Critical factors in vitrification-based cryopreservation of precision-cut liver slices
Guan, Na
Chapter 5

Analysis of Gene Expression Changes to Elucidate the Mechanism of Chilling Injury in Precision-Cut Liver Slices

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Abstract

The exact mechanism of chilling injury (by a decrease of temperature to sub-physiological values), especially in the intact organ, is yet unknown. Precision-cut liver slices (PCLS), which closely resemble the organ from which they are derived, are an ideal in vitro model to study the mechanism of chilling injury in the intact organ.

In the present study we were able to separate chilling injury from other damaging events such as CPA toxicity and ice-crystal injury and performed micro-array analysis of regulated genes. Pathway analysis revealed that different stress responses, lipid/fatty acid and cholesterol biosynthesis and metabolism were affected by chilling. This indicates that the cell membrane might be the primary site and sensor for chilling, which may initiate and amplify downstream intracellular signaling events. Most importantly, we were able to identify gene expression responses from stellate cells and Kupffer cells suggesting the involvement of all liver cell types in the injury. In conclusion, a broad spectrum of previously unknown gene expression changes induced by chilling was identified in the tissue. This is the first report of a systematic investigation on the mechanism of chilling injury in integrated tissue by microarray analysis under conditions in which other sources of injury are minimal.

Keywords: Microarray analysis; chilling injury; cryoprotectants; cryoprotectant toxicity; tissue banking; cryopreservation.
Changes in Gene Expression due to Chilling

Introduction

Successful cryopreservation of cells and tissues is a prerequisite for the building of a tissue bank, facilitating the use of scarce human tissue for research and clinical purposes, and decreasing the use of laboratory animals. Vitrification (cryogenic preservation without ice crystal formation) has been postulated as a promising approach for cryopreservation of intact organs, tissues and cells [1-4]. However, although it avoids ice crystal damage, it is hampered by chilling injury, which refers to the injury due to the decrease of temperature per se in the absence of ice-crystal formation.

The mechanism of chilling injury has been studied in isolated cells [5, 6], but has only sparsely been investigated in intact integrated tissue [7]. These studies with isolated cells (oocytes and spermatozoa) suggested that chilling injury is induced at temperatures below the liquid-to-gel phase transition (LPT) and that it is partly due to the loss of selective permeability of the cell membrane [5, 6]. The relationship between the LPT temperature, membrane lipid composition and the resistance to cold has been extensively studied in oocytes and sperm cells. However, lipid phase transition (LPT) temperature of oocytes and spermatozoa occurs generally above 0 °C. Chilling injury was severe in bovine oocytes already at 16 °C [8]. In contrast to this, short-term (2-24 hrs) exposure of liver and kidney organ slices to 4 °C is not known to induce obvious damage [9, 10] and chilling injury increased gradually in the temperature range between 0 °C -80 °C ([1] and our unpublished results), which is much lower than the LPT temperature. Therefore, it is questionable whether the LPT could be directly responsible for the chilling injury observed during tissue cryopreservation.

Chilling injury occurs independently of ice formation at sub-zero temperatures, in contrast to freezing injury, where extracellular or intracellular ice formation is the major cause of damage. Cryopreservation of sperm and oocytes showed profound effects on cell membrane integrity [11], lipid composition (e.g. of cholesterol, phospholipids, polyunsaturated fatty acids and saturated fatty acids) [12-14], antioxidant enzymes (superoxide dismutase and glutathione peroxidase) [12, 15] and production of reactive oxygen species (ROS) [16]. However, it could not be concluded whether these phenomena were due to chilling injury or to other sources of injury such as CPA toxicity, the formation of ice crystals and osmotic stress. Moreover, to our knowledge nothing is known about the molecular mechanism that controls the adaption to stress caused by chilling after re-warming to physiological temperatures. Furthermore, research is lacking about the response of integrated tissue to chilling.

To understand the mechanism behind chilling injury, in the present study we aimed at separating it from other sources of damage such as osmotic damage, ice crystal formation and toxicity of cryoprotectants. An experiment was designed in which these factors were minimized. This we achieved by selecting low-toxicity mixtures of cryoprotectants that
prevent ice-crystal formation at temperatures as low as -40°C and choosing an appropriate chilling temperature (-15°C) and exposure time (10 min).

Precision-cut liver slices (PCLS) were used as a model to study the mechanisms of chilling injury in integrated tissue. PCLS contain all liver cell types and microstructures that are present in the tissue in vivo, and closely resemble the organ they are derived from. In addition, PCLS have been used as a tool to study the basic molecular mechanisms of drug toxicity [17-19]. PCLS were used to study the mechanism of damage by cryopreservation [20-22], using a fast freezing cryopreservation method. This data gives useful information on the mechanism of damage by fast freezing cryopreservation, but cannot separate damage induced by chilling injury per se from other damage by ice crystal formation and osmotic stress.

Changes at gene expression levels induced by chilling in the PCLS were studied by Affymetrix array. Our study revealed previously unknown early changes in gene expression and signaling pathways induced by chilling, including immunity and stress defense, cell cycle regulation, lipid/fatty acid synthesis and metabolism. Moreover, we observed specific changes in the expression of genes related to heat shock proteins and ribosome biosynthesis. Our findings enhance insight into the molecular basis of chilling injury and could facilitate development of successful vitrification methods by mechanistically-based interventions that avoid loss of cell homeostasis in tissues and organs due to chilling.

Materials and Methods

Chemicals and Reagents

Gentamicin and William’s medium E (WME) supplemented with Glutamax I were purchased from Gibco (Paisley, UK). D-glucose monohydrate was obtained from Sigma-Aldrich (St Louis, MO, USA). TransSend W solution and the proprietary (patented) premixed concentrated Sol Y and Sol Z were a gift from 21st Century Medicine, Fontana, California, USA and were commercially available. The ATP bioluminescence assay kit was purchased from Roche (Mannheim, Germany). All other chemicals were of the highest purity and were commercially available.

Animals and Preparation of Tissue Slices

Male Wistar rats (HsdCpb:WU) (300–350 g) purchased from Charles River (Maastricht, The Netherlands) were maintained under a 12-h light/dark cycle in a temperature- and humidity-controlled room with food (Harlan chow no. 2018, Horst, The Netherlands) and tap water ad libitum. The animal experimental protocol was approved by the Animal Ethical Committee of the University of Groningen.
After anesthetizing the rats with isoflurane/oxygen, the liver was first perfused with TransSend W perfusion medium cooled to 4 °C in situ via the vena cava before the rat was sacrificed and the liver was removed. PCLS were prepared as described previously [23]. In brief, cylindrical cores of liver tissue with a diameter of 5 mm were made by utilizing a hollow drill bit. Cores were sliced with a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) containing ice-cold Krebs-Henseleit saturated with a mixture of 95% oxygen and 5% CO₂. PCLS (about 200 µm thickness and 5 mg wet weight) were stored in ice-cold TransSend W until use.

**Preparation of CPA Working Solutions**

100% working solutions of Sol Y and Sol Z were prepared by adding pre-mixed concentrated CPA solutions to the carrier solution LM5 as suggested by the manufacturer [24]. The final concentrations of all constituents of the CPA solutions used for this study are indicated in Table 1. 100% CPA working solutions were further diluted with LM5 to create the loading step solutions or using LM5 containing 300 mM trehalose to create the unloading step solutions [25]. The composition of LM5 was as in [3, 25], but calcium and magnesium were omitted to avoid precipitation by cryoprotectants.

**CPA Loading/unloading and Induction of Chilling Injury**

Two CPA solutions were used, Sol Y and Sol Z (see table 1). To induce chilling injury, liver slices were first exposed to a series of increasing concentrations of these CPAs (from 0-100% working solution) for CPA loading according to the scheme in Table 2 and subsequently slices were placed in the CPA solution pre-chilled at -15°C and held at that temperature for 10 min (chilling groups: Sol Y+chilling and Sol Z+chilling) using a computer-controlled freezer (IceCube 14s, SY-LAB GmbH, Parkersdorf, Austria). After rewarming to 0°C, the CPAs were gradually removed from the slices by exposing them to a series of CPA solutions with decreasing concentrations (from 100% to 0% working solution). Throughout the CPA loading and unloading process, the slices were placed within 250 ml beakers, which were placed on ice and gently shaken.

To assess the effect of the CPA solutions, separate groups of PCLS (CPA groups: Sol Y and Sol Z) were loaded with the CPA solutions and subsequently unloaded, but not chilled at -15 °C for 10 min. PCLS that did not undergo loading and unloading with CPAs or chilling served as controls (control groups).

After CPA treatment or/and chilling at -15 °C, slices were incubated in 12-well plates (Greiner Bio-One, the Netherlands) under humidified carbogen atmosphere (95% O₂, 5% CO₂) in 1.3 ml WME, supplemented with 25 mM D-glucose and 50 µg/L gentamicin at 37°C under gentle shaking at 90 rpm as described previously (20). Incubation of control slices was started directly after slice preparation. After incubation for 3 hours tissue slices were collected for the determination of the ATP content or RNA isolation. Each experiment
was performed with 4 biological replicates, and within each experiment 4 slices were used per treatment.

Table 1. Composition of 100% working solutions of Sol Y and Sol Z

<table>
<thead>
<tr>
<th></th>
<th>Sol Y</th>
<th>Sol Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Me₂SO (g/L)</td>
<td>223.1</td>
<td>223.1</td>
</tr>
<tr>
<td>Formamide (g/L)</td>
<td>128.6</td>
<td>128.6</td>
</tr>
<tr>
<td>Ethylene glycol (g/L)</td>
<td>168.4</td>
<td>168.4</td>
</tr>
<tr>
<td>3-Methoxy,1,2-propanediol (g/L)</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Sucrose (g/L)</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td>Supercool X-1000* (g/L)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Supercool Z-1000* (g/L)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>5X LM5--* (mL/L)</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Total CPA Molarity</td>
<td>8.8314</td>
<td>8.890</td>
</tr>
</tbody>
</table>

*Patented products of 21st Century Medicine, Inc.

Table 2. Stepwise CPA introduction and removal

<table>
<thead>
<tr>
<th>CPA addition steps</th>
<th>CPA washout steps¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>25% CPA</td>
<td>15 min</td>
</tr>
<tr>
<td>50% CPA</td>
<td>15 min</td>
</tr>
<tr>
<td>75% CPA</td>
<td>15 min</td>
</tr>
<tr>
<td>100% CPA</td>
<td>10 min</td>
</tr>
<tr>
<td>100% CPA</td>
<td>5 min</td>
</tr>
<tr>
<td>75% CPA</td>
<td>10 min</td>
</tr>
<tr>
<td>50% CPA</td>
<td>10 min</td>
</tr>
<tr>
<td>25% CPA</td>
<td>10 min</td>
</tr>
<tr>
<td>LM5</td>
<td>10 min</td>
</tr>
</tbody>
</table>

¹Trehalose was present at a concentration of 300 mM in all of these washout steps.

ATP Determination

Viability of slices from each treatment group was determined by ATP content. For ATP determination, slices were collected individually in 1 mL 70% ethanol / 2 mM EDTA (pH 10.9), snap-frozen in liquid nitrogen and stored at -80 °C until analysis. After homogenization using a Mini-BeadBeater-8 (BioSpec, Bartlesville, OK, USA), the slices were centrifuged at 13,200 rpm for 5 min and the ATP level in the supernatant was determined using the ATP Bioluminescence assay kit CLS II (Roche diagnostics, Mannheim, Germany) according to the manufacturer’s protocol.

DSC Measurement
A Perkin Elmer Pyris 1 differential scanning calorimeter (DSC) equipped in a programmable cryogenic unit (Consarctic, Gottingen, Germany) was used to monitor crystallization in the slices during cooling to -60 °C and warming [23]. Sol Y and Sol Z themselves do not crystallize during cooling to -60°C and warming. Thermograms of the slices impregnated with CPA were recorded from 4°C to -60 °C at a cooling rate of 60 °C/min, holding at -40 °C for 12 min and from -60 °C to 4°C a warming rate of 5 °C/min.

RNA Isolation and Microarray

Four liver slices from the same treatment group in each experiment were collected in one eppendorf tube and snap-frozen in liquid nitrogen. Total RNA was extracted from the liver slices with TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Then the extracted RNA was purified with Purelink RNA Micro Kit Reagents (Invitrogen) following the manufacturer’s instructions. The purity and concentration of RNA were measured by using a NanoDrop ND-1000 Spectrophotometer ((Isogen Life Science, Isogen IJsselstein, the Netherlands). The RNA Integrity Number (RIN) as a measure for RNA integrity was subsequently assessed by an Agilent 2100 Bioanalyzer and the RNA 6000 Nano kit (Agilent Technologies, Inc., CA, USA). The RIN of all samples was greater than 8.0. cDNA synthesis and hybridization were conducted as described previously [18]. The Rat Genome 230 2.0 Array containing 31099 probe sets was used to assess mRNA expression.

Data Processing and Analysis

Data normalization was done by the RMA method on a NuGO blackbox (see http://nbx1.nugo.org) [26] and by applying a correction for biological variation between samples. In brief, the geometric mean of intensity values among all samples from the same experiment was calculated for each probe set and intensity values of each sample were then determined relative to the geometric mean. Statistical analysis was performed with a NuGO blackbox using the Limma R-package [27]. Within the Limma package statistical analysis was performed and different treatment groups were compared which resulted in values for Fold Change (FC), P value and false discovery rate (FDR). Differentially expressed genes were defined as FDR <0.05. All data will be uploaded on Gene Expression Omnibus not later than 4 months after publication.

Principal Component Analysis

Principal component analysis was conducted with ArrayTrack 3.5.0 (NCTR/FDA, AR) (http://www.fda.gov/nctr/science/centers/toxicoinformatics/ArrayTrack/) [28] with the normalized data, and results were exported to Spotfire (Spotfire AB, Göteborg, Sweden) for enhanced visualization. PCA was calculated for the regulated genes based on multigroup comparison of all treatments and using a False Discovery Rate (FDR) value of 0.05.
Pathway analysis

Metacore™ (Version 6.9) (GeneGo, St. Joseph, MI, US) and PathVisio 2.0.11 was used to perform pathway analysis. PathVisio 2.0.11 was used to visualize the pathway of cholesterol biosynthesis. The rat gene database of November 2010 and the rat pathways of September 2010 were used.

Results

Influence of CPAs and Chilling on the Viability of PCLS Indicated by ATP Levels

As shown in Fig. 1, both Sol Y and Sol Z appeared to be non-toxic since the ATP content of liver slices treated with CPA was >95% of control slices. After cooling the slices to -15 °C for 10 min., rewarming and subsequent incubation at 37 °C for 3 h, the ATP content was decreased by 20-30% compared to that of slices that only underwent CPA loading/unloading, indicating moderate chilling injury. This temperature and time schedule were selected after a set of pilot experiments, to induce 20-30% ATP decrease (data not shown). By limiting the extent of chilling injury to this level we intended to avoid massive necrosis or apoptosis in the slices, which would impede identification of more subtle initial changes in gene expression that are caused by chilling.

![Graph showing ATP content of liver slices](image)

**Figure 1.** ATP content of liver slices after treatment with CPA (Sol Y or Sol Z) or CPA + chilling at -15 °C, relative to slices incubated in WME medium directly after slicing (n=4). Data are expressed as Mean± SD for values obtained with slices from four different livers, **p<0.01 when compared with its CPA solution control.

Differential scanning calorimetry revealed that in slices loaded with Sol Y or Sol Z ice crystal formation was totally prevented until -40 °C, indicating that there was no damage induced by ice crystal formation in the slices (Figure 2).
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Figure 2. DSC thermograms of (A) Sol Y and (B) Sol Z cooling from 4 °C to -40 °C, holding at -40 °C for 12 min and then warming up to 4 °C.

Principal Component Analysis (PCA)

Gene expression profiles of the precision-cut liver slices chilled at -15 °C were determined by the Affimetrix whole rat genome array. Five experimental groups were analyzed: one control group, two CPA controls (slices treated with Sol Y and Sol Z respectively) and two chilling groups (Sol Y + chilling and Sol Z + chilling, chilled after CPA treatment with Sol Y or Sol Z respectively) with each group containing four biological replicates.

Principal Component Analysis was used to visualize the gene expression profiles of the regulated genes in the samples. The PCA plot in Fig. 3 based on the 3292 genes that were significantly regulated (FDR<0.05) after a multiple group comparison, shows that samples with similar treatment (control, CPA treatment or CPA + chilling) tended to cluster together. There were minimal differences between the two CPAs, Sol Y and Sol Z, both in the CPA treatment groups and in the chilling groups. Although viability based on ATP was not affected by loading and unloading with Sol Y and Sol Z, PCA indicates that the CPA treatment induced considerable changes in gene expression as compared to the controls as indicated in Fig. 3. However, additional gene expression changes induced by chilling could be clearly separated from those induced by the CPAs.
Effect of Chilling on Gene Expression

To increase the statistical power, contrast analysis was performed after combining samples loaded with Sol Y and Sol Z (n=8) from the same treatment since the PCA analysis showed similar profiles of gene expression induced by the two CPA solutions. The total number of genes that was changed significantly (FDR ≤ 0.05) due to the different treatments is shown in Table 3.

By comparing the chilling group to the CPA control group, it was found that 1108 genes were changed significantly (FDR<0.05) due to chilling, among which 30 genes were up-regulated with a FC>1.5, and only 6 genes were down-regulated with a FC<-1.5. With a lower stringency for the FC cut-off (FC>1.2), 251 genes were up-regulated and 77 genes were down-regulated, indicating that most of the gene expression changes due to chilling were small but statistically significant.

The effect of CPA treatment alone was determined by comparing the CPA treated control groups to the controls. As shown in Table 3, CPA treatment induced profound changes in gene expression with 1985 genes significantly changed despite the maintenance of viability determined by ATP. Since the effect of the CPAs is not within the scope of this paper, we mainly focus on the gene expression changes only due to chilling. Although the expression of 5S rRNA, a component of 60 S ribosomal subunit, usually is considered as...
one of the housekeeping genes within the array, our results indicated that it was one of the most highly up-regulated genes (FC= 2.58) by chilling (Table 5).

Table 3. Number of differentially expressed genes by CPA treatment or CPA treatment followed by chilling.

<table>
<thead>
<tr>
<th></th>
<th>CPA vs control</th>
<th>CPA chilled vs control</th>
<th>CPA chilled vs CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDR ≤ 0.05</td>
<td>1985</td>
<td>3405</td>
<td>1108</td>
</tr>
<tr>
<td>FDR ≤ 0.05 &amp; FC ≥ 1.2</td>
<td>526</td>
<td>965</td>
<td>251</td>
</tr>
<tr>
<td>FDR ≤ 0.05 &amp; FC ≥ 1.5</td>
<td>92</td>
<td>221</td>
<td>31</td>
</tr>
<tr>
<td>FDR ≤ 0.05 &amp; FC ≥ 1.2</td>
<td>213</td>
<td>547</td>
<td>77</td>
</tr>
<tr>
<td>FDR ≤ 0.05 &amp; FC ≤ -1.5</td>
<td>49</td>
<td>196</td>
<td>6</td>
</tr>
</tbody>
</table>

Note: *FDR refers to False Discovery Rate, FC refers to Fold Change.

The significantly regulated genes were calculated by combining the data from Sol Y and Sol Z from 4 biological replicates.

Effect of Chilling on Gene Expression of Heat Shock Proteins (HSPs) and Heat Shock Factors (HSFs)

It is generally believed that chilling can cause protein denaturation and misaggregation [29]. As expected, genes encoding for heat-shock proteins were up-regulated due to chilling as listed in Table 4. Interestingly, the expression of α Crystallin B (Cryab), which is also a marker for the activation of hepatic stellate cells [30] and HSP40 (Dnajb1) was not induced by CPA treatment but only by chilling. Hspb6, Hspb11, Dnajc12 and Hspa4 were only induced by CPA treatment but not further changed by chilling.

Also HSF-1 (Heat Shock factor-1), one of the transcriptional activators of HSP genes, was only up-regulated due to chilling but not by CPA treatment alone. Interestingly, Cirp (cold-inducible RNA-binding protein) and Rbm3 (RNA binding motif protein 3), two cold shock proteins identified in mammalian cells were not changed due to chilling. Rbm3 was down-regulated due to CPA treatment (FC= -1.9).

Hmox-1 (HSP32) was down-regulated after CPA treatment and was induced subsequently due to chilling, but did not reach the basal level. Up-regulation of Hmox-1 is considered to be one of the major defense mechanisms against oxidative stress and tissue injury [31], and it functions more as an antioxidant protein than as a molecular chaperone. Some studies showed that oxidative effects were observed during cryopreservation due to lipid peroxidation [15].

Table 4. Differentially regulated HSP and HSF genes.
**Effect of Chilling on Genes Encoding for RNA and for Proteins and Regulators involved in Ribosome Biosynthesis**

As in Table 5, 5S rRNA, generally considered as a house keeping gene, was up-regulated after chilling. Unfortunately other ribosomal RNAs (18s rRNA and 28s rRNA) are not present in the Rat Genome 230 2.0 Array. The expression of C-Myc, the regulator of ribosome biogenesis and protein synthesis [32], was also increased only after chilling. Other ribosomal proteins or regulatory factors controlling rRNA maturation were further up-regulated after chilling but the increases were not significant.

**Other Genes related to Stress Responses**

mRNA expression of Ddit 3 (FC=1.54), DNA damage inducible transcript 3 and Gadd45b (FC:1.38), growth arrest and DNA-damage-inducible gene, indicative of DNA damage or stressful conditions and induction of cell cycle arrest [33, 34], was increased.

**Pathway Analysis**

The most affected pathways were identified by analyzing the genes that were significantly regulated due to chilling (FDR<0.05 with FC >1.2 or FC<-1.2) with Metacore. The top 10 up- or down-regulated pathways are listed in Table 6.

The observed changes in up-regulated pathways covered a broad spectrum of cellular activities including immune responses, cell cycle regulation and MAPkinase signaling. Several genes in the MAP Kinase family were significantly regulated by chilling, amongst others C-Jun (FC=1.27) and C-Fos (FC=1.58). In contrast, two genes of the p38 family: MAPK14 (P38alpha) (FC: -1.25) and MAPK3 (FC: -1.21) were down-regulated due to...
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CPA exposure and their expression was not further changed due to chilling. Both C-Jun and C-Fos are genes that are activated transiently and rapidly in response to a wide variety of cellular stimuli and are major components of Activator Protein-1 (AP-1), which is an important transcription factor controlling a variety of cellular processes (e.g. differentiation, proliferation and apoptosis). AP-1 is involved in the regulation of G1/S cell transition (Table 6A pathway 1), or cellular metabolism (Table 6A pathway 9), IL1 and IL22-signaling (Table 6A pathway 3 and 8).

**Table 5.** Differentially regulated genes encoding for RNA and proteins involved in ribosome biosynthesis.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>3/2*</th>
<th>2/1*</th>
<th>3/1*</th>
</tr>
</thead>
<tbody>
<tr>
<td>rRNA</td>
<td>Taf1d TATA box binding protein (TBP)-associated factor, RNA polymerase I, D, 41kDa</td>
<td>1.23</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5S rRNA</td>
<td>5S ribosomal RNA</td>
<td>2.58</td>
<td>1.72</td>
<td>4.47</td>
</tr>
<tr>
<td>Rrp8</td>
<td>ribosomal RNA processing 8, methyltransferase, homolog (yeast)</td>
<td>—</td>
<td>1.18</td>
<td>1.21</td>
</tr>
<tr>
<td>Regulatory genes</td>
<td>Taf5 TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor</td>
<td>—</td>
<td>1.54</td>
<td>1.77</td>
</tr>
<tr>
<td></td>
<td>Rpap2 RNA polymerase II associated protein 2 TAF13 RNA</td>
<td>—</td>
<td>1.32</td>
<td>1.47</td>
</tr>
<tr>
<td></td>
<td>Taf13 polymerase II, TATA box binding protein (TBP)-associated factor</td>
<td>—</td>
<td>—</td>
<td>1.21</td>
</tr>
<tr>
<td>Ribosomal</td>
<td>Mrpl47 mitochondrial ribosomal protein L47</td>
<td>—</td>
<td>1.54</td>
<td>1.64</td>
</tr>
<tr>
<td>Proteins</td>
<td>Mrpl49 mitochondrial ribosomal protein L49</td>
<td>—</td>
<td>1.28</td>
<td>1.21</td>
</tr>
<tr>
<td>Regulator</td>
<td>Mrp63 mitochondrial ribosomal protein 63</td>
<td>—</td>
<td>1.14</td>
<td>1.23</td>
</tr>
<tr>
<td>C-Myc</td>
<td>myelocytomatosis oncogene</td>
<td>1.32</td>
<td>—</td>
<td>1.43</td>
</tr>
</tbody>
</table>

*FDR=0.06

It is interesting to see that G1/S transition of the cell cycle was both positively and negatively regulated (Table 5A, Pathway 1). CDK6 (FC=1.23), p21 (FC=1.21), p27KIP1 (FC=1.25) and C-Myc (FC=1.32) were all up-regulated by chilling. CDK6 positively regulates the G1/S transition [35], while, on the other hand, p21 and p27KIP1 inhibit cell cycle progression.

The highest ranked down-regulated pathways are related to cholesterol transport, unsaturated fatty acid biosynthesis and fatty acid oxidation and lipid metabolism (Table 6B). The most strongly regulated pathway (“FXR-regulated cholesterol and bile acids cellular transport”) includes the expression of Abcb1a (Mdr1a) that was increased by chilling (FC=1.33), and the expression of Abcg5 (FC=1.65) and Abcg8 (FC=1.52) that was decreased by chilling.

As shown in Fig. 4, mRNA levels of 5 genes encoding for enzymes (Dhcr7, Sc5d1, Sc4mo1, Cyp51 and Lss) in the rattus norvegicus cholesterol biosynthesis pathway were significantly decreased, while the expression of HMG CoA reductase (Hmgcr), the rate
controlling enzyme in the cholesterol biosynthesis pathway was up-regulated significantly (FDR<0.05).

Table 6. Significantly altered pathways due to chilling at -15 °C for 10 min.

<table>
<thead>
<tr>
<th>#</th>
<th>Pathway Maps</th>
<th>Ratio</th>
<th>pValue</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Cell cycle_ESR1 regulation of G1/S transition</td>
<td>7/33</td>
<td>8.747E-09</td>
</tr>
<tr>
<td>2</td>
<td>Transcription_Role of VDR in regulation of genes involved in osteoporosis</td>
<td>8/61</td>
<td>3.868E-08</td>
</tr>
<tr>
<td>3</td>
<td>Immune response_IL-1 signaling pathway</td>
<td>7/44</td>
<td>7.284E-08</td>
</tr>
<tr>
<td>4</td>
<td>Immune response_Antigen presentation by MHC class II</td>
<td>4/12</td>
<td>2.292E-06</td>
</tr>
<tr>
<td>5</td>
<td>Development_TGF-beta-dependent induction of EMT via MAPK</td>
<td>6/47</td>
<td>2.544E-06</td>
</tr>
<tr>
<td>6</td>
<td>Cell cycle_Influence of Ras and Rho proteins on G1/S Transition</td>
<td>6/53</td>
<td>5.225E-06</td>
</tr>
<tr>
<td>7</td>
<td>Development_WNT signaling pathway. Part 2</td>
<td>6/53</td>
<td>5.225E-06</td>
</tr>
<tr>
<td>8</td>
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<td>Peroxisomal branched chain fatty acid oxidation</td>
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*aRatio: Number of genes significantly altered in the pathway compared to the total number of genes in the pathway.
Changes in Gene Expression due to Chilling

Figure 4. Cholesterol Biosynthesis. Gene expression data mapped on the Rattus norvegicus cholesterol biosynthesis pathway. Fold changes are represented as a gradient and visualized per gene in the box on the left. Red boxes on the right indicate a significance of FDR<0.05. Light green boxes on the right indicate not significant. The dark gray boxes with red lining represent the genes that were not detected by microarray analysis.

Discussion

Studies on the mechanism of chilling injury during cryopreservation have been hampered by concurrent damaging events such as CPA toxicity, ice-crystal injury and osmotic stress. In order to minimize or even avoid the effect of CPA toxicity and of ice-crystal formation, Sol Y and Sol Z were selected from a number of CPA solutions provided by 21st Century Medicine based on their low toxicity (> 95% recovery of ATP) and ability to prevent ice formation at temperatures as low as -40 °C. Moderate chilling injury was induced by holding PCLS loaded with CPA at -15 °C for 10 min, inducing a decrease in ATP content of approximately 25% after removing the CPA and incubation for 3 h at 37 °C.

PCA analysis of the data obtained by microarray analysis indicated that the chilling effect was clearly distinct from the effect of CPA treatment, which shows that the gene expression changes due to chilling per se can be identified.

The analysis of significantly regulated genes indicated up-regulation of genes encoding for heat shock proteins and heat shock factors. It is generally accepted that heat shock
proteins are induced in response to stress and that some of them function as chaperones by
binding to denatured proteins, thereby preventing their aggregation and assisting in their
refolding into native, functional conformations [36]. The observed up-regulation of heat
shock protein expression may thus be an indication that chilling indeed causes protein
denaturation. The reason for the unchanged expression of two genes (Crip and Rbm3)
encoding cold-shock protein might be explained by the fact that they were identified at by
cooling down mammalian cells to sub-physiological temperature such as 32 °C but above 0
°C. [37]. The expression changes have never been studied at cooling down to subzero
temperature, which is the general case for cryopreservation experiment. Our results
indicated that cellular machinery might respond differently to cold stress at different
temperature.

One of the most interesting up-regulated genes was 5S rRNA which is thought to
enhance protein synthesis by stabilization of the ribosome structure. Interestingly, an
additional study using RT-PCR showed that 18S rRNA was also up-regulated due to
chilling (manuscript in preparation). Although rRNAs were shown to be induced during
liver regeneration [38], the response of ribosomal genes to chilling stress has not been
published before to the best of our knowledge. Recently, Quinn [39] observed that
ribosomal genes were significantly up-regulated by heat stress in the Arctic char and they
suggested that ribosome biogenesis was activated during recovery from heat stress. The
transcription of the gene for 5S rRNA is directly activated by Myc, which was up-regulated
due to chilling, via interaction with the RNA pol III-specific factor TFIIB [40]. In addition,
Myc globally regulates ribosomal biogenesis through a multitude of mechanisms including
direct up-regulation of ribosomal RNA synthesis, ribosomal protein components and
proteins required for the processing of rRNA [32]. All together, these results suggest that
ribosomal biosynthesis is enhanced after chilling, which may lead to enhanced biosynthesis
of proteins required after protein denaturation by chilling. Other ribosomal proteins or
regulatory factors controlling rRNA maturation were induced by CPA treatment and not
further induced due to chilling. We may speculate that the CPA treatment induced these
genes to such an extent that the need for the enhanced ribosomal biosynthesis after chilling
was already fulfilled.

As expected, Metacore analysis showed that apoptosis related pathways are not in the
top 10 up-regulated pathways, which indicates that cell death was not a dominant event in
this experimental set-up for enabling the discovery of early events of chilling injury that
occur before massive cell death.

The up-regulated pathways were indicative of stress responses. Genes and signaling
pathways including immunity and defense, such as cytokine production (IL1) and antigen
presentation by MHC class II were up-regulated by chilling.

In addition to activation of inflammatory genes, it is well recognized that cellular stress
leads to activation of the stress related MAP Kinase signaling pathway. Activators of MAP
Kinase include heat [41], osmotic stress, and freezing and thawing [42]. The MAPK superfamily consists of three main signaling pathways: C-Jun N-terminal Kinases (JNKs), the extracellular signal-regulated protein kinases (ERKs) and p38 family kinases. The three MAP kinase pathways appeared to respond differently to chilling. As shown in Fig. 5, the expression of C-Jun was up-regulated by chilling. The JNK pathway is a major route for regulation of C-Jun, which can form a complex with C-Fos, to form AP-1. Since the P38 MAPK pathway was not changed by chilling, the JNKs signaling pathway is probably responsible for the chilling-induced up-regulation of C-Jun. Additionally, activation of ERK might be responsible for the found up-regulation of C-Fos [43].

The cell cycle at G1/S transition was both positively and negatively affected. A possible explanation for this ambivalent finding could be that cell proliferation was arrested in one cell type due to DNA damage (Ddit3) and was initiated in another cell type.

Pathway analysis showed significant changes in cholesterol and lipid homeostasis as a result of chilling. It has been reported that after cryopreservation of boar sperm the cholesterol content was decreased while the total lipid content was increased [12]. Cholesterol is mainly present in the plasma membrane playing an important role in maintaining its integrity and fluidity [44]. Changes in cholesterol content during vitrification were suggested to exert profound effects on the cell membrane and cell physiology [45]. The micro-array results as obtained in the present study provide several clues for the regulatory mechanism behind the cellular cholesterol management induced by chilling.

First of all, 5 genes encoding for enzymes involved in the cholesterol biosynthesis were down-regulated after chilling (Fig 4), which may induce a reduction in cholesterol content in the cell. In line with this, the expression of HMG CoA reductase known to be negatively regulated by cholesterol content [46] is increased. Secondly, the expression of mdr1a was increased. Although multidrug-resistant P-glycoprotein (MDR) is mainly known as a drug transporter, it also has been suggested as being a sensor for cholesterol content in the plasma membrane [47], and is required for the cholesterol trafficking from the plasma membrane to the endoplasmic reticulum [48]. Increased expression of Mdr1a may be indicative for an increased need of the cell for cholesterol. Furthermore, the expression level of Abcg5 and Abcg8 was decreased due to chilling. Abcg5 and Abcg8 are heteromeric ATP binging cassette (ABC) transport proteins and largely mediate the biliary secretion of cholesterol [49]. The decrease in hepatic mRNA expression of Abcg5 and Abcg8 was shown to correlate with a decrease in biliary cholesterol secretion rates in mice [50] and with an increase of cholesterol levels in the liver [51]. Therefore, it may be speculated that the significantly decreased mRNA expression of Abcg5/Abcg8 is a defensive/protective response of PCLS to the reduced sterol content after chilling [52].
Figure 5. Changes in expression of genes in the Metacore™ pathway “Development_TGF-beta-dependent induction of EMT via MAPK”. Differentially expressed genes are indicated by thermometers, Red=up-regulated. Blue=down-regulated. 1=chilling compared to CPA, 2=chilling compared to control and 3=CPA compared to control.

Our results indicated that both peroxisome proliferator-activated receptor regulated lipid metabolism, fatty acid oxidation, and polyunsaturated fatty acid (PUFA) biosynthesis (Table 6B, pathway 8-10) were decreased. The net results would be a decrease in the percentage of PUFA and accumulation of saturated fatty acid. Similar results have been found in boar semen cryopreservation [12]: the lipid composition of boar semen was changed as a result of an increase of the total lipid content and a decreased proportion of PUFA, whereas a significantly reduced amount of cholesterol was found after cryopreservation [12]. Beirao et. al. reported that the viability after cryopreservation was positively related to the amount of unsaturated fatty acid of the sperm of a teleost, Sparus aurata [53].
Inhibition of fatty acid oxidation could also be one of the factors determining the decreased ATP content induced by chilling. The carnitine palmitoyltransferase 1A (cpt1A), essential for fatty acid oxidation, was down-regulated, causing the long-chain fatty acids available for mitochondrial ATP production to be reduced.

In studies on cryopreservation of human sperm [15] and bovine spermatozoa [54], oxidative stress was observed due to lipid peroxidation during cryopreservation. Those experiments used the conventional slow freezing method for cryopreservation and were not able to identify the cause of lipid peroxidation: chilling or ice crystal formation. The present study did not show a response of any of the genes that are believed to respond to oxidative stress due to chilling except for Hmox 1.

In conclusion, a broad spectrum of changes in different cell types in the tissue at the gene expression level was identified that was specifically due to chilling after successfully separating chilling injury from other damaging events. We found that chilling triggered stress responses in PCLS including induction of heat shock proteins, cell-cycle G1/S regulation, reduction of cholesterol synthesis, increase of protein synthesis and decrease of fatty acid oxidation. We speculate that these changes reflect membrane damage, protein denaturation, and possible DNA induced by chilling. Our data supports earlier findings that the cell membrane is the primary site and sensor for chilling as it undergoes changes in the lipid composition, fluidity and protein structure, thereby initiating and amplifying downstream intracellular signaling events. Most importantly, none of genes encoding for CYP450 and other metalizing enzymes was significantly expressed. This result is reasonable and indicates the initial changes by chilling do not involve the regulation of genes encoding metabolizing enzymes, which is good news for using the cryopreserved slices in drug metabolism and toxicology study.

This is the first effort to investigate the mechanism of chilling injury in integrated tissue by using microarray analysis to identify gene expression changes specific to chilling under conditions in which other sources of injury are absent based on classical measures of injury. It not only starts to disclose the secret of chilling injury but also potentially provides indications for mechanism-based interventions in order to improve the vitrification outcome of cells, tissues and organs. Moreover our study might give possible scientific explanations to the observations (e.g. lipid content composition changes) during cryopreservation of reproductive cells.

The findings reported here, of course, represent only a first step in understanding the full gamut of chilling injury. Additional studies are needed to investigate, for example, the effects of chilling at lower temperatures and/or longer exposure times. An integrative approach including changes at the protein level and the function and interactions of the key elements in a temporal fashion is also required to fully understand the cell physiology of chilling injury.
Chapter 5

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Conflict of Interest statement

The authors, N.G., S.A.B., G.M.M.G., and I.A.M.de G declare that there are no conflicts of interest. G.M. Fahy is employed by 21st Century Medicine, Inc., which sells related vitrification solution and ice blockers, and is the designer of Sol Y, Sol Z, LM5, and TransSend W.

References

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