

# Nonmetabolizable Glucose Compounds Impart Cryotolerance to Primary Rat Hepatocytes

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## ABSTRACT

We herein report a novel method for the cryopreservation of hepatocytes using a non-metabolizable glucose derivative in an attempt to mimic the natural cryoprotective adaptations observed in freeze-tolerant frogs. Primary rat hepatocytes were loaded with 3-O-methyl glucose (3OMG) through endogenous glucose transporters without evident toxicity. The 3OMG-loaded hepatocytes were then frozen in a controlled rate freezer down to  $-80^{\circ}\text{C}$  and stored in liquid nitrogen at  $-196^{\circ}\text{C}$ . Hepatocytes cryopreserved with a relatively small amount of intracellular 3OMG ( $<0.2\text{ M}$ ) showed high post-thaw viability and maintained long-term hepatospecific functions, including synthesis, metabolism, and detoxification. Metabolite uptake and secretion rates were also largely preserved in the cryopreserved hepatocytes. This is the first study to demonstrate the use of the non-metabolizable glucose derivative 3OMG in hepatocyte cryopreservation.

## INTRODUCTION

PRESERVATION AND STORAGE OF LIVING MATERIALS are becoming increasingly important given recent developments in tissue engineering, cell transplantation, and stem cell biology. The preservation of primary hepatocytes is of particular importance given the major steps that have been taken recently in the development of cell-based treatments for liver diseases, including bioartificial liver devices, hepatocyte transplantation, and *ex vivo* gene therapy.<sup>1-4</sup> In order for these technologies to reach their full potential, isolated hepatocytes must be easily and effectively stored for on demand use. The challenging task of developing an appropriate long-term biostabilization option for primary hepatocytes still needs to be addressed.

Most traditional approaches of cryopreservation protocols rely on the addition of toxic, penetrating cryopro-

TECTIVE AGENTS (CPAs) such as dimethyl sulfoxide (DMSO) and glycerol.<sup>5</sup> Unfortunately, this approach is not satisfactory for several reasons. First, the high concentrations (1~2 M) of CPAs required are toxic.<sup>6</sup> Second, the use of CPAs involves cumbersome processing steps, such as multi-step addition and removal, in order to minimize toxicity and osmotic injuries.<sup>7,8</sup> Accordingly, there has been a pressing need for improved cryopreservation techniques using small amounts of non-toxic CPAs and simple single-step loading and removal procedures.

In freeze-tolerant wood frogs, freezing triggers the rapid breakdown of liver glycogen reserves to produce a large amount of glucose, which is then circulated to the whole body. The glucose concentration exceeds 200 mM in some tissues and helps preserve the structural integrity of the frog's cells and organs during freezing.<sup>9-11</sup> Based on this key element of cryoprotection in freeze-tolerant

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frogs, we hypothesized that glucose would be an effective CPA for mammalian cells. However, D-glucose is rapidly metabolized and thus escapes accumulation in sufficient quantities to afford protection. Furthermore, excessive exposure to glucose may cause metabolic derangements and lead to a loss of cellular viability and function.<sup>11</sup> One novel solution to these problems is the use of a non-metabolizable glucose derivative instead of metabolically active D-glucose.

Small carbohydrates have been reported to reduce the damage caused by freezing, freeze-drying, and desiccation in a number of biological systems.<sup>12,13</sup> They are believed to work by reducing the formation of lethal intracellular ice crystals and stabilizing cell membranes and proteins.<sup>14-16</sup> Trehalose and sucrose in particular have been shown to be excellent cryoprotectants for a number of mammalian cell types.<sup>13,17-19</sup> Unfortunately, permeabilization of the plasma membrane is needed for oligosaccharides, such as sucrose or trehalose, to reach both sides of membrane and afford full protection.<sup>13</sup>

Although there are several possible approaches for loading sugars into cells, such as the thermotropic lipid-phase transition,<sup>17</sup> genetic engineering,<sup>20</sup> and protein engineering,<sup>13</sup> all suffer from being invasive and cumbersome. Glucose analogues do not suffer from this problem because their uptake in mammalian cells is specifically facilitated by the GLUT family of glucose transporters.<sup>21</sup> Several known compounds mimic D-glucose and are efficiently transported by the GLUT family, but are not metabolizable by the cell. Two well-described examples are 3-O-methyl-glucose (3OMG)<sup>22</sup> and 2-deoxy-glucose (2DG).<sup>23</sup> Both 3OMG and 2DG enter the cell through GLUT family members and can accumulate in the cytoplasm at an appreciable level. Once inside, 3OMG is thought to be metabolically inert, while 2DG is phosphorylated by hexokinase (glucokinase in hepatocytes). The resulting 2DG-6-PO<sub>4</sub> inhibits hexokinase activity and may interfere with normal glucose metabolism.<sup>23</sup>

The present study was designed to evaluate the use of non-metabolizable glucose compounds as novel CPAs for hepatocytes. The findings of this study clearly demonstrate the protective effect of 3OMG in the cryopreservation of primary hepatocytes.

## MATERIALS AND METHODS

### *Cell culture*

Primary rat hepatocytes were isolated from female Lewis rats (Charles River Laboratories, Wilmington, MA) by a procedure previously described.<sup>24</sup> Typically, about  $2.0 \times 10^8$  cells were recovered from each liver. The average viability as judged by trypan blue exclusion was  $91.4 \pm 2.4\%$ . All animal procedures were performed

in accordance with National Research Council guidelines and approved by the subcommittee on Research Animal Care at the Massachusetts General Hospital. Hepatocyte culture conditions have been described elsewhere.<sup>6</sup>

### *Measurement of glucose uptake*

Isotonic uptake solutions containing 200 mM 3OMG (3-O-methyl-glucose, Sigma, St. Louis, MO) were prepared by adding dry 3OMG to one part D-glucose-free Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD) and two parts distilled water, for a total osmolality of 310 mOsm/kg. Isolated hepatocytes were pelleted by centrifugation at 250xg for 5 min and the supernatant was decanted. Uptake was initiated by adding warm uptake solution with 10  $\mu$ Ci/mL (0.16  $\mu$ M) 3-O-methyl-<sup>3</sup>H-D-glucose (Perkin Elmer, Boston, MA) to obtain  $2 \times 10^6$  cell/mL. Cells were incubated at 37°C and samples were taken at 1, 5, 15, 30, and 60 min. At 60 min, the remaining cells were collected by centrifugation at 250xg for 5 min, supernatant decanted, and resuspended with warm D-glucose-free DMEM to wash out intracellular 3OMG. Washed cells were incubated at 37°C and samples were taken at 1, 5, 15, and 30 min. The uptake and efflux were terminated by dilution with a 20-fold excess of cold PBS supplemented with 100  $\mu$ M phloretin (Sigma) to block transport.<sup>25</sup> Cells were immediately collected on a wet membrane filter (1.2  $\mu$ m pore, Millipore, Billerica, MA) and washed with 20 mL of the above cold-blocking solution. Cell-associated radioactivity was assessed in 7 mL of Ultima Gold LSC-cocktail (Packard BioScience, Meriden, CT) using a Beckman LS 6000IC Scintillation Counter (Beckman Coulter, Fullerton, CA). Total protein was determined using a micro protein determination kit (Sigma).

### *Viability assay*

Cells were incubated with isotonic D-glucose-free DMEM supplemented with 200 mM D-glucose, 200 mM 3OMG, or 200 mM 2DG (Sigma) for 60 min at 37°C to load the sugars. Viability after incubation was determined using a Live/Dead Viability/Cytotoxicity kit (Molecular Probes, Eugene, OR). Cells were collected by centrifugation, resuspended in PBS containing 0.8  $\mu$ M calcein AM and 2  $\mu$ M ethidium homodimer-1, and incubated for 15 min at ambient temperature. Viable cells were quantified using a Beckton-Dickinson FACSCalibur flow cytometer (San Jose, CA) as described elsewhere.<sup>13</sup> The viability was shown as percentage of glucose-free control.

### *MTT assay*

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium 6 bromide) assays were performed using an MTT Cell Proliferation Assay kit (American Type Culture Collection, Manassas, VA). Hepatocytes were seeded on col-

lagen-coated, 96-well culture plates with 100  $\mu\text{L}$  isotonic D-glucose-free DMEM medium supplemented with 200 mM 3OMG or 2DG for 60 min at 37°C. Cells incubated with D-glucose-free DMEM without supplement were used as control. At the end of each treatment, 10  $\mu\text{L}$  of MTT solution (5 mg/mL) were added and the cells incubated for 2 h at 37°C. Detergent solution (100  $\mu\text{L}$ ) was then added, the samples were incubated overnight at 25°C, and the absorption at 570 nm was measured in a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA).

### *Cryopreservation and thawing*

Isolated hepatocytes were incubated with isotonic D-glucose-free DMEM with 200 mM 3OMG, 2DG, sucrose, or D-glucose for 60 min at 37°C, as described above. Cells incubated with D-glucose free DMEM without supplement were used as control. Following incubation, cells were pelleted by centrifugation for 5 min, supernatant decanted, and resuspended in cold HypoThermosol solution (HTS) (Biolife Solutions, Binghamton, NY) with 200 mM 3OMG, 2DG, sucrose, or D-glucose ( $1 \times 10^6$  cell/mL). HTS without sugar supplement was used for control samples. Cell suspensions were transferred to 1 mL cryogenic vials (Nalge Company, Rochester, NY) and placed in a controlled-rate freezer (KRYO 10, Planer, Middlesex, United Kingdom). Samples were then cooled at  $-1^\circ\text{C}/\text{min}$  to  $-6^\circ\text{C}$ , at which temperature the vials were seeded to induce the formation of extracellular ice by application of cold forceps to the exterior of the cryovials. This step was followed by a 10 min holding period. Samples were then cooled at  $-1^\circ\text{C}/\text{min}$  to  $-80^\circ\text{C}$  and transferred to liquid nitrogen ( $-196^\circ\text{C}$ ) for storage lasting 1 to 7 days. Following storage, samples were rapidly thawed in a 37°C water bath for 2 min with gentle agitation. The samples were then diluted 1:10 in D-glucose-free DMEM and incubated for 10 min at 37°C to wash out the loaded sugar compounds. Samples were then centrifuged, supernatant decanted, and resuspended in culture medium. The viability of the cryopreserved cells was determined immediately after thawing using a trypan blue exclusion assay and expressed as a percent of the unfrozen control otherwise treated identically.

### *Fluorescence staining of actin filament*

Hepatocytes cultured in a collagen sandwich configuration were fixed using 4% paraformaldehyde (PFA) for 30 min, followed by permeabilization for 5 min in 0.1% Triton X-100 (Sigma). Cells were stained for 30 min with 3 mM rhodamine phalloidin (Molecular Probes) in PBS with 1% bovine serum albumin (BSA, Sigma). Following incubation, the samples were visualized with a Zeiss Axiovert 200 inverted microscope equipped with Cy 3.5 filter sets (Carl Zeiss, München-Hallbergmoos, Ger-

many), and images were captured with the AxioVision 4.0 software suite (Carl Zeiss).

### *Functional assays of hepatocytes after cryopreservation*

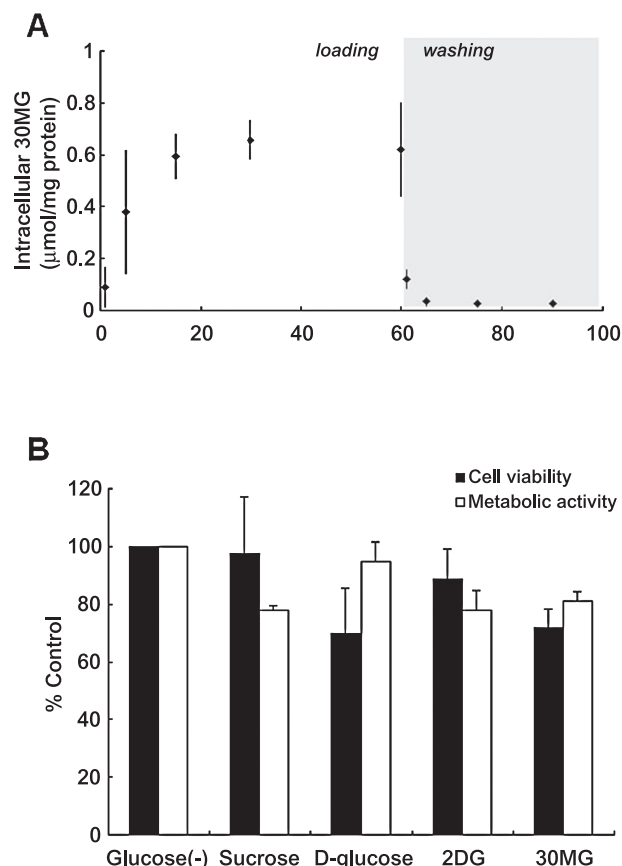
Immediately after thawing, hepatocytes (prefrozen cell number:  $2 \times 10^6/\text{dish}$ ) were seeded into p35 dishes and cultured in a collagen sandwich configuration described elsewhere.<sup>24</sup> Culture medium was changed daily for 14 days, and the collected supernatants were saved for albumin, urea, and metabolite assays. Albumin concentration was analyzed by enzyme-linked immunosorbent assay (ELISA) as previously described.<sup>24</sup> Urea concentration was determined via reaction with diacetyl monoxime using a standard blood urea nitrogen assay kit (Sigma). 3-Methylcholanthrene (3-MC, Sigma) induced CYP activities were assessed based on the time-dependent formation of resorufin from ethoxyresorufin due to isoenzyme P4501A1 activity (EROD assay), as described elsewhere.<sup>6</sup> 3-MC induced hepatocyte cultures received 2 mL of medium containing 2  $\mu\text{M}$  of 3-MC 48 h prior to the assay on days 3 and 7. Rate of resorufin formation, as calculated from the early linear increase in the fluorescence curve (resorufin vs. time), was defined as CYP activity and expressed as nmol/min. The DNA content of each dish was determined<sup>24</sup> at the end of the culture period and concentration values were calculated per  $\mu\text{g}$  DNA to normalize them to the number of viable hepatocytes.

### *Metabolite uptake and secretion*

Uptake and secretion rates were determined for a number of metabolites during the first week of culture after freezing. These included glycerol, triglycerides, glucose, lactate, ornithine, and nineteen of the proteinogenic amino acids (all but tryptophan). Metabolite concentrations were measured in the collected supernatants of fresh and 3OMG-frozen cells, as well as in matched cell-free controls. Daily uptake and secretion rates were then calculated by subtracting the concentrations in the control samples from those in the cell-exposed samples. Amino acids were fluorescently labeled using the AccQ-Tag derivatization system (Waters Co., Milford, MA) and quantified by high-performance liquid chromatography (Waters Model 2695 with Model 474 detector) as described elsewhere.<sup>26</sup> Glycerol, triglyceride, glucose, and lactate concentrations were measured using commercially available kits (Sigma). The DNA content measured at the end of the culture period was used to normalize the uptake and secretion rates for each dish.

### *Statistics and data analyses*

Each experiment was performed at least three times in triplicate. Data are expressed as means  $\pm$  standard errors.



**FIG. 1.** (A) Kinetics of 3OMG uptake and efflux on hepatocytes. Hepatocytes were incubated with 200 mM 3OMG for 60 min and then washed with sugar-free medium for 30 min. Samples were collected at different time points. The amount of intracellular 3OMG was normalized to total protein amount. Values are the means  $\pm$  se for at least five replicates. (B) Cell viability (black bar) and metabolic activity (white bar) of hepatocytes after incubation with various sugars for 60 min. Cells incubated in sugar-free medium were used as control, and the values were shown as the means  $\pm$  se percentage of the controls ( $n = 9$ ).

Statistical significance was calculated using a two-tailed student's  $t$  test for paired data and analysis of variance (ANOVA) as applicable. The threshold for statistical significance was considered  $p < 0.05$ .

## RESULTS

### *Uptake of 3OMG*

The amount of intracellular 3OMG loading by hepatocytes was examined using tritium-labeled 3OMG. A significant amount of 3OMG was taken rapidly into the cells, and a plateau of 62  $\mu\text{mol/mg}$  total protein was reached within approximately 30 min (Fig. 1A). By incubation in glucose-free medium, intracellular 3OMG was washed out within approximately 10 min to nearly 0  $\mu\text{mol/mg}$  total protein (Fig. 1A). The intracellular 3OMG concentration was estimated from the cell number and the mean osmotically active isotonic volume, assuming a uniform internal distribution of 3OMG.<sup>27,28</sup> The osmotically active isotonic volume is a theoretical value representing the volume of water that can be removed from a cell if it is placed in an infinitely concentrated solution.<sup>29</sup> The calculated concentration of intracellular 3OMG after 60 min of loading was  $165.0 \pm 34.1$  mM (Table 1), which roughly corresponded to the concentration of 3OMG in the extracellular solution (200 mM).

### *Cellular viability and metabolic activity after sugar loading*

Cellular viability of hepatocytes after sugar loading was examined in order to evaluate possible toxicity. The viability showed no significant differences between cells incubated with sucrose, D-glucose, 2DG, and 3OMG (Fig. 1B). The MTT assay, a colorimetric analysis based on the activity of mitochondrial dehydrogenase, was also performed on cells incubated with the various sugars. Equal numbers of the cells were used for the MTT assay, so the assay was considered to reflect metabolic activity as well as cellular viability. The metabolic activity of the hepatocytes was approximately 80-90% of no-sugar control after incubation with sucrose, D-glucose, 2DG, and 3OMG, indicating that the sugar manipulations were minimally toxic to primary hepatocytes (Fig. 1B).

### *Effects of glucose compounds on viability and attachment of cryopreserved hepatocytes*

To evaluate the beneficial effects of non-metabolizable glucose compounds during cryopreservation, we measured the post-thaw viability of sugar-loaded hepatocytes

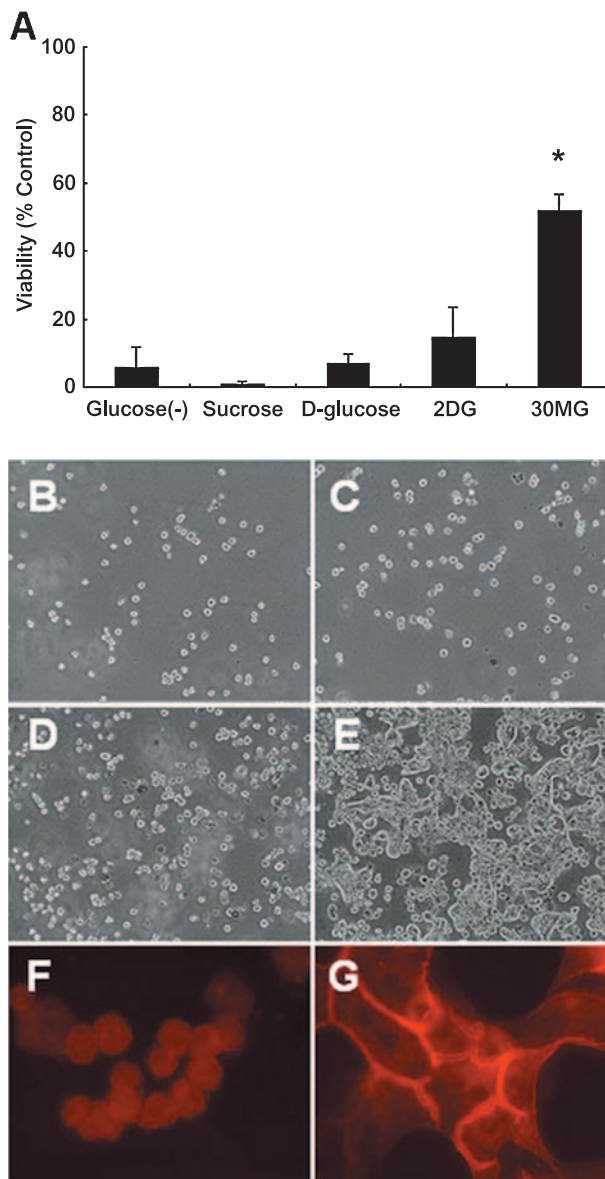
**TABLE 1. INTRACELLULAR CONCENTRATION OF 3OMG IN HEPATOCYTES AFTER LOADING WITH 200 mM 3OMG**

	Loading (60 min)	Washing (30 min)
Measured sugar ( $\mu\text{mol/mg protein}$ )	$0.62 \pm 0.13$	$0.02 \pm 0.01$
Calculated concentration (mM)	$165.0 \pm 34.1$	$6.6 \pm 2.7$

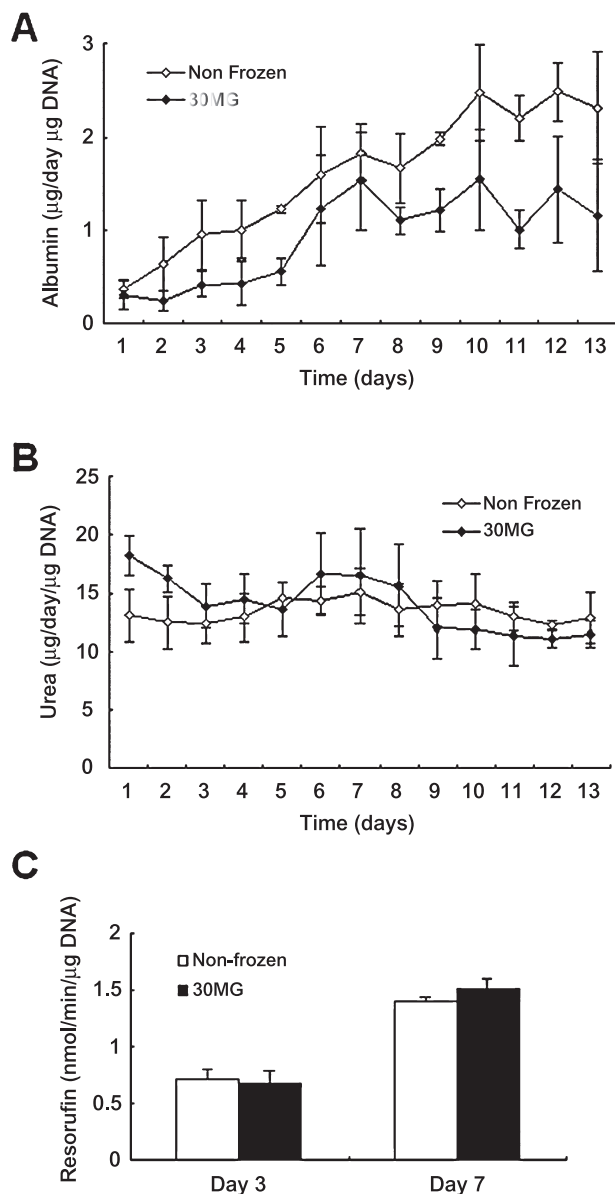
as compared to respective non-frozen samples. Controls cells were incubated in glucose-free DMEM (no-sugar control), sucrose (non-permeable/non-intracellular control), and D-glucose (permeable but metabolizable control). The post-thaw viability of all controls was extremely low (<10%). The 2DG-loaded hepatocytes showed somewhat greater viability (15%). On the other hand, 3OMG-loaded cells showed by far the best viability (>50%) among all groups, with statistically significant differences ( $p < 0.01$ ) (Fig. 2A). To examine the attachment efficiency, frozen-thawed hepatocytes were seeded on a collagen gel. No-sugar control, sucrose-loaded, and D-glucose-loaded hepatocytes rarely attached, remained spherical, and eventually died (Fig. 2B–D), whereas 3OMG-loaded primary hepatocytes attached, spread, and adopted the typical cuboidal shape of normal fresh hepatocytes (Fig. 2E). Moreover, hepatocytes cryopreserved without sugar completely lost their cellular polarity and cytoskeletal organization (Fig. 2F), while 3OMG-loaded hepatocytes showed normal localization of actin filaments (F-actin) at lateral intercellular contacts and the apical canalicular membrane (Fig. 2G).

#### Long-term function of hepatocytes after cryopreservation

To demonstrate that cryopreserved hepatocytes retain their function, we seeded frozen-thawed hepatocytes in a collagen sandwich culture system and measured hepatospecific functions for 14 days. The sandwich culture is a long-term culture technique that results in stable and differentiated hepatocytes.<sup>30,31</sup> We evaluated the albumin synthesis, urea production, and cytochrome P450 (CYP) activity of frozen-thawed hepatocytes as markers of synthetic, metabolic, and detoxification abilities in the hepatocytes. Albumin production of 3OMG-loaded and cryopreserved hepatocytes stabilized following 7 days in culture (Fig. 3A). The average daily albumin production (days 7–13) from 3OMG-loaded hepatocytes was approximately 60% of non-frozen control ( $1.29 \pm 0.19 \mu\text{g}$  and  $2.14 \pm 0.26 \mu\text{g}$ , respectively), a statistically significant difference ( $p = 0.014$ ). Urea synthesis of 3OMG-loaded and cryopreserved hepatocytes was comparable to those of non-frozen control hepatocytes (daily avg.  $12.85 \pm 1.84 \mu\text{g}$  and  $13.58 \pm 0.74 \mu\text{g}$ , respectively) (Fig. 3B), and statistical analysis revealed no significant difference ( $p = 0.51$ ). The CYP activity was measured on days 3 and 7 after thawing. The activities of 3OMG-loaded and cryopreserved hepatocytes were equivalent to non-frozen control without statistically significant difference ( $p > 0.3$ ) (Fig. 3C). No-sugar control, sucrose-loaded, and D-glucose-loaded hepatocytes completely lost these functions in 5 days, and all values were under detectable ranges.



**FIG. 2.** (A) Post-thaw viability of cryopreserved hepatocytes. The protective abilities of 3OMG, 2DG, sucrose, and D-glucose were evaluated by the viability of frozen-thawed hepatocytes loaded with various sugars. The values were shown as the means  $\pm$  se percentage of the non-frozen controls for at least six replicates. The viability of 3OMG-loaded hepatocytes was higher than that of each of the other groups ( $*p < 0.01$ ). (B–E) Typical phase-contrast images of cryopreserved hepatocytes at 48 h after thawing. Cells were seeded and cultured in a collagen sandwich culture. No-glucose control (A), sucrose-loaded (B), and D-glucose-loaded (C) hepatocytes remained in spheroid shape, while 3OMG-loaded cells (D) attached and were well spread (original magnification  $\times 100$ ). (F and G) Rhodamine phalloidin staining of cryopreserved hepatocytes (original magnification  $\times 400$ ). (F) No-glucose control hepatocytes completely lost polarity and structure. (G) Actin filaments (F-actin) were found at their normal sites at both the lateral intercellular contacts and the apical canalicular membrane in 3OMG-loaded hepatocytes. (Color images available online at <[www.liebertpub.com/ten](http://www.liebertpub.com/ten)>.)



**FIG. 3.** Albumin (A) and urea (B) production by frozen-thawed 3OMG-loaded hepatocytes (closed circles) and non-frozen control hepatocytes (open circles). Cells were cultured in a collagen sandwich for 14 days, and media collected daily were analyzed for albumin and urea. 3OMG-loaded hepatocytes maintained high synthetic functions comparable to non-frozen control. All values were normalized by viable cell number (DNA content) and shown as the mean  $\pm$  se ( $n = 9$ ). (C) Cytochrome P450 activity of 3OMG-loaded hepatocytes (black bar) and non-frozen control hepatocytes (white bar) on days 3 and 7 ( $n = 9$ ). 3OMG-loaded and cryopreserved hepatocytes retained comparable detoxification activity to non-frozen control hepatocytes.

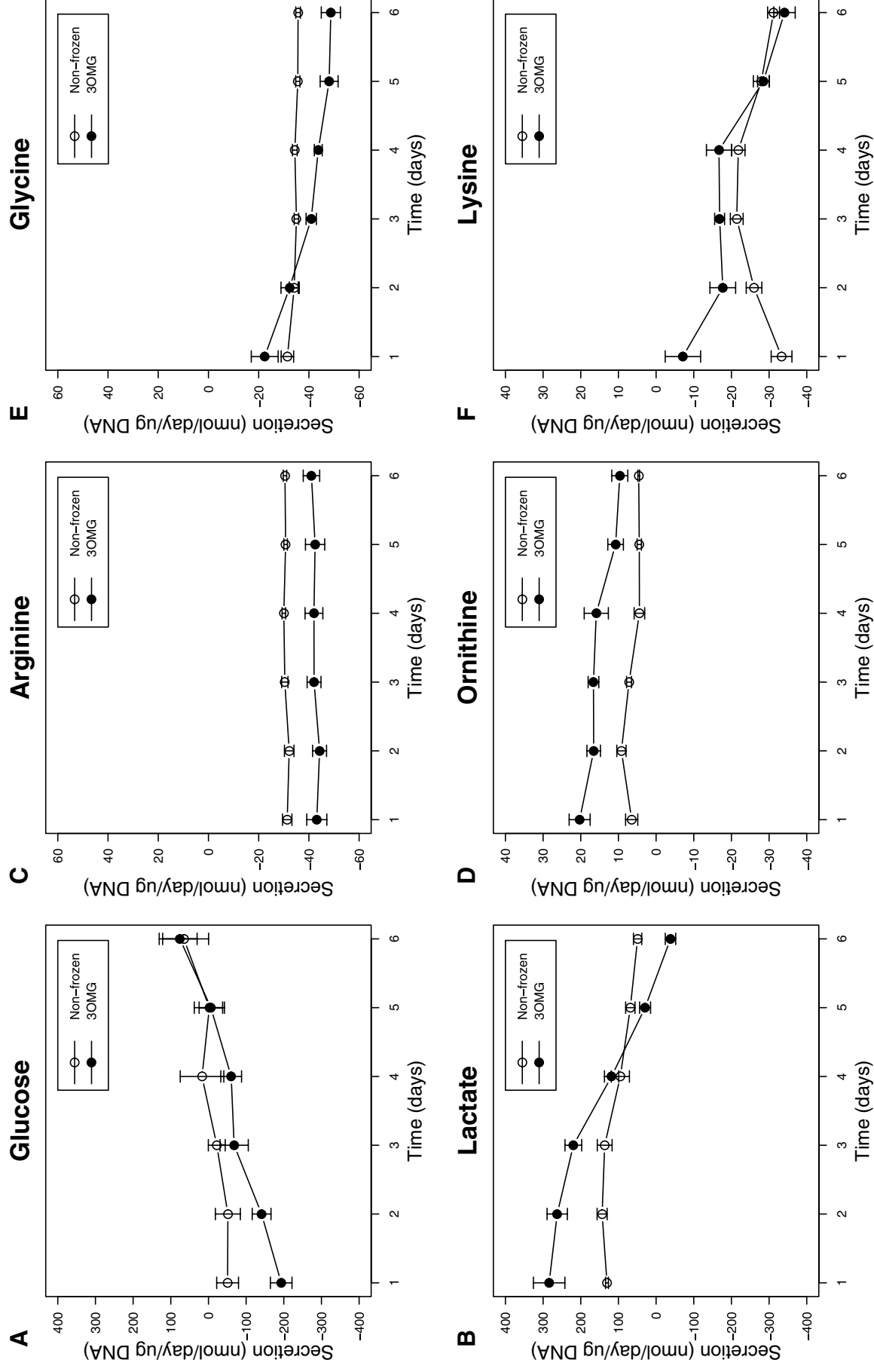
### Metabolite uptake and secretion

Uptake and secretion rates were measured for various metabolites in order to evaluate the effects of 3OMG loading and cryopreservation on protein, carbohydrate, and lipid metabolism. Uptake and secretion rates were largely preserved in 3OMG-cryopreserved hepatocytes when compared to control samples. The only exceptions were glycolysis, the urea cycle and two other amino acids (Fig. 4). Glucose uptake was initially higher in the frozen cells, but by day 5 the uptake had dropped to nearly 0 in both frozen and control cells (Fig. 4A). Lactate secretion was relatively stable in the fresh cells, but dropped significantly in the frozen cells over the time course of 6 days (Fig. 4B). Arginine uptake and ornithine secretion were also consistently higher in the cryopreserved hepatocytes (Fig. 4C and D). For ornithine, the differences were strong enough to be observed even in the unnormalized data, despite fewer viable cells present in the cryopreserved samples. For arginine, the difference is somewhat misleading since both control and cryopreserved samples consumed all of the arginine available in the medium. Transient differences were observed in two amino acids. Glycine uptake was initially lower in the frozen hepatocytes, but by day 5 both frozen and control cells were consuming all of the glycine in the medium (Fig. 4E). A similar but not identical trend was observed for lysine uptake (Fig. 4F). The other examined metabolites were remarkably unaffected by the freezing procedure.

## DISCUSSION

This report demonstrates the novel use of 3-O-methylglucose (3OMG), a non-metabolizable glucose derivative, as an effective cryoprotectant for primary hepatocytes. Primary rat hepatocytes frozen with intracellular 3OMG had high post-thaw viability and maintained near-normal metabolic, synthetic, and detox functions in long-term culture. The simple and convenient protocols used to achieve these results highlight several significant advantages of 3OMG over conventional cryoprotectants. These include minimal toxicity, single-step loading and removal procedures, and effectiveness at low concentrations.

The primary motivation for this study was an observation from nature. In several species of freeze-tolerant frogs, plasma and tissue glucose levels rise nearly 100-fold in response to dermal ice formation. As the frog begins to freeze, stored glycogen is rapidly depolymerized by the liver, releasing massive amounts of glucose into the circulation and increasing plasma concentrations from 1–5 mM to >200 mM.<sup>9–11</sup> The membrane transport capacity of glucose is also enhanced in a number of tissues during the first few hours of freezing, allowing intracellular glucose levels to increase where needed most.<sup>32</sup> In



**FIG. 4.** Stable changes in the uptake and secretion of selected metabolites in 3OMG-frozen (closed circles) and nonfrozen control (open circles) hepatocytes. Cells were cultured in a collagen sandwich configuration and supernatants were collected daily. All values were normalized by viable cell number (DNA content) and are shown as means  $\pm$  se ( $n = 6$ ). Most metabolites tested were not significantly different between frozen and control hepatocytes and are not shown. Uptake of glucose and secretion of lactate were initially higher in the frozen cells (**A, B**). By day five glucose uptake had dropped to zero in both conditions (**A**). Lactate secretion remained stable in the non-frozen cells, but dropped to nearly zero in the frozen cells by day five (**B**). Arginine uptake and ornithine secretion were stably increased in the frozen cells across all six days (**C, D**). Glycine and lysine uptake were initially depressed in the frozen cells, but recovered by day five (**E, F**).

the frogs, glucose is believed to act as a natural cryoprotectant, altering the thermodynamic properties of the cells and stabilizing their membranes and macromolecules. It may also provide a needed energy source for tissue recovery and repair during the thawing process. These adaptations allow the frog to survive freezing down to  $-8^{\circ}\text{C}$  for several weeks.<sup>11</sup>

Our study of cryopreservation using 3OMG and other glucose derivatives is based on the interesting observation that glucose is essential for freeze tolerance in the frogs, but performs rather poorly as a cryoprotectant for mammalian cells. One possibility is that freeze-tolerant frogs have evolved metabolic adaptations protecting them from the cellular damage and metabolic derangements associated with high-glucose concentrations.<sup>11</sup> Another possibility is that mammalian cells simply metabolize glucose too quickly for it to accumulate sufficiently in the cytoplasm. We hypothesized that a metabolically inactive glucose derivative would have similar cryoprotective properties but avoid both of these potential problems. Glucose, sucrose, 2-deoxy-glucose (2DG), and 3-O-methyl-glucose (3OMG) were eventually selected for testing on the basis of their metabolic activity, membrane transport characteristics, and availability.

In hepatocytes and several other cell types, extracellular glucose is rapidly carried across the plasma membrane by the high capacity transporter GLUT-2. Since both 3OMG and 2DG are structurally very similar to glucose, they are also carried by GLUT-2. As shown in Figure 1, intracellular 3OMG can reach an estimated 165 mM when the cells are incubated in a 200 mM extracellular solution. After thawing, the loaded 3OMG can be quickly washed out through GLUT-2 to minimize any deleterious long-term effects it may have. Of the sugars tested, only sucrose is not transported by GLUT-2. Since it is also much too large to cross the plasma membrane passively, it acts solely as an extracellular cryoprotectant.

Of the sugars tested, only 3OMG is metabolically inactive. 2DG has been reported to significantly decrease the metabolic activity of fibroblasts and cancer cells by inhibiting glycolysis.<sup>23,33</sup> In our study, however, neither 2DG nor D-glucose impaired the metabolic activity of hepatocytes, probably because hepatocytes rely less on glycolysis than most cells and can release glucose from glycogen stores. Although 2DG and D-glucose did not seem to affect cellular viability and metabolic activity, they were weak cryoprotectants compared to 3OMG. Since both 2DG and D-glucose are rapidly metabolized, the concentration in the cytosol may not have been sufficient to protect the cells.

Cryopreservation of primary hepatocytes is still challenging despite considerable effort with the limited supply of available hepatocytes.<sup>34</sup> Few studies have reported both high yields after thawing and long-term maintenance

of hepatocyte-specific function.<sup>6,35</sup> Of the sugars tested in this study, only 3OMG-loaded hepatocytes showed long-term survival and maintenance of hepatospecific functions (albumin synthesis, urea production, and cytochrome P450 detoxification) comparable to non-frozen controls. The results indicate that 3OMG protects the cellular structures, organelles, and enzymes required to complete these complex biochemical processes.

The hepatocytes also maintained intact metabolic systems after cryopreservation with 3OMG. Most of the metabolites associated with energy production, amino acid processing, protein synthesis, and lipid metabolism were minimally affected. Since these metabolic functions are essential for the hepatocytes, the results are encouraging for 3OMG-loaded hepatocytes to be used in cell-based bioartificial devices. However, the metabolic changes observed for glycolysis, and the urea cycle may indicate partially impaired mitochondrial function.<sup>36</sup> The increased glucose uptake and lactate secretion of the cryopreserved cells suggests a shift away from oxidative phosphorylation and a greater reliance on glycolysis for energy production. Arginine uptake and ornithine secretion were also higher in the cryopreserved hepatocytes suggesting a block in the mitochondrial portion of the urea cycle. Since the cryopreserved hepatocytes had near normal urea production, it may be that urea production in recently cryopreserved cells is dominated by the simple cleavage of arginine rather than the full urea cycle.

Various strategies may be explored for the further optimization and application of 3OMG-based cryopreservation. The uptake of 3OMG through GLUT-2 could be controlled by changing various biochemical conditions, such as hypoxia,<sup>37</sup> glucose starvation,<sup>38</sup> and chemical exposure.<sup>23,39-41</sup> Further optimization of physiochemical conditions including cooling and warming rates, storage temperature, and carrier solutions could also lead to increased cellular viability and function in cells cryopreserved with 3OMG. We believe that 3OMG is best used with hepatocytes, since they strongly express GLUT-2, a high-capacity transporter that allows high concentrations of 3OMG to be loaded in a short time.<sup>42</sup> Nevertheless, 3OMG has potential for a wide range of other mammalian cell types since it can also be transported through GLUT-1, which is expressed by most mammalian cells.

It would be interesting to compare 3OMG and glucose in the cryopreservation of whole wood frogs or isolated wood frog cells and tissues. In nature, wood frogs rarely survive freezing to temperatures below  $-8^{\circ}\text{C}$ .<sup>11</sup> In experimental systems, isolated wood frog hepatocytes are almost completely protected by 0.2 M glucose as long as the freezing temperature is not too far below  $0^{\circ}\text{C}$ . Even at very high concentrations, glucose is only moderately protective at typical cryopreservation temperatures.<sup>11</sup> This temperature limit was not observed in our studies using 3OMG in mammalian hepatocytes. Testing 3OMG

in wood frogs might help explain some of these observations.

Given the limitations of glucose in wood frogs, it is somewhat surprising that 3OMG is such an effective cryoprotectant for mammalian hepatocytes frozen down to liquid nitrogen temperatures. There are many possible explanations. Successfully freezing an entire organism, or even an organ, is much more difficult than freezing isolated cells. Frog survival during freezing is likely limited by a cell type or biological structure other than hepatocytes. Damage to neural or vascular structures below  $-8^{\circ}\text{C}$  might kill the frog even if its hepatocytes are perfectly protected. Significant differences also exist between the freezing protocols used in our study and in the wood frog hepatocyte studies. Direct comparison would require testing both glucose and 3OMG in wood frogs using otherwise identical protocols.

One interesting possibility is that 3OMG is an intrinsically better cryoprotectant than glucose. Biophysical studies of loading, freezing dynamics, membrane stabilization, and glass transition could help support or refute this idea. Another interesting possibility is that 3OMG is somewhat less toxic than glucose at the high concentrations reached during ultra-low temperature cryopreservation. To test this idea, studies could compare the accumulation of sugar-induced damage or the dysregulation of metabolism in 3OMG and glucose-loaded cells.

In summary, the present study demonstrates the use of the non-metabolizable glucose derivative 3OMG as a novel cryoprotective agent for primary hepatocytes. Unlike conventional cryoprotectants, it is minimally toxic, effective in very small amounts, and convenient to load and remove in single-step procedures. With further research, 3OMG-based cryopreservation may be extended to other cell types and freezing regimes.

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