

# Ischaemic Preconditioning and Intermittent Clamping does not Influence Mediators of Liver Regeneration in a Human Hepatocyte Model of Ischaemia-Reperfusion Injury

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## INTRODUCTION

In recent decades, hepatic resection has become an optimal treatment choice for both primary and secondary malignancies. In the operative setting, blood loss resulting from liver parenchyma transection is limited by applying vascular occlusion. Hence, ischaemia-reperfusion injury (IRI) is an inevitable phenomenon that results following liver surgery. As a consequence, parenchymal cell injury and liver dysfunction of varied severity leads to significant morbidity and mortality post-surgery<sup>[1-3]</sup>. The severity of injury following liver surgery can range from mildly elevated serum aminotransferases to more severe injury presenting as post-liver resection insufficiency<sup>[4]</sup>, which can progress to multi-organ failure and death. In addition, IRI significantly decreases the rate of DNA synthesis and impairs liver regeneration following hepatectomy<sup>[5,6]</sup>.

Liver regeneration is a complex and multi-factorial process that is mediated by interactions between regenerative cytokines, growth factors and metabolic demand of the liver following surgery and IRI. Briefly, following hepatectomy, the regeneration process is initiated by the regenerative cytokine network which includes tumour necrosis factor-alpha (TNF- $\alpha$ ) and interleukin (IL)-6<sup>[7-9]</sup>. Following stimulation from cytokine triggering mechanisms<sup>[10-12]</sup>, TNF- $\alpha$  binds to its receptor on non-parenchymal liver cells and stimulates the production of IL-6<sup>[13]</sup>, via the activation of the transcription factor nuclear factor-kappa B (NF- $\kappa$ B)<sup>[14-16]</sup>. IL-6 acts directly on hepatocytes by binding to the IL-6 receptor complex and induces the translocation of signal transducer and activator of transcription-3 (STAT-3) to the nucleus. Following this, the two main growth-promoting signalling systems, hepatocyte growth factor (HGF) and its receptor (Met) and

## ABSTRACT

**AIM:** The role of ischaemic preconditioning (IPC) on liver regeneration following surgery remains inconclusive. The aim of the study was to assess the effect of IPC on mediators involved in regeneration produced by human hepatocytes, using an in-vitro hypoxia-reoxygenation model to mimic ischaemia-reperfusion injury (IRI).

**METHODS:** Following extraction from samples obtained from liver resection ( $n=5$ ), confluent culture flasks of hepatocytes were subjected to IRI (1 hour hypoxia +1 hour reoxygenation), IPC prior to IRI (10 minutes hypoxia +10 minutes reoxygenation +1 hour hypoxia +1 hour reoxygenation), intermittent clamping (IC, 15 minutes hypoxia +5 minutes reoxygenation x3 +1 hour reoxygenation) and compared to controls. The production of various mediators was determined over 48 hours.

**RESULTS:** Interleukin (IL)-8, granulocyte-colony stimulating factor (G-CSF) and IL-1-receptor antagonist (IL-1ra) were produced by hepatocytes. IPC prior to IRI did not influence the production of IL-8, G-CSF and IL-1ra by hepatocytes compared to IRI over the study period. Similarly, IC did not significantly influence the profile of these mediators compared to IRI in this model.

**CONCLUSION:** IPC, as well as IC did not influence the production of pro-regenerative mediators in a hepatocyte model of IRI. The role of IPC in liver regeneration remains to be determined.

the epidermal growth factor (EGF) receptor and its large family of ligands which includes transforming growth factor- $\alpha$  (TGF- $\alpha$ ) and EGF<sup>[17,18]</sup>, initiate a cascade of events that leads to progression of the cell cycle, culminating in the synthesis of DNA and subsequent cellular mitosis.

Due to the inevitability of IRI in liver surgery, various investigators have attempted to elucidate methods to limit the detrimental effects of IRI and improve liver regeneration of the remnant liver. The technique of ischaemic preconditioning (IPC) involves a short period of vascular occlusion of both the arterial and portal venous inflow to the liver followed by reperfusion prior to a more prolonged inflow occlusion during parenchymal transection. The beneficial effects of IPC on the liver following IRI include decrease in severity of liver necrosis<sup>[19]</sup>, anti-apoptotic effects<sup>[20]</sup>, preservation of liver microcirculation<sup>[21,22]</sup>, and improvement in survival rate<sup>[23]</sup>, and more recently, its role in liver regeneration is currently being evaluated. In the clinical setting, besides IPC, IC is also used to limit the blood loss during liver resection. IC of 15 to 30 min of ischaemia followed by 5 to 10 min of reperfusion has been shown to reduce both liver and lung injury<sup>[24]</sup>.

The hypothesis of this study is that IPC up-regulates the production of cytokines and growth factors by human hepatocytes involved in liver regeneration. Following extraction, hepatocytes were exposed to hypoxia and reoxygenation settings to mimic ischaemia and reperfusion, and the effects of IPC on the production of cytokines and growth factors were determined.

## MATERIALS AND METHODS

### Hepatocyte extraction and culture

Human hepatocytes were extracted from liver specimens obtained from the Hepatobiliary and Transplantation Unit, St. James' s University Hospital, Leeds. Ethical approval for this study was obtained from the Leeds (East) Regional Ethics Committee (reference number 03/316). Written, informed consent was obtained from all patients included in this pilot study providing specimens.

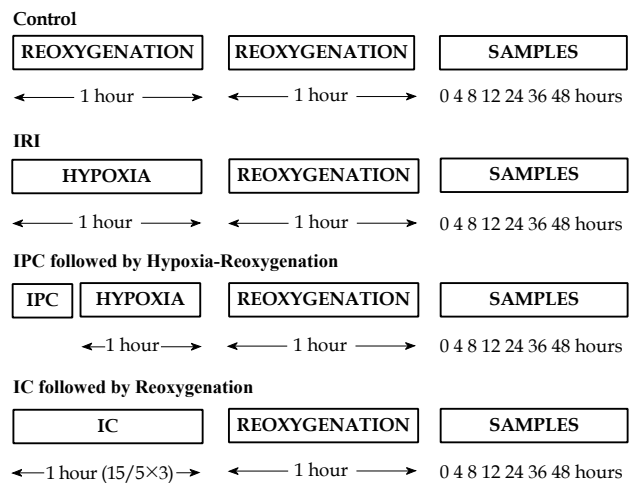
Hepatocytes were isolated using a two step perfusion and digestion method described by Seglen<sup>[25]</sup>. This methodology was used due to the familiarity of this isolation technique and proven consistency of isolating good hepatocyte viability. After obtaining the liver specimen, 1 litre of sterile phosphate-buffered saline (PBS) at 4°C was syringed through visible blood vessels on the cut surface to flush out the patient's blood and its constituents. The specimen was then transported in University of Wisconsin transplantation fluid on ice (4-8°C) to the laboratory. All procedures were performed in a laminar-flow hood to minimise the risk of contamination. The liver specimen was placed on a warm stage and held in place with pipette tips. The visible veins were cannulated with the perfusion tubing. The liver specimen was perfused with liver perfusion medium (Invitrogen, Paisley, UK) through several cannulae at 60-100 mL/min at 37°C for 30 min. The tissue was then perfused with liver digest media (Invitrogen, Paisley, UK) at 60-100 mL/min at 37°C for 30 min. The digested liver was then cooled by immersion in suspension buffer (Dulbeccos minimum essential medium and 2% bovine serum albumin). The liver specimen was then cut open and cells released by gentle scraping with a scalpel. The undigested material and any large fragments are removed by filtration through a coarse sieve (pore size 1 mm) into a freezer-cooled beaker, followed by a cell dissociation sieve (pore size 250  $\mu$ m). The filtrate was collected in a freezer-cooled beaker and divided into freezer-cooled 50 mL tubes for centrifugation. The cell suspension was centrifuged at 50 g, at 4°C, for 2 min, to pellet the dense viable hepatocytes. Each re-suspended hepatocyte

solution was gently diluted to 50 mL and centrifuged again. The pellets were then pooled. The number and viability of the hepatocytes was estimated using a standard Trypan Blue viability assay and counted on a haemocytometer. Viable hepatocytes were cultured with a starting density of  $1.25 \times 10^5$  cells/cm<sup>2</sup> on collagen IV coated 75 cm<sup>2</sup> culture flasks in Hepatozyme (Invitrogen, Paisley, UK) media.

### Hepatocyte characterisation

Characterisation of in-vitro hepatocytes was based on the staining with anti-Hepatocyte paraffin-1 (clone OCH1E5, Dako, UK)<sup>[26,27]</sup>, which is specific for hepatocyte mitochondria, and is considered to be the most specific and sensitive marker of both normal and neoplastic hepatocytes. In addition, hepatocyte paraffin-1 is the most sensitive and specific positive marker of hepatocellular differentiation<sup>[28]</sup>.

Hepatocytes were grown on 13 mm diameter glass cover-slips and washed twice with PBS before fixing. Cells were fixed for staining with 50:50 acetone/methanol at -20°C for 10 min. These fixed cells were washed once with PBS and incubated with 1 in 10 Casein solution and 10% normal horse serum for 30 minutes. Antibodies were diluted in 2% Casein, 2% normal goat serum in PBS. Hepatocyte paraffin-1 and isotype matched control (2.7  $\mu$ L/mL) were incubated on the slides for 1 h (21°C, humidified atmosphere). Slides were washed 3 times with PBS with agitation. Dako's FITC conjugated goat anti mouse IgG was used as a secondary (1:100 dilution in 2% normal goat serum, 2% casein in PBS). Staining was observed using a microscope equipped with fluorescence. Photographs were taken using a digital camera (JVC, KY-F1030).



**Figure 1** Ischaemia and reperfusion protocols used in this study.

### Experimental design

**IRI:** The ischaemia and reperfusion periods were determined at 1 hour of hypoxia and 1 hour of re-oxygenation, respectively (Figure 1). All hypoxia and reoxygenation experiments involved the use of a microaerophilic chamber with O<sub>2</sub> concentration routinely measured and found to be <0.1%. This chamber contained inlet gases of nitrogen and carbon dioxide at 37°C. An appropriate amount of Hepatozyme media were placed inside the chamber for 24 h prior to the experiments, to allow oxygen to diffuse out of the media. Following this, flasks that required hypoxia treatment had their oxygenated media removed, and were incubated in the microaerophilic chamber for 1 hour in hypoxic media. The control flasks experienced an identical number of media changes, but otherwise remained in the 5% CO<sub>2</sub> incubator at 37°C for identical time periods. At the end of the hypoxic period, flasks were removed from the chamber, and the

media discarded. The media was also discarded from the control. Following this, samples had Hepatozyme media added for the reoxygenation period, and were returned to the 5% CO<sub>2</sub> incubator. Following the 1 hour reoxygenation period, 100 µml of media were collected at relevant time points and stored in eppendorfs at -80°C until processed.

**IPC and IC:** The IPC protocol was determined at 10 min of hypoxia followed by 10 min of reoxygenation. Culture flasks of IPC had their media removed, and were incubated in a microaerophilic chamber for 10 min in hypoxic media. After hypoxic preconditioning, these samples were removed from the chamber, and the hypoxic media was replaced with Hepatozyme media. They were placed back in the 5% CO<sub>2</sub> incubator at 37°C for the reoxygenation preconditioning period for 10 min. The samples were then exposed to 1 h of hypoxia and 1 hour of reoxygenation.

The IC protocol in this experiment was set at 15 min of hypoxia followed by 5 min of reoxygenation, and this cycle was performed a total of 3 times, to equal 1 hour. During the hypoxia phase of IC, culture flasks were incubated in a microaerophilic chamber with O<sub>2</sub> concentration <0.1% for 15 min in hypoxic media. After the hypoxic phase, these flasks were removed from the chamber, and the hypoxic media was replaced with Hepatozyme media. They were placed back in the 5% CO<sub>2</sub> incubator at 37°C for 5 min for the reoxygenation period. This cycle was then repeated two further cycles. The samples were then exposed to 1 hour of reoxygenation.

#### Multiplex cytokine and growth factor analysis

Growth factors and cytokines involved in the liver regeneration cascade that were assessed in this study were IL-1β, IL-1ra, IL-6, IL-8, EGF, TGF-α, granulocyte-colony stimulating factor (G-CSF) and TNF-α. Cytokine analysis kit [Human Cytokine LINCOplex kit (Linco, cat no. hCYTO-60K)] was used. Assays were run in duplicates according to the manufacturers' protocol. Data was collected using the Luminex® 100™ IS System (Luminex, cat no. CN-L003-01).

#### Statistical analysis

All concentration values are presented as the mean (standard error of the mean). Comparison between groups at each time-point over the 48 h period was assessed using the Mann Whitney U Test. Statistical analyses were performed using the SPSS for Windows™ version 15.0 (SPSS Inc, Chicago, Ill, USA), and statistical significance was taken at the 5% level.

## RESULTS

### Hepatocytes

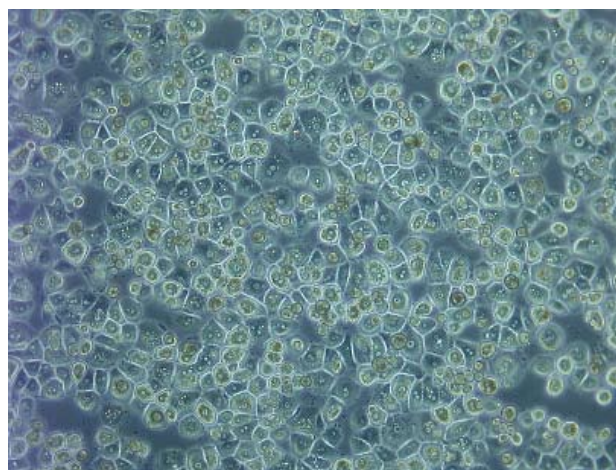
Following extraction, hepatocytes were cultured in culture flasks with Hepatozyme media (Figure 2) with viability of all extractions ranging from 70% to 80%. Following all extractions (*n*=5), hepatocytes were stained for anti-Hepatocyte paraffin-1 (Figure 3). Hepatocytes labelled by this antibody display a distinct, granular cytoplasmic staining pattern, which is occasionally ring-like and was present diffusely throughout the hepatocyte cytoplasm without canalicular accentuation.

### Effects of IRI, IPC and IC

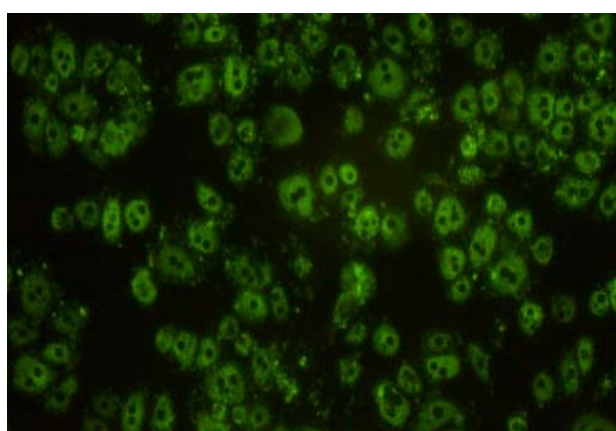
Of the mediators measured, IL-1ra, IL-8 and G-CSF were produced by the hepatocytes at a measurable level in all the groups.

#### IL-1ra

**Controls vs IRI:** Besides a reduction in the production of IL-



**Figure 2** Hepatocytes in Hepatozyme media (phase contrast x200).



**Figure 3** Immunohistochemistry demonstrating the staining of anti-Hepatocyte paraffin-1 in human hepatocytes (fluorescence x200).

1ra at time 0, there was a general increase in IL-1ra production by hepatocytes up to 24 h following IRI compared to controls. Nevertheless, there was no significant difference in the production of IL-1ra by IRI exposure compared to controls (Table 1).

**IRI vs IPC:** Following IPC prior to IRI, there was an overall decrease in production of IL-1ra by hepatocytes after the 0 hour time-point compared to IRI. However, IPC did not significantly influence the IL-1ra profile compared to IRI.

**IRI vs IC:** IC did increase the production of IL-1ra at certain (0, 8 and 36) time-points compared to IRI during the 48 hour period. In the remaining time-points, IC decreased the production of IL-1ra. Nevertheless, IC did not significantly alter the production of IL-1ra compared to IRI.

#### IL-8

**Controls vs IRI:** Besides at 4 h following IRI where there was a 56% increase in IL-8 production, there was an overall trend for IRI to decrease the production of IL-8 by hepatocytes over the 48 hour period compared to controls (Table 2). There was no significant difference in IL-8 profile between the IRI and control groups.

**IRI vs IPC:** IPC prior to IRI on hepatocytes resulted in an overall reduction of IL-8 concentration levels compared to IRI over the 48 hour study period. However, there was no significant difference in the IL-8 profile between the IRI and IPC-treated groups.

**Table 1** Production level of IL-1ra by hepatocytes that underwent IRI, IPC and IC at their respective time points.

Time (Hours)	Control [Mean (SEM) pg/mL]	IRI		IPC		IC	
		Mean (SEM) pg/m	P-value <sup>§</sup>	Mean (SEM) pg/mL	P-value <sup>§</sup>	Mean (SEM) pg/mL	P-value <sup>χ</sup>
0	7.30 (0.02)	1.25 (0.64)	NS	5.97 (3.50)	NS	3.05 (0.16)	NS
4	10.03 (3.07)	13.00 (3.58)	NS	6.73 (4.08)	NS	4.13 (1.91)	NS
8	22.55 (12.07)	14.94 (4.76)	NS	7.20 (0.82)	NS	22.73 (0.64)	NS
12	24.91 (12.04)	26.63 (12.41)	NS	17.39 (4.43)	NS	22.95 (10.87)	NS
24	22.85 (17.82)	17.45 (7.52)	NS	16.45 (6.88)	NS	15.36 (5.45)	NS
36	14.53 (4.59)	16.44 (3.74)	NS	12.96 (5.06)	NS	25.84 (10.66)	NS
48	28.30 (12.62)	17.96 (6.10)	NS	11.39 (5.67)	NS	12.23 (3.99)	NS

<sup>§</sup>: P-value was derived from Mann Whitney U test between the IRI and control groups; <sup>§</sup>: P-value was derived from Mann Whitney U test between the IPC and IRI groups; <sup>χ</sup>: P-value was derived from Mann Whitney U test between the IC and IRI groups; NS: Not significant.

**Table 2** Production level of IL-8 by hepatocytes that underwent IRI, IPC and IC at their respective time points.

Time (Hours)	Control [Mean (SEM) pg/mL]	IRI		IPC		IC	
		Mean (SEM) pg/mL	P-value <sup>§</sup>	Mean (SEM) pg/mL	P-value <sup>§</sup>	Mean (SEM) pg/mL	P-value <sup>χ</sup>
0	6.88 (3.43)	2.31 (0.57)	NS	1.72 (0.45)	NS	2.05 (0.45)	NS
4	6.47 (1.55)	10.10 (3.82)	NS	5.45 (0.58)	NS	6.77 (1.60)	NS
8	14.78 (3.04)	9.81 (1.46)	NS	9.13 (0.96)	NS	10.22 (2.24)	NS
12	15.71 (2.37)	12.80 (2.43)	NS	10.65 (1.68)	NS	13.23 (3.66)	NS
24	23.71 (2.57)	16.89 (3.09)	NS	16.34 (3.52)	NS	18.25 (2.81)	NS
36	28.90 (2.39)	23.21 (3.23)	NS	20.92 (3.66)	NS	18.87 (1.86)	NS
48	34.77 (6.47)	27.78 (5.91)	NS	25.96 (4.68)	NS	29.81 (4.58)	NS

<sup>§</sup>: P-value was derived from Mann Whitney U test between the IRI and control groups; <sup>§</sup>: P-value was derived from Mann Whitney U test between the IPC and IRI groups; <sup>χ</sup>: P-value was derived from Mann Whitney U test between the IC and IRI groups; NS: Not significant.

**Table 3** Production level of G-CSF by hepatocytes that underwent IRI, IPC and IC at their respective time points.

Time (Hours)	Control [Mean (SEM) pg/mL]	IRI		IPC		IC	
		Mean (SEM) pg/mL	P-value <sup>§</sup>	Mean (SEM) pg/mL	P-value <sup>§</sup>	Mean (SEM) pg/mL	P-value <sup>χ</sup>
0	2.89 (2.59)	1.12 (0.41)	0.050	0.56 (0.44)	NS	1.05 (0.60)	NS
4	2.84 (0.78)	1.96 (2.50)	NS	1.38 (0.64)	NS	1.16 (0.40)	NS
8	3.32 (2.34)	1.68 (0.73)	NS	1.25 (0.46)	NS	1.61 (0.57)	NS
12	3.99 (1.20)	1.52 (0.48)	NS	1.82 (1.03)	NS	1.70 (0.72)	NS
24	4.83 (1.68)	2.61 (0.98)	NS	2.05 (0.98)	NS	2.93 (1.15)	NS
36	4.45 (3.31)	5.23 (2.83)	NS	2.72 (0.89)	NS	2.81 (1.08)	NS
48	5.08 (2.59)	5.83 (2.25)	NS	4.66 (1.85)	NS	4.97 (1.64)	NS

<sup>§</sup>: P-value was derived from Mann Whitney U test between the IRI and control groups; <sup>§</sup>: P-value was derived from Mann Whitney U test between the IPC and IRI groups; <sup>χ</sup>: P-value was derived from Mann Whitney U test between the IC and IRI groups; NS: Not significant.

**IRI vs IC:** There was an overall trend for the IC group to decrease the production of IL-8 compared to the IRI group, with the exception at the 8 hour time-point where a marginal increase of 4% was noted. However, there was no significant difference in IL-8 profile between the IC and IRI groups.

### G-CSF

**Controls vs IRI:** In comparison to untreated controls, there was an overall trend for IRI to decrease the production of G-CSF by hepatocytes over the first 24 h. However, only at the 0 hour time-point did IRI significantly decrease the production levels of G-CSF compared to controls ( $p=0.050$ ). In the subsequent 24 h, there was a non-statistical significant increase in the profile of G-CSF following IRI (Table 3).

**IRI vs IPC:** Apart from the 12 hour time-point, IPC prior to IRI resulted in an overall reduction of G-CSF production by hepatocytes at all the remaining time-points over 48 h. Nevertheless, although there was a general trend for IPC-treatment to decrease the production of G-CSF compared to IRI, this observation was not statistically significant between the groups.

**IRI vs IC:** Besides the 12 and 24 hour time-points, there was an overall decrease in the production of G-CSF recorded in the IC group compared to the IRI group. The most marked decrease was noted at the 36 hour time-point, where a 46% reduction was observed.

However, IC did not significantly influence the profile of G-CSF compared to IRI.

## DISCUSSION

Liver regeneration is of clinical significance in view of the increasing number of major liver resections and the increasing use of marginal donor liver allografts for transplantation. Successful patient outcome often depends on liver regeneration, particularly in patients with cirrhotic and steatotic livers. Regeneration of the liver following IRI and major liver surgery is a complex process that involves the integration of a network of cytokines, growth factors, kinases, transcription factors and metabolic demands of the liver. The up-regulation of cytokines and growth factors involved in the liver regeneration cascade by surgical methods, such as IPC, is an interesting and feasible option. In addition to its beneficial effects during liver surgery by preventing blood loss, IPC and IC may also promote the production of mediators involved in liver regeneration.

In this study, isolated human hepatocytes in culture produced IL-1ra, IL-8 and G-CSF in all four groups. There was an overall trend for IPC to decrease the production levels of IL-1ra, IL-8 and G-CSF compared to IRI over the 48 hour study period. Nevertheless, this trend of IPC to reduce all these mediators was not statistically significant. Similar to IPC, IC did not significantly influence the production of IL-1ra, IL-8 and G-CSF by isolated hepatocytes in vitro compared to IRI.

### IL-8

IL-8 has been identified as a unique neutrophil-activating cytokine following hepatic IRI<sup>[29,30]</sup>. Kupffer cells play an important role in neutrophil-mediated injury by producing IL-8 and recruiting neutrophils into the liver during hepatic IRI<sup>[31]</sup>. Neutrophils contribute to hepatocyte apoptosis and can subsequently delay liver regeneration. However, IL-8 also functions as a survival factor for several types of cells, including the liver<sup>[32]</sup>. Hanson and co-workers recently demonstrated that IL-8 protected liver cells in mice from galactosamine and endotoxin-mediated apoptosis<sup>[33]</sup>. The underlying mechanism responsible for IL-8 mediated survival function in human hepatocytes is thought to be due to TNF- $\alpha$  signalling via the NF- $\kappa$ B and the phosphatidylinositol 3-kinase/Akt pathway<sup>[32]</sup>. This signalling pathway is also crucial in the liver regeneration process. Colletti *et al* investigated the role of IL-8 on rat hepatocytes proliferation *in-vitro* and demonstrated a significant increase in proliferation following IL-8 exposure<sup>[34]</sup>.

In comparison to the present study, IRI did not significantly influence the profile of IL-8 in hepatocytes *in-vitro*. In human studies of liver resection, IL-8 plasma concentrations were significantly higher in patients subjected to the Pringle manoeuvre compared with patients who were not at 2 h post-liver resection<sup>[35]</sup>. With respect to the role of IL-8 following IRI, the timing, concentration levels and interaction with other cytokines could determine the role of IL-8 in contributing to hepatocyte apoptosis, survival or proliferation.

To date, there are few published studies assessing the effect of IPC on the production of IL-8 following liver surgery and IRI, and none on liver regeneration. In patients undergoing liver resection, Chouker and co-workers reported no significant difference in systemic IL-8 production in patients pre-treated by IPC compared to patients who were not subjected to IPC<sup>[35]</sup>. In the present study, IPC did not significantly influence the production of IL-8 in isolated hepatocytes in culture. Similarly, there is currently no available data with respect to the effect of IC on IL-8 following IRI and on liver regeneration. In the present study, IC did not significantly influence the profile of IL-8 compared to IRI in hepatocytes *in-vitro*.

### IL-1ra

IL-1ra is an acute phase protein that inhibits the inflammatory effects of IL-1 $\alpha$  and IL-1 $\beta$  following IRI<sup>[36]</sup>, which results in reduction of liver injury and necrosis following hepatectomy<sup>[37]</sup>. Although no study has formally assessed the effect of IL-1ra on markers of liver regeneration, the role of IL-1ra in liver regeneration could be an “indirect effect” by antagonizing the inhibitory effects of IL-1 on hepatocyte proliferation.

Some authors have proposed that IPC promotes liver regeneration by stimulating the production of IL-1ra. Barrier *et al* studied the genetic changes caused by IPC during hepatectomy in living human liver donors and observed that the IL-1ra gene was significantly over expressed in the IPC group compared to the non-IPC group<sup>[37]</sup>. This study also reported that the amount of anti-apoptotic protein Bcl-2 and inducible NO synthase were significantly expressed in the IPC group. Hence, the production of IL-1ra, Bcl-2 and NO by IPC counters the pro-inflammatory and pro-apoptotic effects generated during IRI. The genetic expression profile was determined from micro-array analysis of post-reperfusion liver biopsies following transplantation<sup>[37]</sup>.

Furthermore, IPC has been shown to inhibit the IL-1 $\beta$  release through the over expression of IL-10, possibly mediated by NO, following partial hepatectomy in obese rats with steatotic livers<sup>[38]</sup>. Franco-Gou *et al* observed that IPC, similar to IL-1ra pre-

conditioning, reduced the release of IL-1 and protected against lung damage following reduced size liver transplantation in rats<sup>[39]</sup>. The underlying mechanism for lung protection was thought to be related to NO synthesis and IL-1ra activation.

In the current study, both IPC and IC did not significantly influence the profile of IL-1ra in hepatocytes *in-vitro*. Similar to IL-8, there are currently no results reporting the effect of IC on the production of IL-1ra following liver surgery.

### G-CSF

G-CSF is a haematopoietic growth factor that stimulates proliferation and differentiation of neutrophil colony-forming cells. G-CSF receptors are expressed by cells from the myeloid lineage as well as a variety of non-haematopoietic cells, including hepatocytes and vascular endothelial cells<sup>[40]</sup>. Previous reports have observed that G-CSF promotes liver regeneration in rodents. Theocharis *et al* described an increase in liver regeneration following partial hepatectomy and thioacetamide-induced hepatic failure in G-CSF conditioned rats<sup>[41,42]</sup>. Inderbitzin and co-workers demonstrated that G-CSF supported liver regeneration in a small for size liver remnant mouse model<sup>[43]</sup>. The underlying mechanism by which G-CSF influences liver regeneration is thought to be by promoting sinusoidal dilatation and increasing hepatic blood flow<sup>[44]</sup>. More recently, Piscaglia and co-workers demonstrated that G-CSF facilitated hepatic regeneration in rats subjected to partial hepatectomy by increasing the migration of bone marrow-derived progenitors to the liver, as well as enhancing the endogenous liver stem cell reaction<sup>[45]</sup>. Hence, G-CSF has both a local and systemic effect on the liver regeneration process.

Hanazaki and co-workers observed a significantly higher G-CSF profile during the reperfusion phase in patients undergoing hepatectomy compared to their pre-operative levels<sup>[46]</sup>. This suggests that G-CSF could be an important marker of IRI and / or stimuli for liver regeneration following hepatectomy.

Hepatocytes produced G-CSF following IRI in the current *in-vitro* study. During the 48 hour period, only at the 0 hour time-point did IRI significantly decrease the production of G-CSF compared to controls in isolated hepatocytes *in-vitro*.

At present, there are no published studies assessing the effect of both IPC and IC on the production of G-CSF following hepatectomy or liver transplantation. The current study did not show any significant difference in the profile of G-CSF following IPC and IC compared to IRI in human hepatocytes *in-vitro*.

### “Yin and Yang” of IPC

**Mediators of liver regeneration:** Studies evaluating the effect of IPC in stimulating the release of mediators involved in liver regeneration have produced conflicting findings.

The initiation of the regenerative response is dependent on the early activation of TNF- $\alpha$  and IL-6 responsive transcription factors. Studies have not consistently shown IPC to increase the production of TNF- $\alpha$  and IL-6. Tsuyama *et al* reported that IPC prolonged survival, suppressed liver necrosis induced by IRI and inhibited the release of TNF- $\alpha$  at 1 hour, and IL-6 at 2 and 5 h of the reperfusion phase in mice<sup>[47]</sup>. Bedirli and others demonstrated that IPC inhibited the production of TNF- $\alpha$ , but not IL-6 during the late (24 and 48 h) phases of reperfusion in rats following partial hepatectomy<sup>[48]</sup>. Using a TNF gene-deleted mice model, Teoh *et al* demonstrated that pre-treatment with low dose IL-6 prior to hepatic ischaemia conferred equivalent hepatoprotection and earlier cell cycle entry as IPC compared to non-IPC-treated mice at 2 h of reperfusion<sup>[49]</sup>.

The differences reported among studies investigating the influence of IPC on the regenerative cytokine network may be due to: type of models (*in-vitro*, *in-vivo* or human) used; different timings of IPC applied; different timings of hepatic ischaemia and reperfusion periods; various hepatectomy models used and assessment of mediators of regeneration at different time-points.

With respect to HGF, Franco-Gou *et al* demonstrated an increase in both liver and plasma HGF levels following IPC in reduced size liver transplantation rat model, and this was associated with an increase in hepatocyte proliferation<sup>[50]</sup>.

## CONCLUSION

In the present study, IPC failed to significantly influence the production of IL-8, G-CSF and IL-1ra using an *in-vitro* human hepatocyte IRI model. Similarly, IC did not significantly alter the profile of these regenerative mediators.

Although there has been increased interest in this strategy, results of IPC in enhancing liver regeneration by up regulating growth-promoting factors has shown conflicting results. Nevertheless, the potential clinical benefit of IPC in major liver surgery should not be discounted. In addition, the effect of IC in influencing IRI and liver regeneration requires further evaluation. By understanding the underlying mechanisms by which IPC influences liver regeneration, other strategies as alternatives to IPC, could be developed to modulate the regenerative pathways in the clinical setting and improve outcomes of patients following liver surgery.

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