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Proteome variation of the rat liver after static cold storage assayed in an *ex vivo* model

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ABSTRACT

Cold storage is a common procedure for liver preservation in a transplant setting. However, during cold ischemia, the liver suffers molecular alterations that can affect its performance. Also, deleterious mechanisms set forth in the storage phase are exacerbated during reperfusion. This study aimed to identify liver proteins associated with injury during cold storage and/or normothermic reperfusion using the isolated perfused rat liver model. Livers from male rats were subjected to either (1) cold storage for 24 h, (2) *ex vivo* normothermic reperfusion for 90 min or (3) cold storage for 24 h followed by *ex vivo* normothermic reperfusion for 90 min. Then, the livers were homogenized and proteins were extracted. Protein expression between each experimental group and the control (freshly resected livers) was compared by two-dimensional (2D) gel electrophoresis. Protein identification was carried out by matrix-assisted laser desorption/ionization time-of-flight spectrometry (MALDI-TOF/TOF) using MASCOT as the search engine. 23 proteins were detected with significantly altered levels of expression among the different treatments, including molecular chaperones, antioxidant enzymes, and proteins involved in energy metabolism. Some of them have been postulated as biomarkers for liver damage while others had been identified in other organs subjected to ischemia and reperfusion injury. The whole data set will be a useful resource for studying deleterious molecular mechanisms that result in diminished liver function during storage and subsequent reperfusion.

1. Introduction

Liver transplantation is a well-recognized treatment for end-stage liver disease and acute liver failure. Preservation of the organ after resection is vital to avoid its irreversible damage. Resection from the donor circulatory system exposes the liver to an ischemic period, in which cellular and molecular phenomena are triggered [19]. As the availability of oxygen is reduced, electron flow through the mitochondrial respiratory chain is interrupted. The lack of oxidative phosphorylation rapidly leads to ATP depletion. In turn, the ATP-dependent sodium/potassium plasma membrane pump is impaired leading to an ionic imbalance and malfunction of transport processes [13]. These events may result in apoptosis or necrosis of hepatocytes, causing liver dysfunction [11].

Preservation methods ameliorate these effects. The most common procedure for liver preservation is static cold storage (SCS) [5,25]. SCS consists of flushing and immersing the liver in a refrigerated preservation solution, which includes energy precursors and other additives. Examples are University of Wisconsin solution, histidine-tryptophan-ketoglutarate (HTK) and Celsior solution [2,25]. Low temperatures reduce metabolic activity, effectively increasing the lifespan of the organ [28,42]. However, after a period of hours, cold

Abbreviations: COMT, catechol-O-methyltransferase; cytokeratin 8, K8; EST, estrogen sulfotransferase; hnRNP K, heterogeneous nuclear ribonucleoprotein K; HTK, histidine-tryptophan-ketoglutarate; IPRL, isolated perfused rat liver; IRI, ischemia/reperfusion injury; L-PK, liver-type pyruvate kinase; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; NDRG2, N-myc downstream-regulated gene 2; SCS, static cold storage; SD, standard deviation; two-dimensional, 2D.

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ischemic injury inevitably occurs which predominantly impacts on the sinusoidal milieu [8]. This damage is a significant determinant of liver failure upon reperfusion [34] and imposes an upper limit on the time of cold storage. In humans, it has been documented that SCS for 12–24 h is a risk factor for graft function and patient survival [1,15]. Rat livers can be stored at low temperatures and transplanted after an average time of 18–24 h with positive outcomes ([6] and references therein). Longer storage times (> 48 h) produce marginal results and past 72 h of storage, the success rate of transplantation rates drop to 0 [10]. Even though the 24 h-preserved rat liver is suitable for transplantation, a number of metabolic functions are impaired, especially in mitochondria. As a consequence, ATP content is severely reduced to 4–50% of its original level [10,23,30]. Also, a gradual loss in activity of substrate-linked respiration has been reported for isolated hepatic mitochondria. This has been related to a time-dependent loss of enzyme complexes of the electron transport chain during cold ischemia [38]. The amount of viable hepatocytes decline over storage time, however, this reduction is not significant in the first 24 h of SCS [6]. Furthermore, gluconeogenic and ureogenic activities are well maintained [30]. Overall, 24 h of cold storage is the accepted upper limit for successful transplantation of the rat liver, as the organ can recover after the procedure.

One way to study the impact of preservation strategies on the procured liver is to connect the organ to an *ex vivo* perfusion machine. In the isolated perfused rat liver (IPRL) model, a refluxing device delivers a reperfusion solution that provides a continuous supply of oxygen, nutrients, and antioxidants at a controlled temperature [7,14]. The IPRL model simulates the reperfusion process that occurs after transplantation. Oxygen reentry causes a burst of toxic reactive oxygen species by uncoupled mitochondria, leading to significant mitochondrial damage and ultimately, cell death. This process, known as ischemia/reperfusion injury (IRI), also includes energy depletion, lipid deposition, ionic imbalance, disruption of cellular architecture, and a decrease in protein synthesis, among many others [26].

In this work, rat livers were stored for 24 h at 4 °C in HTK solution and then, placed in an *ex vivo* reperfusion system for 90 min at 37 °C. A cell-free solution (i.e., absence of blood components) was used to analyze the effect of reperfusion. Variations of the proteome liver by SCS, normothermic reperfusion and their combination were analyzed by 2D gel electrophoresis and relevant proteins were identified by MALDI-TOF. The proteins identified in this work will provide important information for understanding molecular events set forth during cold storage in more depth.

2. Materials and methods

2.1. Animals

Male Wistar rats (250–350 g) were obtained from the Central Animal Building at the Biochemistry and Pharmaceutical Sciences Faculty of the National University of Rosario, Argentina. They were acclimated for 24–48 h in the facilities of CAIC at room temperature (25 °C) and under a 12 h day-night cycle until the moment of surgery. Water and food pellets were supplied *ad libitum*.

The animals were anesthetized with chloral hydrate (Parafarm, Argentina) (500 mg/kg, intraperitoneal). The abdomen was opened by a midline incision and the liver was freed from all ligamentous attachments. In brief, 0.1 mL of saline containing 1000 UI of heparin (Sobrius, Argentina) was injected into the femoral vein, the bile duct was cannulated with a PE-50 catheter (Intramedic, USA) and a 14 G Abbocath catheter was introduced and fixed in the portal vein. Perfusion with Krebs Henseleit buffer (118.0 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 5.0 mM glucose, 2.0 UI/mL heparin) was started immediately at 15 mL/min, the suprahepatic inferior vena cava was cannulated with steel tubing (internal diameter 3 mm) and, after blood wash out, the liver was carefully removed without stopping the perfusion [36]. All protocols were performed according to guidelines for care and use of laboratory animals approved by CONICET and the Bioethics Committee of the Faculty, which are in concordance with international regulations (EU Directive 2010/63/EU).

2.2. Experimental design

The liver samples were obtained from four experimental groups (n = 4 in each group) as follows:

- Control: Sample collection immediately after surgical removal.
- SCS group: Sample collection after 24 h of cold storage in HTK solution at 4 °C.
- IPRL group: Sample collection after 90 min of normothermic *ex vivo* reperfusion.
- SCS + IPRL group: Sample collection after cold storage of livers for 24 h in HTK solution at 4 °C followed by 90 min of normothermic *ex vivo* reperfusion.

After treatment, livers were frozen in liquid nitrogen and stored at −70 °C.

2.3. Static cold storage

After procurement, livers subjected to SCS were flushed for approximately 2 min with 20 mL of cold HTK solution injected manually with a syringe through the catheter in the portal vein. Then, the organs were transferred to flasks containing 80 mL of pre-chilled HTK solution and maintained 24 h at 4 °C. The composition of HTK (Custodiol®, Dr. Franz Köhler Chemie GmbH, Germany) is described in Ref. [35].

2.4. Ex vivo reperfusion

Livers were perfused *ex vivo via* an in house built machine at a constant pressure of 10.3 mm Hg using a recirculating system described in Ref. [4] with minor modifications. Krebs Henseleit buffer plus 2.0 % w/v dextran-70 (code A1847, BioChemica, AppliChem GmbH, Germany) and 300 μM sodium taurocholate was reperfused through the portal vein for 90 min at 37 °C. This composition ensured the normal architecture of the livers after the experimental procedures, as assessed by histopathological analysis of hematoxylin/eosin sections (data not shown). The perfusate was recirculated and oxygenated while passing through oxygen-permeable tubing (silicone tubing, internal diameter 0.078”, T5715-9, Baxter Healthcare Corp., USA) inside an appropriate glass container fed with 95 %O_2 and 5 %CO_2, at a constant pressure of 90 mmHg yielding a perfusate with a pO_2 > 450 mmHg [17].

2.5. Pooled liver samples

Approximately 0.1 g of each frozen liver was homogenized using a potter with the addition of 0.5 mL of extraction buffer (10 mM Tris pH 8, 7 M urea, 2 M thiourea, 4 %w/v CHAPS, 40 mM dithiothreitol and 1 %v/v 3-10NL ampholytes). Endonucleases (0.25 mg/mL DNAse, 0.125 mg/mL RNAse) were added and the suspension was incubated for 10 min in ice. Samples were then sonicated (3 pulses, 5 s each). Debris was eliminated by centrifugation at 4 °C for 20 min at 15,000 g. An aliquot was taken for protein quantification using the Bradford method [9]. Protein concentration readings were confirmed by gel densitometry. For the latter, samples were run in 12 %w/v polyacrylamide gels under denaturing conditions with bovine serum albumin as standard (0.1–5 μg). Gels were stained with Coomassie Blue R-250, scanned and the optical density assessed with the software ImageJ (NIH, USA). Pools were prepared by combining an equal amount of protein per individual liver. Then, the protein concentration of each pool was measured as before.

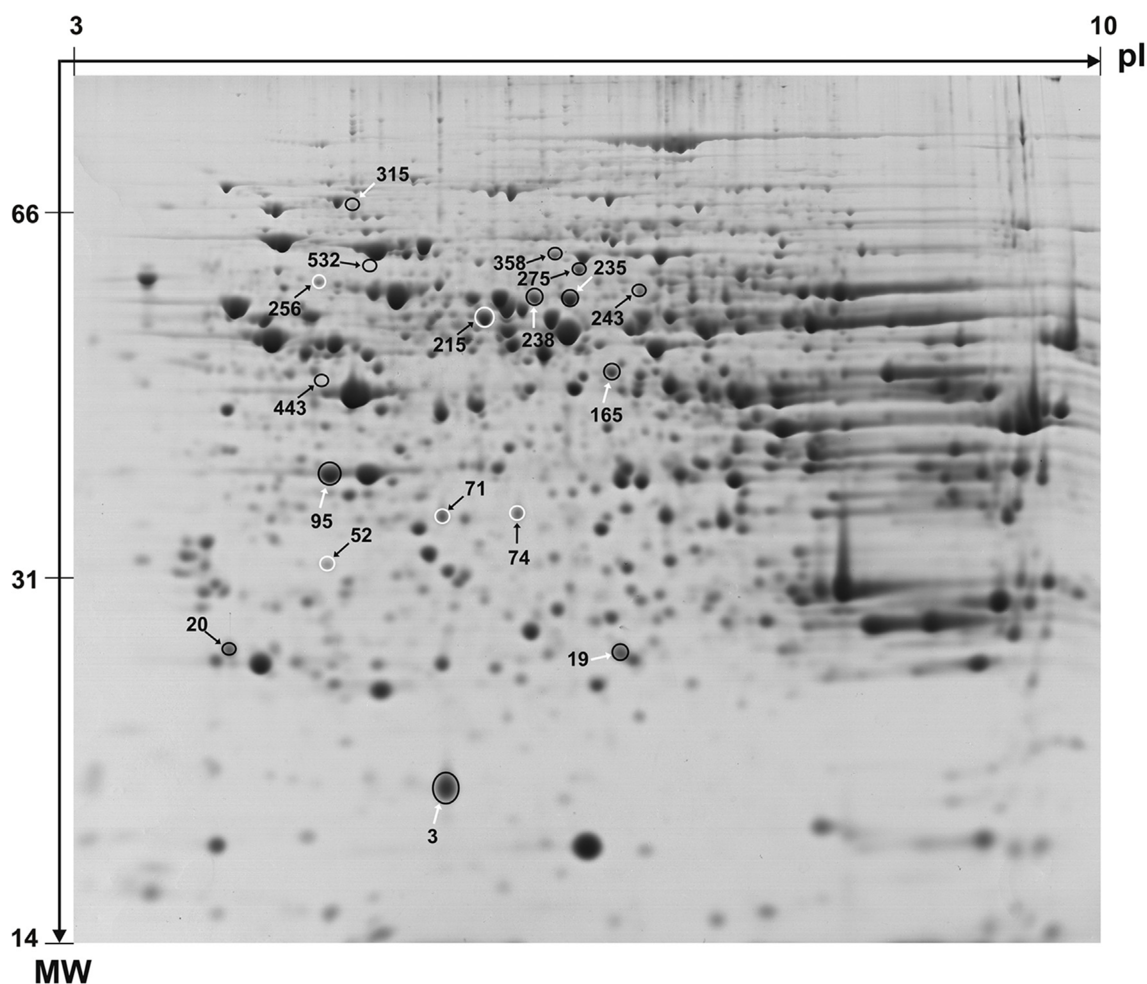

Fig. 1. Image of a 2D gel electrophoresis profile of the rat liver. The first dimension was run using an immobilized pH gradient strip (18 cm, 3–10 non-linear). Strips were loaded with 500 μg of proteins from a pool of four individuals. The second dimension consisted of a 12% polyacrylamide gel under denaturing conditions. The procedure to obtain 2D gels for each group was performed three times. Numbers indicate match number (corresponding to match number in the tables) of differentially expressed proteins as compared to the control group. Black arrows pointing to spots encircled in black indicate spots from the SCS group, black arrows pointing to spots encircled in white indicate spots from the IPRL group and white arrows pointing to spots encircled in black indicate spots from the SCS + IPRL group. Spots that are shared between groups are only referenced once. MW: molecular weight, pI: isoelectric point.

2.6. Two dimensional gel electrophoresis

Immobilized pH gradient strips of pH range 3–10 (non linear), length 18 cm (GE) were used. The strips were hydrated for 20 h at 20 °C with rehydration buffer (7 M urea, 2 M thiourea, 2 %w/v CHAPS, 1.2 % v/v DeStreak reagent (GE) and 0.006 %w/v bromophenol blue) containing 500 μg of total protein from the pooled samples. Strips were focused (29.5 kVh) on a Multiphor™ II System (GE) using a PowerPack 3000 power supply (Bio-Rad). Strips were then equilibrated in equilibration buffer (6 M urea, 75 mM Tris pH 8.8, 30 %v/v glycerol, 2 %w/v sodium dodecyl sulfate, 0.006 %w/v bromophenol blue) in two steps of 15 min. For the first step, dithiothreitol (10 mg/mL) was added and for the second, iodoacetamide (25 mg/mL) was included. The strips were then positioned onto 12% sodium dodecyl sulfate-polyacrylamide gels (12 %w/v acrylamide, 390 mM Tris pH 8.8, 0.1 %w/v SDS). Gels were run using a PROTEAN II xi unit (Bio-Rad) at 15 °C at a constant current of 12 mA. The gels were stained with Coomassie Brilliant Blue R-250 to visualize the proteins. Each pool was analyzed in triplicate. The gels were scanned with an Epson Expression 10000XL scanner at 300 dpi, grayscale 16 bits and focus 0.6 cm.

Spots were detected using the software ImageMaster 2D Platinum 7.0 (GE). Each spot intensity volume (i.e., integration of the optical density over the spot's area) was normalized to the volume of all spots present in the gel to obtain the normalized spot volume (% Vol = individual spot volume/total spot volume × 100). Next, automatic spot matching between all gels was performed. After this process, a group of matched spots was assigned a "match number". Intra-group reproducibility was assessed by correlation analysis in which the %Vol of spots in one gel was plotted against the %Vol of the matched spots in a replicate gel. For every matched spot, the average %Vol and its standard variation in each group was determined. Differentially expressed proteins were evaluated by calculating "fold change" - the ratio of mean %Vol between spots of treated and control samples. Spots with 2-fold or higher quantitative variations (0.5 > %Vol treated sample/% Vol control > 2, unless otherwise stated), a significant result in the one-way ANOVA test ($p < 0.05$) and that were detected in the six gels under comparison were selected for protein identification by mass spectrometry. Candidate matches were manually checked to exclude artifacts (double spots, smears, etc.).

2.7. Mass spectrometry

All following procedures were carried out at the Analytical Biochemistry and Proteomics Unit of the Institut Pasteur in Montevideo. In-gel trypsin digestion and peptide recovery were performed as described before [39]. Peptides were desalted and concentrated using C-

18 ZipTips (Millipore, Billerica, MA, USA) and eluted onto the target plate with 2 μL of α-cyano-4-hydroxycinnamic acid saturated solution dissolved in 50 %v/v acetonitrile and 0.2 %v/v trifluoroacetic acid. Peptide mass-to-charge ratios before and after fragmentation in the collision-induced dissociation chamber were determined by MALDI-TOF in a MALDI TOF/TOF 4800 mass spectrometer (ABSciex). The three (four in one case) most intense peaks in the MS1 scan were chosen for fragmentation. The data was analyzed in MASCOT (sequence query) using the Swiss-Prot database with no taxonomy limitation (557,992 sequences from 13,644 organisms) and with carbamidomethyl-cysteine and oxidation of methionine as variable modifications, one missed cleavage site allowed, peptide mass tolerance 0.08 Da and fragment mass tolerance 0.35 Da. For MS/MS data, protein identifications were evaluated using probability-based MASCOT ions scores. The ions score is $-10*\log(p)$, where p is the absolute probability that the observed match between the experimental data and the database sequence is a random event. Protein score is the sum of the ions scores of the unique peptides identified for that protein. All matched peptides informed in this work have ions scores above the threshold for significance ($p < 0.05$). For combination searches (data including both MS/MS spectra and molecular mass values), the protein score contains contributions from the peptide mass fingerprint and the ions scores. In combination searches, protein hits with a protein score > 70 and $p < 0.05$ for random occurrence were considered significant. For more information on MASCOT scoring, refer to http://www.matrixscience.com/help/scoring_help.html.

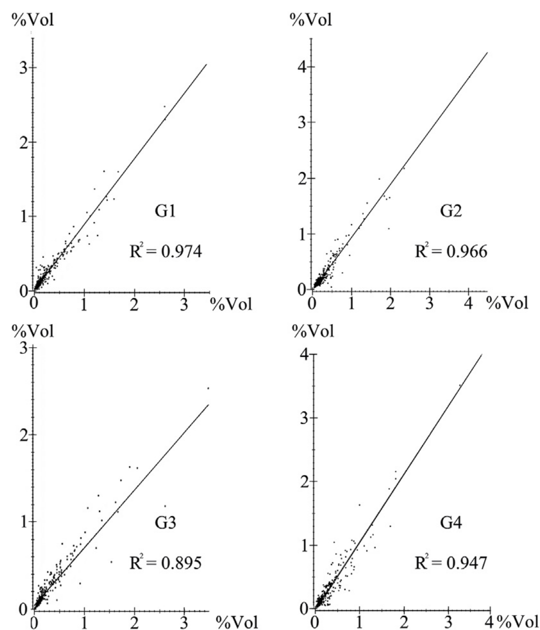

Fig. 2. Scatter plots of %Vol between gels belonging to the same group. Representative scatter plots for each group are shown.
G1: Control group; G2: SCS group; G3: IPRL group; G4; SCS + IPRL group.

3. Results

3.1. Global analysis of gel images of the rat liver proteome subjected to cold storage and/or ex vivo reperfusion

To evaluate the effect of IRI on the rat liver proteome, livers were subjected to SCS followed by normothermic *ex vivo* reperfusion. Proteins were extracted and analyzed by 2D gel electrophoresis and differentially expressed proteins were identified by mass spectrometry. The effect of either SCS (SCS group) or reperfusion (IPRL group) on the rat liver proteome was also studied. The pattern of spot distribution was almost the same across all groups. A representative gel is shown in Fig. 1. In addition, the intra-group %Vol dispersion was low, as revealed by correlation analysis of gels in the same group (Fig. 2). To identify those proteins whose intensity values varied between treatments, a fold-change criterion was used. More than 20 candidates in each comparison met the criteria. However, careful examination of all hits to avoid false positives significantly reduced this number (Fig. 3). For example, candidate spots that were not present in all gels of the triad were discarded. Candidate spots were then analyzed by MALDI-TOF. The generated data was used to interrogate the Swiss-Prot database using MASCOT. All top hits were from *Rattus norvegicus* and had at least one matching peptide from the fragmentation analysis. Results of the mass spectroscopic identification and other relevant data are summarized in Tables 1–3.

3.2. Changes in the rat liver proteome after static cold storage

This preservation strategy involved storage of livers in HTK solution at 4 °C for 24 h. Protein profiles were compared to controls (freshly extracted livers). Six spots showed significant variations in their intensity (Fig. 3). Magnified regions of these spots are shown in Fig. 4, along with bar graphs of the mean %Vol ± standard deviation (SD). Three spots showed a significant increase in intensity while three showed the opposite trend. The identified proteins were N-*myc* downstream-regulated gene 2 (NDRG2, up), catechol-O-methyltransferase (COMT, up), regucalcin (up), two isoforms of serum albumin (down) and L-type pyruvate kinase (L-PK, down) (Table 1).

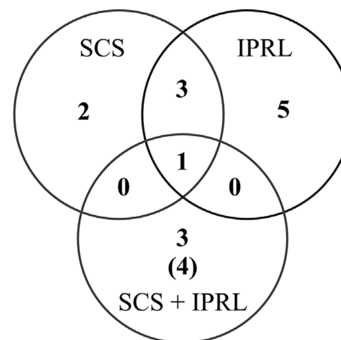

Fig. 3. Number of proteins identified for each group. Numbers in parentheses indicate the number of proteins that were identified after lowering the fold criterion to 1.8.

3.3. Changes in the reperfused rat liver proteome after static cold storage

Many of the molecular effects caused by cold storage are exacerbated when the liver is subjected to reperfusion. To assess the impact of SCS on the reperfused liver, it is necessary to evaluate the effects of the normothermic reperfusion process by itself. Therefore, the proteome of fresh resected livers were compared to that of livers subjected to normothermic reperfusion for 90 min. Nine spots showed a significant variation in their %Vol value (Figs. 3 and 5). Mass spectroscopy analysis revealed the identity of the proteins: heterogeneous nuclear ribonucleoprotein K (hnRNP K, up), cytokeratin 8 (K8, up), COMT (up), two isoforms of estrogen sulfotransferase (EST, up), phospholysine phosphohistidine inorganic pyrophosphate phosphatase (up), NDRG2 (up) and two isoforms of serum albumin (down) (Table 2).

Finally, the proteome of freshly extracted livers were compared to that of livers subjected to SCS followed by normothermic reperfusion. Our 2D gel electrophoresis analysis revealed that only three proteins showed differential expression (Fig. 3). They were all down-regulated and were identified as 2-oxoisovalerate dehydrogenase subunit alpha (mitochondrial), alpha 2u-globulin and peroxiredoxin 3. Since the number of proteins that displayed an altered expression under this condition was low, the fold-criterion was relaxed to 1.8-fold to include

Table 1
Details of proteins from the SCS group identified by mass spectrometry.

Match N°[a]	Identified protein	Accession number[b]	Theoretical mass (kDa)	MASCOT score[c]	Sequence coverage[d] (%)	N° of matched peptides[e]	Densitometric ratio of protein level variation
20	Catechol-O-methyltransferase	P22734	29.6	323/113	56/9	17/2	2.18
243	L-type pyruvate kinase	P12928	62.1	410/164	48/3	25/1	0.49
275	Albumin	P02770	64.3	442/286	43/8	25/4	0.34
358	Albumin	P02770	64.3	163/103	13/4	9/2	0.28
443	NDRG2	Q8VBU2	40.8	136/32	29/6	8/1	n.a.[f]
532	Regucalcin	Q03336	33.4	307/229	45/14	16/3	n.a.

[a] Identification number assigned by the analysis software of spots matched across gels.
[b] Code under which the identified protein is deposited in the Swiss-Prot database.
[c] The first number represents the score from combinatorial searches (MS1 scan data plus fragment ion masses from the three or four most intense peaks in the MS1 scan). The second number indicates the sum of ions scores from fragment ion masses of selected precursor peptides.
[d] Percentage of amino acids spanned by the assigned peptides divided by the sequence length. The first number considers matched precursor peptide masses from the MS1 scan. The second number is the sequence coverage of the peptides chosen for fragmentation that matched to the candidate protein.
[e] The first number is the number of matched *m/z* values of tryptic peptides obtained from an excised spot to theoretical *m/z* values from a candidate protein digested *in silico*. The second value indicates the number of fragmented peptides (out of three or four) whose MS/MS fragment ion masses match to theoretical MS/MS fragment ion masses from the candidate protein.
[f] n.a. Not applicable (division by zero).

more hits in this group. Under this new constraint, five more proteins were identified as being up-regulated (Fig. 6): NDRG2, bifunctional ATP-dependent dihydroxyacetone kinase/fad-amp lyase (cyclizing), protein disulfide isomerase (two isoforms) and regucalcin (Table 3).

4. Discussion

This study aimed to characterize proteome changes of the rat liver after cold storage, not only as a standalone experimental procedure but also to assess its effects on the liver proteome in the reperfusion phase using the IPRL model. As the experimental scheme involves two different treatments (SCS followed by normothermic *ex vivo* reperfusion), proteome variations by either SCS or normothermic reperfusion alone were also analyzed. Interestingly, some proteins showed altered levels both in the SCS group and the IPRL group, even though the treatments are quite different. The expression of these proteins may respond to the absence of blood components, which is a common feature between the SCS and the IPRL group. One spot showing this behavior corresponded to COMT, a highly abundant liver protein responsible for the methylation of catecholamines, whose expression is regulated by hormones. In the rat, during hepatic ischemia, the concentration of plasmatic catecholamines increases and this in turn, may contribute to the rapid changes in COMT levels [49]. The absence of these metabolites may promote changes in COMT expression. It is also well established that albumin content is lowered in a context of acute or chronic hepatic diseases and different types of stress, many mediated by blood components [29]. Along this line, two spots were found corresponding to different isoforms of albumin with lowered intensity values. The finding of elevated levels of NDRG2 in all three groups is worth noticing. The quantity of this protein in the fresh resected liver group was below the level of detection, yet a distinctive spot with a %Vol > 0.12 was detected in the other groups. NDRG proteins have gained much attention due to their multiple roles in cell proliferation, neural differentiation and especially, as a central hub in signaling pathways of the endocrine system. Also, its participation in ischemia-reperfusion injury has been documented in other tissues [40]. Given the various roles of NDRG2 in several normal and pathological cellular pathways, the elevated levels of this protein in liver injury warrant further investigation.

Table 2
Details of proteins from the IPRL group identified by mass spectrometry.

Match N°[a]	Identified protein	Accession number[b]	Theoretical mass (kDa)	MASCOT score[c]	Sequence coverage[d] (%)	No of matched peptides[e]	Densitometric ratio of protein level variation
20	Catechol-O-methyltransferase	P22734	29.6	323/113	56/9	17/2	2.08
52	Phospholysine phosphohistidine inorganic pyrophosphate phosphatase	Q5I0D5	29.2	245/102	54/11	10/2	2.15
71	Estrogen sulfotransferase	P49889	35.4	326/168	65/11	23/3	2.08
74	Estrogen sulfotransferase	P52844	35.5	286/93	62/8	26/2	2.30
215	Cytokeratin 8	Q10758	54.0	224/130	35/4	13/2	2.00
256	Heterogeneous nuclear ribonucleoprotein K	P61980	50.9	236/175	20/5	7/2	2.08
275	Albumin	P02770	64.3	442/286	43/8	25/4	0.36
358	Albumin	P02770	64.3	163/103	13/4	9/2	n.a.[f]
443	NDRG2	Q03336	40.8	136/32	29/6	8/1	n.a.

[a] Identification number assigned by the analysis software of spots matched across gels.
[b] Code under which the identified protein is deposited in the Swiss-Prot database.
[c] The first number represents the score from combinatorial searches (MS1 scan data plus fragment ion masses from the three or four most intense peaks in the MS1 scan). The second number indicates the sum of ions scores from fragment ion masses of selected precursor peptides.
[d] Percentage of amino acids spanned by the assigned peptides divided by the sequence length. The first number considers matched precursor peptide masses from the MS1 scan. The second number is the sequence coverage of the peptides chosen for fragmentation that matched to the candidate protein.
[e] The first number is the number of matched *m/z* values of tryptic peptides obtained from an excised spot to theoretical *m/z* values from a candidate protein digested *in silico*. The second value indicates the number of fragmented peptides (out of three or four) whose MS/MS fragment ion masses match to theoretical MS/MS fragment ion masses from the candidate protein.
[f] n.a. Not applicable (division by zero).

Table 3
Details of proteins from the SCS + IPRL group identified by mass spectrometry.

Match N°[a]	Identified protein	Accession number[b]	Theoretical mass (kDa)	MASCOT score[c]	Sequence coverage[d] (%)	No of matched peptides[e]	Densitometric ratio of protein level variation
3	Alpha 2u-globulin	P02761	18.7	279/173	67/17	15/3	0.34
19	Peroxiredoxin 3	Q9Z0V6	28.3	276/248	29/14	6/3	0.33
95	Regucalcin	Q03336	33.4	378/220	76/14	24/3	1.80
165	2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial	P11960	50.1	380/170	66/9	27/3	0.49
235	Protein disulfide isomerase A3	P11598	56.6	407/207	44/6	26/3	1.81
238	Protein disulfide isomerase A3	P11598	56.6	380/134	48/4	27/2	1.80
315	Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)	Q4KLZ6	59.4	164/108	21/6	7/2	1.91
443	NDRG2	Q03336	40.8	136/32	29/6	8/1	n.a.[f]

[a] Identification number assigned by the analysis software of spots matched across gels.
[b] Code under which the identified protein is deposited in the Swiss-Prot database.
[c] The first number represents the score from combinatorial searches (MS1 scan data plus fragment ion masses from the three or four most intense peaks in the MS1 scan). The second number indicates the sum of ions scores from fragment ion masses of selected precursor peptides.
[d] Percentage of amino acids spanned by the assigned peptides divided by the sequence length. The first number considers matched precursor peptide masses from the MS1 scan. The second number is the sequence coverage of the peptides chosen for fragmentation that matched to the candidate protein.
[e] The first number is the number of matched *m/z* values of tryptic peptides obtained from an excised spot to theoretical *m/z* values from a candidate protein digested *in silico*. The second value indicates the number of fragmented peptides (out of three or four) whose MS/MS fragment ion masses match to theoretical MS/MS fragment ion masses from the candidate protein.
[f] n.a. Not applicable (division by zero).

Two proteins with differential expression compared to the control are exclusive of the SCS group: L-PK and regucalcin. L-PK catalyzes the conversion of phosphoenolpyruvate to pyruvate, which upon entering the Krebs cycle generates citrate, the primary source of acetyl-CoA. Protein levels of L-PK in rat livers are regulated by glucose and oxygen [22]. Diminished glucose availability and hypoxia in SCS are signals that seem to cause the observed changes in L-PK expression. Regucalcin (upregulated) is a calcium binding protein that has multiple roles in

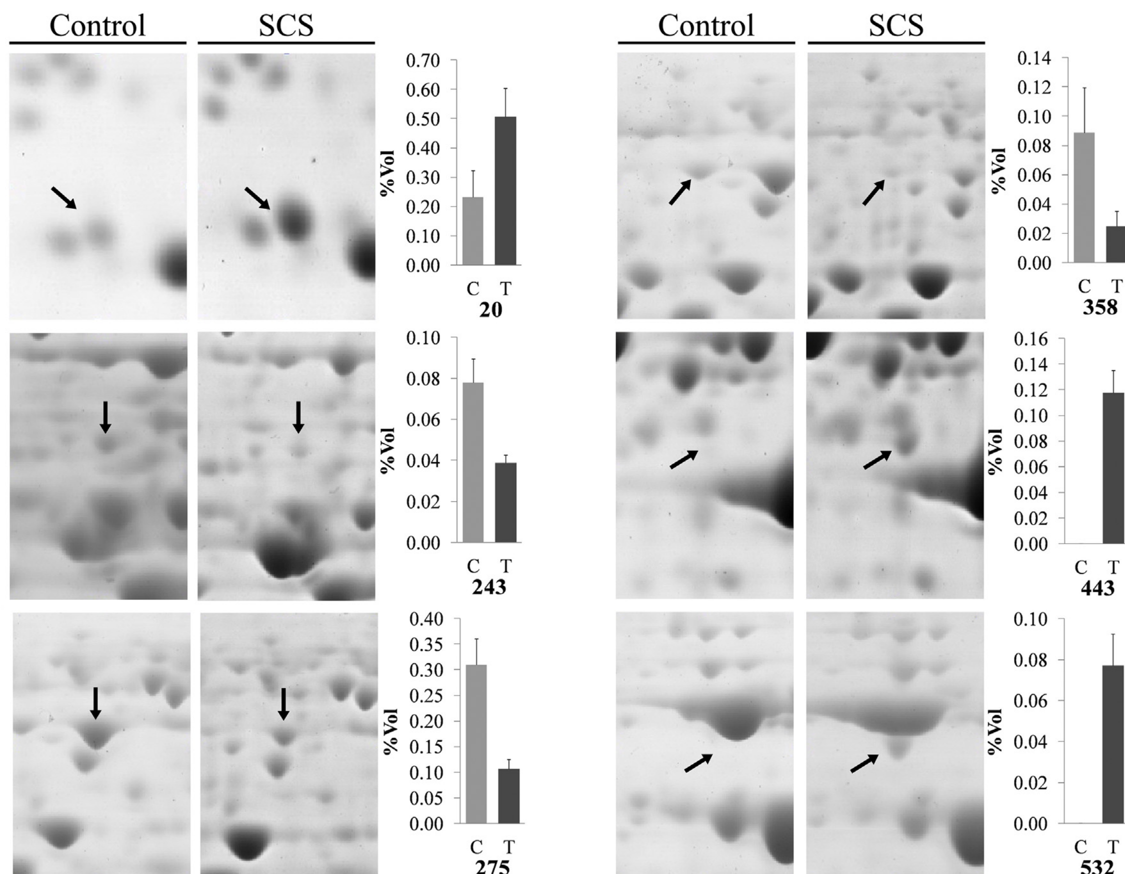

Fig. 4. Magnified regions of 2D gels showing selected proteins in the SCS group. Proteins were extracted from livers subjected to cold storage (4 °C) for 24 h in HTK solution and analyzed by 2D gel electrophoresis. Spots from the SCS group whose %Vol values were significantly different compared to the control group according to the 2-fold criterion are shown. Bars show the mean %Vol ± SD for that particular spot (indicated by arrows in the images) matched in the three technical replicates. C: control (pool of fresh resected livers), T: treated (in this case, pool of livers subjected to SCS). Numbers indicate match number.

Cryobiology 85 (2018) 47–55

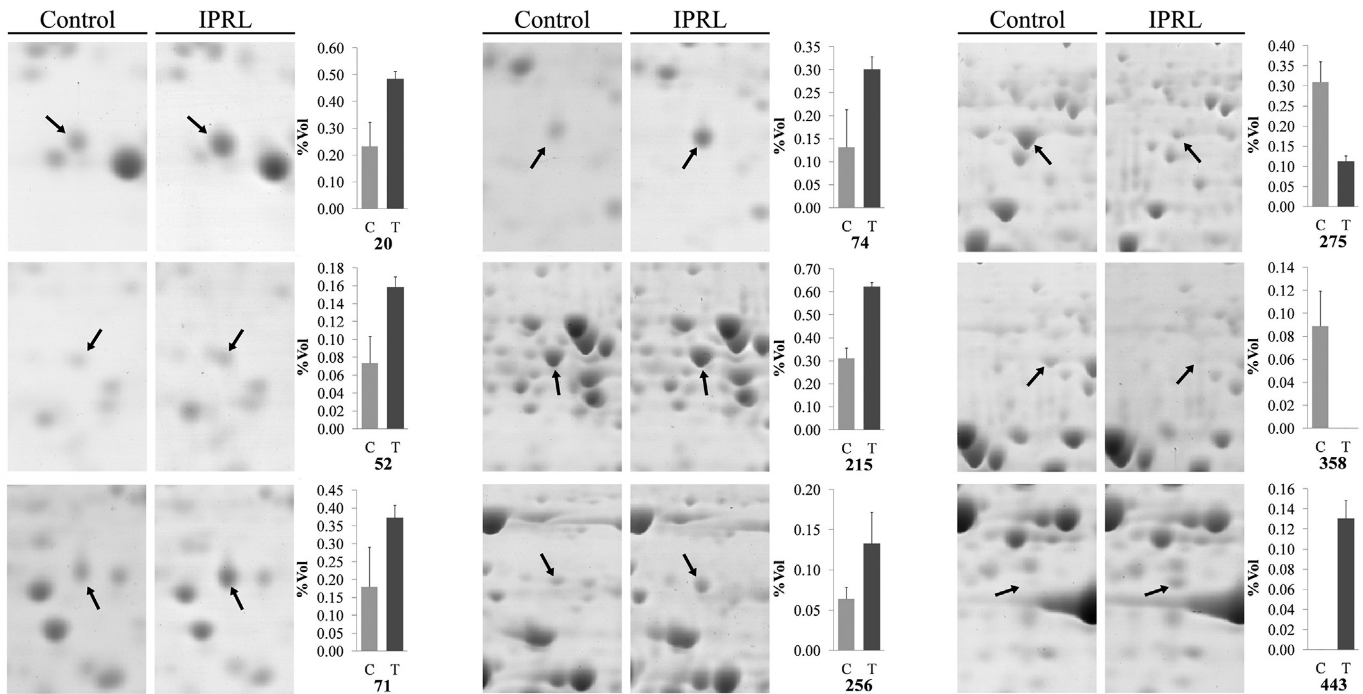

Fig. 5. Magnified regions of 2D gels showing selected proteins in the IPRL group. Proteins were extracted from livers subjected to normothermic reperfusion for 90 min using an *ex vivo* reperfusion machine and then, analyzed by 2D gel electrophoresis. Spots from the IPRL group whose %Vol values were significantly different compared to the control group according to the 2-fold criterion are shown. Bars show the mean %Vol ± SD for that particular spot (indicated by arrows in the images) matched in the three technical replicates. C: control (pool of fresh resected livers), T: treated (in this case, pool of livers subjected to *ex vivo* reperfusion). Numbers indicate match number.

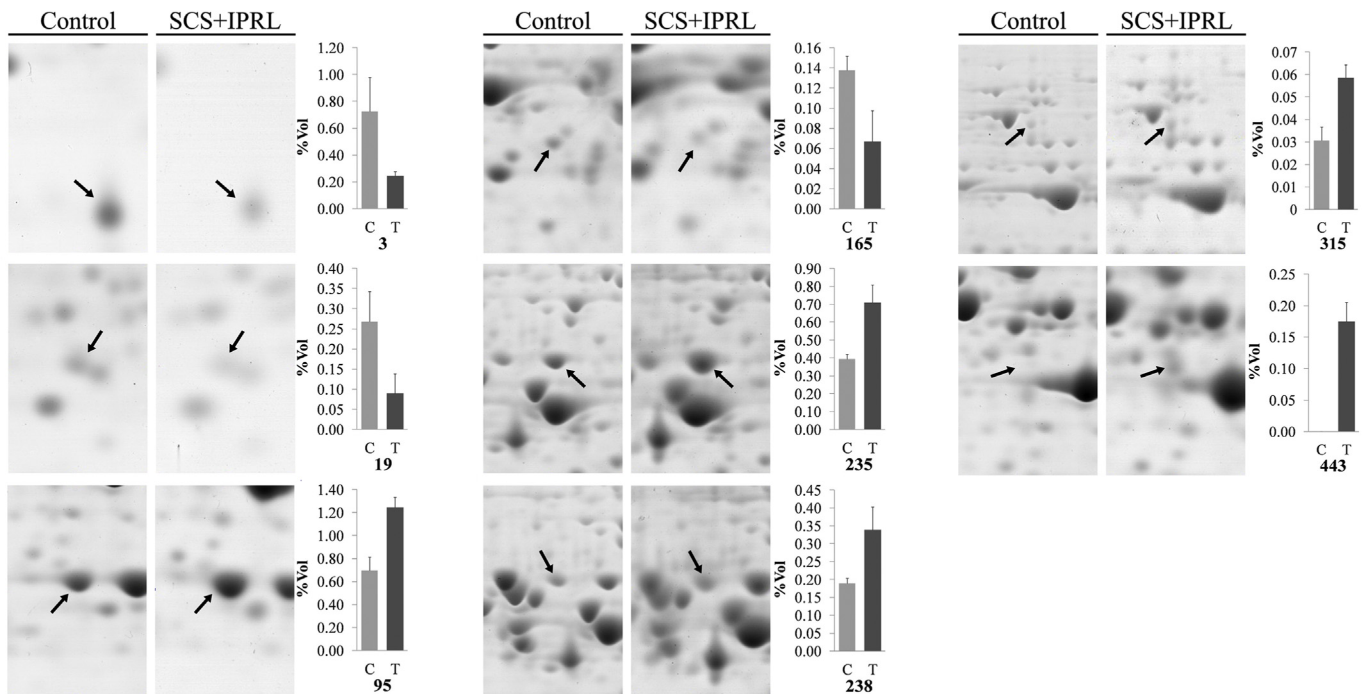

Fig. 6. Magnified regions of 2D gels showing selected proteins in the SCS + IPRL group. Proteins were extracted from livers subjected to cold storage (4 °C) for 24 h in HTK solution followed by normothermic reperfusion for 90 min using an *ex vivo* reperfusion machine and then, analyzed by 2D gel electrophoresis. Spots from the SCS + IPRL group whose %Vol values were significantly different compared to the control group according to the 2 or 1.8-fold criterion are shown. Bars show the mean %Vol ± SD for that particular spot (indicated by arrows in the images) matched in the three technical replicates. C: control (pool of fresh resected livers), T: treated (in this case, pool of livers from the SCS + IPRL group). Numbers indicate match number.

cellular pathways and that has been proposed as a biomarker for liver injury [47]. However, there are no reports of the link between regucalcin upregulation and exposure to low temperatures in the rat model. Interestingly, upregulation of the regucalcin gene during cold adaptation in *Drosophila* has been reported [45].

Five proteins were identified exclusively in the IPRL group. K8 is major component of the intermediate filament cytoskeleton of hepatocytes. Levels of K8 usually spike in response to perfusion stress [27,32,43], which explains our observations. hnRNP K is a transcription factor that targets *cis*-acting elements located in the promoters of the human proto-oncogenes c-Src and c-myc. hnRNP K levels increase slightly in remnant livers after partial hepatectomy in mice [33]. Furthermore, there is a significant relocation of this protein from the cytosol to the nucleus in injured livers within the first hours of surgery. The authors interpreted these results as hnRNP K regulating transcription of immediate-early genes (like c-*myc*, c-*fos* and *egr-1*) and other factors that mediate cell proliferation in regenerating livers. Our results also indicate that hnRNP K plays a role in an early response to liver injury due to mechanical stress. Two isoforms of EST were identified. Expression of EST was found to be induced in IRI and it is thought to protect the liver from injury as male $EST^{-/-}$ mice showed heightened sensitivity to IRI [16]. Phospholysine phosphohistidine inorganic pyrophosphate phosphatase is an enzyme that modifies the *N*-linked phosphorylation status of amine type amino acids. It has been recently shown to regulate histidine phosphorylation in the liver, a rare post translational modification upregulated in liver tumors in mice [18]. Under the conditions of this study, it may regulate protein phosphorylation in response to insults to the organ.

In the SCS + IPRL group, the levels of expression of three proteins were reduced more than two-fold (alpha 2u-globulin, 2-oxoisovalerate dehydrogenase subunit alpha and peroxiredoxin 3). The last two proteins are located in mitochondria. The down regulation of these proteins, in this group only, could be an indication of the declining wealth of mitochondria under cold storage followed by reperfusion, as this organelle is a target of the sudden burst of reactive oxygen species caused by oxygen reentry [26]. To carry out a more exhaustive characterization of the liver proteome subjected to SCS + IPRL, the fold-criterion was lowered from 2 to 1.8. Regucalcin expression was upregulated (this trend was also noted for a different isoform in the SCS group). Two isoforms of protein disulfide isomerase were identified. This family of proteins catalyzes disulfide formation and rearrangement by thiol/disulfide exchange in the endoplasmic reticulum. Interestingly, protein disulfide isomerase was found to be downregulated in a proteomic survey of livers extracted from hypothermic rats [31]. Conversely, upregulation of this protein is linked to endoplasmic reticulum stress caused by IRI, chemical stress or hemorrhagic trauma [3,20,37,48]. Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing) was shown to participate in the antioxidant response of the liver, although its role in this scenario is not yet known [46].

Limitations of the present study ought to be acknowledged. Differential expression was assessed by two-dimensional electrophoresis. This technique has a lower sensitivity than other proteomic approaches such as liquid chromatography-electrospray ionization-tandem mass spectrometry. Thereby, only changes in highly expressed proteins can be detected. Furthermore, the technical variance tends to be high, due to differences in first or second dimension run, staining, image acquisition, etc [44]. As a result, differences in expression must be higher than technical plus biological variance to be detected with sufficient power. This masks true changes in expression below biological and technical variation. One way to decrease biological variance is to pool samples from individuals [21]. It should also be mentioned that livers from same sex same age isogenic rats were used, therefore biological variance should be lower than technical noise, leading to the detection of differentially expressed proteins using fewer gels. However, our conclusions are limited to the averaged sample. In spite of this, it has been established that protein expression in a pool matches the mean expression of the individuals making up the pool for most proteins analyzed with 2D gels [12]. Finally, normothermic reperfusion was performed for 90 min using a cell-free solution. This reperfusion time may not be sufficient to detect proteins involved in a later phase of reperfusion, in which hepatocellular injury is more prominent [24]. This is particularly relevant to energy expensive processes such as protein biosynthesis. However, 90 min is the maximum possible duration of isolated rat liver perfusion with an acellular solution without introducing model-related artifacts (such as vascular resistance) [41] which may have obscured the effects of SCS. Also, excluding blood components from the reperfusion solution does not entirely mimic reperfusion injury in a transplant setting. These limitations notwithstanding, our results revealed a set of proteins that show altered regulation in response to cold ischemia of the liver. The liver proteome after SCS changes if the organ is subjected to normothermic reperfusion. The proteins that were identified in this report participate in many molecular pathways of recognized importance in liver defense mechanisms to cope with injury. As such, further detailed analyses of their role in these pathways are warranted.

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Declarations of interest

None.

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