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DOI:
10.1016/j.cryobiol.2016.11.006

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Document Version
Peer reviewed version

Citation for published version (Harvard):
Nath, J, Smith, T, Patel, K, Ebbs, SR, Hollis, A, Tennant, D, Ludwig, C & Ready, AR 2016, 'Metabolic differences between cold stored and machine perfused porcine kidneys: A 1H NMR based study', Cryobiology. https://doi.org/10.1016/j.cryobiol.2016.11.006

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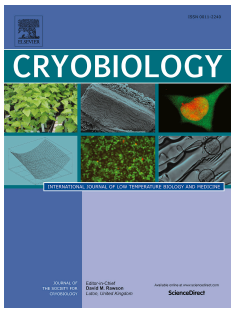

Metabolic differences between cold stored and machine perfused porcine kidneys: A ^{1}H NMR based study

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PII: S0011-2240(16)30439-4

DOI: 10.1016/j.cryobiol.2016.11.006

Reference: YCRYO 3783

To appear in: *Cryobiology*

Received Date: 11 July 2016

Revised Date: 18 October 2016

Accepted Date: 21 November 2016

Please cite this article as: J. Nath, T.B. Smith, K. Patel, S.R. Ebbs, A. Hollis, D.A. Tennant, C. Ludwig, A.R. Ready, Metabolic differences between cold stored and machine perfused porcine kidneys: A ^{1}H NMR based study, *Cryobiology* (2017), doi: 10.1016/j.cryobiol.2016.11.006.

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Metabolic differences between cold stored and machine perfused porcine kidneys: A ^{1}H-NMR based study.

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Key words: hypothermic machine perfusion, kidney, transplantation, metabolism, NMR, organ preservation.

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Abstract

Hypothermic machine perfusion (HMP) and static cold storage (SCS) are the two methods used to preserve deceased donor kidneys prior to transplant. This study seeks to characterise the metabolic profile of HMP and SCS porcine kidneys in a cardiac death donor model.

Twenty kidneys were cold flushed and stored for two hours following retrieval. Paired kidneys then underwent 24 hours of HMP or SCS or served as time zero controls. Metabolite quantification in both storage fluid and kidney tissue was performed using one dimensional ^{1}H-NMR spectroscopy. For each metabolite, the net gain for each storage modality was determined by comparing the total amount in each closed system (i.e. total amount in storage fluid and kidney combined) compared with controls. 26 metabolites were included for analysis.

Total system metabolite quantities following HMP or SCS were greater for 14 compared with controls (all $p<0.05$). In addition to metabolic differences with control kidneys, the net metabolic gain during HMP was greater than SCS for 8 metabolites (all $p<0.05$). These included metabolites related to central metabolism (lactate, glutamate, aspartate, fumarate and acetate).

The metabolic environments of both perfusion fluid and the kidney tissue are strikingly different between SCS and HMP systems in this animal model. The total amount of central metabolites such as lactate and glutamate observed in the HMP kidney system suggests a greater degree of *de novo* metabolic

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activity than in the SCS system. Maintenance of central metabolic pathways may contribute to the clinical benefits of HMP.

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Introduction

Hypothermic Machine Perfusion (HMP) and Static Cold Storage (SCS) are the two methods of kidney preservation that are used widely in clinical practice during the time period between organ retrieval and implantation [16]. A key concept for both preservation modalities is that cellular metabolism, and therefore cellular metabolic requirements, are minimised in these hypothermic conditions and the rate of metabolism reported to be about 5-8% at temperatures below 4°C [29] with a similar decrease in oxygen requirement [1].

The superiority of HMP over SCS is well documented [4,17,22,23,27] but the mechanisms by which this occurs are not clear. Improvement in flow dynamics, with fall in the intra-renal resistance is likely to be one factor but the additional metabolic support derived from the circulation of nutrient-containing perfusion fluid may also help preserve organ function and have a beneficial effect [7,30].

Metabolomic analyses of preservation fluid during HMP using 1D-^{1}H-NMR (One-dimensional proton nuclear magnetic resonance) spectroscopy, by groups including our own, have demonstrated this to be reproducible and highly specific for metabolite identification and quantification [2,10,24]. However, surprisingly, to our knowledge there are no studies that have sought to compare the metabolomic profiles, or metabolome, of HMP and SCS kidneys.

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Porcine kidneys are widely used in transplantation studies owing to their similar physiological and anatomical properties to human organs [9,11]. In addition, the metabolic profiles during periods of HMP for porcine and human kidneys are comparable [24], with a correlation between metabolite profiles during storage and post transplant outcome [2]. For HMP preserved human kidneys, the metabolic profile from perfusates of immediate graft function kidneys differs from those with delayed function [10] and reinforces the concept that significant metabolism occurs during HMP and that metabolism reflects functional outcome.

The aims of this study were twofold. Firstly, to determine the distribution of metabolites between the two different compartments (fluid and tissue) during the organ preservation period. Secondly, to determine the total amount of each metabolite within HMP and SCS kidneys systems after 24 hours of organ storage and through comparison with control kidneys, the metabolic changes that occur.

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Methods

Animal Research

Abattoir/slaughterhouse pig kidneys (F.A. Gill, Wolverhampton, UK) were used and no animals were sacrificed solely for the purposes of this study, negating any need for ethical board approval. Experiments were performed on 22-26 week old male 'bacon weight' pigs, weighing 80-85kg. All experiments were performed following the principles of laboratory animal care according to NIH standards. Animals were sacrificed by electrical stunning and exsanguination. Initial organ preservation was performed following organ retrieval and occurred within 14 minutes of death, replicating deceased cardiac death (DCD) donor conditions. Kidneys were cold flushed (4°C) with 1L SPS-1 (UW) solution at a pressure of 100mmHg. Organs were then stored at 4°C in SPS-1 for 2 hours to replicate the clinical period of organ transportation.

Experimental groups

Paired kidneys were randomly allocated to receive either HMP or SCS for 24 hours. HMP kidneys were perfused with 1L of KPS-1 using the LifePort Kidney Transporter 1.0 (Organ Recovery Systems, Chicago, IL). (Perfusion pressure 30mmHg). SCS Kidneys were submerged in 1L of fresh chilled SPS-1 solution with a surrounding ice bath. Preservation fluid was sampled for each kidney at baseline and 2, 4, 8, 12, 18, and 24 hours. After 24 hours,

organs were rapidly dissected and tissue samples (1cm^3 sections) flash frozen and stored (-80°C). All experiments were performed in a cold room (4°C) to ensure consistency.

Control kidneys

To ascertain metabolism during SCS or HMP storage conditions, baseline values prior to storage conditions were needed (time 0). Large volume tissue sampling precludes effective organ perfusion and therefore 'Control kidneys' were used to establish baseline metabolite levels. These were (n=6) flushed and cold transported in identical fashion to experimental kidneys and tissue samples obtained as described above (i.e. not subjected to 24hrs of SCS or HMP).

Sample processing and metabolite quantification

NMR samples were prepared from storage fluid by mixing 150 µL of 400 mM (pH 7.0) phosphate buffer containing 2 mM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) and 8mM imidazole with 390 µL of each fluid sample and 60 µL of deuterium oxide (D_2O) to reach a final phosphate buffer concentration of 100 mM and a final DSS concentration of 500 µM. After mixing, the 600 µL samples were pipetted into 5mm NMR tubes, sonicated and centrifuged. Technical replicates of samples (x3) were prepared for each timepoint.

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153 For cell extract studies, 500mg of renal cortex was manually cryo-
154 homogenised in liquid nitrogen. 5.1ml of both methanol (-80°C) and
155 chloroform was added to the powdered tissue and samples diluted with
156 4.65ml of dH_20 at 4°C. Samples were centrifuged to separate into polar and
157 non-polar layers and 1.5ml of the upper polar layer was dispensed into a
158 cryovial and dried. Three technical replicates were performed for each tissue
159 sample. Dried polar residue was then dissolved in 390µL of dH_20 and 210 µL
160 of buffer solution as described above.
161
162 The protocol used for ^{1}H-NMR analysis has been described previously
163 [10,24]. Briefly, this entailed processing on a Bruker AVII 500 MHz
164 spectrometer, acquisition of one dimensional spectra and then metabolite
165 identification and quantification using Matlab based 'Metabolab' software [18]
166 and Chenomx 8.1 (ChenomxInc) software respectively. Metabolites were
167 deemed to be present if they exhibited non-ambiguous spectral patterns or
168 their presence deemed biologically plausible and confirmed on ultra
169 performance liquid chromatography mass spectrometry. Any metabolites that
170 were present in different concentrations in the SCS and HMP fluid (e.g.
171 glucose, gluconate, mannitol, adenine, adenosine etc.) were excluded from
172 comparative analysis. Metabolite quantifications were corrected to allow for
173 sample dilution with sample buffer. When determining concentrations of
174 metabolites using Chenomx, the researchers were blind to the storage group.
175 Quantification of the total amount of metabolite in the storage fluid, tissue and
176 total system was calculated based upon the weight of the kidney at time of
177 sample acquisition and final volume of storage fluid.

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179 *Statistical analysis*

180 For each timepoint, three results were obtained (technical replicates) and the
181 median value used. For comparison of SCS and HMP conditions, analysis
182 was performed using Wilcoxon paired signed rank test (two tailed) as one
183 kidney from each pair was subjected to each condition and normality was not
184 consistent on prior analysis. When comparing SCS or HMP with control
185 kidneys, Mann-Whitney u test (two tailed) was used, as these were non-
186 paired samples. Data were reported as median concentrations and
187 interquartile (IQ) range. All analysis was performed using GraphPad Prism
188 version 6.00 for Mac OS X, GraphPad Software, La Jolla California USA, with
189 $p<0.05$ deemed to be indicative of statistical significance.

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Results

Metabolic optimisation of cadaveric kidneys is a potential target to improve the function of kidneys for transplantation. This study seeks to establish the degree of metabolism, if any, that occurs in the two widely used methods of kidney organ storage prior to transplantation (HMP and SCS).

The total quantity of each metabolite after 24 hours of either HMP or SCS was calculated using ^{1}H-NMR methods and compared with control organs to determine the net metabolic change during each storage method.

We found evidence of metabolite production for both storage modalities with 14 metabolites present in significantly greater quantities in the HMP or SCS system compared with controls (all $p<0.05$) (table 1)(Fig 1, Fig 1(Suppl)). There were significantly more metabolites with a net increase in the HMP system (13/14) compared with the SCS system (7/14) ($p=0.033$).

Table 1. Total amount of metabolite present in each of the storage modalities at time zero (controls) or after 24 hours of preservation (SCS or HMP). Data reported as Median (Interquartile Range), unless stated otherwise. Statistical test: $^{\Psi}$ Mann-Whitney u test (two tailed) $^{\#}$Wilcoxon paired signed rank test (two tailed). *Significant at $p<0.05$.

Fig. 1. Metabolites significantly elevated in the HMP system compared with both SCS and control kidneys. Metabolite levels represent total amounts

(mmol) in the storage fluid, kidney tissue and entire system for porcine kidneys after 24hrs of HMP or SCS or time zero controls. Highly significant (**p<0.01) and significant (*p<0.05) differences between HMP system versus both controls and SCS kidneys.

Eight of the metabolites were significantly elevated in the HMP system compared with both the control and SCS systems (all p<0.05), indicating a greater degree of metabolite production. These included lactate, glutamate, aspartate, fumarate, acetate, myo-inisitol, niacinamide and formate (Fig 1).

Despite the additional 24 hours of organ preservation, albeit in static conditions, the amount of lactate in the SCS system was comparable to controls (1.37 vs 1.11mmol p=0.138). However the amount in the HMP system (2.13mmol) was almost twice the amount of either controls or SCS systems (p=0.002 and p=0.031). However, despite greater amounts overall, the amount present in the HMP tissue (0.76mmol) was actually lower than SCS tissue (1.14mmol) or control tissue (1.11mmol) (p= 0.031 and p=0.002 respectively), reflective of lower intracellular concentrations for HMP kidneys.

The distribution of metabolites between the extracellular storage fluid and tissue samples for both storage conditions are detailed in table 2. As expected, there were greater quantities of metabolites in the circulating HMP fluid compared with the static conditions of SCS at most time-points. After 24 hours, the total amount of metabolite in the perfusate for the HMP kidneys

was significantly greater than the SCS group for (21/26 = 80.8%) of metabolites. Whilst concentrations rose most rapidly in the first 2 hours of perfusion and therefore may be in part due a metabolite washout phenomenon, there was an increase in most metabolites over sequential timepoints as would be expected with on-going production (fig 2a-c).

Table 2. Metabolites present in tissue and storage fluid in HMP or SCS kidney systems at 24 hours. Data reported as Median (Interquartile Range), unless stated otherwise. Statistical test: #Wilcoxon paired signed rank test (two tailed). *Significant at $p<0.05$.

Fig. 2. Concentration of metabolites in the storage fluid of SCS and HMP kidneys over 24 hour time period for four example metabolites. Values plotted as median (interquartile range).

Reduced glutathione is a constituent of both KPS-1 (used in HMP) and SPS-1 (used in SCS) fluids at equal concentrations. Whilst this remained at stable in the SCS environment, the glutathione was clearly consumed by the HMP group and after 8 hours concentrations were 17.6 fold higher in the SCS fluid (1.60mM vs. 0.091mM, p=0.001) (fig 2d). Despite apparent organ uptake of reduced glutathione, there was no evidence of this in the tissue samples from either group.

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Discussion

The aim of this study was to determine any metabolic differences between the two clinically used methods of organ storage in this animal model.

Whilst the calculation of the total amount of metabolite within the system does rely on several assumptions (complete metabolite extraction from tissue and metabolite homogeneity within tissue), we felt this was imperative to draw meaningful comparison between groups and enables the calculation of net metabolite production/consumption in these two closed systems (HMP and SCS).

Although the storage fluid used in each experimental group differs (most notably absence of glucose in the SCS fluid) and therefore caution should be exercised in attributing any differences merely to the parameters of storage (i.e. HMP or SCS), this study was designed to assess metabolism during the two clinically used organ preservation techniques, not merely the storage modality in isolation.

This study clearly demonstrates the presence of major central metabolites such as lactate, glutamate, fumarate, aspartate and acetate at greater levels in the HMP system compared with both controls and SCS (fig. 2). This strongly suggests that these metabolites are being produced during HMP. Furthermore, the accumulation of these metabolites into the circulating

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perfusion fluid demonstrates effective homeostatic mechanisms are active to prevent over accumulation within the local cellular environment.

The list of metabolites reported in this study is not exhaustive and is a limitation of this study. Some interesting substrates (eg glucose) were excluded as this is only present in one of the storage fluids (KPS-1). For others the 1D ^{1}H NMR spectral pattern is either ambiguous or can be hidden under more domineering peaks from other compounds.

The increased total lactate in the HMP system is likely to reflect increased glycolysis in the HMP model. Although new glycolytic activity of the glucose within the HMP fluid is one likely contributor, this is unlikely to the singular cause. This is supported by evidence that the HMP fluid glucose concentrations did not decrease during the study period and replicates findings from previous human studies [10]. However conversion of a proportion of perfusion fluid glucose into lactate through glycolytic pathways has been corroborated by work demonstrating activity of these pathways using ^{13}C labelled glucose tracers[25].

The net gain of glutamate, fumarate, aspartate and acetate during HMP is also intriguing. Whilst identification of responsible metabolic pathways is difficult to ascribe solely with ^{1}H NMR studies, one explanation could be increased oxygen dependent tricarboxylic acid (TCA) cycle activity. Although uniform upregulation of TCA intermediates would support this theory, as discussed, many are not easily identifiable using ^{1}H NMR methods[6] and are

rarely found in equipoise even *in vivo* [14]. Several (^{13}C) NMR studies have reported glutamate as a valid marker of TCA activity [3,5,20].

For some metabolites, the total system amounts for HMP and SCS kidneys were comparable to the controls, suggesting that either *de novo* production does not occur during the 24 hour preservation or that consumption mirrors production (table 1 *supplementary*). However, for metabolites with similar total amounts, the compartment in which they were located varied per metabolite. Some metabolites were entirely contained within the HMP kidney tissue (e.g. ADP, AMP, NAD+) and presumably in the intracellular compartment. Other metabolites were evident in both the tissue and storage fluid but at higher concentrations in the HMP fluid. These discrepancies in metabolite location further highlight that cellular transport processes are active in this environment but that movement of metabolites into the extracellular fluid is not indiscriminate.

Reduced glutathione is a constituent of the preservation fluid KPS-1 and is thought to play a role in the removal of Reactive Oxygen Species (ROS) generated during metabolism [19] In contrast to the SCS kidney, there is a rapid decrease in the concentration of glutathione in the preservation fluid of HMP stored kidneys and is about 5% of the SCS values after 8 hours (fig 1c.). The rate of glutathione depletion observed in this study is similar to a previously reported animal model [28] and is likely to reflect cellular uptake of this protective antioxidant. Interestingly, glutathione concentration remained relatively constant in the SCS kidney group. This further reinforces the

concept that HMP exerts its beneficial effects, at least in part, by providing access to the cellular components of the kidney during perfusion. Absence of reduced glutathione in tissue demonstrates that not only is this protective antioxidant readily absorbed by the kidney during perfusion but that even after a few hours it is not longer available in the reduced state.

Although the number of organs in each experimental group is small (n=7), it is comparable to other porcine kidney transplant reports [8,12,15,21,26,30]. To improve validity, samples were processed in triplicate at each timepoint and over 250 NMR spectra were analysed. One strength of this study is that the kidneys stored by HMP or SCS were paired, i.e. from the same pig, thus minimising any metabolic differences arising from polymorphism in cellular mediators of porcine metabolism. Although this approach does not provide functional outcome information for the preserved organ, previous studies have demonstrated good function for otherwise healthy porcine organs stored by either SCS or HMP for similar time periods[2,8,13,15,21,26].

This study demonstrates that in a porcine model, the distribution and amounts of metabolites vary significantly with the storage method (HMP or SCS). The net gain of many central metabolites during HMP conditions further supports the notion that significant metabolism occurs during HMP and this may contribute to the beneficial role of machine perfusion.

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364 Acknowledgements

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366 This work was funded through grants from University Hospitals Birmingham

367 Charities and Organ Recovery Systems.

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Fig. 1. (suppl) Metabolites with comparable total amounts between SCS and HMP systems but significantly elevated compared with controls. Metabolite levels represent total amounts (mmol) in the storage fluid, kidney tissue and entire system for porcine kidneys after 24hrs of HMP or SCS or time zero controls. Highly significant (**p<0.01) and significant (*p<0.05) differences between HMP and SCS systems versus controls.

Fig. 2. (suppl) Chemical shift used for metabolic quantification. Localised spectral plots for metabolites of interest with shaded figures illustrating metabolite quantification via best-fit analysis using Chenomx metabolite database.

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	Storage Modality			p-Values		
	Control System (mmol)	**SCS System (mmol)**	**HMP System (mmol)**	**Control vs SCS** $^{\Psi}$	**Control vs HMP** $^{\Psi}$	**SCS vs HMP** $^{\#}$
Glutamate	1.54 (1.12- 1.84)	1.38 (1.11- 1.66)	3.97 (3.69- 4.71)	0.731	0.002*	0.031*
Myoinositol	1.18 (1.16- 1.19)	1.29 (1.01- 1.52)	2.16 (1.85- 2.41)	0.731	0.002*	0.031*
Lactate	1.11 (0.976- 1.23)	1.38 (1- 1.75)	2.13 (1.67- 2.71)	0.138	0.002*	0.031*
Hypoxanthine	0.454 (0.356- 0.515)	0.710 (0.641- 0.762)	1.05 (0.909- 1.17)	0.001*	0.002*	0.156
Formate	0.442 (0.274- 0.638)	0.643 (0.589- 0.779)	0.842 (0.750- 0.943)	0.101	0.004*	0.031*
Acetate	0.210 (0.206- 0.212)	0.296 (0.253-0.301)	0.552 (0.494-0.654)	0.234	0.041*	0.031*
Alanine	0.302 (0.243- 0.360)	0.486 (0.339- 0.499)	0.501 (0.368- 0.558)	0.035*	0.015*	0.313
Succinate	0.283 (0.267- 0.297)	0.462 (0.312- 0.52)	0.434 (0.307- 0.541)	0.001*	0.015*	0.844
Inosine	0.588 (0.561- 0.628)	1.08 (0.885- 1.12)	0.185 (0.146- 0.233)	0.001*	0.002*	0.031*
Aspartate	0.114 (0.104- 0.118)	0.107 (0.0879- 0.11)	0.165 (0.140- 0.215)	0.234	0.041*	0.031*
Leucine	0.0476 (0.0441- 0.0517)	0.0667 (0.0513- 0.0820)	0.0693 (0.0495- 0.0773)	0.014*	0.026*	0.688
Niacinamide	0.0196 (0.0181- 0.0207)	0.0289 (0.0243- 0.0319)	0.0490 (0.0420- 0.0557)	0.001*	0.002*	0.031*
Tyrosine	0.0262 (0.0217- 0.0302)	0.0434 (0.0339- 0.0462)	0.0387 (0.0332- 0.0431)	0.001*	0.013*	0.438

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Fumarate	0.00412 (0.00339-0.00418)	0.00308 (0.00145-0.00348)	0.0133 (0.0077-0.0212)	0.064	0.002*	0.031*

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	Storage	Total storage fluid amount (mmol)	p-value[#]	Total kidney tissue amount (mmol)	p-Value[#]
Glutamate	SCS	0.0812 (0.125- 0.155)	0.0312*	0.952 (1.26- 1.58)	0.6875
	HMP	2.72 (2.75- 2.89)		0.94 (1.24- 1.68)	
Myoinositol	SCS	0.316 (0.399- 0.879)	0.0625	0.596 (0.676- 0.853)	0.5625
	HMP	1.05 (1.25- 1.38)		0.653 (0.816- 1.3)	
Lactate	SCS	0.153 (0.205- 0.245)	0.0312*	0.89 (1.14- 1.59)	0.0312*
	HMP	1.15 (1.38- 1.82)		0.521 (0.755- 0.895)	
Hypoxanthine	SCS	0.294 (0.328- 0.404)	0.0312*	0.289 (0.407- 0.424)	0.0625
	HMP	0.705 (0.781- 0.867)		0.189 (0.258- 0.31)	
Formate	SCS	0.132 (0.136- 0.186)	0.4375	0.434 (0.486- 0.545)	0.0312*
	HMP	0.151 (0.16- 0.169)		0.688 (0.599- 0.774)	
Acetate	SCS	0.073 (0.0808- 0.0912)	0.0312*	0.167 (0.201- 0.229)	0.0312*
	HMP	0.239 (0.257- 0.331)		0.252 (0.289- 0.344)	
Alanine	SCS	0.0465 (0.0643- 0.0815)	0.0312*	0.303 (0.415- 0.435)	0.0312*
	HMP	0.253 (0.306- 0.358)		0.116 (0.187- 0.207)	
Succinate	SCS	0.0104 (0.0155- 0.0184)	0.0312*	0.298 (0.446- 0.498)	0.0312*
	HMP	0.104 (0.131- 0.208)		0.203 (0.294- 0.347)	
Inosine	SCS	0.703 (0.852- 0.961)	0.0312*	0.145 (0.182- 0.201)	0.0312*
	HMP	0.0877 (0.108- 0.128)		0.058 (0.0723- 0.109)	
Aspartate	SCS	-	0.0312*	0.0879 (0.107- 0.11)	0.3125
	HMP	0.039 (0.0452- 0.0682)		0.0874 (0.115- 0.155)	
Leucine	SCS	0.00442 (0.00506- 0.00761)	0.0312*	0.0486 (0.0591- 0.0775)	0.0312*
	HMP	0.0285 (0.038- 0.0468)		0.0222 (0.0304- 0.0318)	
Niacinamide	SCS	-	0.0312*	0.0243 (0.0289- 0.0319)	0.0938
	HMP	0.0221 (0.028- 0.0282)		0.0194 (0.0221- 0.0278)	

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Tyrosine	SCS	0.00336 (0.0071- 0.00843)	0.0312*	0.0306 (0.0371- 0.0391)	0.0312*
	HMP	0.0197 (0.0228- 0.0276)		0.0112 (0.0143- 0.0171)	
Fumarate	SCS	-	0.0312*	0.00145 (0.00308- 0.00348)	0.0312*
	HMP	0.00456 (0.00737- 0.00895)		0.00314 (0.00574- 0.0126)	

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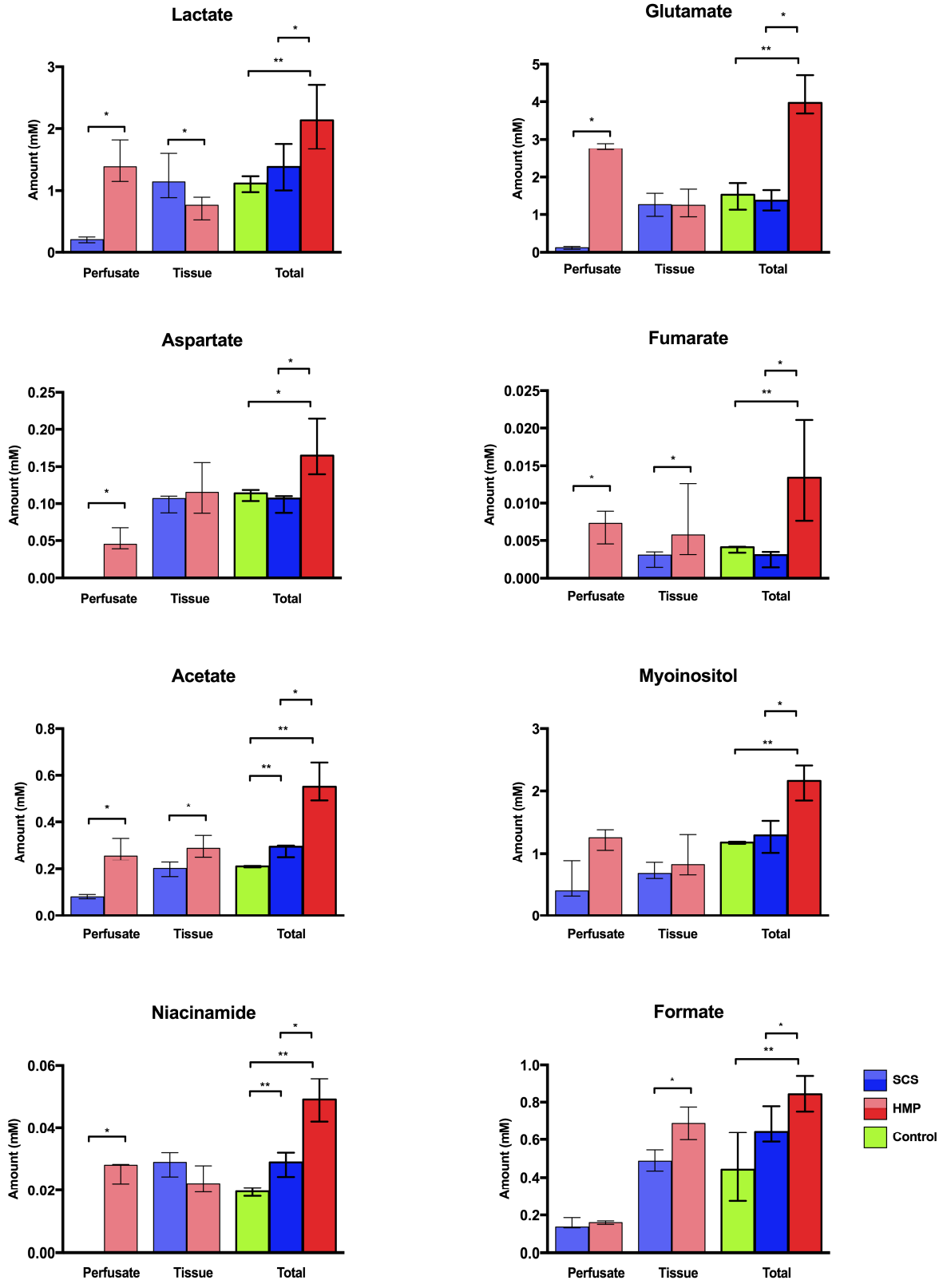

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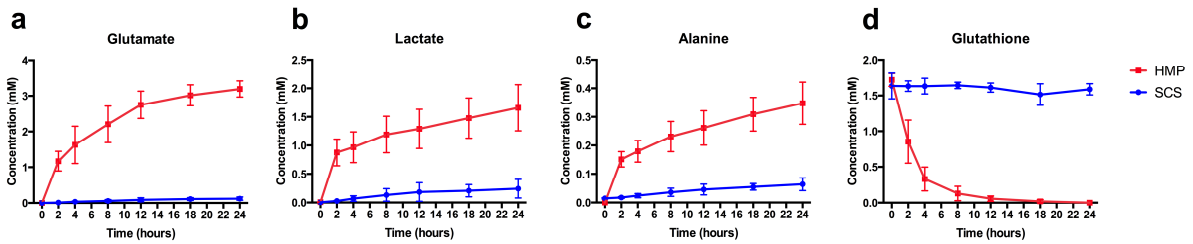

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Metabolic differences between cold stored and machine perfused porcine kidneys: A ^{1}H-NMR based study.

Re: Funding

This work was funded through grants from University Hospitals Birmingham Charities and Organ Recovery Systems.

Kind Regards,

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