-60) cells during cryopreservation and, addition of Nig or Sal, reduced the effects of oxidation on nuclear activities and enhanced cell growth post thaw [8].

It is widely accepted that ROS triggers lipid oxidation through free-radical mechanisms so that intracellular damage can no longer be prevented [9]. ROS generation can be elevated during the cooling and thawing process of the specimens. Chatterjee and Gagnon discovered that levels of superoxide radicals were increased 2.4-fold during cooling and 5.5-fold during thawing of bovine spermatozoa [10]. ROS are naturally known to modulate cellular function via effects on redox signalling pathways [11]. However, they are also involved in oxidative or apoptotic processes during cell cryopreservation [12]. Major targets of ROS degradation are unsaturated fatty acids found in the phospholipid bilayers enveloping cells and intracellular organelles, which affects membrane structure integrity and fluidity [13]. The cytosolic lipidome also plays a major role in maintaining cell functions, such as those of the cardiolipin phospholipids exclusively located on the inner mitochondrial membrane, which are responsible for the stability of enzyme complexes [14]. Oxidation of the cardiolipins can lead to disruption of the membrane, affecting their role as a proton trap and conductor, as well as the process of apoptosis [14]. For example, type 2 diabetes has been associated with peroxidation of the mitochondrial phospholipids via ROS, leading to pancreatic β-cell dysfunction and type- 2 diabetes [15].

More recently, the cryopreservation research and development field have also focused on improving cell based-therapy outcomes such as those involving stem cells [16], and in regenerative medicine studies in which DMSO is used classically as standalone CPA in attempts to minimise cryo-damage at the expense of cytotoxicity [17]. Many studies have also attempted to mitigate the effect of cryo-oxidative damage by testing novel cryo-additive agents with the overall aim to optimise cryo-formulation and reduce ROS effects [18]. One of the major issues with DMSO being used as a CPA is its toxicity [19], and substituting DMSO with alternative CPAs or supplementing with cryo-additive agents has been hampered by their lack of efficacy [17] or lack of cellular uptake [20].

Here, we have established the cryo-lipidomic profile of HL-60 cells as a means of further exploring the biochemical mechanism underpinning the cryopreservation of these cells in DMSO. The main objective was to reverse the cryo-oxidative effects by supplementing the cryo-media with Nig or Sal and thereby re-establish the membrane and intracellular organelle lipidome profile.

## Materials and Methods

*Materials*

HL-60 cells, RPMI-1640 media, fetal bovine serum (FBS), pencillin–streptomycin, sterilised phosphate buffered saline (PBS), DMSO, chloroform, methanol, isopropanol, potassium chloride, formic acid, ammonium formate, Nig and Sal were all purchased from Sigma-Aldrich (Poole, UK). Acetonitrile and water were obtained from Fisher Scientific, Loughborough, UK). Lipid peroxidation (malondialdehyde, MDA) and lactate dehydrogenase (LDH) assays were purchased from Abcam (Cambridge, UK). Progenesis QI, SIMCA-P v13.0, SPECTROstar Nano and MATLAB R2018) software packages were purchased from Nonlinear Dynamics, Umetrics, BMG Labtech and Mathworks, respectively.

*Cell culture and cryopreservation*

HL-60 cells were maintained in culture in RPMI 1460 media prepared with 10% (v/v) FBS and 50 µg/mL penicillin-streptomycin. The study design is shown in Fig. 1. HL-60 cells were cultured to 70% confluence in RPMI 1460 media and pre-incubated for 24 h in 300 µM Nig or 200 µM Sal or Nig (150 µM) / Sal (100 µM) combined. Cells were frozen at 2 x $10^6$ cells/mL in freezing media (90% FBS and 10% DMSO) (Arm 1), in DMSO containing 300 µM Nig (Arm 2), DMSO containing 200 µM Sal (Arm 3) or in Nig (150 µM) /Sal (100 µM) combined (Arm 4). Prior to freezing, HL-60 cells were centrifuged at 100 xg for 5 min and re-suspended in the corresponding freezing medium. Lipidomic and biochemical analysis were performed using 5 replicates per arm. Post thaw, HL-60 cells were harvested for 24 h and biochemical analysis were performed. Lipid extractions were performed 24 h post thaw for the lipidomic analysis (Fig. 1).

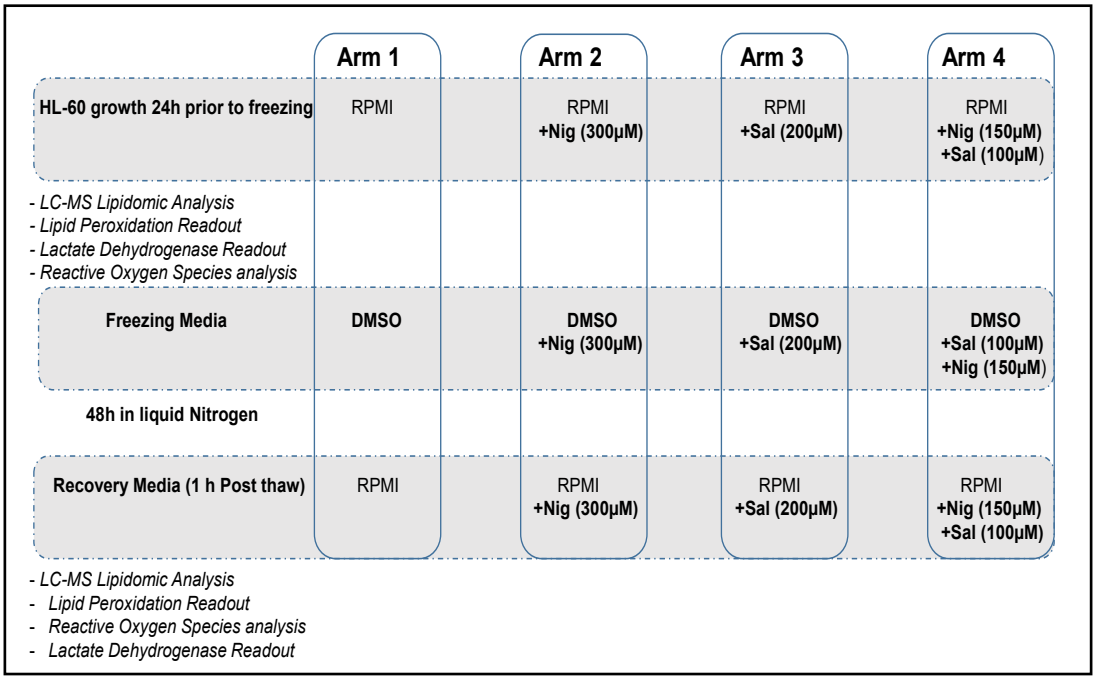



**Fig. 1.** Schematic diagram representing the experimental design of HL-60 cells (n= 5) pre-incubated for 24 hours in either cell culture media +/- Sal (200 µM), Nig (300 µM) or a combination of Sal (100 µM), Nig (150 µM) [Arm1]. Cell were cryopreserved in DMSO alone [Arm 2], DMSO + Nig [Arm 2], DMSO +Sal [Arm 3] or in DMSO + Nig and Sal [Arm 4]. Biochemical assays (LDH, lipid peroxidation and ROS) were performed prior and 1 h post thaw and LC-MS lipidomic analysis was carried out 24h post thaw.

*Extraction of cellular lipids*

For all samples, lipid extraction was performed according to the method of Folch et al. [21]. Briefly, 2 x $10^6$ HL-60 cells were centrifuged at 100 x g for 5 min and the resultant cell pellet was extracted in 3 mL of chloroform/methanol (2/1, v/v). The mixture was then left to stand for 1 h after which it was centrifuged at 1000 x g for 15 minutes to remove precipitated proteins. Samples were partitioned by the addition of 0.8 ml of 0.1 M KCl and centrifuged (400 x g for 5 minutes) to facilitate phase separation. The upper methanol phase was discarded and the lower organic phase containing lipids transferred to a clean glass tube and dried under a gentle stream of nitrogen. The lipid fractions were stored at -20°C prior to lipidomic analysis.

*Liquid chromatography mass spectrometry (LC-MS) analysis of lipids*

Untargeted lipidomic analysis was conducted on a Thermo Exactive Orbitrap mass spectrometer equipped with a heated ESI probe and coupled to a Thermo Accela 1250 ultra-high pressure liquid chromatography (UHPLC) system. All samples were analysed in both positive and negative ion modes over the mass to charge (m/z) range 250–2000. Samples (2 µL) were injected into a Thermo Hypersil Gold C18 column (2.1 mm x 100 mm, 1.9 µm) maintained at 50°C. Mobile phase A consisted of water containing 10 mM ammonium formate and 0.1% (v/v) formic acid. Mobile phase B consisted of 90:10 isopropanol:acetonitrile containing 10 mM ammonium formate and 0.1% (v/v) formic acid. All solvents were LC-MS grade. The initial conditions for analysis were 65%A/35%B. The percentage of mobile phase B was increased from 35-65% over 4 min, followed by 65-100% over 15 min, and then held for 2 min before returning to the starting conditions over 6 min. The flow rate was 400 µL/min [22].

*Lipidomic data processing*

Raw spectral LC-MS data were processed using the Progenesis QI software (version 2.1; Nonlinear Dynamics; Newcastle upon Tyne, UK). Lipids were identified based on their mass to charge ratio through interrogation of HMDB (http://www.hmdb.ca/) and LIPID MAPS (www.lipidmaps.org/). Multivariate statistical analysis was performed using SIMCA-P v13.0 (Umetrics, Umea, Sweden) as a statistical tool. The processed data were transformed using variance stabilisation and the mean abundance within experimental conditions was determined. Lipids were deemed to be altered in abundance following a two-way ANOVA analysis with fold-change differences between groups determined.

After LC-MS processing, data analyses were performed using Excel in order to classify the lipids based on chain length, level of saturation and to perform statistical evaluations. Positive and negative ion mode data were compiled, and the ratios of phospholipids and cardiolipin fatty acid groups prior and post freeze-thaw cycles were evaluated based on their chain length (C) and degree of saturation (:).

*Biochemical assays*

We used the lipid oxidation assay and measurement of extracellular LDH in HL-60 cells in Arm 1 (prior to cryopreservation) and Arm 4 (post-cryopreservation in DMSO + Nig and Sal combination), as shown in Fig. 1. We previously reported lipid oxidation and LDH activities of HL-60 cells when cryopreserved in DMSO +Nig (as per Arm 2) or DMSO +Sal (as per Arm 3) [8].

*Lipid peroxidation*

Lipid peroxidation was determined 1 h post-thaw in duplicate using 2 x $10^6$ cells/mL by measuring the formation of the malondialdehyde-thiobarbituric acid (MDA-TBA) adduct in acidic conditions at 95°C for 1 h. Sample absorbance was measured at 695 nm using the TECAN Spark microplate reader following the manufacturer's instructions. MDA concentrations were expressed in nmol.

*LDH assay*

1 x $10^6$ HL-60 cells/mL were cultured in RPMI 1460 media for 1 h post-thaw in Sal, Nig or Nig + Sal and the supernatants collected by centrifugation at 100 x g at 5°C for 5 min. The LDH assays were performed in duplicate using 50 µL enriched cell culture media and the quantity of nicotinamide adenine dinucleotide (NADH) was detected spectrophotometrically at 450 nm using TECAN Spark microplate reader by mixing NADH detection buffer with the supernatant according to the manufacturer's instructions.

All Biochemical assays were carried out using 5 replicates. Significant differences between groups were determined using Student's t-test for paired and unpaired observations. P values <0.05 were considered significant.

*Confocal microscopy and image analysis*

ROS measurements were carried out using confocal microscopy prior to and post-cryopreservation in DMSO +/- Nig/Sal. For this, 1 x $10^6$ HL-60 cells/mL were cultured in triplicate using RPMI 1460 media containing Sal, Nig or Nig + Sal 24 h prior to, during (in DMSO) and post-cryopreservation for 1, 5 and 24 h post thaw. ROS generation by HL-60 cells was visualized under a Leica TCS SP5 inverted laser scanning microscope (Milton Keynes, UK). The microscope was equipped with 405 diode, argon and HeNe lasers. ROS measurements were performed by staining cells with the DCFDA cellular ROS detection assay kit according to the manufacturer's instructions (Abcam, Cambridge, UK). Leica LAS AF software was used to analyze the images. All measurements were conducted in duplicate and expressed as mean ± standard deviation (S.D.). The differences between the control and 1, 5 and 24 h post thaw groups were calculated as percentages. Statistical tests were deemed significant at $p<0.05$.

## Results

*LC-MS*

The principal component analysis (PCA) scores plot analysis showed that the lipidome profile, analysed in negative and positive modes of HL-60 cells prior to cryopreservation is distinguished from cells cryopreserved in DMSO (Fig. 2). There was a discernible separation between the total lipid profiles of cells cryopreserved in DMSO alone versus DMSO + Nig or Sal or a mixture of Nig and Sal. There was a significant overlap between the groups of cells

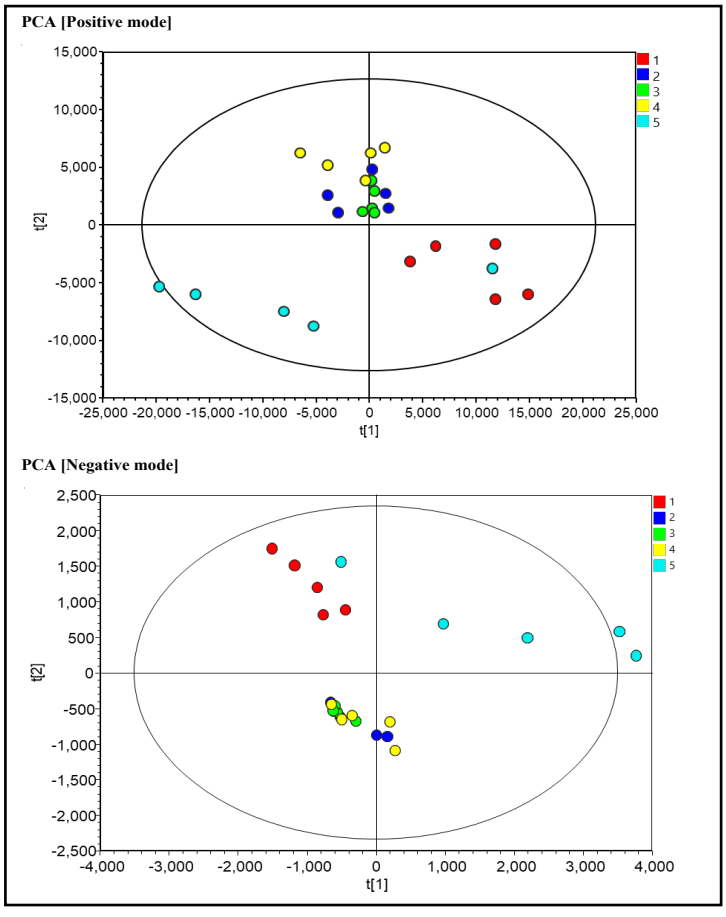



**Fig. 2.** Principal component analysis (PCA) scores plot showing the separation between HL-60 cells (n= 5) total lipidome profile (analysed in positive and negative ion modes) prior and post cryopreservation in cryo-additive agents. HL-60 cells were pre-incubated for 24 hours in cell culture media [5: Blue-sky circles] and cryopreserved in DMSO alone [1: Red circles], DMSO + Nig [2: Blue circles]; in DMSO +Sal [3: Green circles] and cells frozen DMSO + Nig and Sal [4: Yellow circles].

cryopreserved in DMSO + Nig, DMSO + Sal or DMSO + mixture of Nig and Sal and all of these could be differentiated from the control and DMSO. When comparing the five different conditions, the data points representing cells prior to cryopreservation were the most dispersed compared to the other groups (post-cryopreservation), which were more clustered.

Supervised modelling using the orthogonal projections to latent structures discriminant analysis (OPLS-DA) was performed for the positive ion mode lipidomic analyses to differentiate the driving forces among the variables of Sal or Nig versus the Nig + Sal combination. The OPLS-DA scores plot showed that combination of DMSO + Nig and Sal was more strongly separated from DMSO + Sal than from DMSO + Nig, as seen by a greater distance between the groups (Fig. 3). Furthermore, the data points in the DMSO + Nig group showed more variation within the group than the DMSO + Sal group. The OPLS-DA model was used here to establish the lipids specie separation between the five conditions prior to and 24 h post-thaw of HL-60 cells (Fig. 1).

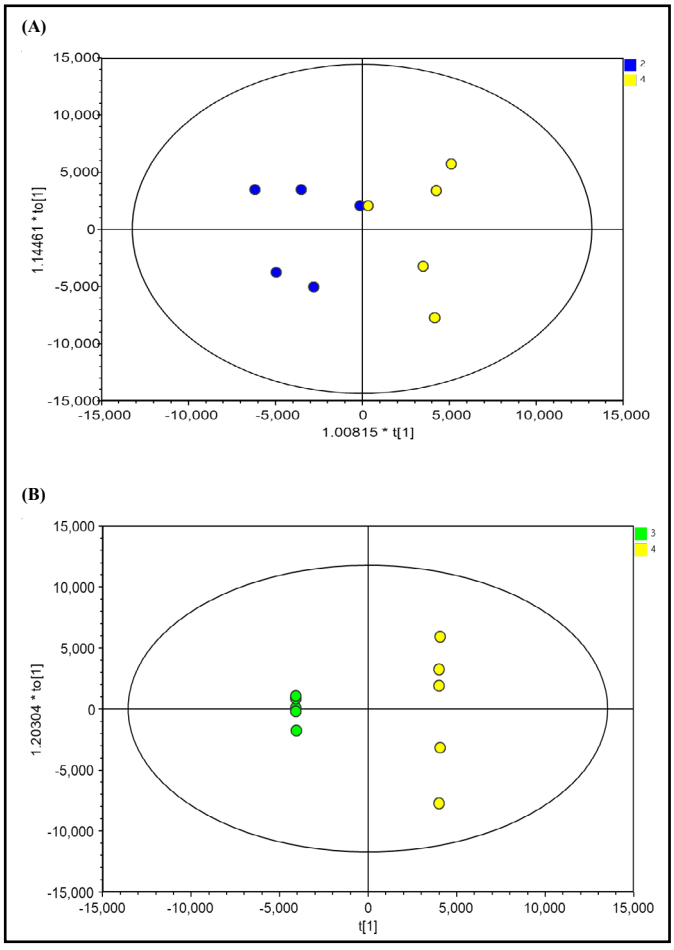



**Fig. 3.** Orthogonal partial least squares (OPLS-DA) Mix Scores Plot analysis of total lipidome (analysed in positive ion mode) profile 24 hours post thaw of HL-60 cells (n= 5). Cell were cryopreserved in DMSO + Nig [2: Blue circles] (A) or in DMSO +Sal [3: Green circles] (B) compare to cells frozen DMSO + Nig and Sal [4: Yellow circles].

LC-MS global analysis of the lipid populations assessing chain length and degree of unsaturated /saturated fatty acids ratio in HL-60 cells prior and post thaw are summarised in Table 1. The fatty acid ratio or fold change in Table 1 is calculated by dividing the mass of the lipids post-cryopreservation by the mass of the same lipid prior freezing HL-60 cells (n=5 per group) in DMSO +/- Nig/Sal. When comparing the ratio of saturated fatty acids (C14:0 to C24:0) prior and post cryopreservation in DMSO + or – Nig or Sal while maintained at a level of 1 to 5 fold when HL-60 cells were cryopreserved in DMSO + Nig, Sal or both (Table 1). The ratio of mono-unsaturated fatty acids (MUFAs) in the overall lipids population with the carbon chain ranging From C14:1 to C20:1 were roughly stable in cells frozen in DMSO averaging 0.7 to 0.8 fold except C18:1 extracted from HL-60 cells cryopreserved in DMSO alone (0.73 ± 0.04). In the presence of DMSO + Nig such ratio rose to 4.35 ± 0.18 folds and was maintained in DMSO + both Nig/Sal equalling 4.02±0.20 fold. Nig seems to maintain the ratio of MUFAs high but DMSO + Sal only showed a modest effect on C18:1 by keeping the ratio to 1.52 ± 0.05 fold (Table 1). The presence of Nig or Sal showed a bigger effect on the MUFA's long chains C22:1 and C24:1 by keeping their ratio between 8 and 14 folds when cryopreserved in DMSO + Nig/Sal (Table 1).

Post-thaw, poly-unsaturated fatty acid (PUFA) of the lipid populations chain length (C) and degree of saturation (:) which are C18:3 & 4, C20:3 & 5 and C22:4 ratios prior and cryopreservation were dramatically decreased when cells were cryopreserved in DMSO alone compared to pre-cryopreservation while attaining similar levels of around 0.8 up to 8.8 fold when Nig and/or Sal were added prior to, during and post-cryopreservation (Table 1). In contrast, the ratios of the C18:3 (15.63 ± 1.05) and C20:4 10.47 ± 0.69 were high when cells were cryopreserved in DMSO alone while only averaging a ratio of 0.8 to 2 post cryopreserving HL-60 in DMSO + Nig and/or Sal (Table 1).

**Table 1.** Ratio of HL-60 cells global lipids distribution based on the number of fatty acids carbon and saturated/unsaturated bonds prior and post cryopreservation in DMSO +/- Nig/Sal. The fold change or ratio of global lipidome is calculated by dividing the lipids mass of the post prior-cryopreservation in DMSO alone or +/- Nig/Sal by the prior-cryopreservation (n=5; Mean +/- SEM). Note: LDL = Low Detection Limit

| Lipid populations Chain length (C) and Degree of saturation (:) | DMSO | DMSO + Nig | DMSO+ Sal | DMSO + Nig/Sal |
|---|---|---|---|---|
| C14:0 | 3.73±0.4 | 0.76±0.04 | 0.75±0.04 | 0.77±0.04 |
| C16:0 | 3.78±0.12 | 1.62±0.07 | 1.56±0.07 | 1.01±0.05 |
| C18:0 | 0.97±0.06 | 1.09±0.02 | 0.96±0.02 | 1.12±0.03 |
| C20:0 | 3.44±0.22 | 3.04±0.09 | 2.65±0.08 | 2.56±0.07 |
| C22:0 | 5.66±0.59 | 0.94±0.05 | 0.88±0.02 | 0.95±0.05 |
| C24:0 | 4.57±0.41 | 180.38±1.00 | LDL | LDL |
| C14:1 | 0.75±0.07 | 0.74±0.04 | 0.71±0.04 | 0.75±0.05 |
| C16:1 | 0.80±0.06 | 0.65±0.06 | 0.69±0.05 | 0.65±0.07 |
| C18:1 | 0.73±0.04 | 4.35±0.18 | 1.52±0.05 | 4.02±0.20 |
| C20:1 | 0.76±0.05 | 0.83±0.06 | 0.78±0.07 | 0.82±0.07 |
| C22:1 | LDL | 7.88±0.33 | 7.69±0.33 | 7.83±0.33 |
| C24:1 | 5.83±0.58 | 14.55±0.28 | 12.35±0.26 | 14.09±0.28 |
| C18:2 | 15.63±1.05 | 0.8±0.06 | 0.8±0.06 | 0.74±0.07 |
| C18:3 | 0.67±0.07 | 1.75±0.06 | 1.63±0.06 | 1.73±0.06 |
| C18:4 | LDL | 5.35±0.27 | 5.02±0.27 | 5.56±0.29 |
| C20:3 | 0.59±0.11 | 6.9±0.25 | 6.61±0.26 | 5.54±0.12 |
| C20:4 | 10.47±0.69 | 2.13±0.05 | 2.04±0.06 | 2.27±0.06 |
| C20:5 | 2.43±0.20 | 5.62±0.11 | 8.08±0.16 | 6.12±0.12 |
| C22:4 | LDL | 6.9±0.15 | 2.04±0.12 | 5.37±0.19 |

When assessing the global fatty acids ratio or fold change prior and post cryopreservation, C14:0 saturated fatty acids were significantly increased in DMSO alone to 3.73 +/- 0.4 folds while averaging 0.75 when HL-60 cells were cryopreserved in Nig, Sal or a combination of Nig and Sal (Table 1). Similar pattern is observed for the saturated fatty acids palmitic acid (C16:0) decreasing from 3.78 +/- 0.12 folds when cryopreserved in DMSO alone to around 0.75 fold in DMSO, Nig and/or Sal post freeze-thaw. The saturated fatty acids C22:0 extracted from HL-60 post cryopreservation in DMSO alone also significantly increased 5.66 +/- 0.59 folds in comparison to cells cryopreserved in DMSO + Nig/Sal averaging 0.9 fold.

In the MUFAs, the sub-population of Palmitoleic acid (C16:1) ratio was slightly high 0.80 ± 0.06 fold when the HL-60 were cryopreserved in DMSO alone and averaging 0.66 fold when Nig and/or Sal were added to the cryo-formulation media (Table 1). The ratio of Nervonic acid (C24:1) sub-population isolated from HL-60 cells post cryopreservation in DMSO was at 5.83±0.58 folds in comparison to unfrozen cells. Post freeze thaw in DMSO with Nig or in combination with Sal increased such a ratio was tripled and averaging ~ 13 folds (Table 1). The ratio of Oleic acid (C18:1) subpopulation was significantly kept low at 0.73 +/- 0.04 fold post cryopreservation in DMSO alone compared to 4 folds in the presence of Nig. When adding Sal to DMSO, the ratio of oleic acid (C18:1) sub-population increased modestly equalling 1.52 ± 0.05 fold but with a higher protective effect of C18:1 compare to DMSO alone (Table 1).

HL-60 cell PUFA levels were significantly altered by cryopreservation and modulated by the presence of the CPAs prior to, during and post cryopreservation (Table 1). The biggest change was observed in relation to Docosatetraenoic acid (C22:4), with undetectable levels post freeze-thaw cycle in DMSO alone. The addition of Nig (300 uM) to DMSO mitigated such an effect by increasing C22:4 level compare to prior cryopreservation to 6.9 ± 0 .15 folds or to 5.37 ± 0.19 in the presence of Nig (150 uM) and Sal (100uM) mixture. Similar ratio changes

were observed for eicosapentaenoic acid (C20:5), γ-Linolenic acid (C18:3), Stearidonic acid C18:4 and Dihomo-γ-linolenic acid (C20:3) whereby the presence of Nig and/Sal increases the PUFA levels. In contrast, the ratio of Arachidonic acid (C20:4) sub-population were modestly increased averaging 2 folds when cells were cryopreserved in DMSO + Nig and/or Sal while dramatically increased to 10.47 +/- 0.69 folds compare to unfrozenHL-60 cells. Similar pattern was noticeable for Linolelaidic acid (C18:2) where DMSO showed a higher protective properties (15 folds) while moderate change in the presence of Nig/Sal was observed only averaging 0.8 fold increase post freeze-thaw (Table 1).

Comparing the overall ratio of unsaturated to saturated fatty acids of the lipid group (Fig. 4), 1.99 +/- 0.03 and 1.53 +/- 0.03 folds were observed when HL-60 cells were cryopreserved in DMSO + Nig and DMSO + Sal respectively. When combining DMSO with Nig and Sal, the ratio of unsaturated to saturated fatty acids global lipid population was kept at a higher level of 2.08 +/- 0.03 folds in comparison to HL-60 cells cryopreserved in DMSO alone equalling 0.49 +/= 0.05 fold (Fig. 4).

Fig. 5 summarises the ratio or fold changes of phospholipid's total number of saturated and unsaturated bonds isolated from HL-60 cells prior and post freeze-thaw in DMSO +/- Nig/Sal. Mass spectrometry lipidome analysis showed that saturated fatty acids or mono-saturated fatty acids increased up to 5 times in DMSO (5.04 +/- 0.36 folds) while only averaging 0.7 fold post freeze-thaw in DMSO + Sal/Nig. Similar pattern is also seen for the phospholipids with the fatty acids number of unsaturated bonds equalling 4 (10.47 +/- 0.69 folds) in DMSO alone versus an average of 3 folds when HL-60 cells were cryopreserved in DMSO + Nig/Sal. In contrast, the ratio of fatty acids equalling 7 unsaturated bonds was unchanged when cryopreserving HL-60 cells in DMSO alone or in the presence of Nig/Sal. Fatty acids with a number of unsaturated bonds higher than 7 ratio's was unchanged when HL-60 cells were cryopreserved in DMSO alone compare to unfrozen cells. Adding Nig, Sal or the combination of both to DMSO, the ratio of these PUFA phospholipids population post freeze is dramatically increased by 6 to 18 folds in relation to prior cryopreservation's levels (Fig. 5).

Similar results were obtained for the cardiolipins population isolated from HL-60 cells 24 h post cryopreservation in DMSO +/- Nig/Sal are summarised in Table 2. The overall ratio of unsaturated (∑Unsat) and saturated (∑Sat) representing the cardiolipin's fatty acids population showed that cryopreserving HL-60 cells in DMSO alone the ratio reached 0.86 +/- 0.10 fold (Fig. 6). A slight increase in the unsaturated/saturated fatty acids ratio was observed in the presence of the cryo-additives Nig and Sal equalling 0.95 +/- 0.09 fold.

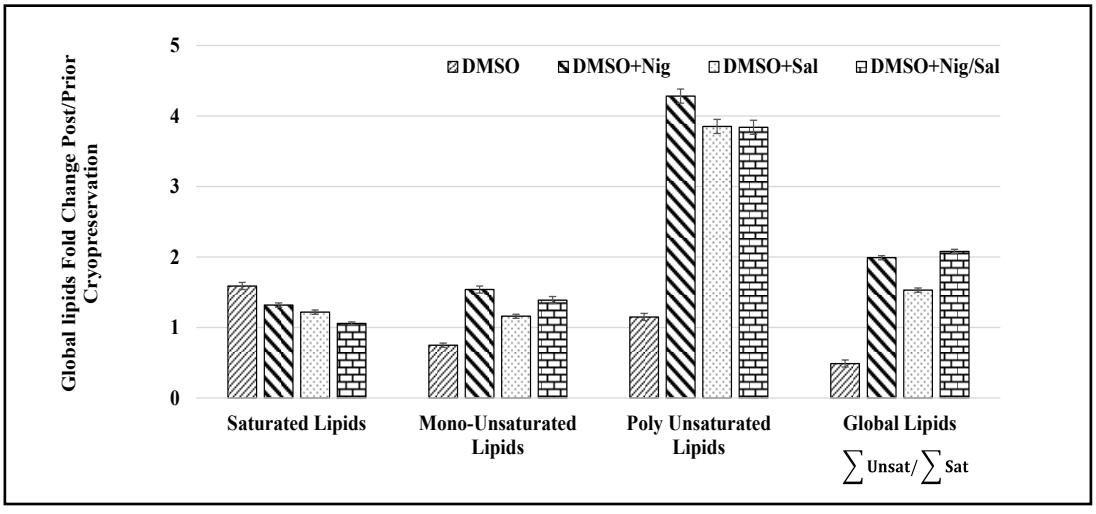



**Fig. 4.** Ratio of the sum of global lipids Unsaturated fatty acids (∑Unsat) and the sum of Saturated fatty (∑Sat) isolated from HL-60 cells prior and post cryopreservation in DMSO alone, DMSO +Nig, DMSO +Sal or DMSO +Nig/Sal (n=5; Mean +/- SEM).

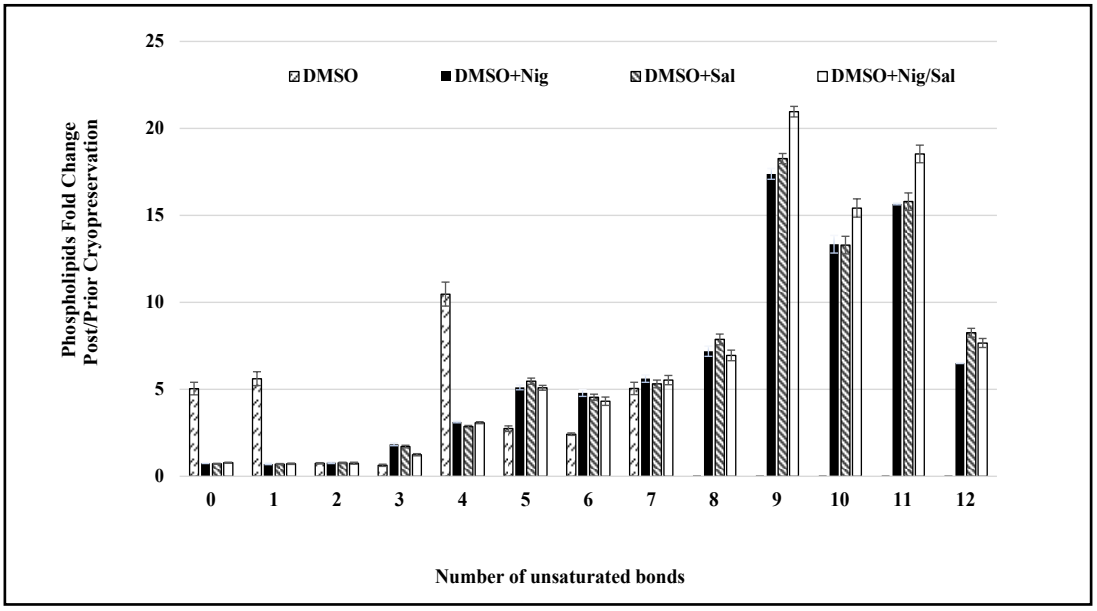



**Fig. 5.** Ratio of HL-60 cells phospholipids number of unsaturated and saturated bonds prior and post cryopreservation in DMSO +/ - Nig/Sal. HL-60 cells were pre-incubated for 24 hours in Nig, Sal or a combination of Nig/Sal prior freezing and cryopreserved in DMSO, DMSO + Nig, DMSO + Sal or DMSO + Nig/Sal. The fold change or ratio of the phospholipids is calculated by dividing the lipids mass of the post prior-cryopreservation in DMSO alone or +/- Nig/Sal by the prior-cryopreservation. (n=5; Mean +/- SEM).

In the presence of Nig the ratio was at 0.97 ± 0.09 fold and 0.94 ± 0.09 fold in the presence of Sal (Fig. 6).

*Biological assays*

The HL-60 cell lipid peroxidation profiles prior to and post cryopreservation in DMSO +/- CPAs (n = 5 replicates) are shown in Fig. 7. A significant increase in the MDA activity was observed as seen prior to cryopreservation of HL-60 cells in DMSO from 11.07 +/- 2.58 nmole and reached 17.1 +/- 3.96 nmole 1 h post thaw with a value of p=0.017. The cryopreservation of HL-60 cells in DMSO with Sal (100 uM) and Nig (150 uM) combined and growing the cells in RPMI medium containing Sal (100 uM) and Nig (150 uM) resulted in restoration of the MDA activity to 10.91 +/- 2.13 nmole 1 h post thaw. The reduced level of lipid oxidation post thaw trended towards significance (p=0.055) as MDA was closer to its level prior cryopreservation (Fig. 7).

**Table 2.** HL-60 cells Cardiolipins ratio is based on the number of fatty acids carbon and saturated/unsaturated bonds prior and post cryopreservation in DMSO +/- Nig/Sal. The fold change or ratio of cardiolipins is calculated by dividing the lipids mass of the post prior-cryopreservation in DMSO alone or +/- Nig/Sal by the prior-cryopreservation (n=5; Mean +/- SEM)

| Cardiolipins Chain length (C) and Degree of saturation (:) | DMSO | DMSO + Nig | DMSO+ Sal | DMSO + Nig/Sal |
|---|---|---|---|---|
| C16:0 | 6.03±0.20 | 1.39±0.20 | 1.58±0.19 | 1.24±0.20 |
| C18:0 | 13.03±0.19 | 5.34±0.19 | 5.89±0.19 | 5.19±0.19 |
| C16:1 | 6.26±0.14 | 1.90±0.14 | 2.28±0.14 | 1.61±0.15 |
| C18:1 | 11.75±0.14 | 5.57±0.14 | 5.73±0.14 | 5.44±0.14 |
| C20:4 | 21.22±0.56 | 11.20±0.59 | 10.71±0.57 | 7.44±0.60 |
| C22:5 | 1.64±0.43 | 0.81±0.44 | 0.87±0.43 | 0.64±0.45 |

As shown in Fig. 8, HL-60 cells cryopreserved in DMSO and seeded 1 h post-thaw showed significantly (p=0.0084) increased extracellular levels of LDH activity (0.0165 +/- 0.0013 mU/mL) compared to the activity (0.0089 +/- 0.0012 mU/mL) prior to cryopreservation. HL-60 cells cryopreserved in DMSO, Sal (100uM) and Nig (150uM) and seeded for 1 h in cell culture media containing Sal (100 μM) and Nig (150 μM) showed a significant (p=0.0084)

reduction of 33% in LDH extracellular activity. The presence of Nig and Sal in the cell culture media prior to cryopreservation, in the freezing media (FBS, 10% DMSO) and post-thaw media reduced LDH activity to its level prior to cryopreservation (0.0128 +/- 0.0197 mU/mL) and increased the protective properties of the cryo-media.

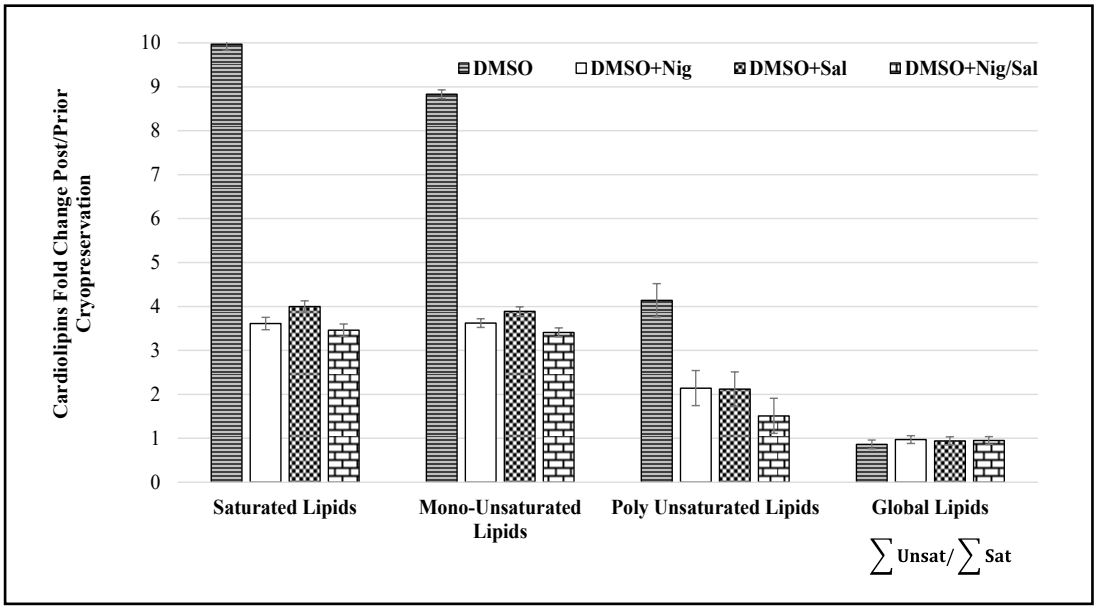



**Fig. 6.** HL-60 cells Cardiolipins ratio of the sum of Unsaturated fatty acids (∑Unsat) and the sum of Saturated fatty (∑Sat) of phospholipid isolated from HL-60 cells prior and post cryopreservation in DMSO, DMSO +Nig, DMSO +Sal or DMSO + Nig/Sal (n=5; Mean +/- SEM).

**Fig. 7.** Lipid peroxidation (MDA) assay was measured in HL-60 incubated prior [Control] and 1 hour post thaw in media with and without Nig (150 μM) + Sal (100 μM) and cryopreserved in DMSO alone [DMSO] or with Nig (150 μM) + Sal (100 μM) [DMSO (+) NS]. The data are represented in mean [n=5 replicates] ± SEM (* P value <0.05).

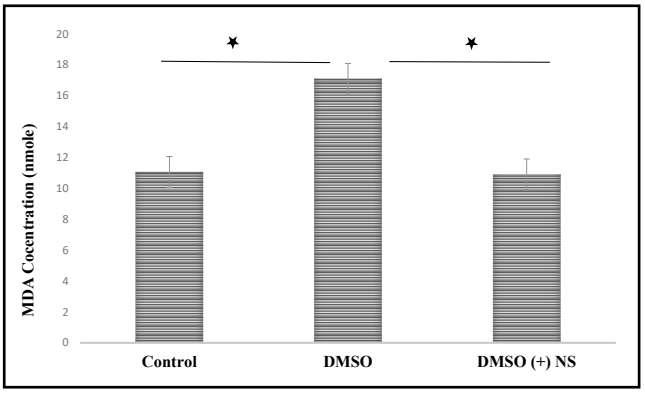
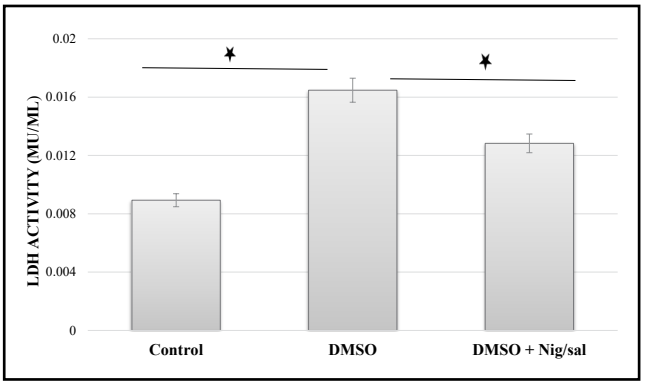



**Fig. 8.** Extra-cellular Lactate Dehydrogenase (LDH) assay of HL-60 were measured prior freezing [Control]. Cells were frozen in DMSO + Nig and Sal and LDH activity were measured 1 hour post thaw in RPMI media only [DMSO], RPMI +Nig (150 μM) + Sal (100 μM) [DMSO + Nig/Sal]. Extracellular LDH activity (mU/ml) and data are presented as a mean [n=5 replicates] ± SEM (* P value < 0.05).



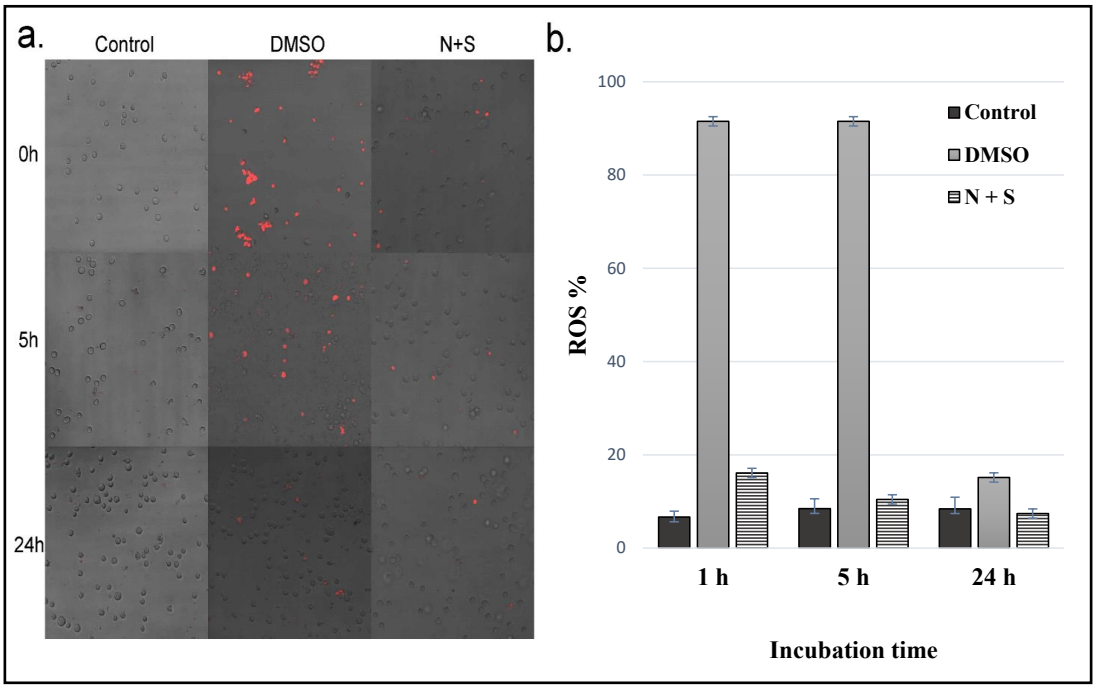



**Fig. 9.** Reactive Oxygen Species (ROS) measurement by confocal microscopy. ROS levels in HL-60 cells were measured prior freezing [Control] and post freezing in DMSO alone or DMSO + Nig and Sal. (a) represents real time ROS levels were also measured 1 hour, 5 hours and 24 hours post thaw of HL-60 cells frozen with DMSO [DMSO] and DMSO +Nig (150 µM) + Sal (100 µM) [N + S]. ROS levels were expressed in percentage (ROS count/Cell number x 100) (b) and data are presented as a mean ± SEM [n=3].

*ROS assay*

Real-time analysis of ROS generation in living HL-60 cells was assessed using confocal microscopy. We investigated whether the addition of the Nig (200 µM) and Sal (100 µM) prior to and 1 h post-and during cryopreservation in DMSO + Nig/Sal would reduce excessive ROS generation by cryo-damage (Fig. 6). HL-60 were thawed in the presence or absence of Nig (150 µM) and Nig (100 µM), and subsequently stained with DCFDA 1, 5 and 24 h post-thaw. Post staining, HL-60 cells were analyzed by confocal microscopy (Fig. 9a) and data are presented as the mean ± SEM percentage ROS count/cell.

The ROS levels in HL-60 cells was 6.6 ±2.4 % prior to cryopreservation (control) and increased to 91.5 ± 1.9 % 1 h post-thaw in DMSO alone (Fig. 9a and 9b). At 1h post thaw, ROS generation increased from 6.6 ± 1.3 % prior to freeze/thaw to 16.1 ± 2.4 % when HL-60 cells were cryopreserved in DMSO containing Nig and Sal while ROS levels reached 91.5 ± 1.9 % for cells cryopreserved in DMSO alone (Fig. 9b). In the 5 h post-thaw measurement, the level of ROS in HL-60 cells rose from 8.4 ± 2.1 % prior to freeze/thaw up to 91.5 ± 1.9 % when cells were cryopreserved in DMSO alone compared to 10.4 ±1.3 % for HL-60 cells cryopreserved in DMSO + Nig and Sal. Finally, 24 h post-thaw, ROS generation in HL-60 cells cryopreserved in DMSO alone (15.1 ± 2.4 %) increase compare to the ROS level prior to cryopreservation (8.4 ±2.5 %). The presence of Nig/Sal prior to, during and post cryopreservation mitigated the effect of cryo-damage by maintaining ROS levels at 7.4 ±0.9 %, which was similar to the value in cells that were not subjected to cryopreservation (Fig. 9b).

## Discussion

This study provides the first detailed lipid and fatty acid composition profiles associated with cryo-oxidation of HL-60 cells, as a means of assessing the effects of CPAs. Previous lipidomic studies were focused on DMSO cytoxicity effects [19] and establishing lipidomic profiles associated with the freeze/thaw cycle [23]. However, the focus over recent years has been on intensifying research in the development of novel cryo-additive agents to preserve cells and/or organs for medical applications [24].

We previously carried out proteomic profiling of nucleated HL-60 cells to demonstrate the anti-oxidative capabilities of Nig and Sal in reducing cryo-oxidative damage at the protein level [8]. This showed increased levels of NADH ubiquinone oxidoreductase 75 kDa subunit in response to HL-60 cryo-stress and to addition of Sal or Nig to DMSO in the cryo-preservation media. We also showed that Hsp70-binding protein 1 decreased in the presence of Nig/Sal and increased when the cells were cryopreserved in DMSO alone [8]. The changes in these proteins indicated changes in the cellular stress response which was mitigate by the presence of Nig or Sal. Here, we performed LC-MS-based lipidome analysis of the same cells to gain further insights into the mechanism of action of these CPAs. Visualization of the data by PCA showed that the combination of DMSO + Nig and Sal could be differentiated from DMSO plus either of these CPAs, and that the DMSO + Nig was closer to the DMSO + Nig and Sal combination compared to the DMSO + Sal treatment. This is consistent with our proteomic analysis in which the levels of a higher number of proteins were modulated by the combination of both CPAs and a greater number of proteins were altered by DMSO + Nig compared to DMSO + Sal [9]. At the functional level, Nig and Sal have both been shown to reduce lipid oxidation, although only Nig was found to reduce protein oxidation/carbonylation post thaw [8].

Changes in composition of lipids post cryopreservation, such as increased levels of phospholipids and triacylglycerol and reduced levels of PUFAs, have been reported in boar semen [6]. Here, we have shown that cryopreserving HL-60 cells in DMSO + Nig and/or Sal helped to maintain a significant increase in PUFAs. More importantly, cell membrane phospholipids [25] and PUFAs are known to be sensitive to oxidation mediated by ROS [9, 26]. The ratio of unsaturated to saturated fatty acids prior/post freeze thaw cycle is generally used as an overall measure on the extent of lipid peroxidation and a previous attempt to attenuate unsaturated fatty peroxidation was carried out using a combination of α-tocopherol and PUFAs [27].

The global analysis of the HL-60 lipidome profiles of cells cryopreserved in DMSO with cryo-additives (Nig or Sal) was similar to that prior to cryopreservation. For example, the unsaturated to saturated fatty acid ratio was higher for cells cryopreserved in DMSO + Nig/Sal in comparison to DMSO alone. Thus, the presence of CPAs such as a Nig and Sal throughout the cryopreservation can help to maintain high levels of the unsaturated fatty acids of the phospholipids for example. PUFA protection from cryo-oxidative damage is paramount as unsaturated fatty acids are known to possess a lower melting point than phospholipids with saturated acyl chains [28] which is crucial in maintaining cell integrity, fluidity and functionality. The present study have also shown that when HL-60 cells were cryopreserved in DMSO + Nig/Sal, the overall ratio of the phospholipid's unsaturated to saturated fatty acids with high number (N>5) of unsaturated fatty acids bound were maintained (or higher) to prior their cryopreservation levels. We have shown here that the use of DMSO, alone as a protective agent, has led to an increase of saturated and mono-unsaturated fatty acids post thaw suggesting an increase on lipids peroxidation [6]. The present lipidome analysis of the human leukaemia post cryopreservation in DMSO alone has shown that the PUFA peroxidation is reflective of the cryo-oxidative status of the cells with an increased level of intracellular reactive oxygen and extracellular Lactate dehydrogenase.

We found that the levels of mono- (C18:1 Oleic acid, C22:1 Erucic acid and C24:1 Nervonic acid) and poly-unsaturated fatty acids (Stearidonic acid C18:4, C22:4 Adrenic acid) were maintained or higher level post cryopreservation in DMSO + Nig/Sal combinations.

Similar anti-oxidative properties of cryo-additives were also previously seen when cells were cryopreserved in DMSO +/- ascorbic acid/pyruvate combined with alpha-tocopherol [29]. Although PUFAs are susceptible to beta-oxidation, Arachidonic acid C20:4 or Linolelaidic acid C18:2 with high degree of unsaturated fatty acids were low prior to cryopreservation and their levels increased when HL-60 cells were cryopreserved in DMSO alone compare to DMSO + Nig and/or Sal. The increased levels in linoleic acid (C18:2) post cryopreservation in DMSO alone could be associated with cryo-stress as linoleic acid is one of the essential fatty acids which is the source of all higher PUFAs synthesis needed post oxidative stress [30]. Moreover, high level of arachidonic acid (C20:4) has been previously shown to be associated with cytotoxicity and cell death [31].

This study is the first to report that cryopreservation using Nig + Sal modulates the mitochondrial cardiolipin composition in HL-60 cells, in a manner similar to the effects on total phospholipids. Overall, the ratio of cardiolipin-related unsaturated to saturated fatty acids post thaw was lowest for DMSO alone compared to adding Nig, Sal or a combination of Nig and Sal. This is likely to be due to increased lipid peroxidation and cytotoxicity effects when cells are cryopreserved in DMSO alone. Saturated fatty acids such as palmitic and stearic acid, were also found to be highest in DMSO compared to Nig/Sal. These data suggest that in the absence of Nig/Sal, induced lipid peroxidation and cell toxicity occurs. Moreover, the unsaturated to saturated cardiolipin-related fatty acid ratio observed here was slightly higher when cells were cryopreserved in DMSO + Nig/Sal compared to DMSO alone. The increased cryo-protective effect of Nig at 300 μM on cardiolipins could be also due to its ability to mitigate against mainly lipid and protein oxidation while Sal mainly reduces proteins oxidation as previously shown by our group [8]. Nevertheless, the limited effect of the cryo-additive agents (Nig/Sal) versus DMSO alone on Cardiolipins, known to be localised in the inner membrane of the mitochondrion [32], raises the issue of disaccharides, when used as cryo-protective agents, intracellular uptake [33].

Although, previous studies on cardiolipins are limited, Vähäheikkilä et al. have shown that cardiolipin peroxidation can lead to thinning of the membrane and conformal changes [32]. Given the highly specific binding of cardiolipins to various mitochondrial associated proteins, the authors suggested that changes in the structural profile could influence both the mitochondrial proton and electron transport mechanisms [32]. Cardiolipin appears to be susceptible to oxidative stress and the resulting dysfunctional mitochondrial membrane protein activities were shown to be associated with major diseases [34, 35]. In addition, cryopreservation can induce mitochondrial ultrastructure and functional changes, which can affect biological functions such as sperm quality [36].

One of the limitations of this study is the potential reflection of the molecular class investigated here on other cell types. This procedure was based on examining the effect of adding Nig/Sal on the lipidomic/biological profiles of one nucleated cell (e.g. HL-60). Therefore, it is possible that a different selection of alternative cellular models would lead to different conclusions from those drawn in this study. Moreover, future use of LC-MS/MS lipidomic analysis combined with cellular sub-fractionation would pinpoint the subtype of lipid population and their intracellular localisation (e. g. Mitochondrial and cardiolipins) subjected to cryo-damage. Nevertheless, similar anti-oxidative patterns were observed where alternative CPAs were used in optimising cryopreservation of cells, tissues and organs [24].

## Conclusion

In summary, the present study we have shown an increased level of protective properties of Nig and Sal whereby the maintenance of increased level of unsaturated fatty acids corresponded to a reduced levels of MDA-lipid oxidation and cytotoxicity levels post

cryopreservation in the mixture of Nig and Sal. A reduced level of lipid peroxidation and cytotoxicity was also shown when Sal or Nig were used separately in the presence of DMSO [8].

The findings presented here could pave away to future translational studies whereby establishing and reversing the molecular/biological pathways associated with cellular cryo-damage could lead not only optimizing cryo-formulation but also to the discovery of a more targeted cryo-protectants. The present study could also influence the development of future cryo-formulants destined for clinical practices in cell/organ transplant-based therapy [37] and/or the food industry.

## Acknowledgements

*Author Contributions*

All authors edited and approved the final version of the manuscript.

M.S., N.A.S.A. performed all experimental manipulations and sample preparation for LC-MS, prepared the tables and figures, and performed the lipidomic analysis. P.D.W., S.R.T., M.K.D. and M.S. performed sample acquisition and data analysis for the lipidomic study. B.F.E.M performed and analysed ROS assays. H.R. designed and supervised the project and performed biological interpretation of the data and N.K.H.S. co-supervised the project. H.R prepared drafted the manuscript and wrote the manuscript. N.K.H.S edited and revised manuscript.

*Funding Sources*

This work was supported by the King Abdul Aziz City for Science and Technology research fund.

## Disclosure Statement

The authors have no conflicts of interest to declare.

## References

1 Anchordoguy TJ, Cecchini CA, Crowe JH, Crowe LM: Insights into the cryoprotective mechanism of dimethyl sulfoxide for phospholipid bilayers. Cryobiology 1991;28:467-473.

2 Van Meer G, de Kroo AIPM: Lipid map of the mammalian cell. J Cell Sci 2011;124:5-8.

3 Levental KR, Malmberg E, Symons JL, Fan YY, Chapkin RS, Ernst R, Levental I: Lipidomic and biophysical homeostasis of mammalian membranes counteracts dietary lipid perturbations to maintain cellular fitness. Nat Commun 2020;11:1339.

4 Gaschlera MM, Stockwell BR: Lipid peroxidation in cell death. Biochem Biophys Res Commun 2017;482:419-425.

5 Iuchi K, Ema M, Suzuki M, Yokoyama C, Hisatomi H: Oxidized unsaturated fatty acids induce apoptotic cell death in cultured cells. Mol Med Rep 2019;19:2767-2773.

6 Cerolini S, Maldjian A, Pizzi F, Gliozzi TM: Changes in sperm quality and lipid composition during cryopreservation of boar semen Gliozzi. Reproduction 2001;121:395-401.

7 Cabrera T, Ramires-Neto C, Belaz KRA, Freitas-Dell'aqua CP, Zampieri D, Tata A, Eberlin MN, Alvarenga MA, Souza FF: Influence of spermatozoal lipidomic profile on the cryoresistance of frozen spermatozoa from stallions. Theriogenology 2018;108:161-166.

8 Al-Otaibi NAS, Cassoli JS, Martins-de-Souza D, Slater NKH, Rahmoune H: Human leukemia cells (HL-60) proteomic and biological signatures underpinning cryo-damage are differentially modulated by novel cryo-additives. Gigascience 2019;8:giy155.

9 Amidi F, Pazhohan A, Shabani Nashtaei M, Khodarahmian M, Nekoonam S: The role of antioxidants in sperm freezing: a review. Cell Tissue Bank 2016;17:745-756.
10 Chatterjee S, Gagnon C: Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol Reprod Dev 2001;59:451-458.
11 Dröge W: Free radicals in the physiological control of cell function. Physiol Rev 2002;82:47-95.
12 Di Santo M, Tarozzi N, Nadalini M, Borini A: Human Sperm Cryopreservation: Update on Techniques, Effect on DNA Integrity, and Implications for ART. Adv Urol 2012;2012:854837.
13 Wong-Ekkabut J, Xu Z, Triampo W, Tang IM, Tieleman DP, Monticelli L: Effect of lipid peroxidation on the properties of lipid bilayers: a molecular dynamics study. Biophys J 2007;93:4225-4236.
14 Vähäheikkilä M, Peltomaa T, Róg T, Vazdar M, Pöyry S, Vattulainen I: How cardiolipin peroxidation alters the properties of the inner mitochondrial membrane? Chem Phys Lipids 2018;214:15-23.
15 Ma ZA: The role of peroxidation of mitochondrial membrane phospholipids in pancreatic β -cell failure. Curr Diabetes Rev 2012;8:69-75.
16 Hornberger K, Yu G, McKenna D, Hubel A: Cryopreservation of Hematopoietic Stem Cells: Emerging Assays, Cryoprotectant Agents, and Technology to Improve Outcomes. Transfus Med Hemother 2019;46:188-196.
17 Awan M, Buriak I, Fleck R, Fuller B, Goltsev A, Kerby J, Lowdell M, Mericka P, Petrenko A, Petrenko Y, Rogulska O, Stolzing A, Stacey GN: Dimethyl sulfoxide: a central player since the dawn of cryobiology, is efficacy balanced by toxicity? Regen Med 2020;15:1463-1491.
18 Len JS, Koh WSD, Tan SX: The roles of reactive oxygen species and antioxidants in cryopreservation. Biosci Rep 2019;39:BSR20191601.
19 Best BP: Cryoprotectant Toxicity: Facts, Issues, and Questions. Rejuvenation Res 2015;18:422-436.
20 Stewart S, He X: Intracellular Delivery of Trehalose for Cell Banking. Langmuir 2019;35:7414-7422.
21 Folch J, Lee M, Sloane Stanley GH: A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497-509.
22 Laudicella VA, Beveridge C, Carboni S, Franco SC, Doherty MK, Long N, Mitchell E, Stanley MS, Whitfield PD, Hughes AD: Lipidomics analysis of juveniles' blue mussels (Mytilusedulis L. 1758), a key economic and ecological species. PLoS One 2020;15:e0223031.
23 Borges ED, Vireque AA: Updating the Impact of Lipid Metabolism Modulation and Lipidomic Profiling on Oocyte Cryopreservation. EMJ 2019;4:79-87.
24 Elliott GD, Wang S, Fuller BJ: Cryoprotectants: A review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. Cryobiology 2017;76:74-91.
25 Philippova M, Resink T, Erne P, Bochkov V: Oxidised phospholipids as biomarkers in human disease. Swiss Med Wkly 2014;144:w14037.
26 Klaiwattana P, Srisook K, Srisook E, Vuthiphandchai V, Neumvonk J: Effect of cryopreservation on lipid composition and antioxidant enzyme activity of seabass (Lates calcarifer) sperm. IJFS 2016;15:157-169.
27 Towhidi A, Parks JE: Effect of n-3 fatty acids and α-tocopherol on post-thaw parameters and fatty acid composition of bovine sperm. J Assist Reprod Genet 2012;29:1051-1056.
28 Murata N, Wada H: Acyl-lipid desaturases and their importance in the tolerance and acclimatization to cold of cyanobacteria. Biochem J 1995;308:1-8.
29 Breininger E, TresaBeconi M: Ascorbic acid or pyruvate counteracts peroxidative damage in boar sperm cryopreserved with or without α-tocopherol. Anim Sci Pap Rep 2014;32:5-13.
30 Innis SM: Fatty acids and early human development. Early Hum Dev 2007;83:761-766.
31 Pompeia C, Lima T, Curi R: Arachidonic acid cytotoxicity: can arachidonic acid be a physiological mediator of cell death? Cell Biochem Funct 2003;21:97–104.
32 Vähäheikkilä M, Peltomaa T, Róg T, Vazdar M, Pöyry S, Vattulainen I: How cardiolipin peroxidation alters the properties of the inner mitochondrial membrane? Chem Phys Lipids 2018;214:15-23.
33 Zhanga M, Oldenhof H, Siemeb H, Wolkers WF: Freezing-induced uptake of trehalose into mammalian cellsfacilitates cryopreservation Biochim Biophys Acta 2016;1858:1400-1409.
34 Paradies G, Petrosillo G, Paradies V, Ruggiero FM: Oxidative stress, mitochondrial bioenergetics, and cardiolipin in aging Free Radic Biol Med 2010;48:1286-1295.
35 Paradies G, Petrosillo G, Paradies V, Reiter RJ, Ruggiero FM: Melatonin, cardiolipin and mitochondrial bioenergetics in health and disease. J Pineal Res 2010;48:297-310.

36 Figueroa E, Lee-Estevez M, Valdebenito I, Watanabe I, Oliveira RPS, Romero J, Castillo RL, Farías JG: Effects of cryopreservation on mitochondrial function and sperm quality in fish. Aquaculture 2019;511:634190.
37 Jang TH, Park SC, Yang JH, Kim JY, Seok JH, Park US, Choi CW, Lee SR, Han J: Cryopreservation and its clinical applications. Integr Med Res 2017;6:12-18.