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Cryopreservation of Hepatocyte (HepG2) Cell Monolayers: Impact of Trehalose

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ABSTRACT

A simple method to cryogenically preserve hepatocyte monolayers is currently not available but such a technique would facilitate numerous applications in the field of biomedical engineering, cell line development, and drug screening. We investigated the effect of trehalose and dimethyl sulfoxide (Me2SO) in cryopreservation of human hepatocellular carcinoma (HepG2) cells in suspension and monolayer formats. HepG2 cell monolayers were incubated for 24 h at varying concentrations of trehalose (50-150 mM) prior to cryopreservation to identify the optimum concentration for such preincubation. When trehalose alone was used as the cryoprotective agent (CPA), cells in monolayer format did not survive freezing while cells in suspension demonstrated 14% viability 24 h after thawing. Only 6-13% of cells in monolayers survived freezing in cell culture medium supplemented with 10% Me2SO, but 42% of cells were recovered successfully if monolayers were preincubated with 100 mM trehalose prior to freezing in the Me2SO supplemented medium. Interestingly, for cells frozen in suspension in presence of 10% Me2SO, metabolic activity immediately following thawing did not change appreciably compared to unfrozen control cells. Finally, Raman spectroscopy techniques were employed to evaluate ice crystallization in the presence and absence of trehalose in freezing solutions without cells because crystallization may alter the extent of injury observed in cell monolayers. We speculate that biomimetic approaches of using protective sugars to preserve cells in monolayer format will facilitate the development of techniques for long-term preservation of human tissues and organs in the future.

INTRODUCTION

The ability to effectively cryopreserve monolayers of hepatocytes on microtiter plates (MTPs) would facilitate cell based applications in drug discovery, cell line development, and bioartificial liver-support systems [2, 50, 52]. Despite several studies on cryopreservation of hepatocyte monolayers [36, 38, 50, 54] currently established cryopreservation protocols involving the use of 10% dimethyl sulfoxide (Me2SO) often yield poor outcomes, and simple methods that maintain high amounts of viable cells after freezing and thawing of monolayers for cell-banking and bioengineering purposes are still underdeveloped. While cryogenic storage of suspended cells is routinely performed in laboratories around the world [34, 47], some cell types (e.g., stem cells) are extremely difficult to cryopreserve in suspension [4]. Furthermore, it is highly desirable to preserve characteristics of adherent cells, for example, as in the case for networks of neurons [31]. In comparison to cryopreservation of suspended cells, protocols for cryopreservation of adherent cell monolayers are significantly underdeveloped. While researchers have recently started to systematically analyze the cryoprotective agent (CPA) requirements for adherent cells [27], there are two principal approaches to cryopreservation of cell monolayers that are independent of the CPA. One approach involves growing cells on small glass slides that are coated with an extracellular matrix (e.g. collagen). The slides are then removed from the cell-culture dish and exposed to a CPA solution in cryogenic vials which are cooled at a controlled rate to the desired temperatures [22, 49]. The second approach involves using passive cooling devices to cryopreserve cells directly in the culture dishes on which they grown (e.g. MTPs) after exposing the monolayers to a CPA [36, 38, 54]. While both protocols result in varying degrees of success in preserving cells in monolayers, protocols to preserve hepatocyte monolayers on MTPs are still underdeveloped.

CPAs play an important role in determining the outcome of the cryopreservation protocol. Along with traditional CPAs such as Me2SO, ethylene glycol, and glycerol, non-reducing sugars such as trehalose and sucrose have been used to improve cryopreservation [6, 24, 26, 37]. Concerns about the toxicity of Me2SO at room temperature warrant the development of alternative CPAs that provide a lower toxicity to the cell [25]. Another commonly used CPA, glycerol, displays lower toxicity to cells than Me2SO, but unfortunately often requires more cumbersome multi-step loading protocols [33, 37]. Use of trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) as a CPA has been explored due to its involvement in ameliorating physiological stresses in several organisms in nature. Trehalose is involved in stress and cold-tolerance of several animals such as cysts of the brine shrimp *Artemia franciscana*, adults of the arctic springtail *Megaphorura arctica*, and the nematode *Aphelenchus avenae* [13, 17, 32]. Unfortunately, in absence of a specific sugar transporter, trehalose is impermeable to the plasma membrane and only small amounts of the sugar are taken up by cells via endocytosis after extended incubation periods with the sugar [23, 43]. Nevertheless, the addition of trehalose to Me2SO based freezing solution was found to increase the viability of human embryonic stem cells and primary hepatocytes in suspension which are known to be challenging to cryopreserve [28, 57], and viable endothelial cells have been obtained after cryopreservation when cells were cultured for 24 h with 0.2 M trehalose prior to freezing [8].

We demonstrate in this study that at a freezing rate of 1 ºC • min^{-1} the commonly used cryoprotective compound Me2SO can be used to promote viability and maintain attachment of HepG2 cell monolayers after freezing and thawing. Our results also show that cryogenic outcomes for these HepG2 monolayers are improved by 24 h of incubation with the non-reducing sugar trehalose prior to freezing in Me2SO-supplemented medium.

MATERIAL AND METHODS

Chemicals. All chemicals for respiration measurements were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ). Low endotoxin α,α-trehalose dihydrate was obtained from Pfanstiehl Inc. (Waukegan, IL). Water for solution preparation was purified with a Milli-Q Reagent Water System (Billerica, MA) to an electrical resistance of 18 mΩ.

Cell Culture. Human hepatocellular carcinoma cells (HepG2) were obtained from the American Type Culture Collection (Manassas, VA) and grown in 75 cm^2 cell culture flasks (Corning Incorporated, Corning, NY). Standard cell culture medium was composed of Opti-MEM I reduced serum medium (Invitrogen, Carlsbad, CA) supplemented with 5.5% fetal bovine serum (FBS) purchased from Atlanta Biologicals Inc. (Flowery Branch, GA), 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (MP Biomedicals, Santa Anna, CA). HepG2 cells were maintained in a humidified atmosphere of 6.5% CO_2 and 93.5% air at 37 °C and the culture medium was renewed every 3 - 4 days. The cells were subcultured every seven days or before reaching 90% confluency. To subculture cells were dissociated using 0.25% trypsin plus 1 mM EDTA in balanced salt solution (Invitrogen, Carlsbad, CA) and reseeded at $1.5 \cdot 10^6$ cells per 75 cm^2 cell culture flasks.

Impact of trehalose on cell proliferation. To assess the effect of trehalose on cell viability and proliferation, cells were seeded at a density of $1 \cdot 10^6$ cells per 60 mm culture dish (Corning Incorporated, Corning, NY) and the medium was exchanged after 24 h against medium containing trehalose concentrations ranging from 0-150 mM. Cells were then exposed to trehalose for 24 h followed by dissociation using 0.25% trypsin plus 1 mM EDTA in balanced salt solution, and the number of viable cells was determined by counting with a hemocytometer

(Hauser and Son, Philadelphia, PN) after 1:1 dilution of the sample with 0.4% trypan blue solution (Sigma-Aldrich, St. Louis, MO). In experiments to investigate sustained impacts of trehalose exposure on cell proliferation, cells that were exposed to trehalose for 24 h were sub-cultured at $1 \cdot 10^6$ cells per 60 mm culture dish in trehalose free standard culture medium for an additional 24 h. The percentage of plated cells was calculated by dividing the number of cells with intact cell membranes in the control and treatment groups by the number of cells initially plated multiplied by 100.

Cryopreservation of HepG2 cells in suspension. For cryopreservation experiments with cells that were exposed to trehalose concentrations ranging from 0-150 mM for 24 h the cells were detached using 0.25% trypsin plus 1 mM EDTA in balanced salt solution (Invitrogen, Carlsbad, CA, USA) and suspended in a freezing solution made as follows: 1 M trehalose was first prepared in cell culture grade water (Invitrogen, Carlsbad, CA) and the stock solution was filtered through a 0.22 µm sterile filter. This trehalose solution was added to Opti-MEM I supplemented with 20% FBS to yield a final concentration of 100 mM trehalose. Cells suspended in this solution were transferred into cryogenic vials (Thermo Fisher Scientific Inc., Waltham, MA) at a concentration of $1 \cdot 10^6$ cells $\cdot$ mL^{-1} and were transferred to -80 ºC inside an isopropanol-based passive freezing device designed to achieve a rate of cooling close to -1°C $\cdot$ min^{-1} (Thermo Scientific Inc., Waltham, MA). After cooling to -80 ºC for 24 h cell samples were rapidly thawed at 37 ºC and 500 µL of this sample was transferred to a 60 mm culture dish containing 4.5 mL of standard culture medium. Thawed cells were cultured for 24 h and then counted after being detached as described above. The percentage of recovered cells was calculated by dividing the number of cells with intact cell membranes in the control (no

preincubation with trehalose) and treatment groups by the number of cells before freezing multiplied by 100.

Cryopreservation of HepG2 cell monolayers. Cells to be frozen in the monolayer format were seeded at $0.25 \cdot 10^6$ cells per well in 500 µL of cell culture medium in 24-well plates (Corning Incorporated, Corning, NY) coated with collagen. To promote attachment of cells after thawing, collagen I from rat tail (Invitrogen, Carlsbad, CA) was diluted to 50 µg/ml in 20 mM acetic acid (Fischer Scientific, Fair Lawn, NJ) and added to each well of the cell culture plate at 5 µg collagen • cm^{-2}. Plates were incubated with the dissolved collagen for 1 h and after this incubation period the collage solution was removed and the plates were rinsed three times with 200 µL Dulbecco's phosphate buffered saline (Thermo Fisher Scientific Inc., Waltham, MA) to remove any residual acetic acid solution. The collagen treated plates were allowed to dry for one hour in a laminar flow hood and stored for less than one week at 4 °C prior to use. Before experimental treatments cells were allowed to attach for at least 1 h to the collagen coated plates in a humidified atmosphere of 6.5% CO_2 and 93.5% air at 37 °C. After the cells adhered firmly to the culture plates in some experiments the medium was exchanged against medium that was supplemented with trehalose. Control cells received no trehalose and experimental cells were incubated with concentrations ranging from 50 to 200 mM trehalose for 24 h. Following the incubation period the culture medium was removed and cells were exposed for five minutes to different formulations of solutions supplemented with CPAs as listed in Table 1. After 5 min the solutions were removed and the plates placed inside a cell freezing container (BioCision, LLC, Larkspur, CA) and transferred to -80 ºC freezer. The freezing container includes a highly thermo-conductive stage (CoolCell® MP plate) placed in direct contact with the underside of the MTP that minimizes the temperature differential between wells and ensures a uniform rate of

thermal energy transfer of 1 ºC • min^{-1} during the freezing process. After 24 h at -80 ºC cells were rapidly thawed by addition of cell culture medium that was warmed to 37 °C.

Metabolic activity and cell membrane integrity after thawing of HepG2 cell monolayers. In contrast to monolayer samples that were cultured for 24 h after thawing, the number of cells with intact membranes could not be determined by trypan blue exclusion immediately after freezing and thawing of the monolayers. HepG2 cells formed aggregates during trypsinization and reproducible cell numbers could not be enumerated. However, this problem was not observed if cells were cultured under standard conditions for 24 h after thawing. Metabolic activity of cells stored at -80 ºC for 24 h was monitored immediately after thawing with alamarBlue™ (BioSource International, Inc., Camarillo, CA). A working solution of alamarBlue™ was prepared by diluting the commercial stock solution 10-fold with standard cell culture medium. Then 0.5 ml of this working solution was added to each well of the 24-well culture plate. A sample of 0.1 ml was removed 2 h after addition of the working solution and transferred to a 96-well plate (Fischer Scientific, Pittsburg, PA). The absorbance at $\lambda = 570$ nm and $\lambda = 600$ nm was measured with an absorbance microplate reader (Epoch 1, BioTek, Winooski, VT) and the percent of reduced alamarBlue™ (RA%) was calculated as: RA% = (((O2 • A1) – (O1 • A2))/((R1 • N2) – (R2 • N1))) • 100, where O1 (80586) is the molar extinction coefficient of oxidized alamarBlue at 570 nm and O2 (117216) is the molar extinction coefficient of oxidized alamarBlue at 600 nm. The values for R1 (155677) and R2 (14652) represent the molar extinction coefficients of reduced alamarBlue at 570 nm and 600 nm and A1 and A2 are the absorbance of test wells measured at 570 nm and 600 nm, respectively. N1 and N2 correspond to the absorbance values measured for negative control wells at 570 nm and 600 nm, respectively.

The membrane integrity of adherent cells immediately after freezing and thawing was determined using a SYTO 13 and propidium iodine membrane-integrity assay which can be performed on attached cells (Molecular Probes, Eugene, OR). The stock solution was prepared by adding 10 µL of a 1 mg • mL^{-1} SYTO 13 solution (aq.) and 5 µL of a 1.0 mg • mL^{-1} propidium iodine solution (aq.) to 10 mL of DMEM (Invitrogen Corporation, Carlsbad, CA). An aliquot of 130 µL of this solution was then added to the attached cells and imaged using an inverted Zeiss microscope (Göttingen, Germany) equipped with dual fluorescence filter set (51004-V2, Chroma Technology, Brattleboro, VT). Images were processed using Image J processing software (National Institutes of Health, Bethesda, MD, USA). The percentage of cells with intact membranes (membrane integrity) was determined by counting live (green) and dead (red) cells on four representative images from the same sample.

Respirometry on HepG2 cells. Oxygen consumption of control HepG2 cells grown for a minimum of ten passages after cryopreservation and of cells directly after thawing was measured at 37 ºC in 2 mL of MiR05 (110 mM sucrose, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 3 mM $MgCl_2$, 0.5 mM EGTA, 0.1% BSA, 20 mM HEPES-KOH, pH 7.1) at a concentration of 1 • 10^6 cells • mL^{-1}. Cells suspended in 10% Me2SO + 5.5% FBS in Opti-MEM I were frozen to -80 ºC, stored for 24 h, and rapidly thawed as described above. The freezing solution was removed after thawing by centrifugation of cells at 175 g for 5 min, and the cell pellet was suspended in standard culture medium and kept on ice until processed for respirometry. Oxygen flux of control cells was determined before freezing and without exposure to the Me2SO contained in the freezing solution. In order to supply mitochondrial substrates cells from both samples were permeabilized by addition of digitonin dissolved in Me2SO at 10 mg • mL^{-1} (final concentration 10 µg • 10^{-6} cells). This digitonin concentration was found to be

sufficient to permeabilize the plasma membrane of HepG2 cells with low impact on the integrity of the outer mitochondrial membrane. Proton leak respiration was initiated by adding 2 mM malate, 10 mM glutamate and 5 mM pyruvate, which supplies electrons to the electron transport system (ETS) via production of NADH by mitochondrial dehydrogenases. To induce oxidative phosphorylation (OXPHOS) 1 mM ADP was added and convergent electron entry to the ubiquinone pool via NADH and $FADH_2$ was initiated by addition of 10 mM succinate. Potential damage to the outer mitochondrial membrane was investigated through addition of cytochrome *c* (10 µM). Leak respiration in presence of ADP and ATP was measured after addition of oligomycin (2 µg • mL)$^{-1}$, and contribution of complex I to leak respiration was recorded after addition of rotenone (0.5 µM). Non-mitochondrial oxygen consumption was recorded after addition of 2.5 µM of antimycin A. Oxygen consumption was measured with an Oxygraph-2K (OROBOROS Instruments, Innsbruck, Austria) and DATLAB software (OROBOROS Instruments) was used for data analysis and acquisition. Oxygen flux (Jo_2; pmol O_2 • s^{-1} • 10^{-6} cells) was calculated as the time derivative of oxygen concentration.

Raman Microspectroscopy. Micro-Raman spectroscopy allows the probing of a small volume by combining a confocal microscope with the spectroscopy system. We have used micro-Raman spectroscopy in this study and refer to the spectra obtained as "Raman spectra". Raman spectra were made with a WITec Alpha300 system (wavelength of incident laser light, λ = 532 nm) and a 10 x objective (WITec Instruments Corp, Knoxville, Tennessee). The incident laser power was carefully tuned to avoid sample damage or laser induced heating and measurements were thus performed at around 25 mW incident laser power. A Linkam cryogenic stage (FDCS 196, Linkam Scientific Instruments, Surrey, UK) was used to cool the CPA solutions at controlled

freezing rates. Raman spectra were obtained at definite intervals during freezing and thawing of the solutions.

Statistical analyses. Data were analyzed with a one-way analysis of variance (ANOVA) on ranks followed by comparison of experimental groups with the appropriate control group (Holm-Sidak method) or 2-way ANOVA followed by comparison of experimental groups with the appropriate control groups (Holm-Sidak method). SigmaPlot 12.5 (Systat Software Inc., San Jose, CA) was used for the analyses.

RESULTS

Effect of trehalose on HepG2 cell proliferation and viability. In order to evaluate the protective potential of trehalose in cryopreservation of HepG2 cells the impact of this sugar on cell viability and proliferation was investigated since increased extracellular osmolyte concentrations inevitably cause osmotic stress to cells. Compared to untreated controls the numbers of viable cells were decreased after 24 h of exposure to the sugar at all concentrations investigated ($p < 0.05$, $n = 9$ experiments). Strikingly, no net increase in cell numbers occurred over 48 h at 150 mM trehalose, the highest concentration investigated, and only 1.06 ± 0.03 (SE) $\bullet 10^6$ viable cells were recovered from $1 \bullet 10^6$ cells plated (Fig. 1A). In addition to the acute effect of trehalose on cell proliferation (a hyperosmotic challenge) prolonged effects that persisted after the sugar was removed (a hypoosmotic challenge) were also observed. Cell numbers in control samples increased to 144 ± 4.2 (SE) % of the original value during 24 h after plating, but in samples that were exposed to concentrations of 100 mM and 150 mM trehalose, prior to seeding, only about 83% of cells were recovered (Fig. 1B, $n = 9$) showing that osmotic challenges in either direction (hypoosmotic or hyperosmotic) causes reductions in cell viability.

Cryopreservation of HepG2 cells in suspension and monolayer formats. We tested whether trehalose provides cellular protection during one freeze thaw cycle to -80 ºC when present as the only cryoprotective agent (CPA). We tested the cryoprotective effects of sugar incubation at concentrations ranging from 0 to 150 mM. The experimental cells (with trehalose) and control cells (without trehalose) were frozen to -80 ºC suspended in a solution composed of cell culture medium supplemented with 20 % FBS and 100 mM trehalose. Under these conditions incubation with 100 mM trehalose prior to freezing provided the highest protection to the plasma membrane ($p < 0.05$, $n = 9$) compared to controls, and 13.8 ± 0.26 (SE) % of cells were recovered 24 h after

thawing and plating of samples frozen in suspension (Fig. 2). Unfortunately, cell monolayers did not survive freezing in this solution (Table 1), and cell viability was not increased after incubation with 100 mM trehalose (data not shown). Three additional freezing solutions were investigated next and HepG2 monolayers were frozen in these solutions with a trehalose preincubation step. Under theses condition 10 % Me2SO provided the same protection in either 90 % FBS or in cell culture medium (Table 1). Due to the fact that FBS is rather expensive we utilized culture medium supplemented with 10 % Me2SO and 5.5 % FBS in the subsequent experiments.

Cryopreservation of HepG2 monolayers after incubation with trehalose. Me2SO is widely used in cryopreservation of cells in suspension but protection of confluent HepG2 cell monolayers was poor. Only 10.5 ± 1.8 (SE) % of control cells before freezing were recovered after thawing and 24 h recovery without trehalose preincubation prior to freezing (Table 1). To investigate if preincubation with trehalose improves viability of HepG2 cell monolayers frozen in presence of Me2SO, alamarBlue assays were performed. In presence or absence of trehalose in the Me2SO based freezing solution, alamarBlue reduction rates immediately after thawing of HepG2 monolayers were statistically unchanged after incubation with trehalose concentrations ranging from 50 to 150 mM as compared to controls without trehalose incubation (Fig. 3). Surprisingly, robust alamarBlue reduction rates were observed after thawing of control samples (without trehalose incubation), which yielded low percentages of recovered cells if cultured for 24 h after thawing (Table 1). Since the active compound, resazurin, in alamarBlue is reduced by cellular dehydrogenases [41] we speculated that most enzymes might be well protected during freezing in presence of 10 % Me2SO and resazurin reduction rates measured immediately after thawing are not indicative of cell viability after cryopreservation of cell monolayers. However, cells

could not be completely detached from the collagen matrix directly after thawing (see Materials and Methods). Therefore, trypan blue exclusion assays were performed on the cells 24 h after thawing and culture under standard conditions.

Cell counts revealed that preincubation with trehalose concentrations ranging from 50 to 200 mM significantly improved recovery of cells frozen as monolayers compared to controls without exposure to trehalose (n = 7-12, $p < 0.05$) (Fig. 4A). Furthermore, preincubation with 100 mM trehalose significantly outperformed all other concentrations evaluated (Fig. 4A). To investigate if addition of trehalose to the freezing solution further improves cryopreservation outcome, the same series of experiments was repeated in presence of 100 mM extracellular trehalose in the freezing solution along with 10% Me2SO (Fig. 4B). Two-way analysis of variance (ANOVA) detected a significant interaction between trehalose preincubation and the presence or absence of trehalose in the freezing buffer ($p = 0.003$) and surprisingly, the addition of the sugar to cell culture medium containing 10 % Me2SO caused a 50% reduction in cell recovery (Fig. 4B). These results were confirmed by live-dead staining performed immediately after thawing (Fig. 5). We observed a lower percentage of cells staining positive for ethidium homodimer (dead cell staining) when HepG2 monolayers were preincubated with 100 mM trehalose and frozen in cell culture medium supplemented with only 10 % Me2SO (Fig. 5A.3), compared to a freezing solution supplemented with 10 % Me2SO and 100 mM trehalose (Fig. 5B.3). The percentage of cells with intact membranes decreased from about 80% to about 60% after the addition of trehalose to the freezing solution (Table 2, $p < 0.001$). However, similar to our alamarBlue data obtained directly after thawing of HepG2 monolayers, no significant differences among the preincubation groups were detected in the stained HepG2 monolayers (n = 4; $p = 0.93$, Table 2).

Respiration of HepG2 cells cryopreserved in suspension format. Based on our alamarBlue data we speculated that cellular dehydrogenases may be preserved during cryopreservation in presence of Me2SO and remain functional directly after thawing. Unfortunately, respiration measurements of cells frozen in the monolayer format were not possible since cells could not be separated from the collagen matrix directly after thawing (see Materials and Methods), and we decided to investigate the impact of freezing on cells in suspension on the activities of mitochondrial dehydrogenases and bioenergetically properties. After the addition of malate plus glutamate and pyruvate to never-frozen, permeabilized HepG2 cells (supplying NADH to complex I) the oxygen flux was 8.1 ± 0.5 (SE) pmol O_2 • sec $^{-1}$ • 10^{-6} cells (n = 4). This value was indistinguishable from cells that were frozen in suspension format to -80 ºC in cell culture medium supplemented with 10 % Me2SO and assessed directly after thawing (Fig. 6). Both never-frozen control and cryopreserved cells showed an about 3.5-fold increase in oxygen flux after engaging the phosphorylation system by addition of 1 mM ADP and a further increase of about 80% was recorded after addition of succinate which supplies electrons to the mitochondrial ubiquinone pool via the bound flavin adenine dinucleotide (FAD) of the succinate dehydrogenase complex. Importantly, the response to cyt *c* addition was indistinguishable between control cells and cryopreserved cells, indicating a well preserved outer mitochondrial membrane immediately after cryopreservation. In summary, directly after thawing all investigated bioenergetics functions of mitochondria were unchanged from control cells (Fig. 6). While we aimed to use alamarBlue and respiration assays as tools to quantify changes in cellular properties immediately after thawing no changes in cellular physiology for HepG2 cells could be detected with these two assays. In presence of 10% Me2SO cryopreservation outcomes apparently depend on events that are difficult to detect directly after thawing and the impact of

trehalose on preservation of HepG2 monolayers could only be demonstrated after 24 h of recovery by trypan blue exclusion assays (see above).

Decrease in ice crystal formation in the presence of trehalose. The behavior of water at low temperatures and the formation of ice crystals have profound effects on cryopreservation [5]. We speculated that the addition of trehalose to the Me2SO based freezing buffer may change the ice-formation characteristics of water, and Raman microspectroscopy is an excellent tool to characterize these changes from a molecular standpoint. Ice formation can be easily detected by observing Raman spectra of water at low temperatures [20]. The phenomenon of ice formation affects the O-H stretching modes of water (2900–3600 cm^{-1} range), which indicates the state of intramolecular O-H pairs and hydrogen bonds. Based on the nature of hydrogen bonds, the O-H stretch region of the water spectrum consists of two principal regions known as symmetric and asymmetric stretches, with Gaussian shaped peaks around 3242 cm^{-1} and 3451 cm^{-1}, respectively [5]. The symmetric peak has been attributed to the O-H vibration in tetra-bounded water molecules that are under influence of fully developed hydrogen bonds, while the asymmetric peak correspond to the O-H vibrations of water molecules that have partially or incompletely developed bonds (hence free water) [9]. In order to understand the impact of trehalose on ice formation in our freezing buffer Raman microspectroscopy was performed without cells present. Onset of ice nucleation during freezing was indicated in by the appearance of a definitive ice peak at 3100 cm^{-1} (Fig. 7). If 100 mM trehalose was added to the 10% Me2SO solution, we observed a significant decrease (>50%) in the intensity of the ice peak at -170 ^{0}C (Fig. 7). This signifies that presence of trehalose at cryogenic temperatures leads to a decrease in ice-crystal formation compared to Me2SO alone. Furthermore, the presence of trehalose affected the state of

345 the water molecules at cryogenic temperatures. A significant decrease of both the symmetric
346 (3242 cm^{-1}) and the asymmetric peaks (3451 cm^{-1}) was observed in presence of trehalose.

Discussion

Animals in the temperate and polar regions of the globe that survive cooling to subzero body temperatures [3, 12, 21, 51] experience reductions in cellular water content, due to the precipitation of water as ice, if the animal fails to maintain a super-cooled state. Extracellular ice formation will lead to osmotic dehydration of cells and tissues which might protect from intracellular ice formation (IIF) an event lethal for most animals. Some animals are known to accumulate compatible osmolytes [59], prior or during the onset of cellular water limitation and some exceptional species are capable of surviving the formation of intracellular ice [46, 48, 55]. Since cells in freeze-tolerant animals experience dehydration in highly organized tissues, we hypothesized that compatible osmolytes in general and protective sugars such as trehalose in particular are useful in cryopreservation of cell monolayers. We have demonstrated in this study that incubation of confluent HepG2 monolayers with 100 mM trehalose prior to freezing increases cell viability by roughly 400% after thawing and recovery.

Trehalose is a non-reducing disaccharide with unique chemical properties that accumulates in some organisms that survive freezing and desiccation in nature [18, 42]. Similar to the proposed functions of trehalose in anhydrobiosis [16], the presence of the sugar during freezing might aid in the protection of membranes and proteins through multiple mechanisms. Three hypotheses have been postulated to account for the mechanisms by which trehalose preserves cellular structures during reductions in water activity: (1) the water replacement hypothesis, which suggests that sugars can replace water molecules by forming hydrogen bonds with polar residues and stabilize cellular structures to prevent denaturation, (2) the preferential exclusion theory which suggests that trehalose attracts water away from macromolecules, compacts the macromolecule and reduces the probability to denature, and (3) the vitrification

hypothesis suggests that the sugar solutions form amorphous glasses, which reduces structural fluctuations throughout freezing [14, 15, 53, 58]. Glass formation (T_g) occurs when a liquid is cooled sufficiently quickly and the viscosity becomes so great that the rearrangement of molecules in the liquid becomes extremely slow or stops, which occurs at approximately -100 ºC for trehalose solutions with weight fractions of water > 0.75 [11, 56].

To exert maximal protective properties trehalose has to be present on both sides of the plasma membrane [24], but unfortunately the sugar is only readily permeable to cells that express a specific transporter in their plasma membrane [10; 29]. One technique to accumulate some levels of intracellular trehalose in mammalian cells is through endocytosis after adding trehalose to the culture medium for extended periods of time [8, 23], and we hypothesized that low levels of trehalose also accumulate intracellular in HepG2 cells after 24 h of exposure to the sugar as demonstrated for J774.Q1 mouse macrophages [23]. In order to successfully load intracellular trehalose as a cryoprotectant in biomedical applications the optimal concentration used during pre-incubations has to be carefully optimized since the sugar can cause acute and prolonged reductions in cell viability and proliferation (Fig. 1). We found that intracellular loaded trehalose in presence of 100 mM extracellular trehalose conferred some protection to cells frozen in solution but no viable cells were recovered if cells were frozen as monolayers (Fig. 2, Tab. 1). Constraints for cells frozen in solution are different from the physical stress cells experience when frozen as monolayers. Furthermore, the formation of intracellular ice may be less harmful to cells in monolayers than for isolated cells frozen in suspension has been demonstrated [1], and some suggest that IIF is preceded by damage to the plasma membrane and IIF is a result of cell injury not the cause [39].

392 The increase in recovered viable cells observed after preincubation with trehalose may
393 not be due only to the cryoprotective properties of the sugar, but also to an indirect effect of
394 osmotic stress, as suggested by [44]. We do agree that osmotic stress prior to dehydration may
395 activate survival pathways that might have protective effects when cells experience dehydration
396 during freezing [7]. Nevertheless, based on extensive research that demonstrates the protective
397 effects of trehalose on membranes and proteins during desiccation and freezing [18, 19] this
398 'preconditioning' effect is in our opinion rather minor compared to the direct cell stabilizing
399 effects of trehalose.

400 The main factors that impact viability of cells after freezing and thawing, in both
401 monolayer and suspension formats, is ice-crystal formation and hyperosmotic stress due to
402 precipitation of water as ice [26, 34, 35]. Water that is below its freezing point is referred to as
403 supercooled and has a higher chemical potential at a given subzero temperature than that of ice
404 or water in a solution that is in equilibrium with ice [35]. As extracellular ice precipitates, a
405 significant increase in the concentration of all other solutes occurs in the remaining liquid water
406 fraction. This might lead to excessive dehydration that may result in irreversible membrane
407 alteration and cell death. The formation of extracellular ice, therefore, results in an increased
408 osmotic pressure in the extracellular space that creates an osmotic gradient across the plasma
409 membrane, which provides a driving force for the efflux of water from the cell [39]. Intracellular
410 ice formation (IIF) may occur during freezing rates that are substantially higher than 1 ºC • min^{-1}
411 but do not approach ultra-rapid freezing rates used in vitrification techniques [34, 35].
412 Extracellular and intracellular ice crystals that form during freezing and thawing may penetrate
413 cellular membranes and will inevitably lead to a reduction in the amount of viable cells that are
414 recovered after cryopreservation. A highly controlled rate of freezing is vital to ensure proper

movement of water across the plasma membrane which allows osmotic dehydration to reach equilibrium with intracellular and extracellular contents in order to prevent IIF [33, 47]. We found that at a freezing rate of 1 ºC • min^{-1} only about 10 % of cells frozen as monolayers could be recovered whereas recovery for cells in suspension at this freezing rate is commonly above 90% (data not shown). Me2SO protects as penetrating CPA and protect cells during cryopreservation in part due to the ability to reduce the concentration of damaging solutes in the unfrozen water fraction during the freezing and thawing processes [35]. However, osmotic dehydration during freezing appears to be more detrimental for cells that are frozen as monolayers than in suspension. Cell monolayer are firmly attached to the extracellular matrix and before the eutectic temperature is reached less able to move into the remaining unfrozen water fraction compared with cells frozen in suspension [40]. The combination of intracellular trehalose and Me2SO might mitigate physical stresses and help to stabilize cellular membranes during freezing of cell monolayers as has been demonstrated for freezing of vesicles [45]. Surprisingly, we found that cellular respiration and resazurin reduction was unchanged immediately after thawing of cells frozen in suspension or in the monolayer format, respectively (Figs. 3, 4). Larsen [30] observed that freezing of skeletal muscle tissue in presence of Me2SO negatively impacts respiration fueled by complex I. These observations indicate that different cell and tissue types respond differently to cryopreservation but that some cellular samples might be stored at -80 ºC prior to assessment of bioenergetics properties. We are currently investigating the optimal conditions and concentrations of trehalose used in preincubation to store isolated mitochondria, cells, and tissues at cryogenic temperatures prior to high-resolution respirometry. A comprehensive study of mitochondrial properties is a time consuming process and cryopreservation of samples prior to high-resolution respirometry would greatly simplify the

experimental workflow and increase the accessibility to use this technology as a diagnostic tool in medicine.

The Raman data sheds light on the effect of presence of trehalose in CPA at the molecular level. Ice formation in extracellular solution can be directly linked to cellular injury at cryogenic temperatures for both attached and suspended cells. Decreased intensity of the ice peak at cryogenic temperatures (Fig. 7) indicates a protective effect of trehalose. The simultaneous decrease of both the symmetric and asymmetric peaks of the water molecules in the spectra in presence of trehalose further indicates that presence of trehalose in CPA is capable of decreasing water activity and thus rendering a protective effect in cryopreservation. In contrast to this observation, we found the highest recovery of viable cells after preincubation with 100 mM trehalose but in absence of additional trehalose in the freezing buffer (Figs. 4, 5). This finding might be specific to hepatocytes since we did not observe a reduction in viability for neuroblastoma cells frozen in the presence of trehalose and Me2SO compared to Me2SO alone (Bailey, Nathan, Menze, unpublished observations). HepG2 cells might be especially sensitive to additional hyperosmotic stresses during freezing and thawing caused by the addition of non-permeating compounds to the freezing buffer. Furthermore, during processing for cryopreservation the extracellular solution containing 100 mM was simply removed and the cells were loaded with Me2SO. The residual trehalose from the incubation medium might be sufficient to confer protection without causing additional hyperosmotic stress during freezing and thawing. In summary our study demonstrates that trehalose incubation facilitates preservation of cells in monolayer format. In order to develop trehalose based techniques for long-term preservation of human tissues and organs in the future more efficient loading protocols for the sugar are likely needed.

Acknowledgments

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Table 1: Composition of solutions employed for freezing of hepatocellular carcinoma (HepG2) cell monolayers and cell recovery after freezing without trehalose pre-incubation.

Composition of freezing solution	**% Live Cell Recovery[1]**
20% FBS + 100 mM trehalose in Opti-MEM I	0.00 ± 0.0
10% Glycerol + 5.5% FBS in Opti-MEM I	6.4 ± 0.9
90% FBS + 10% Me2SO	12.1 ± 2.5*
10% Me2SO + 5.5% FBS in Opti-MEM I	10.5 ± 1.8*

[1]Live cell recovery is expressed in % of cells that were recovered after thawing followed by 24 h of standard cell culture conditions compared to numbers of cells before freezing. Only cells that excluded trypan blue were used to calculate the fraction of viable cells (n = 8-30, ±SE). Asterisks indicate no significant differences in recovery between cells frozen in 10% Me2SO in FBS, or 10% Me2SO in cell culture medium ($p = 0.297$).

Table 2: Membrane integrity of hepatocellular carcinoma cells (HepG2) measured immediately after thawing. Cell monolayers were incubated with different concentrations of trehalose for 24 h before freezing.

Trehalose [mM]	**Buffer 1 (no trehalose)**	**Buffer 2 (100 mM trehalose)**
24 h preincubation	% Membrane integrity[1]	% Membrane integrity[1]
0	80.6 ± 5.0	68.3 ± 2.6
50	87.1 ± 3.6	57.5 ± 3.8
100	90.2 ± 4.2	47.8 ± 8.8
150	69.2 ± 8.4	69.5 ± 6.2

[1]Membrane integrity is expressed in % of cells staining positive for SYTO 13 and negative for propidium iodine divided by the total number of cells. Monolayers were frozen in 10% Me2SO + 5.5% FBS in Opti-MEM I (Buffer 1) or 10% Me2SO + 5.5% FBS in Opti-MEM I + 100 mM trehalose (Buffer 2). Data are based on enumerating cell numbers in random areas of thawed monolayers (n = 4 ±SE). Independent from the concentration of trehalose during preincubation the presence of trehalose in the freezing buffer significantly reduced the number of cells with intact membranes after thawing ($p < 0.001$). No significant differences in membrane integrity were found among the trehalose concentrations used in the 24 h pre-incubation step ($p = 0.093$).

Figure Legends

Fig. 1: Impact of trehalose on cell proliferation. A) Effect at the end of 24 h of trehalose exposure. A reduction in proliferation of human hepatocellular carcinoma cells (HepG2) is observed in culture media supplemented with trehalose. B) Effect 24 h after the removal of trehalose. HepG2 cells transiently treated with trehalose showed prolonged inhibitions in cell proliferation after removal of the sugar. *Statistically significant different to 0 mM trehalose (n = 9, ±SE). Significance level was set at $p \leq 0.05$.

Fig. 2: Percentage of cells recovered after freezing of suspended cells exposed to trehalose for 24 h. HepG2 cells were incubated with trehalose for 24 h and frozen to -80 ºC in culture medium supplemented with 100 mM trehalose and 20 % FBS. Incubation with 100 mM trehalose for 24 h confers highest amount of protection to suspended cells (n = 9, ±SE) measured after 24 h of cell recovery. *Statistically significant different to 0 mM trehalose. Significance level was set at $p \leq 0.05$.

Fig. 3: Cellular alamarBlue reduction immediately after thawing. HepG2 cells were incubated with trehalose for 24 h and frozen in cell culture medium supplemented with 10 % Me2SO (black bars) or with 10 % Me2SO plus 100 mM trehalose (gray bars). No statistically significant differences to the respective controls at 0 mM trehalose were detected immediately after thawing (n = 6-12, ±SE). Significance level was set at $p \leq 0.05$.

Fig. 4: Trehalose pre-incubation increases percentage of cells recovered after freezing of HepG2 monolayers. HepG2 cell monolayers were incubated for 24 h with different concentrations of trehalose and frozen in culture medium supplemented with A) 10 % Me2SO or B) 10 % Me2SO plus 100 mM trehalose. Cell samples were thawed by addition of culture

medium and cell counts were performed after a 24 h recovery period (n = 7-12, ±SE). *Statistically significant different to 0 mM trehalose. #Statistically significant different to all other treatments tested. Significance level was set at $p \leq 0.05$.

Fig. 5: Membrane integrity of HepG2 cells frozen in monolayer format immediately after thawing. HepG2 control cells without trehalose incubation (A,B-1) and cells incubated with 50 mM (A,B-2), 100 mM (A,B,-3), or 150 mM (A,B-4) trehalose were frozen at a rate of 1 ºC per minute to -80 ºC, thawed, and stained for viable (green) and dead (red) cells. Monolayers were frozen in cell culture medium supplemented with 10 % Me2SO (panel A), or with 10 % Me2SO plus 100 mM trehalose (panel B).

Fig. 6: High-resolution respirometry after freezing and thawing of suspended HepG2 cells in presence of 10 % Me2SO. Respiration of hepatocellular carcinoma cells (HepG2) was measure before (black bars) and after cells were frozen to -80 ºC in cell culture medium supplemented with 10 % Me2SO (gray bars). No significant differences in respiration rates were observed immediately after thawing compared to controls before freezing. Oxygen flux is shown after successive additions of cells (HepG2), digitonin (Dig), malate plus glutamate plus pyruvate (MGP), succinate (S), ADP (D), cytochrome c (Cyt-*c*), oligomycin (Omy), rotenone (Rot), and antimycin A (Ama) (n = 4, ±SE).

Fig. 7: Raman microspectroscopy on CPA solutions at -170 ºC. The presence of trehalose leads to a significant reduction in ice-crystal formation as indicated by a reduced ice peak at 3100 cm^{-1}.

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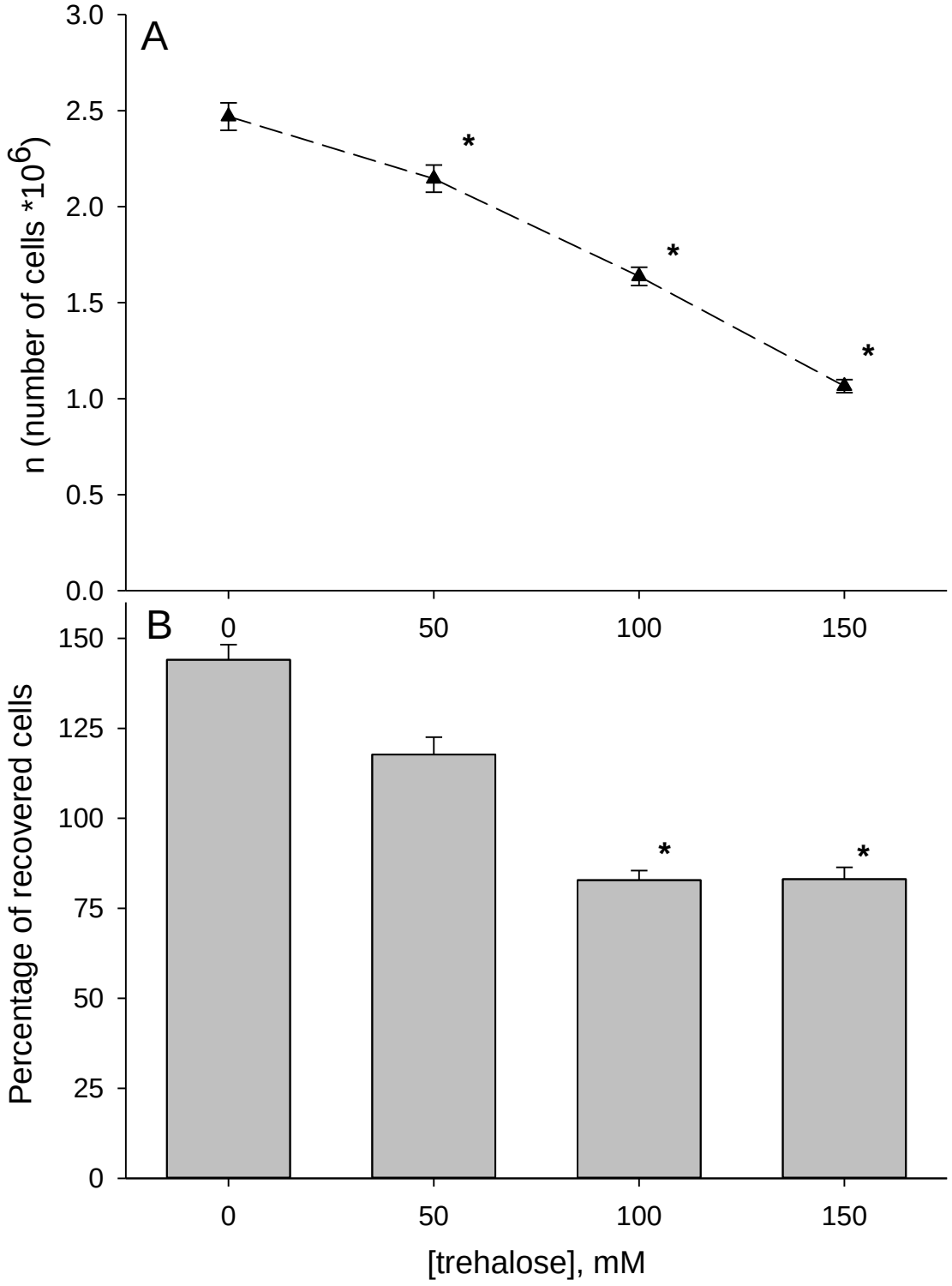
688 **Fig. 1**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n (number of cells *10^6)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
B
Percentage of recovered cells
150
125
100
75
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25
0
*
*
0
50
100
150
[trehalose], mM

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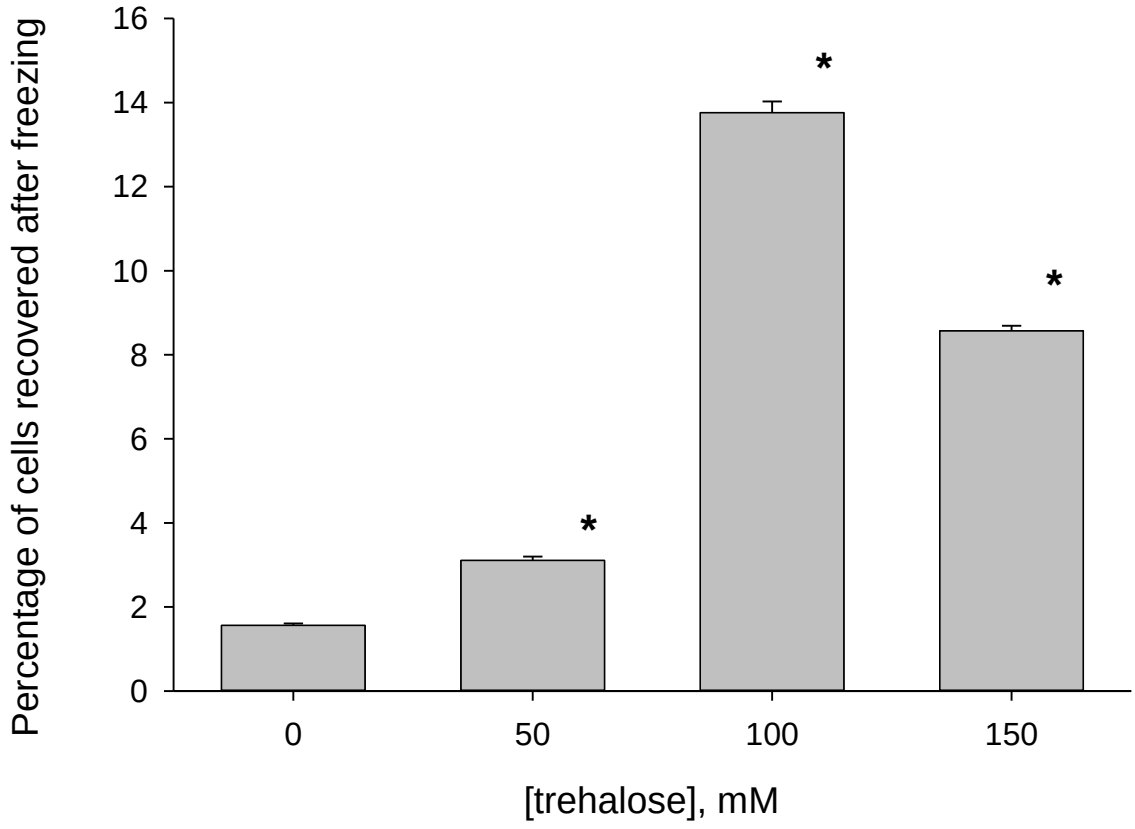
691 **Fig. 2**



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695 **Fig. 3**

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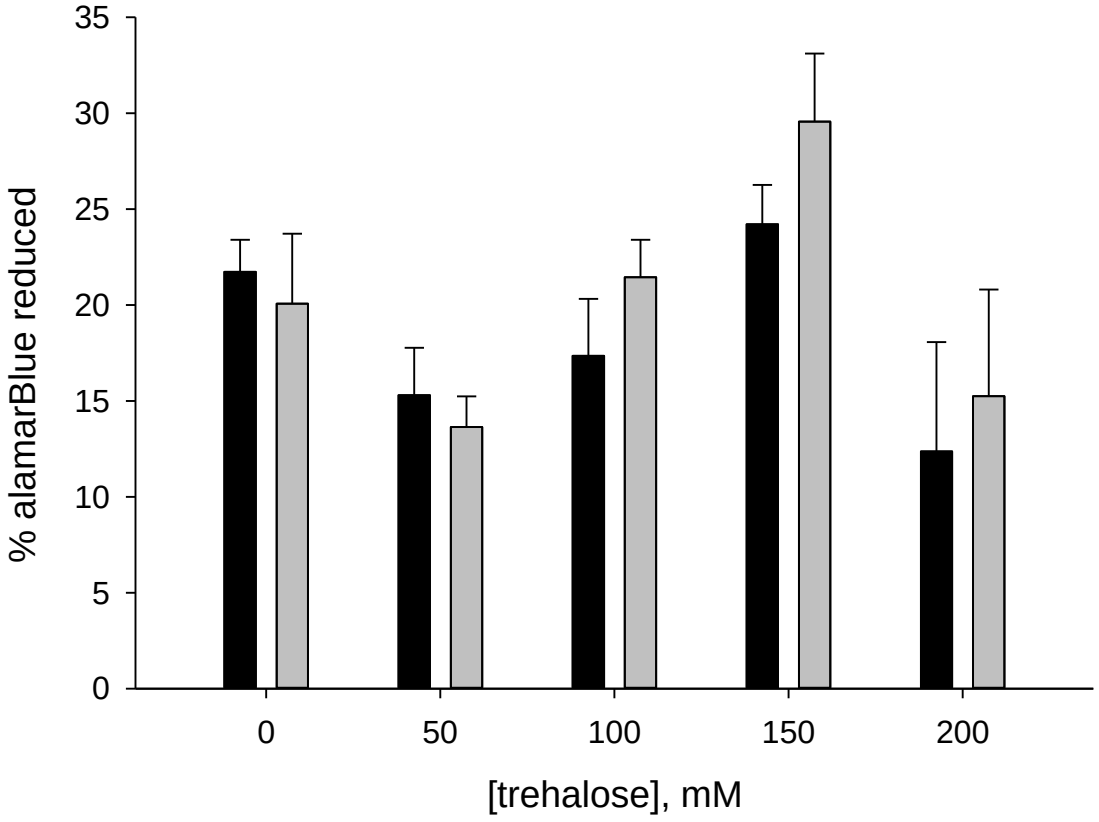

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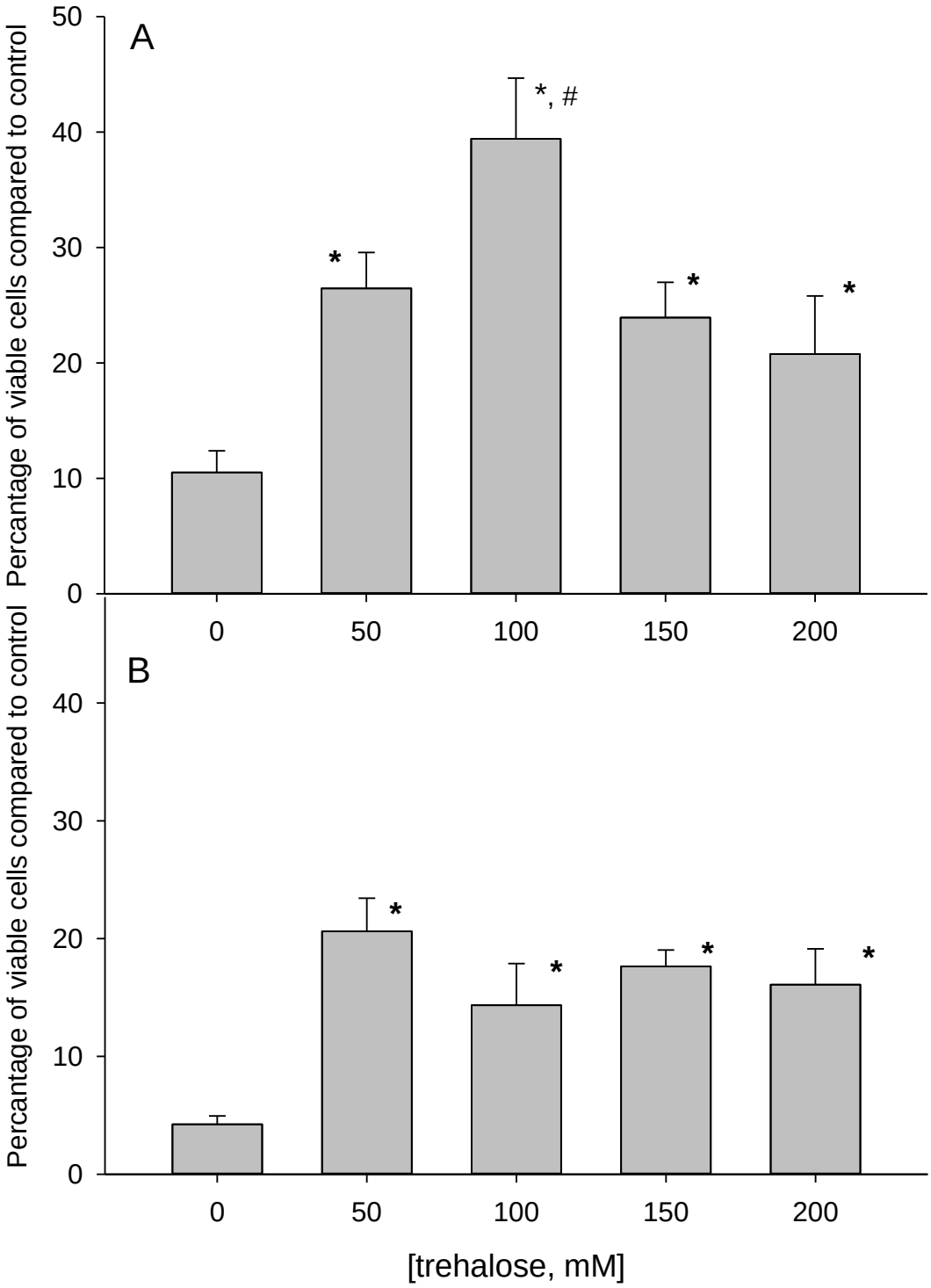
699 **Fig. 4**


A
B
Percantage of viable cells compared to control
Percantage of viable cells compared to control
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0
50
100
150
200
[trehalose, mM]

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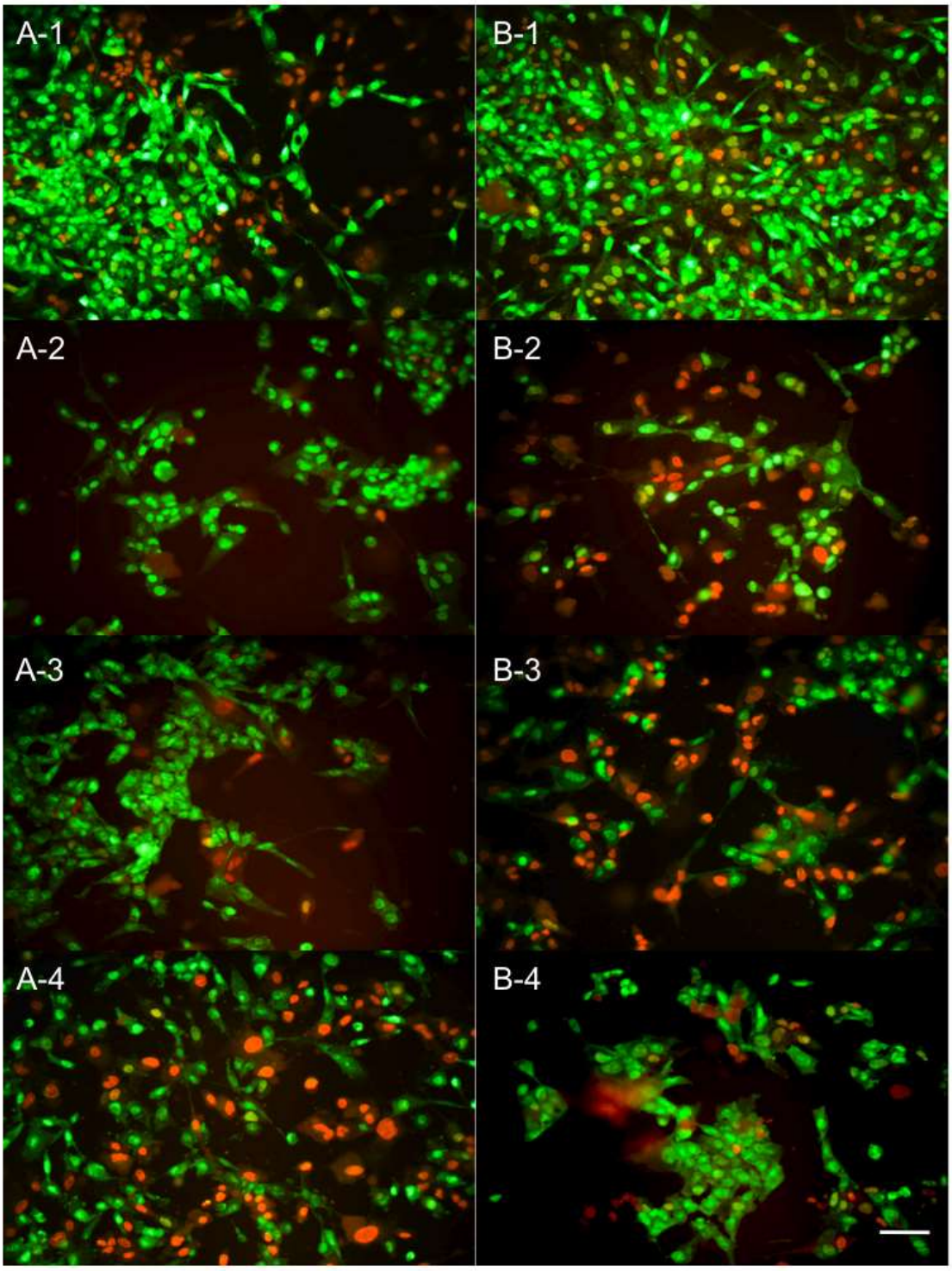
701 **Fig. 5**



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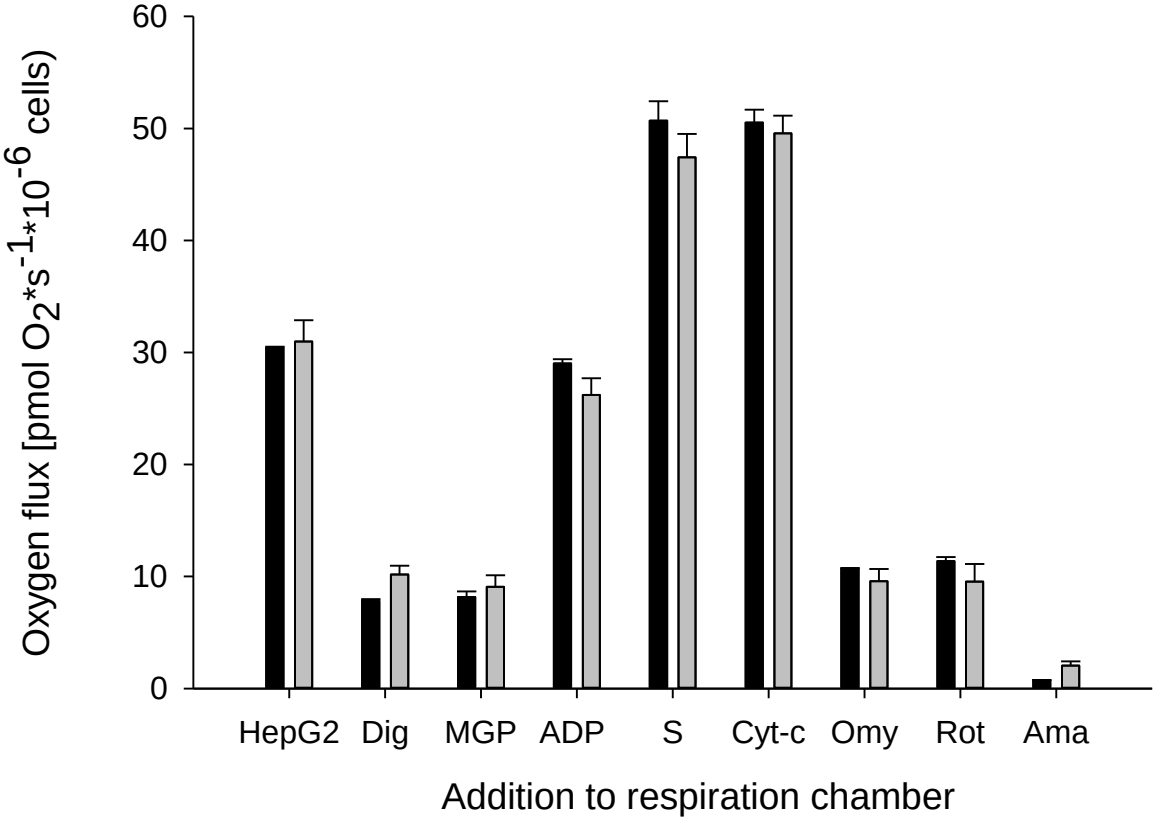

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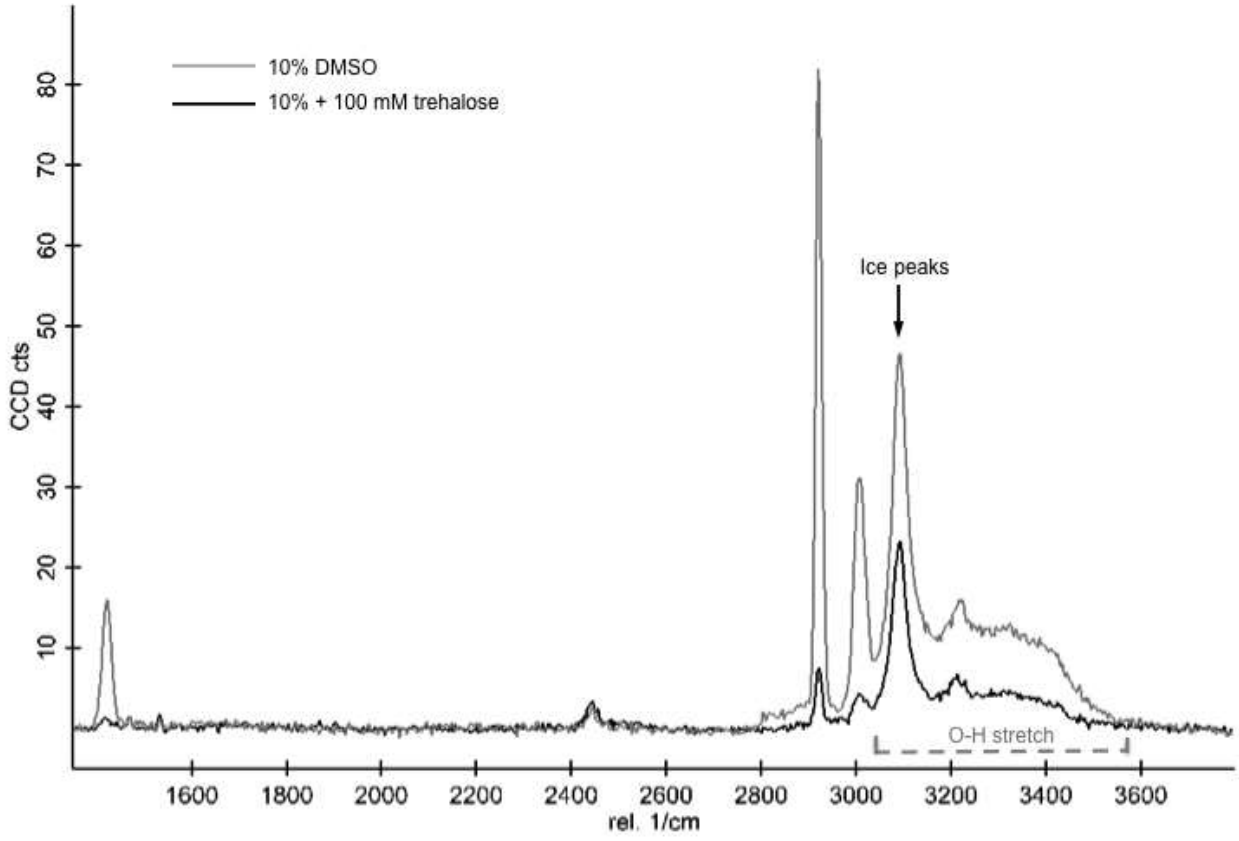

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