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Title: Normothermic machine perfusion (NMP) inhibits proinflammatory responses in the liver and promotes regeneration

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Abbreviations: AST, aspartate transaminase; CS, cold storage; DBD, donation after brain death; DCD, donation after cardio-circulatory death; IRI, ischemia-reperfusion injury; NMP, normothermic machine perfusion, MPO, myeloperoxidase.

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Background and Aims: Liver transplantation is a successful treatment for patients with liver failure. However, organ shortage results in over 11% of patients losing their chance of a transplant due to liver decompensation and death. Ischemia/reperfusion injury (IRI) following conventional cold storage (CS) is a major cause of injury leading to graft loss after liver transplantation. A novel method of organ preservation, normothermic machine perfusion (NMP), provides oxygen and nutrition during preservation and allows aerobic metabolism. NMP has recently been shown to enable improved organ utilization and post-transplant outcomes following a Phase I and a Phase III randomized trial. The aim of the present study is to assess the impact of NMP on reducing IRI and to define the underlying mechanisms.

Methods: We transplanted and compared 12 NMP with 27 CS-preserved livers by performing gene microarray, immunoprofiling of hepatic lymphocytes and immunochemistry staining of liver tissues for assessing necrosis, platelet deposition and neutrophil infiltration, and the status of steatosis after NMP or CS pre- and post-reperfusion.

Results: Recipients receiving NMP grafts showed significantly lower peak AST levels than those receiving CS grafts. NMP altered gene-expression profiles of liver tissue from pro-inflammation to pro-healing and regeneration. NMP also reduced the number of IFN-γ and IL-17 producing T cells and enlarged the $CD4^{pos}CD25^{high}CD127^{neg}FOXP3^{pos}$ Treg pool. NMP liver tissues showed less necrosis and apoptosis in the parenchyma and fewer neutrophil infiltration compared to CS liver tissues.

Conclusion: Reduced IRI in NMP recipients was the consequence of the combination of inhibiting inflammation and promoting graft regeneration.

Introduction

Liver transplantation is the only treatment for patients with end-stage acute and chronic liver failure. It is a successful treatment with a 5-year patient and graft survival rate exceeding 80% (NHS BT report 2016 to 2017). However, organ shortage remains a global problem that results in the removal of over 11% of patients placed on the waiting list due to death or worsening of their condition (1).

The rate of organ donations has increased over the last 10 years. However, this increase is mainly limited to high-risk, so-called 'marginal donors', which largely comprise donors deceased after circulatory death (DCD) (2) older donors, and fatty livers (3). There is evidence that marginal organs have greater vulnerability to ischemia reperfusion injury (IRI), an inevitable process of organ transplantation (4).

A number of experimental and clinical studies have shown that reperfusion of allografts is associated with organ inflammation characterized by the release of inflammatory mediators, up-regulation of adhesion molecules, immune cell infiltration and increased cell death (5–7). This damage affects short and long-term organ outcome by contributing to the development of primary non-function and ischemic cholangiopathy respectively (8).

Normothermic machine perfusion (NMP) is a novel technology that allows preservation and transport of organs from donor hospital to recipient hospital at body temperature while providing the organ with oxygen and nutrients (9). NMP has been introduced into clinical practice with the aim of minimizing IRI and its effects on the liver graft, enabling successful preservation and functional testing of marginal livers, improving the logistics of transplantation by allowing prolonged preservation from 12 to 24 hours, and ultimately increasing the number of livers successfully transplanted. A phase I clinical trial using a novel NMP device (*metra*®, OrganOx Ltd, UK) demonstrated safety of the technology (10). The NMP device also led to a reduction in the levels of liver enzyme aspartate

aminotransferase (AST) in the recipients, which is used as a surrogate marker of liver damage (10). The reduction in allograft damage and improved organ utilization of NMP over CS has been further confirmed in a randomized controlled multicenter trial including seven liver transplant centers from the UK and Europe (Consortium for Organ Preservation in Europe, COPE, www.cope-eu.org), in which a total of 137 NMP and 133 cold storage (CS) grafts were enrolled (11), with 120 NMP and 100 CS livers ultimately transplanted.

The current investigation aims to assess the impact of NMP preservation on the degree of liver allograft IRI compared to conventional CS, and to identify the mechanisms underpinning any observed differences.

Material and Methods

DONORS AND RECIPIENTS

In 2013, we carried out 14 liver transplants using DBD (n = 12) and DCD (n = 2) grafts preserved with NMP, as part of a phase I clinical trial that recruited a total of 20 patients (10). In the current analysis, we included only the 12 DBD grafts and excluded the DCD transplants due to the small number. These livers were matched with 27 CS DBD grafts that were transplanted in the same period. Donors and recipients in both groups were matched according to age, intensive therapy unit (ITU) stay, infections, inotropic support and donor liver steatosis. The recipients were also matching for their original liver diseases, with a similar proportion of alcoholic related liver diseases, autoimmune liver diseases (autoimmune hepatitis, primary sclerosing cholangitis and primary biliary cholangitis), HCV infection, HCC, inherited hepatic cholestasis and drugs induced acute liver failure [paracetamol overdose (POD) and non-POD], they also presented with a similar severity of liver disease, representing by MELD scores assessed at the time listing for liver transplant. Their demographic and clinical data were summarized in Tables 1 and 2 (Table 1 for donors and

Table 2 for recipients respectively). This study was approved by the Ethics Committees of St Thomas's Hospital (REC Reference: 09/H0802/100) and Dulwich Ethics Committee Approvals were also obtained from National Health Service Blood and Transplant (NHSBT), National Research Ethics Committee, and the Medicines and Healthcare Products Regulatory Authority. The trial was registered with the ISRCTN (14355416)

LIVER PERFUSATE AND ISOLATION OF HEPATIC MONONUCLEAR CELLS (HMC)

We previously showed that intrahepatic lymphocytes (HMC) obtained through liver perfusion are representative of liver-resident lymphocytes (12). Donor livers were perfused at the end of NMP or CS with preservation solution as previously described (10). In brief, in NMP the perfusion was stopped at the end of preservation and the organ was cooled by rapid flashing of 2 liters of cold HTK solution (Custodiol-HTK, Essential Pharmaceuticals, Ewing, NJ) (10). An additional 1 liter was used to further flush the grafts and the clear perfusate was collected for mononuclear cell extraction. In CS livers, grafts were perfused with 1 liter of UW solution at the end of preservation and the perfusate was similarly collected. All perfusates were processed within 6 hours by centrifugation to reduce the volume. The final volume of 30 ml of HMC rich solution was used to isolate HMC by density gradient centrifugation (Lymphoprep, GE, Sweden) (12). HMC isolated after NMP (8 out of the 12 cases) were compared to those after CS (20 out of 27 grafts).

PHENOTYPIC AND FUNCTIONAL ANALYSIS OF HMC USING FLOW CYTOMETRY

HMCs were stained for 20 min at 4°C degree using monoclonal antibodies (mAbs) specific for CD3-PE-Cy7, CD4-APC-Cy7, CD8-PercP-Cy5.5, CD19-APC, CD25-PE,

CD127-FITC (Cambridge, UK) and FOXP3-FITC (eBioscience, San Diego, USA). For intracellular cytokine detection HMC were stimulated with PMA/Ionomycin/Brefeldin (eBioscience) for 4 hours, fixed (Cytofix/CytoPerm eBiosciences) then stained using mAbs specific for IL-2, IL-10, IL-17, IL-22, IL-23, IFN-γ and TNF-α (BioLegend, San Diego, USA). 7AAD was used to exclude dead cells from the analysis. Cells were acquired on a BD Canto II (BD, Bioscience, San Jose, USA). Analysis was performed using Flowjo (Tree Star Inc., USA).

LIVER BIOPSIES

Tru-cut liver biopsies were performed on NMP and CS grafts at the end of preservation (pre-reperfusion) and 60 min following reperfusion (post-reperfusion, Figure 1. Panel A). A 2-3-mm portion of the needle biopsy liver cylinder was immediately snap frozen in liquid nitrogen and transferred to -80°C degree freezer. The remaining cylinder was formalin fixed and paraffin embedded (FFPE).

LIVER TISSUE RNA EXTRACTION AND PROCESSING

For total RNA extraction, cryopreserved liver tissue samples were homogenized in TRIzol reagent (Invitrogen) using a pestle and nuclease-free 1.5-ml reaction tubes (Ambion). Total RNA was then extracted following the manufacturers guidelines, and quality was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies).

LIVER TISSUE MICROARRAY GENE EXPRESSION ANALYSIS

Microarray data were analyzed using Illumina microarrays: expression data were computed using BeadStudio data analysis software (Illumina) and subsequently processed employing quantile normalization using the Lumi bioconductor package. Next, a step of conservative probe-filtering was used to excluding those probes with a detection P value

greater than 0.05 in all samples which resulted in the selection of a total of 27084 probes from the original set of 29377. Differential expression was assessed by using linear models and empirical Bayes moderated t-statistics. Linear Models for Microarray Analysis (LIMMA) R-package software was used for the analysis of gene expression microarray data. Group comparisons and determinations of false discovery rates (FDR computation using Benjamini-Hochberg procedure) were performed (13). All liver tissue microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GSE112713). To further explore the functional relationships between groups we dissected the molecular pathways contained in the microarray differential gene expression data set employing both Gene Set Enrichment Analysis (GSEA) using canonical pathways C2 geneset collection (14).

qPCR on liver tissue

qPCR was conducted employing the ABI 7900 Sequence Detection System and TaqMan LDA microfluidic plates (Applied Biosystems). DNA was removed from total RNA preparations using Turbo DNA-free DNAse treatment (Ambion), and RNA was then reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). To quantify transcript levels, target gene Ct values were normalized to the housekeeping gene (GAPDH) to generate ΔCt values. 88 genes were analysed in total.

IMMUNOHISTOCHEMISTRY STAINING AND HISTOLOGICAL IMAGING

Pre- and post-reperfusion biopsies were fixed in 10% buffered formalin, paraffin embedded, then cut into 4 µm-thick sections fixed to microscope slides. Tissue sections were stained with routine haematoxylin and eosin (H&E), as well as immunohistochemical (IHC) staining for myeloperoxidase (MPO), using a fully automated IHC Leica BOND-MAX immunostainer (Leica Biosystems, Newcastle, UK), and each biopsy was scored using

standard criteria (see supplementary S1). The signal was detected with a Leica Bond Polymer Refine Detection kit (Novocastra, Newcastle, UK). The antibodies used were obtained from Abcam Plc. (Cambridge, UK).

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, USA). Data were presented as mean values +/- SEM, and statistical significance was determined using the Mann Whitney U test when comparing two unpaired/unmatched groups; the Kruskal-Wallis test when comparing multiple unpaired/unmatched groups with post hoc Dunns multiple comparison tests; and the Two-Way ANOVA (Ordinary) with Sidak's multiple comparisons test when comparing multiple unmatched groups with more than one grouping variable. All tests carried out were nonparametric. Statistical significance was considered when: $*P \leq 0.05$.

Results

CLINICAL DATA SHOWED REDUCED IRI

Consistent with the clinical data derived from two clinical trials (10,11), peak AST within 7 days was significantly lower in the NMP group compared with the CS group ($p<0.01$) (Table 2) . Additionally, peak INR within 7 days was lower in NMP group compared to CS group ($p=0.07$). The other biochemical post-operative parameters that include alkaline phosphatase (ALP) and total bilirubin together with post-transplant clinical parameters such as the days of ITU stay, the rates of acute rejection and one year graft and recipient survival, which were comparable between the two groups. The data are shown in Tables 1 and 2.

NMP UP-REGULATED TISSUE REGENARATION GENES AND DOWN-REGULATED PRO-INFLAMMATORY GENES

In order to gain a global picture of how NMP protects hepatocytes and maintains allograft function, we assessed the gene expression profiles of NMP (n=11) and CS (n=11) liver biopsies taken after preservation (pre-reperfusion) and after transplantation (post-reperfusion) by using microarray analysis (Figure 1. Panel A).

19,416 genes were assessed with a cut-off for significance of 1.5-fold change and of <25% false discovery rate (FDR). We assessed the gene expression changes that occurred from pre-reperfusion to post-reperfusion for both NMP and CS liver tissues; we also assessed the unique genes changed in each cohort and the changes common to both. For both NMP and CS there were more genes down regulated (CS: 3550; NMP: 2402; Both: 2212) than up-regulated post-reperfusion (CS: 256; NMP: 306; Both: 243). Among those 306 up-regulated genes in NMP there are genes that relate to tissue regeneration and platelet function, but not many genes which relate to immune cell functions. In contrast, there was a high representation of immune-related genes within the 256 up-regulated in CS, in particular pro-inflammatory cytokines and genes involved in humoral immunity, neutrophil chemotaxis and platelet function. A similar functional representation was seen for down-regulated genes in the 2402 NMP genes and the 3550 CS genes. The genes that commonly changed in both NMP and CS contained a moderate number of immune-related genes; both pro- and anti-inflammatory, neutrophil- and coagulation-related genes, and those involved in tissue regeneration (Figure 1. Panel B).

We then investigated the differences in gene expression between NMP and CS at the time of pre- and post-reperfusion. The differences were greater at the pre-reperfusion stage (568 up- and 574 down-regulated genes in NMP relative to CS) compared to those in post-

reperfusion stage (213 up- and 219 down-regulated genes in NMP relative to CS) (Figure 1. Panels C and D). To investigate what biological pathways are altered due to these changes in gene expression, we analysed the gene data using GSEA canonical pathway analysis using a cut-off for significance of <25% false discovery rate (FDR). NMP showed a down regulation of 123 pathways pre-reperfusion and 74 pathways post-reperfusion relative to CS (see supplementary S2 and S3). Pathways down-regulated in pre-reperfusion included "allograft rejection", "graft versus host disease", 7 platelet/coagulation pathways, and 16 immune pathways including "TH1TH2", "IFNG signalling", "PD1 signalling", "IL-2", "IL-12", "IL-6", and "CCR5". Pathways down-regulated in post-reperfusion included 3 platelet/coagulation pathways and 12 immune pathways including "IL-17", "neutrophils", "IFNFLAM", "BCR", "integrin 2", "leukocyte transendothelial migration", and "PD1 signalling".

In order to validate our findings from the microarray analysis we performed quantitative PCR (qPCR) on 88 genes that were either up- or down-regulated in NMP livers relative to CS livers; including genes involved in stress response, growth and regeneration, apoptosis, metabolism, and immune responses.

We also investigated a number of IRI-related genes previously described by us (5). The majority of genes showed a clear differential expression between livers that were maintained by NMP and those stored using CS. A number of genes involved in response to stress and cell death or apoptosis were up-regulated in CS but not in NMP livers post-reperfusion (Figure 2. Panel A), examples of which include the genes for thrombomodulin (THBD) and IFN-γ (IFNG) (Figure 2. Panel B). NMP livers showed a significant up-regulation of genes involved in immune-trafficking compared to CS in both Pre- and Post-reperfusion stages; such genes included CXCL9, CXCL10, and CXCL11 (Figure 2. Panel B).

$CD4^{pos}CD25^{high}CD127^{neg}FOXP3^{pos}$ REGULATORY T CELLS WERE MORE ABUNDANT IN NMP GRAFTS

We have previously shown that intrahepatic lymphocytes obtained through liver perfusion are representative of liver-resident lymphocytes (12). We assessed the impact of NMP on the proportions of HMC compared to CS using flow cytometric analysis of liver perfusates collected after each respective preservation (pre-reperfusion, Figure 1. Panel A).

Viability of HMC from both NMP and CS liver grafts was constantly higher than 95%. Approximately 9 to 50 x 10^6 living HMC were isolated from NMP liver grafts (n = 8) and 40 to 60 x10^6 from CS grafts (n = 21).

The proportion of hepatic $CD3^{neg}CD19^{pos}$ B cells, $CD3^{pos}CD4^{pos}$ T cells and $CD3^{pos}CD56^{pos}$ NKT-like cells were comparable amongst NMP and CS grafts. The proportion of $CD3^{neg}CD56^{pos}$ NK cells tended to be higher in CS than in NMP grafts (p=0.08) (Figure 3. Panels A and B). By contrast, the proportion of $CD3^{pos}CD8^{pos}$ T cells (41.97%±16.03 versus 18.6%±8.2, p=0.0005) (Figure 3. Panel B) and $CD4^{pos}CD25^{high}CD127^{neg}FOXP3^{pos}$ regulatory T cells (Tregs) (4.36%±3.27 versus 1.9%±1.8, p=0.0156) were significantly higher in NMP than in CS liver grafts (Figure 3. Panel C).

NMP GRAFTS CONTAINED FEWER PRO-INFLAMMATORY CYTOKINE-PRODUCING T CELLS THAN CS GRAFTS

Intrahepatic T cells are a rich source of T helper and regulatory cytokines; chemical mediators capable of promoting inflammation, cellular regeneration, fibrosis, or immune tolerance. Using flow cytometry, we next assessed the effect of NMP on Th1 (IL-2, IL-17, IFN-γ), Th2 (IL-4) and Treg (IL-10 and TGF-β) cytokine production by intrahepatic $CD4^{pos}$ and $CD8^{pos}$ T cells (Figure 3. Panels D and E). NMP had significantly lower proportions of

$CD4^{pos}$ T cells producing IL-4 ($p<0.05$), IL-2 ($p<0.001$), IFN-γ ($p<0.05$) and IL-17 ($p<0.0001$). There was no change in the production of the regulatory cytokines IL-10 (Figure 3. Panel E) or TGF-β (data not shown). Furthermore, NMP significantly decreased the proportions of $CD8^{pos}$ T cells producing IFN-γ ($p<0.001$), while modest reductions in IL-17 were also observed. There were no changes in IL-2 production by $CD8^{pos}$ T cells after NMP (Figure 3. Panel E).

DONOR ICU-STAY INFLUENCED T CELL FUNCTION IN NMP GRAFTS

We then sought the associations between HMC function and allograft functions. Among HMC collected after NMP, there was a positive correlation between the days of donor ICU stay and the frequency of IL-17 producing $CD4^{pos}$ T cells ($R^2=0.69$, $p=0.01$) (Figure 3. Panel F). There was no correlation between donor infection status, BMI and/or fatty liver score and HMC functions.

COLD ISCHEMIA TIME WAS THE MAIN FACTOR INFLUENCING ALLOGRAFT FUNCTION

In the setting of CS, there was a positive correlation between peak AST levels and the duration of cold ischemia ($R^2=0.34$, $p=0.0071$), and a negative correlation between IL-10 producing $CD4^{pos}$ T cells and duration of cold ischemia ($R^2=0.56$, $p=0.01$) (Figure 3. Panel F).

Although we did not observe a correlation between the frequency of IFN-γ-producing $CD8^{pos}$ T cells and peak AST levels as reported by us in 2015 for CS grafts, after combining the CS and NMP cases, there was a positive correlation between the frequency of IFN-γ producing $CD8^{pos}$ T cells and peak AST levels ($R^2=0.4$, $p<0.05$) (data not shown). We did not

observe any correlations with donor parameters, including age, BMI, days of ICU stay, infection status and fatty liver score.

REDUCED NEUTROPHIL INFILTRATION AND CELL DEATH FOLLOWING NMP

By performing tissue staining on biopsies collected pre- and post-reperfusion, we sought evidence to support the hypothesis that reduced IRI is the consequence of NMP (H&E staining not shown), since both pre- and post-reperfusion biopsies showed fewer apoptotic/necrotic cells in NMP compared to CS, although apoptosis/necrosis was increased in post-reperfusion biopsies in both NMP and CS conditions compared to pre-reperfusion (Figure 4. Panels A and B, Figure 5. Panel A).

The number of neutrophil clusters tended to be lower in pre-reperfusion compared with post-reperfusion biopsies in both NMP and CS (Figure 5. Panel B). However, NMP livers showed lower numbers of clusters compared with CS livers in both pre- and post-reperfusion stages (Figure 5. Panel B).

We also evaluated neutrophil infiltrate by MPO single epitope staining. The NMP group had significantly fewer of MPO^{pos} cells in the liver lobules, in both pre- ($p = 0.0002$) and post- ($p = 0.036$) reperfusion biopsies compared to CS allografts, although the reduction of MPO^{pos} cell staining was more pronounced at the pre-reperfusion stage (Figure 5. Panel C).

STEATOSIS STATUS DID NOT CHANGE AFTER NMP

Finally, we assessed the impact of NMP on the fatty content of the liver grafts. We did not observe significant differences in terms of steatosis, both small and large droplet (data not shown), between pre- and post-reperfusion biopsies.

DISCUSSION

NMP has emerged as an effective tool for liver graft preservation, since it has the ability of avoiding cold ischemia and reducing IRI sequelae as shown in a phase I clinical trial (10) and a recent randomized multicentre controlled clinical trial (11). It is assumed that the beneficial effect of NMP is the consequence of a combination of factors, including perfusion at body temperature, oxygenation, and the addition of nutrients and anti-oxidants, which maintain the liver as closely as possible to physiological conditions. In the current study, we showed that the beneficial effect of NMP was derived from the combination of the following: maintaining liver metabolism, inhibiting inflammation and reducing cell death.

We have confirmed that NMP did have an impact on graft quality by reducing IRI sequelae in liver grafts, in the form of lower peak AST levels in the first postoperative week compared to those receiving CS grafts. AST is a well-defined surrogate marker for long term graft function and survival (15). This is consistent with the previously reported data (10,11), confirming that NMP is indeed able to reduce the degree of IRI. Although the impact of NMP on one-year graft and patient survival was comparable between NMP and CS groups, longer period of follow-up would be more desirable.

By collecting liver tissues at two key time points, pre-reperfusion (post NMP) and post-reperfusion, we assessed the gene expressing patterns in three ways: 1) differences occurred from pre-reperfusion to post-reperfusion for both NMP and CS liver tissues; 2) differences between NMP and CS at pre- and post-reperfusion; 3) the unique genes changed in each cohort and the changes common to both.

When NMP and CS were assessed separately in terms of the gene expression changes that occurred post-reperfusion relative to pre-reperfusion, NMP liver showed significant gene activity in the areas of growth, metabolism and tissue repair, while CS livers showed more immune-related gene activity post-reperfusion. In order to directly compare NMP to CS, the

differences in gene-expression profiles were assessed in both pre- and post-reperfusion biopsies. NMP showed the greatest difference from CS at the pre-reperfusion stage, in particular, the down-regulation of pathways such as “allograft rejection”, “graft versus host disease”, and “platelet/coagulation”, we believe that the alterations in these pathways possibly contributed to the beneficial effect of NMP to minimise IRI. It is worth pointing out that some of the pathways down-regulated in NMP have been defined previously as being involved in inflammation in the setting of transplantation (16), such as “IFNG signalling” (17), “IL-2” (18), “IL-6” and “IL-12” (19). In combination with “PD1 signalling” (20), the down-regulation of these pathways could suppress the initiation of IRI and reduce cell apoptosis. More importantly, “PD1 signalling” was continuously down-regulated post-reperfusion, along with several others with known roles in IRI, including “platelet/coagulation” (21), “neutrophils” (22) and ‘IL-17” (23).

In addition, both NMP and CS promoted up-regulation of pathways including “differentiation”, “regeneration” and “healing”. Taking together the fact that NMP down-regulated inflammation, the overall impact of NMP should favour the preservation of grafts in good quantity by minimizing inflammatory and cell death events associated with IRI.

While confirming the alterations of 88 key genes following NMP by qPCR, we further evaluated the expression levels of individual genes (Figure 2. Panel B). Of these genes, the CXC chemokine family genes, CXCL9, CXCL10 and CXCL11, known to elicit their chemotactic functions by interacting with the chemokine receptor CXCR3 (24), changed their expression in synchrony. Their levels were significantly higher in NMP tissues than in CS at both time points, although CXCL11’s level was higher at post-reperfusion only. It is known that IFN-γ induces the expression of CXCL9 (25), its up-regulation was not hindered by the reduced levels of IFN-γ in NMP grafts. The CXC family was recently shown in mouse to have a novel role in converting CD4 T effectors into CD4/CD8 double negative regulatory T

cells and their migration to the liver (26), hence this pathway might contribute to the reduced IRI.

Next, we tested the hypothesis that inhibited gene expression defined by microarray could be linked to their translation status, i.e. cytokine secretion by donor derived T cells to inhibit inflammation. We collected hepatic lymphocytes at the end of NMP, the same time point when pre-reperfusion liver biopsies were taken. Therefore, we were able to evaluate the impact of NMP directly on donor hepatic lymphocytes, the major player in graft rejection and tolerance induction (27) by comparing with lymphocytes collected from CS grafts. We observed reduced numbers of pro-inflammatory cytokines IFN-γ and IL-17 producing $CD4^{pos}$ and $CD8^{pos}$ T cells which are known to be involved in the process of IRI (28, 29). In conjunction with reduced AST levels in recipients, it is logical to speculate that down-regulated pathways "IFNG signalling" and "IL-17" contribute to the overall impact of NMP in reducing IRI. The other possibility is that the altered ratio of Tregs to T effectors provides a counterbalance for the tissue damaging effects of IL-17 producing T effectors in NMP grafts. This assumption is based on an unexpected finding that $CD4^{pos}CD25^{high}CD127^{neg}$ $FOXP3^{pos}$ regulatory T cells (Tregs) pool was enlarged following NMP. In the NMP liver grafts, Tregs were about 2.3 folds compared to those in CS livers, indicating that preservation using NMP was able to retain or even increase Treg number. It is well recognised that Tregs could reduce IRI (30). In view of the fact that the liver contains approximately 10^{10} lymphocytes of which two thirds are T cells, and donor derived lymphocytes are constantly released into the recipient's circulation and could still be detected even 30 years after engraftment (31), donor-derived Tregs are likely to be involved in the process of induction of graft acceptance. Comparing the attempt of generating *ex vivo* Tregs from recipients pre-liver transplantation, with target number of 10 x 10^6 /kg recipient body weight (The ONE study UK Treg trial: https://clinicaltrials.gov/ct2/ show/NCT02129881), donor derived Tregs

outnumber those derived from the recipient. Once the outcome of this trial is released, further comparison of the efficacy of Tregs derived from either donors or recipients can be carried out.

Regarding the immunoregulatory cytokine IL-10, we observed a negative correlation between the frequency of IL-10 producing $CD4^{pos}$ T cells and cold ischemia time, indicating that CS could influence HMC immunoregulatory function in contrast with NMP, which has the potential to enhance the production of IL-10 through an enlarged Treg pool.

The other important finding is that down-regulated genes in "neutrophils" pathways did lead to a significant reduction in neutrophil infiltration in NMP grafts. This reduction was observed in both pre- and post-reperfusion liver tissues. Giving the important role played by platelets on IRI and inflammation (32), we believe that the reduction in "platelet/coagulation" gene expression may have contributed to the reduced IRI in NMP recipients, and we intend to investigate this further in future studies.

Attenuation of neutrophil infiltration in pre-reperfusion biopsies in NMP livers is of interest. Leucocyte infiltration in DBD donors is well documented as results of the systemic inflammatory response associate with brain death (33). It is possible that NMP reduces neutrophil levels by the fact that there is circulation during the preservation time and possibly to an anti-inflammatory hepatic environment, discussed earlier. More work is necessary to elucidate neutrophil movements during NMP and it's impact on graft outcome.

Research in recent years has provided rich data to elucidate the interaction between platelets and neutrophils (34), in the context of liver transplantation, by forming complexes platelets and neutrophils jointly drive IRI in the liver (35). Therefore, reducing neutrophil infiltration will lead to controlling the formation of this complex and ameliorating IRI.

One of the key goals of NMP is to improve the quality of marginal liver grafts which are currently labelled as unusable and rejected by all liver transplantation centres due to their high fat content (>60%) (36), we also assessed whether the fat content could be reduced by NMP. However, our investigation was restricted by the fact that all the liver grafts included in the current study were usable and the majority of them contain very low to no fatty content (Table 1). No change in fat content was seen following NMP. Recently, Liu Q et al (37) assessed lipid metabolism and function of discarded human livers with steatosis (up to 33.3%) after 24-hour NMP. When the period of NMP finished, the fatty content was almost unchanged, indicating that NMP is unlikely to impact on liver fat content when it is at an acceptable level and only a high proportion of fat, i.e., >60% should be treated by NMP and assessed.

In conclusion, the data of the current report have provided mechanistic support to the clinical application of NMP. By minimising inflammation, cell death and, promoting liver regeneration and healing, NMP might be an ideal tool to rescue those currently discarded marginal allografts, including DCD, and potentially to increase the donor pool.

Figure legends

Figure 1. Microarray showing gene expression profiles of NMP liver grafts compared to CS grafts indicating reduced inflammation and increased regeneration. A. Schematic diagram showing the timing of liver-tissue biopsies, the first time point was after a period of preservation by either CS or NMP right before reperfusion in the donor (pre-biopsy), and the second time point was 60 minutes after reperfusion (post biopsy); B. Venn diagram showing the changes in gene expression (up and down-regulated) in post-reperfusion relative to pre-reperfusion biopsies, as analysed by the R/Bioconductor package GO stats. In both NMP and CS, the number of genes significantly altered were displayed along with a list of the functional categories most represented by these genes; C and D. Volcano plots showing the changes in expression (up and down) of all the genes obtained by microarray analysis, in NMP liver grafts relative to CS liver grafts at the pre-reperfusion stage (C) and post-reperfusion stage (D). The grey area denotes significantly changed genes, the number of those genes was displayed, and examples were displayed with arrowed labels. Significance was decided by a cut-off of 1.5-fold change and of <25% FDR. CS: cold storage; NMP: normothermic machine perfusion; FDR: false discovery rate.

Figure 2. qPCR confirming gene expression findings by microarray. A. A heat map showing the expression levels of 88 genes, tested by qPCR from NMP and CS liver biopsies taken pre- and post-reperfusion (11 cases per group), as well as a qPCR assay control; Red denotes up-regulated expression and green is down-regulated expression; genes grouped by function were outlined by the dashed lines. B. ΔCt values (inversely proportional to gene levels) for 8 genes in liver biopsies from: Pre CS and Pre NMP (pre-reperfusion), Post CS and Post NMP (post-reperfusion);; groups were compared using Kruskal-Wallis test (* $P \leq 0.05$ and ** $P \leq 0.01$).

Figure 3. Hepatic lymphocytes from NMP liver grafts exhibiting an anti-inflammatory phenotype. A. Representative flow cytometry density plot showing T cell markers of CD4 and CD8 expression on T cells from HMC. Frequencies of $CD4^{pos}$ T, $CD8^{pos}$ T, B, NK, NKT-like cells (B), and Treg cells (C) within HMC from NMP and CS livers pre-reperfusion; D. Representative flow cytometry contour plot showing IFN-γ expression by T cell from HMC (right) and fluorescence-minus-one (FMO) control with HMC stained with all antibodies except anti-IFN-γ; E. Frequencies of IL-4, IL-10, IL-2, IFN-γ and IL-17 producing $CD4^{pos}$ and $CD8^{pos}$ T cells within HMC from NMP and CS livers; F. Linear regression analysis from left to right showing the positive correlation between the percentages of IL-17 producing $CD4^{pos}$ T cells in NMP livers and the days of donor staying in intensive care unit (ICU); a positive correlation between the peak levels of recipient aspartate aminotransferase (AST) and cold ischemic time (CIT); and a negative correlation between percentages of IL-10 producing $CD4^{pos}$ T cells in CS livers and CIT. CS: cold storage; NMP: normothermic machine perfusion; HMC: hepatic mononuclear cells; FS: forward scatter.; groups were compared using Two-Way ANOVA with Sidak's multiple comparisons test (* $P \leq 0.05$ and *** $P \leq 0.001$).

Figure 4. Reduced immune cell infiltrates in NMP liver tissue. Representative micrographs of MPO (A) stained biopsies from NMP and CS liver grafts at pre- and post-reperfusion stages, showing more disseminated immune cell infiltrate in CS compared to NMP livers, which is identified as neutrophils by MPO immunohistochemical staining. Neutrophil infiltration in the hepatic lobules was reduced in NMP liver grafts. Representative micrographs showing MPO staining in the hepatic lobules (B) of biopsies from NMP and CS liver grafts at pre- and post-reperfusion stages. High power fields were randomly selected for each case from the lobular region. MPO: myeloperoxidase.

Figure 5. IRI was reduced in NMP liver grafts compared to CS. A. Percentage of cases with cell death, apoptosis (score 1 or 2) and necrosis (score 3, 4, or 5) from NMP and CS liver grafts at both pre- and post-reperfusion stages. B. Percentage of cases with absence (score 0) or presence of neutrophil clustering (score 1 – 5) from NMP and CS grafts at both pre- and post-reperfusion stages. C. Proportions of MPO^{pos} cells in the hepatic lobule from NMP and CS grafts at both pre- and post-reperfusion stages. IRI: ischemia-reperfusion injury; groups were compared using Kruskal-Wallis test (* $P \leq 0.05$, ***$P \leq 0.001$).

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