Implications of Differential Stress Response Activation Following Non-Frozen Hepatocellular Storage

William L. Corwin,¹⁻³ John M. Baust,^{1,3} John G. Baust,^{1,2} and Robert G. Van Buskirk¹⁻³

Hepatocytes are critical for numerous cell therapies and *in vitro* investigations. A limiting factor for their use in these applications is the ability to process and preserve them without loss of viability or functionality. Normal rat hepatocytes (NHEPs) and human hepatoma (C3A) cells were stored at either 4°C or 37°C to examine post-processing stress responses. Resveratrol and salubrinal were used during storage to determine how targeted molecular stress pathway modulation would affect cell survival. This study revealed that storage outcome is dependent upon numerous factors including: cell type, storage media, storage length, storage temperature, and chemical modulator. These data implicate a molecular-based stress response that is not universal but is specific to the set of conditions under which cells are stored. Further, these findings allude to the potential for targeted protection or destruction of particular cell types for numerous applications, from diagnostic cell selection to cell-based therapy. Ultimately, this study demonstrates the need for further in-depth molecular investigations into the cellular stress response to bioprocessing and preservation.

Introduction

OPTIMAL PROCESSING AND preservation of hepatocytes is critical, given the multitude of *in vitro* applications currently employed. Presently, hepatocytes are used for a number of analyses such as the study of metabolism, drug-drug interactions, hepatotoxicity, and transplantation.¹⁻³ Furthermore, hepatocytes and hepatocyte derivatives are also used for a host of cell-based therapies, including transplantation, tissue engineering, and bioartificial liver support systems.⁴⁻⁶ It has become apparent that a limiting factor for these applications, as well as future uses of these biologics from continued advances in biotechnology and cell-based therapy, is proper bioprocessing and preservation to maintain *in vivo*-like functionality.

It is well established in the literature that a molecular-based response contributes significantly to the loss of function and cellular demise associated with the stresses of the processing and preservation procedures.⁷⁻²¹ This molecular-based response is predominantly associated with activation of the apoptotic cell death cascade; however, more recent studies have demonstrated a molecular-based form of necrotic cell death as well.²²⁻²⁴ There remains the question of whether the molecular response of cells exposed to these stressors is universal or whether it is dependent upon the multitude of different factors involved (i.e., cell type, storage

solution, temperature). The answer to this question would have major implications for numerous current and future approaches to bioprocessing and cold storage best practices. Further, this knowledge would help to direct future research in this area as more studies continue to examine the molecular pathways responsible for failure of these systems.

To probe this question, primary rat hepatocytes and C3A cells, a human hepatocellular carcinoma cell line (a clonal derivative of the Hep G2 cell line), were selected for this study. These cell types were chosen in an effort to compare the post-bioprocessing molecular stress response in two similar, yet molecularly different, systems. Further, two compounds (salubrinal and resveratrol) were utilized to examine the effect of specific pathway modulation on bioprocessing outcome.

Salubrinal has been shown to protect cells against apoptosis induced by endoplasmic reticulum (ER) stress mediated through the unfolded protein response (UPR) pathway.^{25,26} The UPR pathway is activated following an accumulation of mis-folded proteins in the ER lumen. The UPR includes two functions; the first is an attempt to correct the problem through a decrease in protein translation and an upregulation of folding and chaperone proteins. If the stress is prolonged and severe, then the second function, apoptotic cell death, is initiated. Specifically, salubrinal functions to inhibit eukaryotic translation initiation factor 2 subunit

¹Institute of Biomedical Technology, and ²Department of Biological Sciences, Binghamton University, Binghamton, New York.

³CPSI Biotech, Owego, New York.

α (eIF2 α) phosphatase enzymes, thus keeping eIF2 α in a phosphorylated or active conformation, which inhibits translation and the further accumulation of proteins in the ER lumen.^{27,28} Numerous reports have demonstrated the significant effects that salubrinal addition can have on cell systems through the modulation of this ER stress related pathway.^{15,25,27,29,30} The UPR pathway has become increasingly important for study as more and more reports have demonstrated its involvement in numerous cell systems following a host of different stress stimuli.³¹ We hypothesized that through the use of salubrinal, the pro-apoptotic signaling of the UPR could be modulated to improve storage outcomes for hepatocytes.

Resveratrol, a compound found in the skin of red grapes and other fruits, has been the focus of myriad reports detailing a range of potential effects from life-extending properties to the demonstration of an anti-cancer effect *in vitro*, as well as beneficial cardiovascular results.^{32–38} It is believed that resveratrol exerts its effects primarily through the activation of genes that mimic caloric restriction, and while still under investigation, it is thought this is achieved through the activation of Sirtuin 1. A recent report implicated the involvement of the UPR pathway in resveratrol's anti-cancer effects;³⁹ additionally, other studies have demonstrated the potential connection of ER stress and the UPR as a mediator of resveratrol's effects.^{25,40–43} While the exact mechanism of action of resveratrol is still being elucidated, its recent link to the UPR pathway, coupled with its reported anti-cancer effects, have made it an attractive agent for this study. Given resveratrol's reported anti-cancer effects, we hypothesized that a differential response of the two cell types used in this study might be induced, yielding a negative effect in C3A cells and a positive effect in normal hepatocytes.

The current study focused on determining whether modifying molecular response pathway activation, particularly the UPR, using these two chemical modulators, has an effect on the survival of hepatic cells following bioprocessing and storage. Further, utilization of the two cell types allowed for the determination of similarities or differences in the stress response which, in turn, may provide insight into the universality versus specificity of the molecular response of hepatic cells to the stresses of bioprocessing.

Methods and Materials

Cell culture

Human hepatoma (C3A) cells (American Type Culture Collection, Manassas, VA) were maintained under standard culture conditions (37°C, 5% CO₂/95% air) in minimal essential medium with Earle's salts, L-glutamine, and nonessential amino acids (EMEM) (Caisson Laboratories, North Logan, UT) supplemented with 10% Fetal Bovine Serum (Atlanta Biologicals, Lawrenceville, GA), 1% penicillin/streptomycin and 1mM sodium pyruvate (Caisson). Cells were propagated in Falcon T-75 flasks from passage 8 through 20 and media was replenished every 2 days of cell culture. Primary rat hepatocytes (Invitrogen, Carlsbad, CA) were thawed from liquid nitrogen storage and directly seeded into tissue culture plates pre-coated with collagen prior to experimentation, as these cells cannot be subcultured.

Hypothermic storage

Cells were seeded into 96-well tissue culture plates (35,000 cells per well for both primary hepatocytes and C3A cells) and cultured for 48 h into a monolayer. Culture media was decanted from experimental plates and replaced with 100 μ l/well of the pre-cooled (4°C) media (complete growth media, HBSS with calcium and magnesium, or ViaSpan). Cultures were maintained at 4°C for 18 h to 6 days. Following cold storage, the media was decanted, replaced with 100 μ l/well of room temperature (~25°C) complete culture media and placed into standard culture conditions (37°C, 5% CO₂) for recovery and assessment.

Normothermic storage

Cells were seeded into 96-well tissue culture plates (35,000 cells per well for both primary hepatocytes and C3A cells) and cultured for 48 h into a monolayer. Culture media was decanted from experimental plates and replaced with 100 μ l/well of the warm (37°C) media (complete growth media or HBSS with calcium and magnesium). Cultures were maintained at 37°C in a sealed bag with excess air extruded before closing, for 1 to 7 days. Following storage, the media was decanted, replaced with 100 μ l/well of warm (37°C) complete culture media, and placed into standard culture conditions (37°C, 5% CO₂) for recovery and assessment.

Cell viability assay

To assess cell viability, the metabolic activity assay, alamarBlue™ (Invitrogen), was utilized. Cell culture media was decanted from the 96-well plates and 100 μ l/well of the working alamarBlue™ solution (1:20 dilution in HBSS) was applied. Samples were then incubated for 60 min ($\pm$ 1 min) at 37°C in the dark. Fluorescence levels were analyzed using a Tecan SPECTRAFluorPlus plate reader (TECAN, Austria GmbH). Relative fluorescence units were converted to a percentage compared to normothermic controls set at 100%. Readings were taken immediately following removal from storage, as well as at 24 and 48 h of recovery.

Modulation studies

Chemical modulation of molecular pathways was conducted through the use of salubrinal (UPR-specific inhibitor) and resveratrol. Salubrinal (EMD Chemicals Inc., Gibbstown, NJ) was added to hypothermic or normothermic media at a working concentration of 25 μ M immediately before utilization. Resveratrol (EMD Chemicals Inc.) was utilized at a concentration of 25 μ g/ml (110 μ M) and a lower concentration of 2.5 μ g/ml (11 μ M) (data not shown, except in Fig. 2B). All chemicals were diluted in DMSO prior to utilization, and DMSO controls were conducted to ensure no effect of the dilution vehicle.

Fluorescence microscopy

Samples in 96-well plates were assessed for the presence of live, necrotic, or apoptotic cells through triple labeling using Hoechst (81 μ M), propidium iodide (9 μ M), and YoPro-1 (0.8 μ M) (Molecular Probes, Eugene, OR), respectively.

Probes were added to samples and incubated in the dark for 20 min prior to imaging. All fluorescence images of labeled cells were obtained at 1, 4, 8, 24, 28, 32, and 48 h post-storage using a Zeiss Axiovert 200 fluorescent microscope with the AxioVision 4 software (Zeiss, Germany).

Flow cytometric analysis

Counts of the live (unlabeled), necrotic (propidium iodide-labeled [$1.5\ \mu\text{M}$]) and apoptotic (YoPro-1-labeled [$0.1\ \mu\text{M}$]) cells were obtained using microfluidic flow cytometry (Millipore). Probes were added to each sample and incubated in the dark for 20 min prior to cell collection. Samples were labeled, collected, and analyzed at 1, 4, 8, 24, 28, 32, and 48 h post-storage using the CytoSoft 5.2 software for the Guava PCA-96 system.

Western blot analysis

Cells were cultured in 60 mm Petri dishes to form a monolayer. Cell culture media was removed and replaced with 4 ml of pre-cooled (4°C) media, and dishes were placed at 4°C for 18 h. Following cold storage, media was decanted and replaced with 4 ml of room temperature culture media, and placed into standard culture conditions (37°C , 5% CO_2) for recovery. Cell lysates were collected at 1, 4, 8, and 24 h post-storage using ice-cold radio-immunoprecipitation assay cell lysis buffer with protease inhibitors. Samples were homogenized by vortex mixing and centrifuged at 15,000 rpm for 15 min at 4°C . Protein concentrations were quantified using the bicinchonic acid protein assay (Thermo Fisher Scientific, Rockford, IL) and a Tecan SPEC-TRAFluorPlus plate reader. Equal amounts of protein (70–100 μg) for each sample were loaded and separated on a 10% SDS-PAGE gel (Bio-Rad, Hercules, CA). Proteins were transferred to PVDF membranes (Bio-Rad) and blocked with a 1:1 mixture of NAPTM-Blocker (G-Biosciences, Maryland Heights, MO) with 0.05% Tween-20 in PBS for 2 h at room temperature. Membranes were incubated at 4°C overnight in the presence of each antibody: anti-human caspase 9, anti-human caspase 3, anti-human PARP, anti-human Bid, anti-human calnexin, anti-human AIF, and anti-human GAPDH (Cell Signaling Technology, Danvers, MA). Membranes were then washed three times with 0.05% Tween-20 in PBS and exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Membranes were again washed three times with 0.05% Tween-20 in PBS before detection with the LumiGLO[®]/Peroxide chemiluminescent detection system (Cell Signaling Technology). Membranes were visualized using a Fujifilm LAS-3000 luminescent image analyzer. Equal protein loading was achieved through initial quantification of all samples and confirmed by Ponceau S staining of PVDF membranes prior to blocking as well as probing for GAPDH levels.

Data analysis

Viability experiments were repeated a minimum of three times with an intra-experiment repeat of seven replicates. Western blots, flow cytometry, and fluorescence microscopy were all conducted on a minimum of three separate experiments. Standard errors were calculated for viability values and single-factor analysis of variance (ANOVA)

and Student's *t*-tests were utilized to determine statistical significance.

Results

Effect of hypothermic exposure on human hepatoma (C3A) cell survival

To examine the response of human hepatoma cells to cold exposure, C3A cells were stored at 4°C and examined post-storage for remaining viability as a measure of cell survival using the metabolic activity indicator, alamarBlueTM. The addition of resveratrol and salubrinal to three storage solutions (complete culture media, HBSS, and ViaSpan) was performed to determine their effects on C3A cells during hypothermic storage. C3A cells stored hypothermically for 18 h in the three storage solutions demonstrated a profound difference in post-storage viability (Fig. 1A). Under the 'complete media' storage condition, there was a profound loss in sample viability, with an immediate post-storage survival of $5.8 \pm 1.7\%$. With subsequent culture in complete media, cells exhibited a slow repopulation, only growing to $17.6 \pm 5.2\%$ of control at 48 h post-storage. In contrast, cells stored in HBSS and ViaSpan for 18 h remained highly viable, with greater than 90% survival of the populations immediately post-storage.

Incorporation of the chemical modulators resveratrol and salubrinal had profound effects on the 'complete media' storage condition. The addition of resveratrol yielded a complete loss of viability immediately post-storage, with minimal re-growth after 48 h of culture. Conversely, the inclusion of salubrinal resulted in a marked improvement in cell survival when compared to the 'complete media' alone condition immediately following storage (5.7% vs. 24.1%, respectively). Further, this beneficial effect became increasingly significant throughout the 48 h of recovery as the population re-grew to $\sim 85\%$ of the pre-storage control. The addition of resveratrol to HBSS resulted in a significant loss ($\sim 80\%$ decrease) in viability (95.6% vs. 11.8%), while the addition of salubrinal demonstrated no appreciable difference when compared to HBSS alone. Similarly, the inclusion of resveratrol and salubrinal in the ViaSpan storage regime had no appreciable effect.

To further investigate the effect of hypothermic storage and molecular modulation on C3A cells, the storage interval was increased to 24 h, and samples were again analyzed via alamarBlueTM (Fig. 1B). The increase in storage time resulted in an almost complete loss of cell viability for all the 'complete media' storage conditions, regardless of whether a chemical agent was added. Extension of the storage interval also resulted in a decreased cell survival of $78.8 \pm 12.2\%$ for the HBSS storage condition. The addition of resveratrol to HBSS resulted in effectively a complete loss of cell survival ($3.3 \pm 1.2\%$); conversely, salubrinal supplementation of HBSS yielded a highly protective effect as the population remained fully viable immediately post-storage ($98.4 \pm 3.9\%$), an $\sim 25\%$ increase over the HBSS alone condition. Cells stored in ViaSpan for 24 h demonstrated a similar outcome as those stored for 18 h; no considerable difference was observed between the three conditions, with all cells remaining highly viable ($>90\%$) immediately post-storage.

FIG. 1. Metabolic activity of human hepatoma (C3A) cells following 18 or 24 hours of hypothermic storage. C3A cells were placed at 4°C in either: Complete Growth Media, HBSS with Ca^{++} and Mg^{++} , or ViaSpan for 18 or 24 h (A) and (B), respectively, alone or with addition of 25 $\mu\text{g}/\text{mL}$ resveratrol or 25 μM salubrinal. Resultant cell viability analyses were conducted at 0, 24, and 48 h post-storage using the metabolic activity indicator, alamarBlue™. ($n=3, \pm \text{SEM}$).

Comparison of human hepatoma (C3A) cell vs. primary hepatocyte survival following cell storage

Following the establishment of the viability profiles for C3A cells after hypothermic storage, a comparison of C3A cells and primary hepatocytes (NHEPs) was conducted to examine if similar results would be obtained when utilizing the same modulators. Further, the incorporation of a normothermic storage regime was added to determine if alterations in storage temperature would result in different outcomes for the two cell types. In examining hypothermic storage in HBSS, storage intervals in which a profound loss in viability ($>50\%$) was observed (2 days for C3A cells and 18 h for NHEPs) were utilized to determine if salubrinal addition would have an effect on storage outcome. The re-

sults revealed that salubrinal addition had a highly positive effect on C3A cell survival in HBSS ($61.7 \pm 7.4\%$ viable) following 2 days of cold storage, as compared to HBSS alone which resulted in a complete loss of cell viability ($1.2 \pm 0.4\%$) (Fig. 2A). Conversely, salubrinal had no significant effect on NHEP survival following 18 h of cold storage (HBSS: $48 \pm 8.0\%$ vs. HBSS + salubrinal: $55.9 \pm 12.6\%$, $p = \text{NS}$).

When comparing resveratrol addition to HBSS during 18 h of cold storage, a profound effect was observed in both cell types (Fig. 2B). C3A cells stored in HBSS alone remained highly viable after 18 h of hypothermic storage with a post-storage survival of $95.6 \pm 5.0\%$. The addition of resveratrol to this condition resulted in a significant decrease in post-storage viability, to $12.1 \pm 4.1\%$. Interestingly, NHEPs stored under these conditions yielded the opposite effect, as seen in

FIG. 2. Viability comparison between stored human hepatoma cells and normal hepatocytes. C3A and NHEP cells were placed at either 4°C or 37°C in HBSS with or without the addition of 25 μ g/ml resveratrol or 25 μ M salubrinal. (A) C3A cells following 48 h of hypothermic storage and NHEP cells following 18 h of hypothermic storage with and without the addition of salubrinal. (B) C3A and NHEP cells following 18 h of hypothermic storage with and without the addition of resveratrol. (C) C3A and NHEP cells following 3 days of hypoxic normothermic storage with and without the addition of salubrinal. (D) C3A and NHEP cells following 1 day of hypoxic normothermic storage with and without the addition of resveratrol, ($n=3$, $\pm$ SEM).

C3A cells. NHEP survival following 18h of cold storage in HBSS alone resulted in post-storage viability of $48.1 \pm 8.0\%$. The inclusion of resveratrol in this storage condition resulted in an improved cell survival to $71.3 \pm 10.4\%$, an increase of $\sim 23\%$ (Fig. 2B). A lower resveratrol concentration of 11 μ M was utilized for this set of experiments, as no significant effect was seen when the standard concentration (110 μ M) was used.

Following observation of the different responses of the two cell types to the addition of the chemical modulators during hypothermic storage, a regime of hypoxic normothermic storage was utilized to examine if alterations in temperature would affect storage outcome. The post-storage viability of C3A cells observed following 3 days of warm storage was $87.6 \pm 3.0\%$ (Fig. 2C). Interestingly, salubrinal addition, which had a positive effect during hypothermic storage, resulted in a negative effect under normothermic storage. The resultant viability when salubrinal was added under this storage condition was $40.4 \pm 1.6\%$, an $\sim 47\%$ decrease in survival. When NHEPs were examined following 3

days of hypoxic normothermic storage, the results revealed a similarly high viability ($85.4 \pm 6.0\%$). The addition of salubrinal resulted in a positive effect on cell survival for NHEPs with a post-storage viability of $113.4 \pm 11.6\%$, an increase of nearly 28%. This was an interesting observation given that salubrinal had no significant effect during hypothermic storage of NHEPs. Further, salubrinal addition to C3A cells had the opposite effect (cytotoxic) during normothermic storage.

A final comparison of the effect of adding resveratrol to HBSS between these cell types was conducted following one day of hypoxic normothermic storage (Fig. 2D). The viability of C3A cells following 1 day of warm storage in HBSS was found to be $93.7 \pm 3.0\%$. The inclusion of resveratrol to this condition resulted in a decrease in survival of $\sim 17\%$, yielding a post-storage viability of $77.0 \pm 2.9\%$. Similarly, NHEPs stored in HBSS alone for 1 day resulted in high post-storage viability ($95.0 \pm 6.7\%$). Further, resveratrol addition had a negative effect on storage outcome, decreasing survival $\sim 12\%$ to a post-storage viability of $83.3 \pm 4.1\%$.

TABLE 1. SUMMARY OF VIABILITY OUTCOMES FOR HEPATOCYTE CELL STORAGE

Media	Agent	Cold Stress Regime		Normothermic Stress Regime	
		C3A	Hepatocyte	C3A	Hepatocyte
Media	Resveratrol	-- (18 h)	++ (18 h)	-- (7 d)	-- (1 d)
	Salubrinal	++ (18 h)	Ø (18 h)	-- (7 d)	+ (6 d)
HBSS	Resveratrol	-- (18 h)	++ (18 h)	-- (1 d)	-- (1 d)
	Salubrinal	++ (2 d)	Ø (18 h)	-- (2 d)	++ (3 d)
ViaSpan	Resveratrol	++ (4 d)	++ (18 h)	N/A	N/A
	Salubrinal	++ (4 d)	Ø (18 h)	N/A	N/A

Key: –, Negative; +, Positive; Ø, No effect; (#d), # days stress exposure; --, Highly negative; ++, Highly positive; N/A, Not available.

Given the multitude of factors and variables examined in this study, an overview of experimental outcomes is presented in Table 1. The summary data illustrate that targeted modulation using resveratrol and salubrinal has differential effects both between and within the two cells types during storage. Additionally, Table 1 shows the results for storage of C3A cells and NHEPs in their respective complete media and ViaSpan following hypothermic storage. Interestingly, the results seen with HBSS storage differed from the results obtained when these chemical modulators were utilized in the other storage media. The results showed that a single chemical modulator such as resveratrol had a beneficial effect on C3A cells during hypothermia in ViaSpan, but a negative effect when used in HBSS. A number of other instances where the incorporation of chemical modulators had differential effects depending on the condition and cell type were noted in this study (Table 1). These data illustrate how factors such as cell type, storage media, storage temperature, and molecular modulation are critical aspects that determine outcome, and how changing any single factor can yield a completely different result.

Flow cytometric analysis of cell death populations following cell storage

Following the establishment of cell viability profiles in the various model systems, flow cytometric analysis was conducted to examine if and how apoptotic and necrotic cell death populations changed over the 48-h, post-storage interval. The fluorescent probes propidium iodide (necrosis) and Yo-Pro-1 (apoptosis) were utilized at 1, 4, 8, 24, 28, 32, and 48 h post-storage to determine how the relative size of these populations changed as a result of the chemical modulation during HBSS storage.

Apoptotic analysis of C3A cell storage for 24 h at 4°C in HBSS alone, and with the addition of resveratrol or salubrinal, is presented in Figure 3A. Determination of the apoptotic percentage for pre-storage normothermic controls (~3%) provided a baseline for apoptosis in this population. After 24 h of cold storage in HBSS, C3A cells showed a low level of apoptosis (~2%–3%) throughout the first 8 h of recovery. Interestingly, a peak of $10.8 \pm 1.9\%$ was noted at 28 h post-storage, with an elevated level of apoptosis found throughout the 24–48 h time points demonstrating significantly delayed onset of a molecular-based cell death response to the stresses associated with cold storage. When resveratrol was added during storage, a reduced level of apoptosis was observed throughout the 48 h post-storage,

whereas necrotic levels were found to increase. This was likely due to the negative effect that resveratrol had on the cells under this storage regime, as noted by the low survival for this condition (Fig. 1B) and the high level of necrosis (Fig. 3B). Interestingly, when salubrinal was added under this storage condition, a near complete blockage of apoptosis was found throughout the 48 h post-storage (Fig. 3A). These data suggest that salubrinal has a beneficial effect on cell survival, potentially mediated through an anti-apoptotic route.

In addition to apoptotic analysis, samples were probed for necrotic cell death (Fig. 3B). Baseline necrotic levels for the pre-storage normothermic control were found to be $7.3 \pm 1.3\%$ for C3A cells. In comparison, the HBSS alone storage condition resulted in an initial elevation in necrosis of $34.9 \pm 11.8\%$ at 1 h post-storage, which gradually decreased over the 48-h recovery interval to $14.4 \pm 3.5\%$. A significant increase in necrosis was found with resveratrol within 1 h post-storage ($98.0 \pm 0.3\%$). This population remained elevated throughout the post-storage recovery interval, decreasing in the later time points as the necrotic population began to lyse and disintegrate in culture. When HBSS with salubrinal was used, a low level of necrosis (below 20%) was seen throughout the entire 48-h, post-storage assessment interval.

Following analysis of cell death populations subsequent to hypothermic storage, examination of cell response to the same conditions when stored for 48 h in a hypoxic normothermic state was conducted. Analysis of apoptotic populations of C3A cells stored in the same storage media as for the hypothermic studies demonstrated a similar baseline level of apoptosis for the pre-storage normothermic controls (Fig. 3C). C3A samples stored at 37°C in HBSS alone demonstrated a similar trend as their hypothermic counterpart with a delayed peak in apoptosis occurring at 24 h post-storage ($24.1 \pm 1.3\%$) and remaining elevated at 28 and 32 h of recovery, followed by a return to baseline by 48 h. The inclusion of resveratrol resulted in a near complete blockage of apoptosis during the initial 8 h of recovery. The level of apoptosis rose by 24 h and continued climbing until reaching a peak of 8.7% at 48 h post-storage. Interestingly, the addition of salubrinal during normothermic storage resulted in a similar outcome (blocking of apoptosis) as seen under the hypothermic storage model. While blocking apoptosis, salubrinal was ultimately found to have a negative effect on overall cell survival for this condition (Table 1).

Analysis of necrotic populations subsequent to storage revealed that C3A cells stored in HBSS alone resulted in a

FIG. 3. Flow cytometric analysis of cell death population following storage. C3A cells were placed at 4°C or 37°C in either: HBSS, HBSS + 25 µg/ml resveratrol, or HBSS + 25 µM salubrinal for 24 h (hypothermia) or 48 h (normothermia). Following the storage interval, flow cytometric analysis of the apoptotic (Yo-Pro-1) and necrotic (propidium iodide) populations was conducted at 1, 4, 8, 24, 28, 32, and 48 h of recovery. **(A)** Apoptotic population analysis following 24 h of hypothermic storage in either HBSS, HBSS + 25 µg/ml resveratrol, or HBSS + 25 µM salubrinal. **(B)** Necrotic population analysis following 24 h of hypothermic storage in either HBSS, HBSS + 25 µg/ml resveratrol, or HBSS + 25 µM salubrinal. **(C)** Apoptotic population analysis following 48 h of hypoxic normothermic storage in either HBSS, HBSS + 25 µg/ml resveratrol, or HBSS + 25 µM salubrinal. **(D)** Necrotic population analysis following 48 h of hypoxic normothermic storage in either HBSS, HBSS + 25 µg/ml resveratrol, or HBSS + 25 µM salubrinal. ($n=3, \pm$ SEM)

low level of necrosis initially at 1 h post-storage (Fig. 3D). Analysis of the three conditions revealed that for HBSS, HBSS with resveratrol, and HBSS with salubrinal, necrotic levels were found to be $24.7 \pm 1.3\%$, $64.4 \pm 3.2\%$, and $54.8 \pm 2.9\%$, respectively, at 1 h post storage (Fig. 3D). All the experimental conditions demonstrated similar patterns with an initial peak in necrosis that decreased over the recovery interval to near control levels by 48 h post-storage. These analyses in combination demonstrate the potential anti-apoptotic mechanism by which resveratrol and salubrinal may have their effects, both beneficial and detrimental depending on the condition under which samples are stored.

Fluorescent microscopy analysis of cell death populations following sample storage

To confirm the results of the flow cytometric cell death analysis, fluorescent microscopy was conducted utilizing the same probes (propidium iodide and Yo-Pro-1) for necrotic and apoptotic cells, respectively. A triple stain using Hoechst 33342 was also included as a viability probe. The

analysis was conducted to visually and qualitatively confirm the data obtained via flow cytometry. A representative image of this analysis is presented in Figure 4 in which C3A cells stored under four conditions (control, HBSS, HBSS with resveratrol, and HBSS with salubrinal) were visualized following 24 h of hypothermic storage and 24 h of recovery.

Through this analysis, the data obtained during flow cytometry were visually confirmed. The control micrograph revealed a high level of viable cells (blue-labeled cells) and low baseline levels of apoptosis (green) and necrosis (red). The HBSS condition yielded an increased level of both apoptosis and necrosis (high incidence of green and red labeled cells) which correlated well with the flow cytometry results. C3A cells stored in HBSS with resveratrol displayed a high level of necrosis with nearly all cells found to be propidium-iodide-positive (red) and only a small percentage viable (blue). The cells stored in HBSS with salubrinal displayed a similar trend as observed via flow cytometry, with almost no apoptosis observed and necrosis the primary mode of cell death. Further, the level of overall cell death was found to be less than that of HBSS alone.

Fluorescent Microscopy 24 Hours Post 24 Hours Hypothermic Storage (C3A)

FIG. 4. Fluorescent microscopic analysis of cell death population following storage. C3A samples were placed at 4°C in either: Control, HBSS, HBSS + resveratrol (25 µg/ml), HBSS + salubrinal (25 µM) for 24 h. Fluorescent micrographs were taken after 24 h of recovery and probed for viable (Hoechst, blue), apoptotic (Yo-Pro-1, green), and necrotic (propidium iodide, red) populations.

Effect of chemical modulators on protein levels after cell storage

Following viability and cell death analyses, an examination into post-storage protein levels was performed to determine what effect the chemical modulators had on apoptotic and ER stress-related proteins. Proteins associated with apoptotic and UPR pathway activation were isolated from C3A cells at 1, 4, 8, and 24 h post-storage and utilized for Western blot analysis. Accordingly, C3A cells stored at 4°C for 18 h in either HBSS, HBSS with resveratrol, or HBSS with salubrinal were probed for the apoptosis-related proteins pro-caspase 9, pro-caspase 3, and Bid (Fig. 5A). The C3A cells stored in HBSS alone or in HBSS with salubrinal, which were found to remain highly viable when stored at 4°C for 18 h (Fig. 1A), revealed little change in the levels of pro-caspase 9, pro-caspase 3, and Bid post storage (Fig. 5A). In contrast, more pronounced changes in proteins levels were observed when cells were stored in HBSS with resveratrol, where significant decreases (cleavage) of pro-caspase 9, pro-caspase 3, and Bid levels were observed by 1 h post-storage, which continued to decrease at 4 and 8 h, followed by a return to control levels by 24 h. The cleavage of these proteins further supported the notion of pro-apoptotic signaling during the recovery interval. This data further demonstrates that resveratrol, which was found to have a negative effect under this condition, may in part exert its effect through a molecular based, pro-apoptotic mechanism.

The cold storage interval was extended to 48 h to further examine the effect of salubrinal on the cell proteins (Fig. 5B). The results of the storage of cells in HBSS with resveratrol were not included as there was a complete loss of viability

precluding protein analysis. As with the 18-h samples, cells stored for 48 h were probed for proteins associated with apoptotic activation. The resultant data revealed a number of changes in protein levels with apoptotic activation in the HBSS condition (Fig. 5B). As observed after 18 h, the cleavage of pro-caspase 9, pro-caspase 3, and Bid were evident following 48-h storage. It was seen, however, in more of a delayed-onset fashion with the decreases in protein levels not evident until later in the recovery interval. Further, analysis of the apoptotic-associated protein AIF (apoptosis-inducing factor) revealed a peak immediately at 1 h post-storage for HBSS alone with elevated levels remaining at 4 and 8 h post-storage. AIF is an inducer of apoptosis and as such, an increase in its level is associated with pro-apoptotic signaling. While C3A cells stored in HBSS with salubrinal also showed an increased level of AIF compared to the control, the initial peak seen with HBSS alone was not observed. The reduced levels of AIF combined with little to no observed cleavage of pro-caspase 9, pro-caspase 3, and Bid suggested a lack of apoptotic activation for this condition. In addition, analysis of the levels of PARP (poly ADP-ribose polymerase), a DNA repair enzyme whose cleavage is indicative of apoptotic activation, was also conducted. The HBSS storage condition revealed a complete loss of PARP throughout all the time points examined. Cells stored in HBSS with salubrinal revealed an initial retention of PARP at 1 h post-storage before decreasing over the subsequent recovery period. Last, calnexin levels were examined to assess the involvement of the ER stress response and UPR pathway activation. Calnexin acts as a chaperone that functions to maintain proper protein folding. Calnexin cleavage has been reported as an indicator of the signaling of an apoptotic response as a result of ER related stress

FIG. 5. Western blot analysis of cell death-related proteins following storage. C3A cells were placed at 4°C for 18 and 48 h, or 37°C for 48 h, in either: HBSS, HBSS + 25 µg/ml resveratrol, or HBSS + 25 µM salubrinal. Proteins were isolated at 1, 4, 8, and 24 h post-storage. **(A)** C3A samples were probed for pro-apoptotic proteins (Bid, pro-caspase 9, pro-caspase 3) following 18 h of hypothermic storage. **(B)** C3A samples were probed for pro-apoptotic proteins (Bid, pro-caspase 9, pro-caspase 3, PARP, and AIF) and UPR pathway (calnexin) following 48 h of hypothermic storage. **(C)** C3A samples were probed for pro-apoptotic proteins and UPR involvement following 48 h of hypoxic normothermic storage.

(i.e., the pro-apoptotic signaling of the UPR). Western blot analysis of calnexin levels revealed that, in the HBSS-alone samples, a pronounced cleavage was observed over the post-storage interval, whereas this was not found in the HBSS with salubrinal samples.

C3A cells were also examined via Western blot analysis after 48 h of hypoxic normothermic storage to determine the molecular activation patterns of cell death (Fig. 5C). Interestingly, the observation of increased pro-apoptotic signaling (i.e., caspase cleavage) that was seen in conditions experiencing profound losses in viability, as revealed by metabolic activity analysis, was not detected under this storage condition. The addition of resveratrol and salubrinal were both shown to have a profound negative impact on cell survival in HBSS storage at normothermic temperatures. However, little to no cleavage of procaspases 9 and 3 was observed. These results suggest that these compounds may exert their effects through a caspase-independent, apoptotic mechanism following extended normothermic storage. Further, the examination of calnexin revealed a more pronounced cleavage at earlier time points for both the resveratrol- and salubrinal-containing samples, implying the activation of the UPR pathway. Taken together, these data suggest that the same compound added to a storage solution (HBSS) may activate different molecular stress response mechanisms, depending upon the specific storage conditions.

Conclusions

In this study, two different hepatocellular cell types were investigated for their response to stresses associated with bioprocessing and biopreservation. The conditions of hypothermia and hypoxic normothermia were utilized as mechanisms to simulate the stressors these cells experience during cold storage and warm ischemia and reperfusion, respectively. Further, the use of resveratrol and salubrinal allowed for an examination of the effects of specific chemical modulators of the cellular molecular stress response on sample quality and storage outcome.

The results of these investigations demonstrate that numerous factors affect storage outcome. The variables examined in this study, including cell type, storage temperature, storage length, storage solution, and chemical modulators, were all found to have an impact on the final outcome (viability and mechanism of cell death). These findings suggest that there does not appear to be a universal molecular cell storage stress response; instead, the response is dependent upon the specific set of factors involved in the bioprocessing event. For instance, the data revealed that the addition of resveratrol had a completely different effect on cell survival between the C3A cells and the hepatocytes. Given the complex nature of molecular cell signaling, it is difficult to postulate what specific differences account for this opposing result. While cancer cells have developed numerous mechanisms for avoiding cell death through changes in molecular response pathways, it would be premature to suggest that this is a cancer-specific phenomenon. Further, it was found that the response of the samples, even within the same cell type, could differ depending upon the stress factors involved including temperature, time, and medium. These findings differed from what would be expected from a molecular modulator, such as an antioxidant or other chemical

agent, where the agent typically has either a cytoprotective or cytotoxic effect, but not both.

The dual-effect phenomenon found in this study has a variety of implications. First and foremost, these findings suggest that proper bioprocessing requires a tailored approach to achieve the desired outcome of fully viable and functional cells. This may be a particularly important concern when considering the complexity of whole organ bioprocessing, where a number of different cell types that may not respond in the same way to a specific solution or chemical modulation are present. To this end, preliminary studies on normal human hepatocytes have demonstrated outcomes similar to the rat NHEP data presented here. Additionally, the differences observed between normal and cancerous hepatocytes may also have a number of implications. These data suggest the potential to specifically target the protection or destruction of cancerous or noncancerous cells in various settings such as anti-cancer, molecular-based treatments; specific maintenance of cancerous cells in a tumor biopsy; or screening out teratoma formation during the production of induced pluripotent stem cells.

Another interesting observation from these data was the potent anti-apoptotic effect of salubrinal. Interestingly, this blockage of apoptosis was found to occur regardless of whether salubrinal had a beneficial or detrimental effect on cell survival. This finding supports the idea of a cell death continuum in which necrosis and apoptosis are considered extreme opposite ends of a continual, molecular-based cell death spectrum. A number of recent studies have begun to show that necrosis has a molecular or programmed mechanistic route for completion and it is not simply an unregulated process of cellular lysis.^{22–24} This may provide an explanation for the dual-action phenomenon of salubrinal where its anti-apoptotic effect results in higher cell survival under some conditions, and conversely, a push of cells towards a molecular-driven necrotic demise in other instances of greater stress. Further, the ability of resveratrol to initially block apoptosis, but at the same time increase necrosis while also significantly delaying the peak in apoptosis in C3A cells stored under normothermic conditions supports this cell death continuum hypothesis.

As our results showed that resveratrol and salubrinal, two compounds that act through different mechanisms, exhibited dual cytoprotective or cytotoxic effects depending on the numerous factors previously discussed, we hypothesized that there was a potential link after bioprocessing stress. Several recent studies by others have also begun detailing a potential link between resveratrol and ER stress.^{25,32,39–45} Interestingly, the literature from the last few years also contains data demonstrating either a protective role or a pro-apoptotic role for resveratrol, both of which are mediated through ER stress response signaling, similar to that found in this study. Several of these studies also point to the ER's ability to trigger apoptosis through the pro-apoptotic signaling of the UPR pathway as a potential mechanism for the specific anti-cancer effect of resveratrol.^{25,32,39,42}

The data presented in this study, along with that in the recent literature and in on-going human NHEP studies, demonstrate that ER stress and the subsequent UPR acts as an intermediary or "cell fate switch" that determines whether to trigger cell survival or a cell death response, depending on the environmental factors involved in cell storage. Our data show that, by modifying cellular stress

response pathways and the cell environment, it may be possible to target certain cells or tissues specifically to achieve a multitude of outcomes. Extending cell survival and function could allow for improvement in a number of areas ranging from transplantation to bioprocessing to the use of bioreactors. It is clear that the future of cell-based techniques, technologies, and therapies lies in a deeper understanding of the molecular pathways responsible for the cellular responses to the stresses of bioprocessing and biopreservation.

Author Disclosure Statement

Funding provided through NIH Grants: 5R43DK083800-02 and 1R43DK091952-01A1. No competing financial interests exist.

References

- Giri S, Bader A. Improved preclinical safety assessment using micro-BAL devices: The potential impact on human discovery and drug attrition. *Drug Discov Today* 2011;16:382–397.
- Hughes RD, Mityr RR, Dhawan A. Current status of hepatocyte transplantation. *Transplantation* 2012;93:342–347.
- Knobeloch D, Ehnert S, Schyschka L, et al. Human hepatocytes: Isolation, culture, and quality procedures. *Methods Mol Biol* 2012; 806:99–120.
- Sgroi A, Serre-Beinier V, Morel P, Buhler L. What clinical alternatives to whole liver transplantation? Current status of artificial devices and hepatocyte transplantation. *Transplantation* 2009;87:457–466.
- Kumar A, Tripathi A, Jain S. Extracorporeal bioartificial liver for treating acute liver diseases. *J Extra Corpor Technol* 2011;43:195–206.
- Yu Y, Fisher JE, Lillegard JB, et al. Cell therapies for liver diseases. *Liver Transpl* 2012;18:9–21.
- Baust JG. Concepts in biopreservation. In: *Advances in Biopreservation*, Baust JG, Baust JM, Eds. Boca Raton: CRC Press. 2007:1–14.
- Baust JM. Properties of cells and tissues influencing preservation outcome: Molecular basis of preservation-induced cell death. In: *Advances in Biopreservation*, Baust JG, Baust JM, Eds. Boca Raton: CRC Press. 2007:63–87.
- Baust JM, Snyder KK, Van Buskirk RG, Baust JG. Changing paradigms in biopreservation. *Biopreserv Biobank* 2009;7:3–12.
- Baust JM, Vogel MJ, Van Buskirk R, Baust JG. A molecular basis of cryopreservation failure and its modulation to improve cell survival. *Cell Transplant* 2001;10:561–571.
- Brockbank KGM, Taylor MJ. Tissue Preservation. In: *Advances in Biopreservation*. Baust JG, Baust JM, Eds. NY: Taylor & Francis. 2007:157–196.
- Cosentino LM, Corwin W, Baust JM, et al. Preliminary report: Evaluation of storage conditions and cryococktails during peripheral blood mononuclear cell cryopreservation. *Cell Preserv Technol* 2007;5:189–204.
- Taylor MJ. Biology of cell survival in the cold: The basis for biopreservation of tissues and Organs. In: *Advances in Biopreservation*, Baust JG, Baust JM, Eds. Boca Raton: CRC Press. 2007:15–62.
- Van Buskirk RG, Snyder KK, Baust JM, et al. Hypothermic storage and cryopreservation. The issues of successful short-term and long term preservation of cells and tissues. *Bio Process Intl* 2004;2:42–49.
- Corwin WL, Baust JM, Baust JG, et al. The unfolded protein response in human corneal endothelial cells following hypothermic storage: Implications of a novel stress pathway. *Cryobiology* 2011;63:46–55.
- Corwin WL, Baust JM, Vanbuskirk RG, et al. In vitro assessment of apoptosis and necrosis following cold storage in a human airway cell model. *Biopreserv Biobank* 2009;7:19–27.
- Gomez-Lechon MJ, Lahoz A, Jimenez N, et al. Evaluation of drug-metabolizing and functional competence of human hepatocytes incubated under hypothermia in different media for clinical infusion. *Cell Transplant* 2008;17:887–897.
- Li L, Li CM, Zhang BY, et al. Apoptosis of rat liver in cold preservation with custom-designed KYL solution. *Hepatobil Pancreat Dis Int* 2007;6:497–503.
- Vairetti M, Ferrigno A, Bertone R, et al. Apoptosis vs. necrosis: Glutathione-mediated cell death during rewarming of rat hepatocytes. *Biochim Biophys Acta* 2005;1740:367–374.
- Fu T, Guo D, Huang X, et al. Apoptosis occurs in isolated and banked primary mouse hepatocytes. *Cell Transplant* 2001;10: 59–66.
- Matsushita T, Yagi T, Hardin JA, et al. Apoptotic cell death and function of cryopreserved porcine hepatocytes in a bioartificial liver. *Cell Transplant* 2003;12:109–121.
- Han J, Zhong CQ, Zhang DW. Programmed necrosis: Backup to and competitor with apoptosis in the immune system. *Nat Immunol* 2011;12:1143–1149.
- Christofferson DE, Yuan J. Necroptosis as an alternative form of programmed cell death. *Curr Opin Cell Biol* 2010;22:263–268.
- Wu W, Liu P, Li J. Necroptosis: An emerging form of programmed cell death. *Crit Rev Oncol Hematol* 2011;82:249–258.
- Liu BQ, Gao YY, Niu XF, et al. Implication of unfolded protein response in resveratrol-induced inhibition of K562 cell proliferation. *Biochem Biophys Res Commun* 2010;391:778–782.
- Fullwood MJ, Zhou W, Shenolikar S. Targeting phosphorylation of eukaryotic initiation factor-2 α to treat human disease. *Prog Mol Biol Transl Sci* 2012; 106:75–106.
- Wu CT, Sheu ML, Tsai KS, et al. Salubrinal, an eIF2 α dephosphorylation inhibitor, enhances cisplatin-induced oxidative stress and nephrotoxicity in a mouse model. *Free Radic Biol Med* 2011;51:671–680.
- Kessel D. Protection of Bcl-2 by salubrinal. *Biochem Biophys Res Commun* 2006;346:1320–1323.
- Drexler HC. Synergistic apoptosis induction in leukemic cells by the phosphatase inhibitor salubrinal and proteasome inhibitors. *PLoS One* 2009;4:e4161.
- Zhang P, Hamamura K, Jiang C, et al. Salubrinal promotes healing of surgical wounds in rat femurs. *J Bone Miner Metab* 2012;30:568–579.
- Hetz C. The unfolded protein response: Controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol* 2012;13:89–102.
- Huang TT, Lin HC, Chen CC, et al. Resveratrol induces apoptosis of human nasopharyngeal carcinoma cells via activation of multiple apoptotic pathways. *J Cell Physiol* 2011;226:720–728.
- Dudley J, Das S, Mukherjee S, et al. Resveratrol, a unique phytoalexin present in red wine, delivers either survival signal or death signal to the ischemic myocardium depending on dose. *J Nutr Biochem* 2009;20:443–452.
- Hwang JT, Kwak DW, Lin SK, et al. Resveratrol induces apoptosis in chemoresistant cancer cells via modulation of AMPK signaling pathway. *Ann NY Acad Sci* 2007;1095:441–448.
- Lagouge M, Argmann C, Gerhart-Hines Z, et al. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1 α . *Cell* 2006;127: 1109–1122.
- Roy P, Kalra N, Prasad S, et al. Chemopreventive potential of resveratrol in mouse skin tumors through regulation of mitochondrial and PI3K/AKT signaling pathways. *Pharm Res* 2009 26:211–217.
- Shakibaei M, John T, Seifarth C, et al. Resveratrol inhibits IL-1 β beta-induced stimulation of caspase-3 and cleavage of PARP in human articular chondrocytes in vitro. *Ann NY Acad Sci* 2007;1095:554–563.

38. Trinchieri NF, Nicotra G, Follo C, et al. Resveratrol induces cell death in colorectal cancer cells by a novel pathway involving lysosomal cathepsin D. *Carcinogenesis* 2007;28:922–931.
39. Wang FM, Galson DL, Roodman GD, et al. Resveratrol triggers the pro-apoptotic endoplasmic reticulum stress response and represses pro-survival XBP1 signaling in human multiple myeloma cells. *Exp Hematol* 2011;39:999–1006.
40. Li C, Wang L, Huang K, et al. Endoplasmic reticulum stress in retinal vascular degeneration: Protective role of resveratrol. *Invest Ophthalmol Vis Sci* 2012;53:3241–3249.
41. Chinta SJ, Poksay KS, Kaundinya G, et al. Endoplasmic reticulum stress-induced cell death in dopaminergic cells: Effect of resveratrol. *J Mol Neurosci* 2009;39:157–168.
42. Park JW, Woo KJ, Lee JT, et al. Resveratrol induces pro-apoptotic endoplasmic reticulum stress in human colon cancer cells. *Oncol Rep* 2007;18:1269–1273.
43. Tabata Y, Takano K, Ito T, et al. Vaticanol B, a resveratrol tetramer, regulates endoplasmic reticulum stress and inflammation. *Am J Physiol Cell Physiol* 2007; 293 (1). C411–8.
44. Yoon DH, Kwon OY, Mang JY, et al. Protective potential of resveratrol against oxidative stress and apoptosis in Batten disease lymphoblast cells. *Biochem Biophys Res Commun* 2011;414: 49–52.
45. Um HJ, Bae JH, Park JW, et al. Differential effects of resveratrol and novel resveratrol derivative, HS-1793, on endoplasmic reticulum stress-mediated apoptosis and Akt inactivation. *Int J Oncol* 2010;36:1007–1013.

Address correspondence to:

John G. Baust, Ph.D.

UNESCO Professor and Director

Institute of Biomedical Technology

Science 3 Suite 144

State University of New York

Binghamton, New York 13902

E-mail: JBaust@Binghamton.edu