

University of Louisville

---

From the Selected Works of Michael A. Menze

---

August 12, 2014

## Cryopreservation of hepatocyte (HepG2) cell monolayers: Impact of trehalose

Blake Stokich, *Eastern Illinois University*

Quinn Osgood

David Grimm, *Eastern Illinois University*

Shhyam Moorthy

Nilay Chakraborty, et al.



Available at: [https://works.bepress.com/michael\\_menze/44/](https://works.bepress.com/michael_menze/44/)

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22

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

Blake Stokich<sup>1</sup>, Quinn Osgood<sup>2</sup>, David Grimm<sup>1</sup>, Shhyam Moorthy<sup>3§</sup>, Nilay Chakraborty<sup>2</sup> and  
Michael A. Menze<sup>1</sup>

<sup>1</sup>Department of Biological Science, Eastern Illinois University, Charleston, IL 61920

<sup>2</sup>Department of Mechanical Engineering, University of Michigan, Dearborn, MI 48128

<sup>3</sup>Department of Biological Sciences, Division of Cellular, Developmental and Integrative  
Biology, Louisiana State University, Baton Rouge, LA 70803

<sup>§</sup>Present address: MD Anderson Cancer Center, Cancer Biology Program, Graduate School of  
Biomedical Sciences (GSBS), The University of Texas Health Science Center at Houston

Address for correspondence: Michael A. Menze, Tel.: 217-581-6386; Fax: 217-581-7141; Email:  
[mmenze@eiu.edu](mailto:mmenze@eiu.edu); co-corresponding author: Nilay Chakraborty; Email: [nilay@umich.edu](mailto:nilay@umich.edu)

Keywords: adherent cell monolayer; osmotic stress; respiration (OXPHOS); DMSO

23 **ABSTRACT**

24 A simple method to cryogenically preserve hepatocyte monolayers is currently not available but  
25 such a technique would facilitate numerous applications in the field of biomedical engineering,  
26 cell line development, and drug screening. We investigated the effect of trehalose and dimethyl  
27 sulfoxide (Me2SO) in cryopreservation of human hepatocellular carcinoma (HepG2) cells in  
28 suspension and monolayer formats. HepG2 cell monolayers were incubated for 24 h at varying  
29 concentrations of trehalose (50-150 mM) prior to cryopreservation to identify the optimum  
30 concentration for such preincubation. When trehalose alone was used as the cryoprotective agent  
31 (CPA), cells in monolayer format did not survive freezing while cells in suspension  
32 demonstrated 14% viability 24 h after thawing. Only 6-13% of cells in monolayers survived  
33 freezing in cell culture medium supplemented with 10% Me2SO, but 42% of cells were  
34 recovered successfully if monolayers were preincubated with 100 mM trehalose prior to freezing  
35 in the Me2SO supplemented medium. Interestingly, for cells frozen in suspension in presence of  
36 10% Me2SO, metabolic activity immediately following thawing did not change appreciably  
37 compared to unfrozen control cells. Finally, Raman spectroscopy techniques were employed to  
38 evaluate ice crystallization in the presence and absence of trehalose in freezing solutions without  
39 cells because crystallization may alter the extent of injury observed in cell monolayers. We  
40 speculate that biomimetic approaches of using protective sugars to preserve cells in monolayer  
41 format will facilitate the development of techniques for long-term preservation of human tissues  
42 and organs in the future.

43

## 44 INTRODUCTION

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

67 CPAs play an important role in determining the outcome of the cryopreservation  
68 protocol. Along with traditional CPAs such as Me2SO, ethylene glycol, and glycerol, non-  
69 reducing sugars such as trehalose and sucrose have been used to improve cryopreservation [6,  
70 24, 26, 37]. Concerns about the toxicity of Me2SO at room temperature warrant the development  
71 of alternative CPAs that provide a lower toxicity to the cell [25]. Another commonly used CPA,  
72 glycerol, displays lower toxicity to cells than Me2SO, but unfortunately often requires more  
73 cumbersome multi-step loading protocols [33, 37]. Use of trehalose ( $\alpha$ -D-glucopyranosyl- $\alpha$ -D-  
74 glucopyranoside) as a CPA has been explored due to its involvement in ameliorating  
75 physiological stresses in several organisms in nature. Trehalose is involved in stress and cold-  
76 tolerance of several animals such as cysts of the brine shrimp *Artemia franciscana*, adults of the  
77 arctic springtail *Megaphorura arctica*, and the nematode *Aphelenchus avenae* [13, 17, 32].  
78 Unfortunately, in absence of a specific sugar transporter, trehalose is impermeable to the plasma  
79 membrane and only small amounts of the sugar are taken up by cells via endocytosis after  
80 extended incubation periods with the sugar [23, 43]. Nevertheless, the addition of trehalose to  
81 Me2SO based freezing solution was found to increase the viability of human embryonic stem  
82 cells and primary hepatocytes in suspension which are known to be challenging to cryopreserve  
83 [28, 57], and viable endothelial cells have been obtained after cryopreservation when cells were  
84 cultured for 24 h with 0.2 M trehalose prior to freezing [8].

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

## 90 MATERIAL AND METHODS

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

95 *Cell Culture.* Human hepatocellular carcinoma cells (HepG2) were obtained from the American  
96 Type Culture Collection (Manassas, VA) and grown in 75 cm<sup>2</sup> cell culture flasks (Corning  
97 Incorporated, Corning, NY). Standard cell culture medium was composed of Opti-MEM I  
98 reduced serum medium (Invitrogen, Carlsbad, CA) supplemented with 5.5% fetal bovine serum  
99 (FBS) purchased from Atlanta Biologicals Inc. (Flowery Branch, GA), 100 units/ml penicillin,  
100 100  $\mu$ g/ml streptomycin, and 250 ng/ml amphotericin B (MP Biomedicals, Santa Anna, CA).  
101 HepG2 cells were maintained in a humidified atmosphere of 6.5% CO<sub>2</sub> and 93.5% air at 37 °C  
102 and the culture medium was renewed every 3 - 4 days. The cells were subcultured every seven  
103 days or before reaching 90% confluency. To subculture cells were dissociated using 0.25%  
104 trypsin plus 1 mM EDTA in balanced salt solution (Invitrogen, Carlsbad, CA) and reseeded at  
105  $1.5 \cdot 10^6$  cells per 75 cm<sup>2</sup> cell culture flasks.

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

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

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

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

136 *Cryopreservation of HepG2 cell monolayers.* Cells to be frozen in the monolayer format were  
137 seeded at  $0.25 \cdot 10^6$  cells per well in 500  $\mu$ L of cell culture medium in 24-well plates (Corning  
138 Incorporated, Corning, NY) coated with collagen. To promote attachment of cells after thawing,  
139 collagen I from rat tail (Invitrogen, Carlsbad, CA) was diluted to 50  $\mu$ g/ml in 20 mM acetic acid  
140 (Fischer Scientific, Fair Lawn, NJ) and added to each well of the cell culture plate at 5  $\mu$ g  
141 collagen  $\cdot$  cm<sup>-2</sup>. Plates were incubated with the dissolved collagen for 1 h and after this  
142 incubation period the collage solution was removed and the plates were rinsed three times with  
143 200  $\mu$ L Dulbecco's phosphate buffered saline (Thermo Fisher Scientific Inc., Waltham, MA) to  
144 remove any residual acetic acid solution. The collagen treated plates were allowed to dry for one  
145 hour in a laminar flow hood and stored for less than one week at 4 °C prior to use. Before  
146 experimental treatments cells were allowed to attach for at least 1 h to the collagen coated plates  
147 in a humidified atmosphere of 6.5% CO<sub>2</sub> and 93.5% air at 37 °C. After the cells adhered firmly  
148 to the culture plates in some experiments the medium was exchanged against medium that was  
149 supplemented with trehalose. Control cells received no trehalose and experimental cells were  
150 incubated with concentrations ranging from 50 to 200 mM trehalose for 24 h. Following the  
151 incubation period the culture medium was removed and cells were exposed for five minutes to  
152 different formulations of solutions supplemented with CPAs as listed in Table 1. After 5 min the  
153 solutions were removed and the plates placed inside a cell freezing container (BioCision, LLC,  
154 Larkspur, CA) and transferred to -80 °C freezer. The freezing container includes a highly  
155 thermo-conductive stage (CoolCell® MP plate) placed in direct contact with the underside of the  
156 MTP that minimizes the temperature differential between wells and ensures a uniform rate of



157 thermal energy transfer of  $1\text{ }^{\circ}\text{C} \cdot \text{min}^{-1}$  during the freezing process. After 24 h at  $-80\text{ }^{\circ}\text{C}$  cells  
158 were rapidly thawed by addition of cell culture medium that was warmed to  $37\text{ }^{\circ}\text{C}$ .

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

179           The membrane integrity of adherent cells immediately after freezing and thawing was  
180 determined using a SYTO 13 and propidium iodine membrane-integrity assay which can be  
181 performed on attached cells (Molecular Probes, Eugene, OR). The stock solution was prepared  
182 by adding 10  $\mu\text{L}$  of a 1  $\text{mg} \cdot \text{mL}^{-1}$  SYTO 13 solution (aq.) and 5  $\mu\text{L}$  of a 1.0  $\text{mg} \cdot \text{mL}^{-1}$   
183 propidium iodine solution (aq.) to 10 mL of DMEM (Invitrogen Corporation, Carlsbad, CA). An  
184 aliquot of 130  $\mu\text{L}$  of this solution was then added to the attached cells and imaged using an  
185 inverted Zeiss microscope (Göttingen, Germany) equipped with dual fluorescence filter set  
186 (51004-V2, Chroma Technology, Brattleboro, VT). Images were processed using Image J  
187 processing software (National Institutes of Health, Bethesda, MD, USA). The percentage of cells  
188 with intact membranes (membrane integrity) was determined by counting live (green) and dead  
189 (red) cells on four representative images from the same sample.

190 *Respirometry on HepG2 cells.* Oxygen consumption of control HepG2 cells grown for a  
191 minimum of ten passages after cryopreservation and of cells directly after thawing was measured  
192 at 37 °C in 2 mL of MiR05 (110 mM sucrose, 60 mM potassium lactobionate, 20 mM taurine, 10  
193 mM  $\text{KH}_2\text{PO}_4$ , 3 mM  $\text{MgCl}_2$ , 0.5 mM EGTA, 0.1% BSA, 20 mM HEPES-KOH, pH 7.1) at a  
194 concentration of  $1 \cdot 10^6$  cells  $\cdot \text{mL}^{-1}$ . Cells suspended in 10% Me2SO + 5.5% FBS in Opti-MEM  
195 I were frozen to -80 °C, stored for 24 h, and rapidly thawed as described above. The freezing  
196 solution was removed after thawing by centrifugation of cells at 175 g for 5 min, and the cell  
197 pellet was suspended in standard culture medium and kept on ice until processed for  
198 respirometry. Oxygen flux of control cells was determined before freezing and without exposure  
199 to the Me2SO contained in the freezing solution. In order to supply mitochondrial substrates  
200 cells from both samples were permeabilized by addition of digitonin dissolved in Me2SO at 10  
201  $\text{mg} \cdot \text{mL}^{-1}$  (final concentration 10  $\mu\text{g} \cdot 10^{-6}$  cells). This digitonin concentration was found to be

202 sufficient to permeabilize the plasma membrane of HepG2 cells with low impact on the integrity  
203 of the outer mitochondrial membrane. Proton leak respiration was initiated by adding 2 mM  
204 malate, 10 mM glutamate and 5 mM pyruvate, which supplies electrons to the electron transport  
205 system (ETS) via production of NADH by mitochondrial dehydrogenases. To induce oxidative  
206 phosphorylation (OXPHOS) 1 mM ADP was added and convergent electron entry to the  
207 ubiquinone pool via NADH and FADH<sub>2</sub> was initiated by addition of 10 mM succinate. Potential  
208 damage to the outer mitochondrial membrane was investigated through addition of cytochrome *c*  
209 (10 μM). Leak respiration in presence of ADP and ATP was measured after addition of  
210 oligomycin (2 μg • mL)<sup>-1</sup>, and contribution of complex I to leak respiration was recorded after  
211 addition of rotenone (0.5 μM). Non-mitochondrial oxygen consumption was recorded after  
212 addition of 2.5 μM of antimycin A. Oxygen consumption was measured with an Oxygraph-2K  
213 (OROBOROS Instruments, Innsbruck, Austria) and DATLAB software (OROBOROS  
214 Instruments) was used for data analysis and acquisition. Oxygen flux (J<sub>O<sub>2</sub></sub>; pmol O<sub>2</sub> • s<sup>-1</sup> • 10<sup>-6</sup>  
215 cells) was calculated as the time derivative of oxygen concentration.

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

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

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

231

## 232 RESULTS

233 *Effect of trehalose on HepG2 cell proliferation and viability.* In order to evaluate the protective  
234 potential of trehalose in cryopreservation of HepG2 cells the impact of this sugar on cell viability  
235 and proliferation was investigated since increased extracellular osmolyte concentrations  
236 inevitably cause osmotic stress to cells. Compared to untreated controls the numbers of viable  
237 cells were decreased after 24 h of exposure to the sugar at all concentrations investigated ( $p <$   
238  $0.05$ ,  $n = 9$  experiments). Strikingly, no net increase in cell numbers occurred over 48 h at 150  
239 mM trehalose, the highest concentration investigated, and only  $1.06 \pm 0.03$  (SE)  $\cdot 10^6$  viable cells  
240 were recovered from  $1 \cdot 10^6$  cells plated (Fig. 1A). In addition to the acute effect of trehalose on  
241 cell proliferation (a hyperosmotic challenge) prolonged effects that persisted after the sugar was  
242 removed (a hypoosmotic challenge) were also observed. Cell numbers in control samples  
243 increased to  $144 \pm 4.2$  (SE) % of the original value during 24 h after plating, but in samples that  
244 were exposed to concentrations of 100 mM and 150 mM trehalose, prior to seeding, only about  
245 83% of cells were recovered (Fig. 1B,  $n = 9$ ) showing that osmotic challenges in either direction  
246 (hypoosmotic or hyperosmotic) causes reductions in cell viability.

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

255 thawing and plating of samples frozen in suspension (Fig. 2). Unfortunately, cell monolayers did  
256 not survive freezing in this solution (Table 1), and cell viability was not increased after  
257 incubation with 100 mM trehalose (data not shown). Three additional freezing solutions were  
258 investigated next and HepG2 monolayers were frozen in these solutions with a trehalose  
259 preincubation step. Under these conditions 10 % Me2SO provided the same protection in either  
260 90 % FBS or in cell culture medium (Table 1). Due to the fact that FBS is rather expensive we  
261 utilized culture medium supplemented with 10 % Me2SO and 5.5 % FBS in the subsequent  
262 experiments.

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

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

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

300           *Respiration of HepG2 cells cryopreserved in suspension format.* Based on our  
301 alamarBlue data we speculated that cellular dehydrogenases may be preserved during  
302 cryopreservation in presence of Me2SO and remain functional directly after thawing.  
303 Unfortunately, respiration measurements of cells frozen in the monolayer format were not  
304 possible since cells could not be separated from the collagen matrix directly after thawing (see  
305 Materials and Methods), and we decided to investigate the impact of freezing on cells in  
306 suspension on the activities of mitochondrial dehydrogenases and bioenergetically properties.  
307 After the addition of malate plus glutamate and pyruvate to never-frozen, permeabilized HepG2  
308 cells (supplying NADH to complex I) the oxygen flux was  $8.1 \pm 0.5$  (SE)  $\text{pmol O}_2 \cdot \text{sec}^{-1} \cdot 10^{-6}$   
309 cells ( $n = 4$ ). This value was indistinguishable from cells that were frozen in suspension format  
310 to  $-80$  °C in cell culture medium supplemented with 10 % Me2SO and assessed directly after  
311 thawing (Fig. 6). Both never-frozen control and cryopreserved cells showed an about 3.5-fold  
312 increase in oxygen flux after engaging the phosphorylation system by addition of 1 mM ADP  
313 and a further increase of about 80% was recorded after addition of succinate which supplies  
314 electrons to the mitochondrial ubiquinone pool via the bound flavin adenine dinucleotide (FAD)  
315 of the succinate dehydrogenase complex. Importantly, the response to cyt *c* addition was  
316 indistinguishable between control cells and cryopreserved cells, indicating a well preserved outer  
317 mitochondrial membrane immediately after cryopreservation. In summary, directly after thawing  
318 all investigated bioenergetics functions of mitochondria were unchanged from control cells (Fig.  
319 6). While we aimed to use alamarBlue and respiration assays as tools to quantify changes in  
320 cellular properties immediately after thawing no changes in cellular physiology for HepG2 cells  
321 could be detected with these two assays. In presence of 10% Me2SO cryopreservation outcomes  
322 apparently depend on events that are difficult to detect directly after thawing and the impact of



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

325 *Decrease in ice crystal formation in the presence of trehalose.* The behavior of water at low  
326 temperatures and the formation of ice crystals have profound effects on cryopreservation [5]. We  
327 speculated that the addition of trehalose to the Me2SO based freezing buffer may change the ice-  
328 formation characteristics of water, and Raman microspectroscopy is an excellent tool to  
329 characterize these changes from a molecular standpoint. Ice formation can be easily detected by  
330 observing Raman spectra of water at low temperatures [20]. The phenomenon of ice formation  
331 affects the O-H stretching modes of water (2900–3600  $\text{cm}^{-1}$  range), which indicates the state of  
332 intramolecular O-H pairs and hydrogen bonds. Based on the nature of hydrogen bonds, the O-H  
333 stretch region of the water spectrum consists of two principal regions known as symmetric and  
334 asymmetric stretches, with Gaussian shaped peaks around 3242  $\text{cm}^{-1}$  and 3451  $\text{cm}^{-1}$ , respectively  
335 [5]. The symmetric peak has been attributed to the O-H vibration in tetra-bounded water  
336 molecules that are under influence of fully developed hydrogen bonds, while the asymmetric  
337 peak correspond to the O-H vibrations of water molecules that have partially or incompletely  
338 developed bonds (hence free water) [9]. In order to understand the impact of trehalose on ice  
339 formation in our freezing buffer Raman microspectroscopy was performed without cells present.  
340 Onset of ice nucleation during freezing was indicated in by the appearance of a definitive ice  
341 peak at 3100  $\text{cm}^{-1}$  (Fig. 7). If 100 mM trehalose was added to the 10% Me2SO solution, we  
342 observed a significant decrease (>50%) in the intensity of the ice peak at -170  $^{\circ}\text{C}$  (Fig. 7). This  
343 signifies that presence of trehalose at cryogenic temperatures leads to a decrease in ice-crystal  
344 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 ( $3242\text{ cm}^{-1}$ ) and the asymmetric peaks ( $3451\text{ cm}^{-1}$ ) was observed in presence of trehalose.

## 347 **Discussion**

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

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

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

375 To exert maximal protective properties trehalose has to be present on both sides of the  
376 plasma membrane [24], but unfortunately the sugar is only readily permeable to cells that  
377 express a specific transporter in their plasma membrane [10; 29]. One technique to accumulate  
378 some levels of intracellular trehalose in mammalian cells is through endocytosis after adding  
379 trehalose to the culture medium for extended periods of time [8, 23], and we hypothesized that  
380 low levels of trehalose also accumulate intracellular in HepG2 cells after 24 h of exposure to the  
381 sugar as demonstrated for J774.Q1 mouse macrophages [23]. In order to successfully load  
382 intracellular trehalose as a cryoprotectant in biomedical applications the optimal concentration  
383 used during pre-incubations has to be carefully optimized since the sugar can cause acute and  
384 prolonged reductions in cell viability and proliferation (Fig. 1). We found that intracellular  
385 loaded trehalose in presence of 100 mM extracellular trehalose conferred some protection to  
386 cells frozen in solution but no viable cells were recovered if cells were frozen as monolayers  
387 (Fig. 2, Tab. 1). Constraints for cells frozen in solution are different from the physical stress  
388 cells experience when frozen as monolayers. Furthermore, the formation of intracellular ice may  
389 be less harmful to cells in monolayers than for isolated cells frozen in suspension has been  
390 demonstrated [1], and some suggest that IIF is preceded by damage to the plasma membrane and  
391 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  $1\text{ }^{\circ}\text{C} \cdot \text{min}^{-1}$   
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

415 movement of water across the plasma membrane which allows osmotic dehydration to reach  
416 equilibrium with intracellular and extracellular contents in order to prevent IIF [33, 47]. We  
417 found that at a freezing rate of  $1\text{ }^{\circ}\text{C} \cdot \text{min}^{-1}$  only about 10 % of cells frozen as monolayers could  
418 be recovered whereas recovery for cells in suspension at this freezing rate is commonly above  
419 90% (data not shown). Me2SO protects as penetrating CPA and protect cells during  
420 cryopreservation in part due to the ability to reduce the concentration of damaging solutes in the  
421 unfrozen water fraction during the freezing and thawing processes [35]. However, osmotic  
422 dehydration during freezing appears to be more detrimental for cells that are frozen as  
423 monolayers than in suspension. Cell monolayer are firmly attached to the extracellular matrix  
424 and before the eutectic temperature is reached less able to move into the remaining unfrozen  
425 water fraction compared with cells frozen in suspension [40]. The combination of intracellular  
426 trehalose and Me2SO might mitigate physical stresses and help to stabilize cellular membranes  
427 during freezing of cell monolayers as has been demonstrated for freezing of vesicles [45].  
428 Surprisingly, we found that cellular respiration and resazurin reduction was unchanged  
429 immediately after thawing of cells frozen in suspension or in the monolayer format, respectively  
430 (Figs. 3, 4). Larsen [30] observed that freezing of skeletal muscle tissue in presence of Me2SO  
431 negatively impacts respiration fueled by complex I. These observations indicate that different  
432 cell and tissue types respond differently to cryopreservation but that some cellular samples might  
433 be stored at  $-80\text{ }^{\circ}\text{C}$  prior to assessment of bioenergetics properties. We are currently investigating  
434 the optimal conditions and concentrations of trehalose used in preincubation to store isolated  
435 mitochondria, cells, and tissues at cryogenic temperatures prior to high-resolution respirometry.  
436 A comprehensive study of mitochondrial properties is a time consuming process and  
437 cryopreservation of samples prior to high-resolution respirometry would greatly simplify the

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

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

461 **Acknowledgments**

462 This research was funded by an Eastern Illinois University Council on Faculty research (CFR)  
463 Grant to M.A.M., and an Eastern Illinois University Honors College Undergraduate Research,  
464 Scholarship, and Creative Activities Grants (URSCA) to B.S., and a University of Michigan  
465 Rackham Replacement Research Grant to N.C. (U039417). Appreciation is extended to Prof.  
466 Steven Hand (Louisiana State University) for serving as the honors thesis advisor for S.M. and  
467 for his advice and suggestions on the manuscript. We also thank Dr. Chakraborty's graduate  
468 student Mian Wang for his help with Raman Spectroscopy.



469

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

472

---

<b>Composition of freezing solution</b>	<b>% Live Cell Recovery<sup>1</sup></b>
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*

---

473

474 <sup>1</sup>Live cell recovery is expressed in % of cells that were recovered after thawing followed by 24 h  
475 of standard cell culture conditions compared to numbers of cells before freezing. Only cells that  
476 excluded trypan blue were used to calculate the fraction of viable cells ( $n = 8-30, \pm SE$ ). Asterisks  
477 indicate no significant differences in recovery between cells frozen in 10% Me2SO in FBS, or  
478 10% Me2SO in cell culture medium ( $p = 0.297$ ).

479

480

481

482

483

484

485

486

487

488

489

490

491

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

495

<b>Trehalose [mM]</b>	<b>Buffer 1 (no trehalose)</b>	<b>Buffer 2 (100 mM trehalose)</b>
24 h preincubation	% Membrane integrity <sup>1</sup>	% Membrane integrity <sup>1</sup>
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

496

497 <sup>1</sup>Membrane integrity is expressed in % of cells staining positive for SYTO 13 and negative for  
 498 propidium iodine divided by the total number of cells. Monolayers were frozen in 10% Me2SO +  
 499 5.5% FBS in Opti-MEM I (Buffer 1) or 10% Me2SO + 5.5% FBS in Opti-MEM I + 100 mM  
 500 trehalose (Buffer 2). Data are based on enumerating cell numbers in random areas of thawed  
 501 monolayers ( $n = 4 \pm SE$ ). Independent from the concentration of trehalose during preincubation  
 502 the presence of trehalose in the freezing buffer significantly reduced the number of cells with  
 503 intact membranes after thawing ( $p < 0.001$ ). No significant differences in membrane integrity  
 504 were found among the trehalose concentrations used in the 24 h pre-incubation step ( $p = 0.093$ ).

505

506

507 **Figure Legends**

508 **Fig. 1: Impact of trehalose on cell proliferation.** A) Effect at the end of 24 h of trehalose  
509 exposure. A reduction in proliferation of human hepatocellular carcinoma cells (HepG2) is  
510 observed in culture media supplemented with trehalose. B) Effect 24 h after the removal of  
511 trehalose. HepG2 cells transiently treated with trehalose showed prolonged inhibitions in cell  
512 proliferation after removal of the sugar. \*Statistically significant different to 0 mM trehalose ( $n =$   
513  $9, \pm SE$ ). Significance level was set at  $p \leq 0.05$ .

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

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

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

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

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

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

546 **Fig. 7: Raman microspectroscopy on CPA solutions at -170 °C.** The presence of trehalose  
547 leads to a significant reduction in ice-crystal formation as indicated by a reduced ice peak at  
548  $3100 \text{ cm}^{-1}$ .

549 **References:**

- 550 [1] J.P. Acker, and L.E. McGann, Protective effect of intracellular ice during freezing?  
551 *Cryobiology* 46 (2003) 197-202.
- 552 [2] E. Alexandre, C. Viollon-Abadie, P. David, A. Gandillet, P. Coassolo, B. Heyd, G. Manton,  
553 P. Wolf, P. Bachellier, D. Jaeck, and L. Richert, Cryopreservation of adult human  
554 hepatocytes obtained from resected liver biopsies. *Cryobiology* 44 (2002) 103-13.
- 555 [3] F. Ali, and D.A. Wharton, Cold tolerance abilities of two entomopathogenic nematodes,  
556 *Steinernema feltiae* and *Heterorhabditis bacteriophora*. *Cryobiology* 66 (2013) 24-9.
- 557 [4] A.F. Beier, J.C. Schulz, D. Dorr, A. Katsen-Globa, A. Sachinidis, J. Hescheler, and H.  
558 Zimmermann, Effective surface-based cryopreservation of human embryonic stem cells  
559 by vitrification. *Cryobiology* 63 (2011) 175-85.
- 560 [5] C. Branca, S. Magazu, G. Maisano, and P. Migliardo, Anomalous cryoprotective  
561 effectiveness of trehalose: Raman scattering evidences. *J Chem Phys* 111 (1999) 281-  
562 287.
- 563 [6] K.G. Brockbank, L.H. Campbell, E.D. Greene, M.C. Brockbank, and J.G. Duman, Lessons  
564 from nature for preservation of mammalian cells, tissues, and organs. *In Vitro Cell Dev*  
565 *Biol Anim* 47 (2011) 210-7.
- 566 [7] M.B. Burg, J.D. Ferraris, and N.I. Dmitrieva, Cellular response to hyperosmotic stresses.  
567 *Physiol Rev* 87 (2007) 1441-74.
- 568 [8] L.H. Campbell, and K.G. Brockbank, Culturing with trehalose produces viable endothelial  
569 cells after cryopreservation. *Cryobiology* 64 (2012) 240-4.
- 570 [9] D.M. Carey, and G.M. Korenowski, Measurement of the Raman spectrum of liquid water. *J*  
571 *Chem Phys* 108 (1998) 2669-75.

- 572 [10] N. Chakraborty, M.A. Menze, J. Malsam, A. Aksan, S.C. Hand, and M. Toner,  
573 Cryopreservation of spin-dried mammalian cells. PLoS One 6 (2011) e24916.
- 574 [11] T. Chen, A. Fowler, and M. Toner, Literature review: supplemented phase diagram of the  
575 trehalose-water binary mixture. Cryobiology 40 (2000) 277-82.
- 576 [12] M.S. Clark, and M.R. Worland, How insects survive the cold: molecular mechanisms-a  
577 review. J Comp Physiol B 178 (2008) 917-33.
- 578 [13] M.S. Clark, M.A. Thorne, J. Purac, G. Burns, G. Hillyard, Z.D. Popovic, G. Grubor-Lajsic,  
579 and M.R. Worland, Surviving the cold: molecular analyses of insect cryoprotective  
580 dehydration in the Arctic springtail *Megaphorura arctica* (Tullberg). BMC Genomics 10  
581 (2009) 328.
- 582 [14] J.S. Clegg, P. Seitz, W. Seitz, and C.F. Hazlewood, Cellular responses to extreme water  
583 loss: the water-replacement hypothesis. Cryobiology 19 (1982) 306-16.
- 584 [15] J.H. Crowe, L.M. Crowe, and D. Chapman, Preservation of membranes in anhydrobiotic  
585 organisms: the role of trehalose. Science 223 (1984) 701-3.
- 586 [16] J.H. Crowe, F.A. Hoekstra, and L.M. Crowe, Anhydrobiosis. Annu Rev Physiol 54 (1992)  
587 579-99.
- 588 [17] J.H. Crowe, J.F. Carpenter, and L.M. Crowe, The role of vitrification in anhydrobiosis.  
589 Annu Rev Physiol 60 (1998) 73-103.
- 590 [18] J.H. Crowe, Trehalose as a "chemical chaperone": fact and fantasy. Adv Exp Med Biol 594  
591 (2007) 143-58.
- 592 [19] L.M. Crowe, J.H. Crowe, A. Rudolph, C. Womersley, and L. Appel, Preservation of freeze-  
593 dried liposomes by trehalose. Arch Biochem Biophys 242 (1985) 240-7.

- 594 [20] J. Dong, J. Malsam, J.C. Bischof, A. Hubel, and A. Aksan, Spatial distribution of the state  
595 of water in frozen mammalian cells. *Biophys J* 99 (2010) 2453-9.
- 596 [21] D. Doucet, V.K. Walker, and W. Qin, The bugs that came in from the cold: molecular  
597 adaptations to low temperatures in insects. *Cell Mol Life Sci* 66 (2009) 1404-18.
- 598 [22] S.L. Ebertz, and L.E. McGann, Cryoinjury in endothelial cell monolayers. *Cryobiology* 49  
599 (2004) 37-44.
- 600 [23] G.D. Elliott, X.H. Liu, J.L. Cusick, M. Menze, J. Vincent, T. Witt, S. Hand, and M. Toner,  
601 Trehalose uptake through P2X7 purinergic channels provides dehydration protection.  
602 *Cryobiology* 52 (2006) 114-27.
- 603 [24] A. Eroglu, M.J. Russo, R. Bieganski, A. Fowler, S. Cheley, H. Bayley, and M. Toner,  
604 Intracellular trehalose improves the survival of cryopreserved mammalian cells. *Nat*  
605 *Biotechnol* 18 (2000) 163-7.
- 606 [25] G.M. Fahy, Cryoprotectant toxicity neutralization. *Cryobiology* 60 (2010) S45-53.
- 607 [26] A. Fowler, and M. Toner, Cryo-injury and biopreservation. *Ann N Y Acad Sci* 1066 (2005)  
608 119-35.
- 609 [27] A. Fry, and A. Higgins, Measurement of Cryoprotectant Permeability in Adherent  
610 Endothelial Cells and Applications to Cryopreservation. *Cell Mol Bioengin* 5 (2012) 287-  
611 298.
- 612 [28] E. Katenz, F.W. Vondran, R. Schwartlander, G. Pless, X. Gong, X. Cheng, P. Neuhaus, and  
613 I.M. Sauer, Cryopreservation of primary human hepatocytes: the benefit of trehalose as  
614 an additional cryoprotective agent. *Liver Transpl* 13 (2007) 38-45.
- 615 [29] T. Kikawada, A. Saito, Y. Kanamori, Y. Nakahara, K. Iwata, D. Tanaka, M. Watanabe, and  
616 T. Okuda, Trehalose transporter 1, a facilitated and high-capacity trehalose transporter,

617 allows exogenous trehalose uptake into cells. Proc Natl Acad Sci U S A 104 (2007)  
618 11585-90.

619 [30] S. Larsen, C. Wright-Paradis, E. Gnaiger, J.W. Helge, and R. Boushel, Cryopreservation of  
620 human skeletal muscle impairs mitochondrial function. Cryo Letters 33 (2012) 170-6.

621 [31] W. Ma, T. O'Shaughnessy, and E. Chang, Cryopreservation of adherent neuronal networks.  
622 Neurosci Lett 403 (2006) 84-9.

623 [32] K.A.C. Madin, and J.H. Crowe, Anhydrobiosis in nematodes: Carbohydrate and lipid  
624 metabolism during dehydration. J Exp Zool 193 (1975) 335-42.

625 [33] P. Mazur, R.H. Miller, and S.P. Leibo, Survival of frozen-thawed bovine red cells as a  
626 function of the permeation of glycerol and sucrose. J Membr Biol 15 (1974) 137-58.

627 [34] P. Mazur, Freezing of living cells: mechanisms and implications. Am J Physiol 247 (1984)  
628 C125-42.

629 [35] P. Mazur, Principles of cryobiology. in: B.J. Fuller, N. Lane, and E.E. Benson, (Eds.), Life  
630 in the frozen state, CRC Press, Boca Raton, FL, 2004, pp. 3-65.

631 [36] G.C. McKay, C. Henderson, E. Goldie, G. Connel, C. Westmoreland, and M.H. Grant,  
632 Cryopreservation of rat hepatocyte monolayers: cell viability and cytochrome P450  
633 content in post-thaw cultures. Toxicol In Vitro 16 (2002) 71-9.

634 [37] H.T. Meryman, Cryopreservation of living cells: principles and practice. Transfusion 47  
635 (2007) 935-45.

636 [38] Y. Miyamoto, S. Enosawa, T. Takeuchi, and T. Takezawa, Cryopreservation in situ of cell  
637 monolayers on collagen vitrigel membrane culture substrata: ready-to-use preparation of  
638 primary hepatocytes and ES cells. Cell Transplant 18 (2009) 619-26.



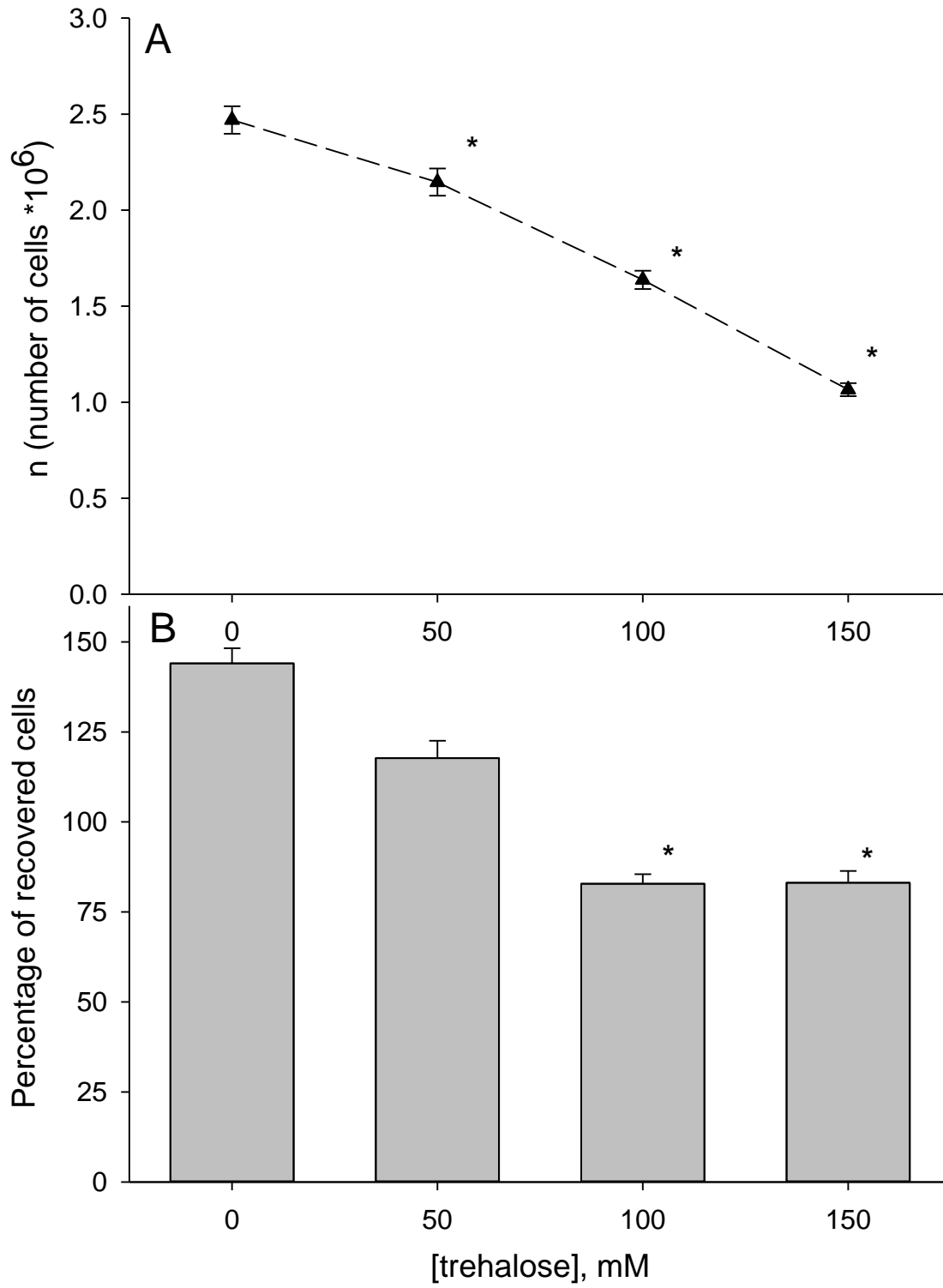
- 639 [39] K. Muldrew, and L.E. McGann, The osmotic rupture hypothesis of intracellular freezing  
640 injury. *Biophys J* 66 (1994) 532-41.
- 641 [40] T. Nei, Mechanism of freezing injury to erythrocytes: effect of initial cell concentration on  
642 the post-thaw hemolysis. *Cryobiology* 18 (1981) 229-37.
- 643 [41] J. O'Brien, I. Wilson, T. Orton, and F. Pognan, Investigation of the Alamar Blue (resazurin)  
644 fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem* 267  
645 (2000) 5421-6.
- 646 [42] S. Ohtake, and Y.J. Wang, Trehalose: current use and future applications. *J Pharm Sci* 100  
647 (2011) 2020-53.
- 648 [43] A.E. Oliver, K. Jamil, J.H. Crowe, and F. Tablin, Loading human mesenchymal stem cells  
649 with trehalose by fluid phase endocytosis. *Cell Preserv Technol* 2 (2004) 35-49.
- 650 [44] C. Pribenszky, G. Vajta, M. Molnar, Y. Du, L. Lin, L. Bolund, and J. Yovich, Stress for  
651 stress tolerance? A fundamentally new approach in mammalian embryology. *Biol Reprod*  
652 83 (2010) 690-7.
- 653 [45] A.S. Rudolph, and J.H. Crowe, Membrane stabilization during freezing: the role of two  
654 natural cryoprotectants, trehalose and proline. *Cryobiology* 22 (1985) 367-77.
- 655 [46] R.W. Salt, Intracellular freezing in insects. *Nature* 193 (1962) 1207-1208.
- 656 [47] G. Seth, Freezing mammalian cells for production of biopharmaceuticals. *Methods* 56  
657 (2012) 424-31.
- 658 [48] B.J. Sinclair, and D. Renault, Intracellular ice formation in insects: unresolved after 50  
659 years? *Comp Biochem Physiol A Mol Integr Physiol* 155 (2010) 14-8.
- 660 [49] J.H. Son, K.H. Kim, Y.K. Nam, J.K. Park, and S.K. Kim, Optimization of cryoprotectants  
661 for cryopreservation of rat hepatocyte. *Biotechnol Lett* 26 (2004) 829-33.

- 662 [50] D.J. Stevenson, C. Morgan, E. Goldie, G. Connel, and M.H. Grant, Cryopreservation of  
663 viable hepatocyte monolayers in cryoprotectant media with high serum content:  
664 metabolism of testosterone and kaempherol post-cryopreservation. *Cryobiology* 49  
665 (2004) 97-113.
- 666 [51] K.B. Storey, and J.M. Storey, Natural freeze tolerance in ectothermic vertebrates. *Annu Rev*  
667 *Physiol* 54 (1992) 619-37.
- 668 [52] C. Terry, A. Dhawan, R.R. Mitry, and R.D. Hughes, Cryopreservation of isolated human  
669 hepatocytes for transplantation: State of the art. *Cryobiology* 53 (2006) 149-59.
- 670 [53] S.N. Timasheff, The control of protein stability and association by weak interactions with  
671 water: how do solvents affect these processes? *Annu Rev Biophys Biomol Struct* 22  
672 (1993) 67-97.
- 673 [54] P. Watts, and M.H. Grant, Cryopreservation of rat hepatocyte monolayer cultures. *Hum Exp*  
674 *Toxicol* 15 (1996) 30-37.
- 675 [55] D. Wharton, and D. Ferns, Survival of intracellular freezing by the Antarctic nematode  
676 *Panagrolaimus davidi*. *J Exp Biol* 198 (1995) 1381-7.
- 677 [56] J. Wolfe, and G. Bryant, Freezing, drying, and/or vitrification of membrane- solute-water  
678 systems. *Cryobiology* 39 (1999) 103-29.
- 679 [57] C.F. Wu, H.C. Tsung, W.J. Zhang, Y. Wang, J.H. Lu, Z.Y. Tang, Y.P. Kuang, W. Jin, L.  
680 Cui, W. Liu, and Y.L. Cao, Improved cryopreservation of human embryonic stem cells  
681 with trehalose. *Reprod Biomed Online* 11 (2005) 733-9.
- 682 [58] G. Xie, and S.N. Timasheff, The thermodynamic mechanism of protein stabilization by  
683 trehalose. *Biophys Chem* 64 (1997) 25-43.

684 [59] P.H. Yancey, M.E. Clark, S.C. Hand, R.D. Bowlus, and G.N. Somero, Living with water  
685 stress: evolution of osmolyte systems. Science 217 (1982) 1214-22.

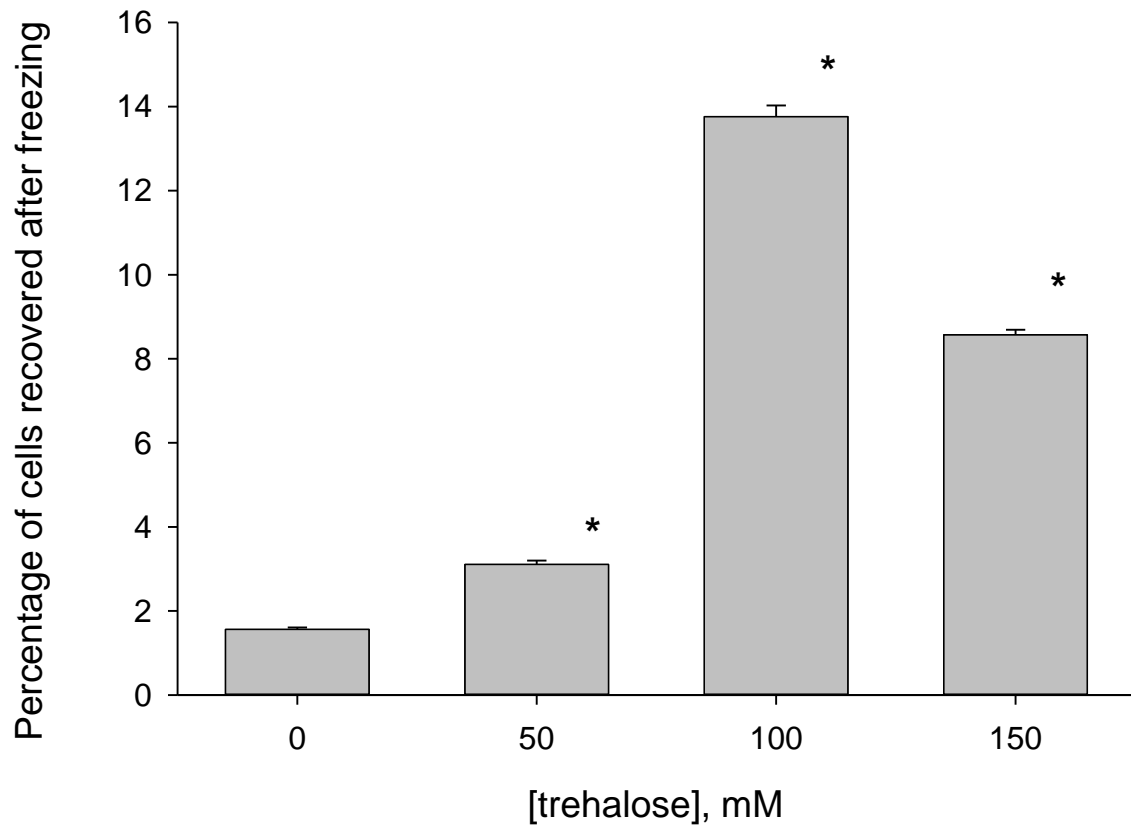
686

687



690

691 **Fig. 2**



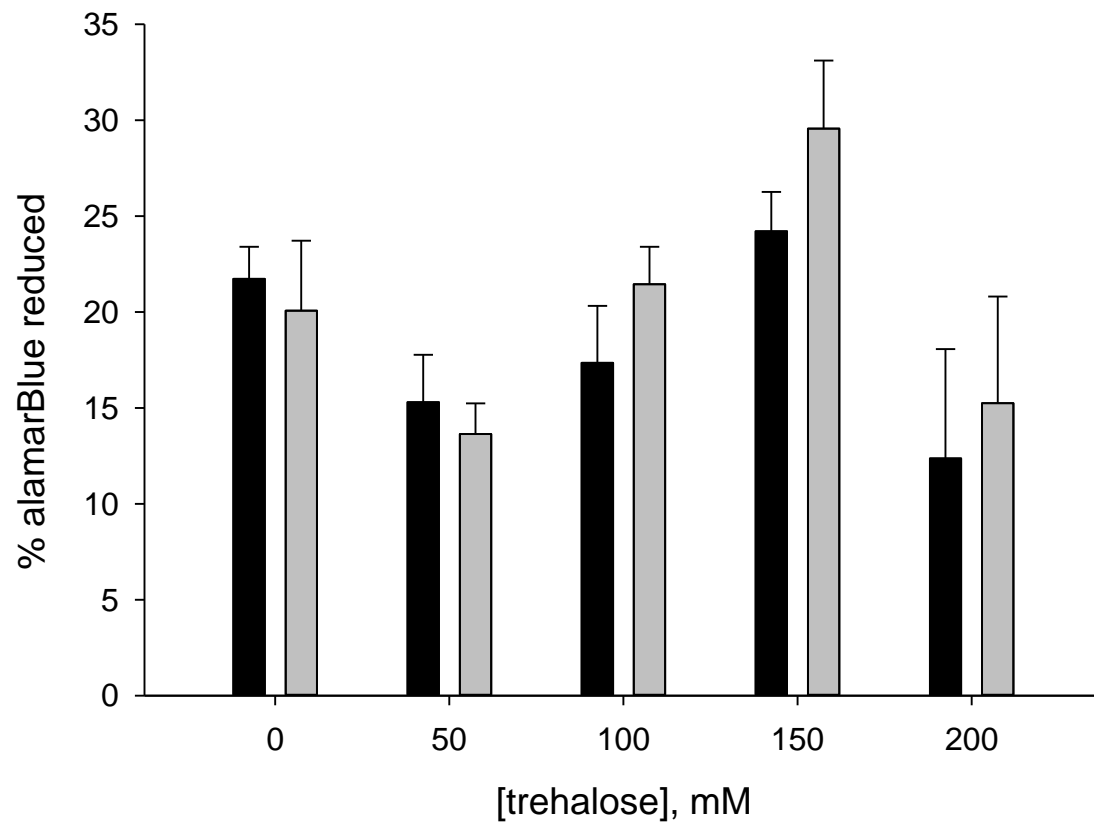
692

693

694

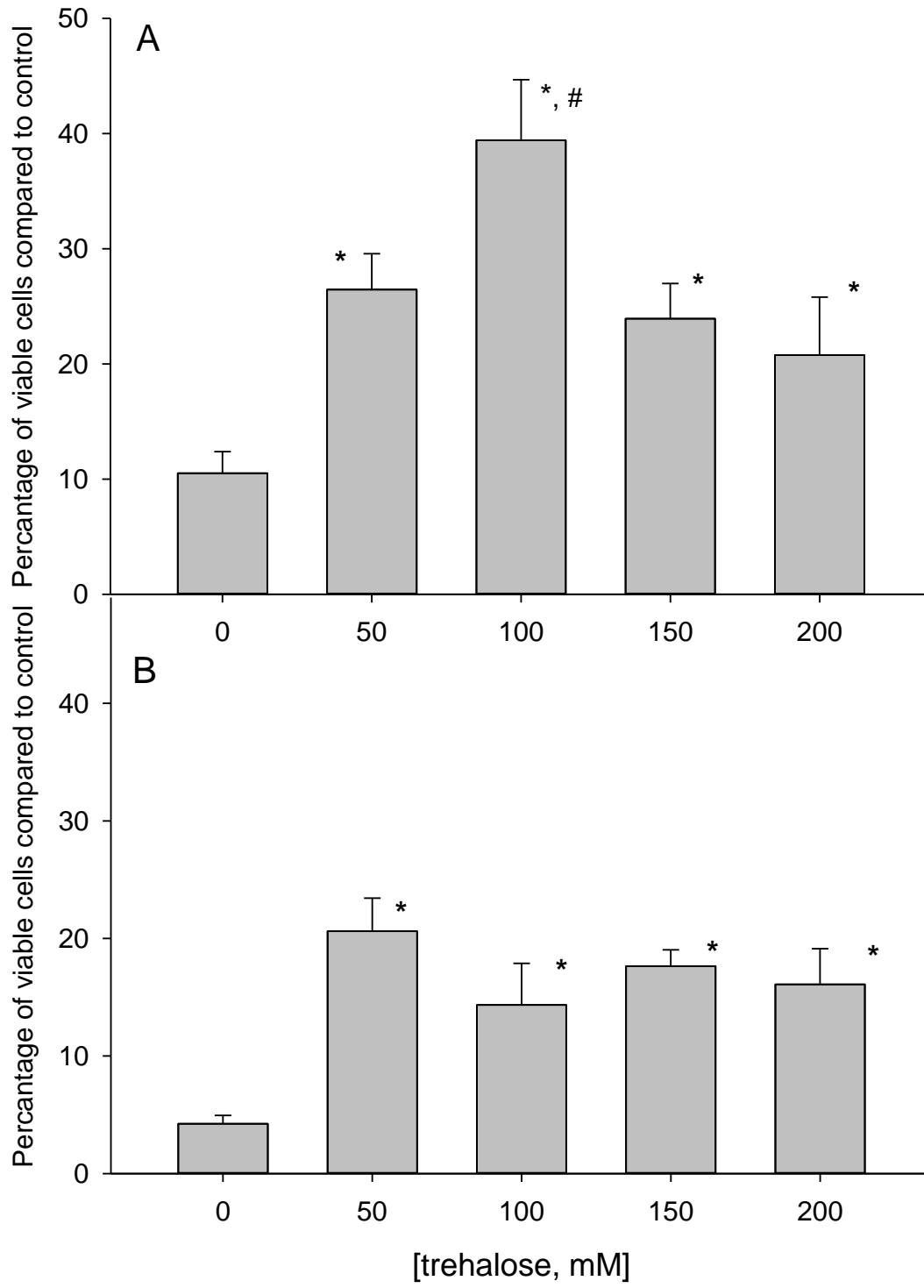
695 **Fig. 3**

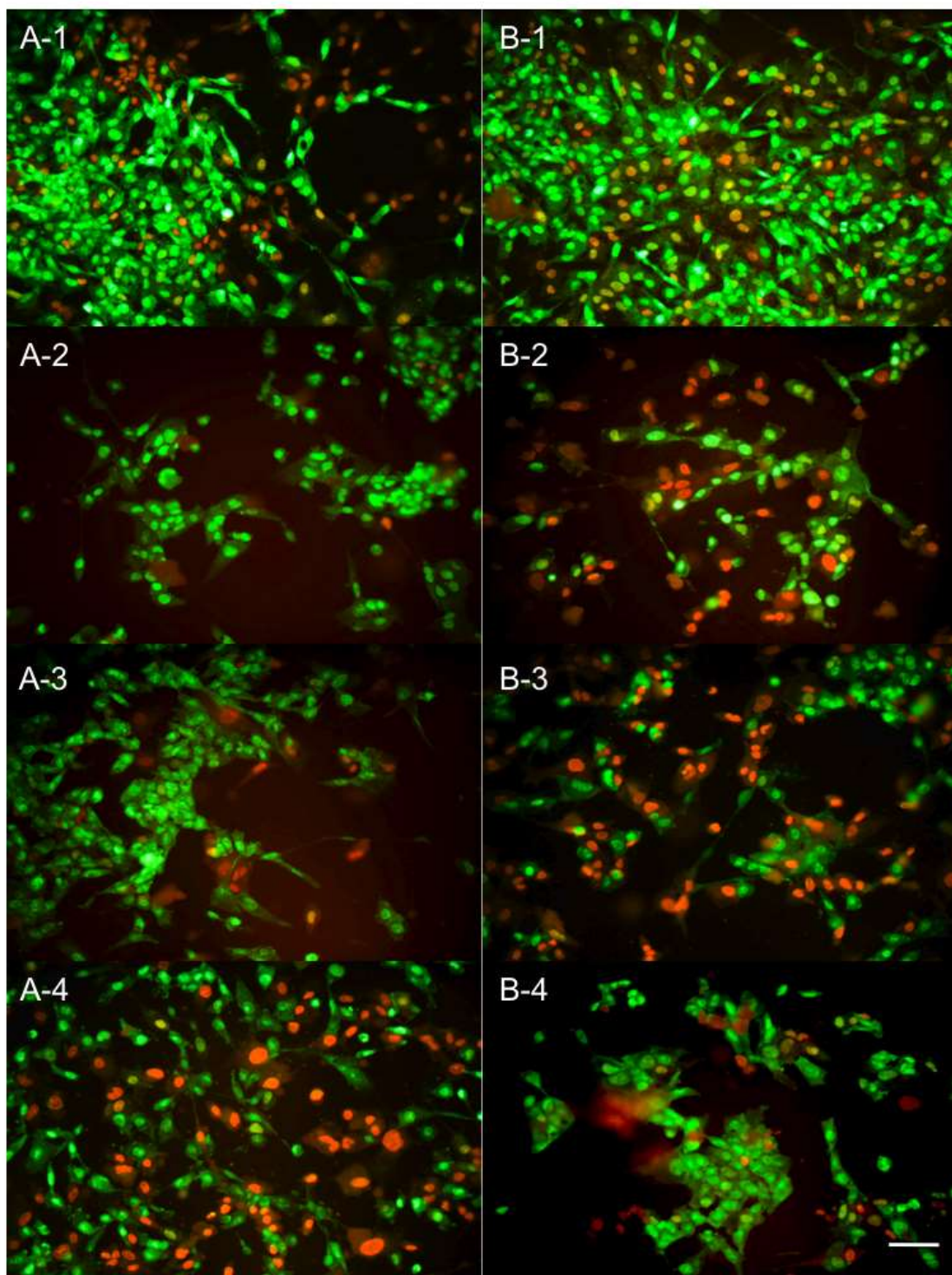
696



697

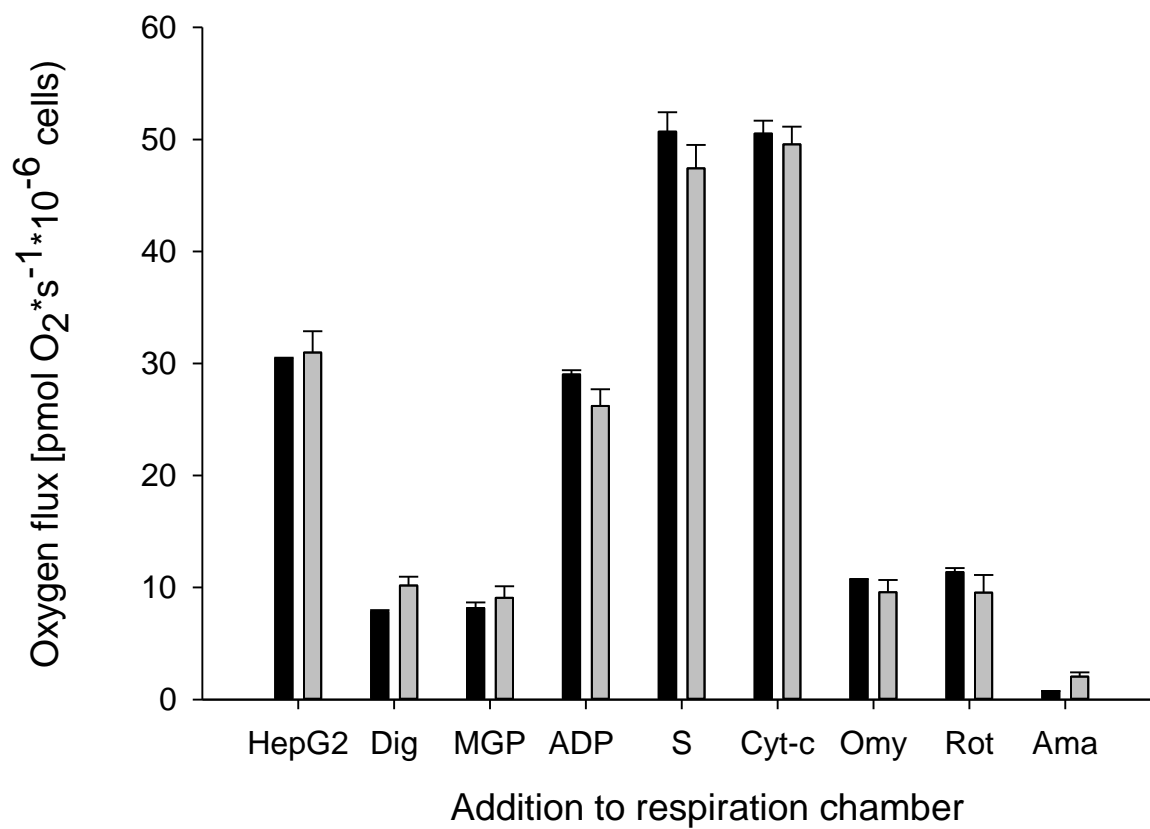
698







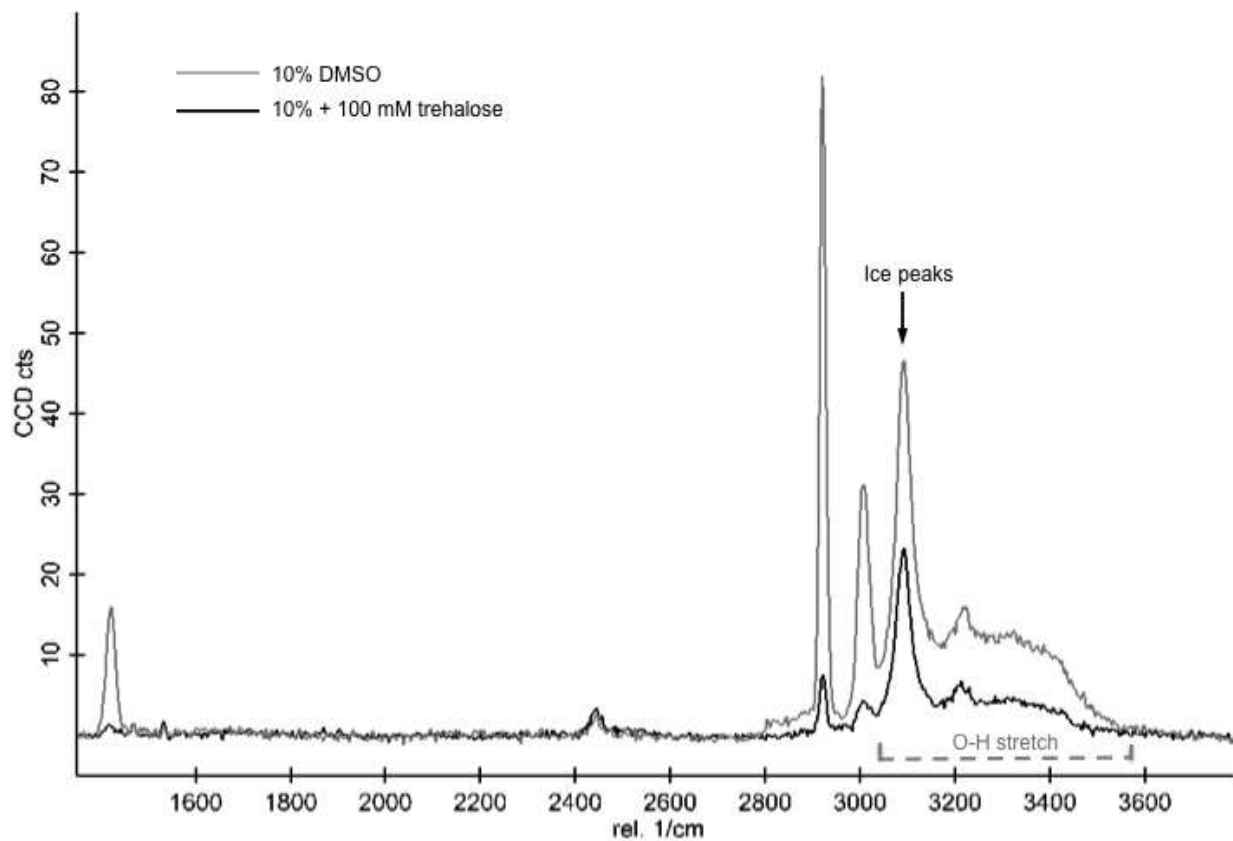
703 **Fig. 6**



704

705

706 **Fig. 7**



707

708

709

710