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HIGHLIGHTS

- We investigated the effect of pharmacologic preconditioning with HDD, LDD and anti-CD163-dex on ischemia/reperfusion injury.
- Liver cell apoptosis and necrosis were analyzed by stereological quantification.
- Anti-CD163-dex and high dose dexamethasone reduces the number of apoptotic cells following ischemia/reperfusion injury.

ARTICLE INFO

ABSTRACT

Aim: The Pringle maneuver is a way to reduce blood loss during liver surgery. However, this may result in ischemia/reperfusion injury in the development of which Kupffer cells play a central role. Corticosteroids are known to have anti-inflammatory effects. Our aim was to investigate whether a conjugate of dexamethasone and antibody against the CD163 macrophage cell surface receptor could reduce ischemia/reperfusion injury in the rat liver.

Methods: Thirty-six male Wistar rats were used for the experiments. Animals were randomly divided into four groups of eight receiving anti-CD163-dexamethasone, high dose dexamethasone, low dose dexamethasone or placebo intravenously 18 h before laparotomy with subsequent 60 min of liver ischemia. After reperfusion for 24 h the animals had their liver removed. Bloods were drawn 30 min and 24 h post ischemia induction. Liver cell apoptosis and necrosis were analyzed by stereological quantification.

Results: After 24 h' reperfusion, the fraction of cell in non-necrotic tissues exhibiting apoptotic profiles was significantly lower in the high dose dexamethasone (p = 0.03) and anti-CD163-dex (p = 0.03) groups compared with the low dose dexamethasone and placebo groups. There was no difference in necrotic cell volume between groups. After 30 min of reperfusion, levels of haptoglobin were significantly higher in the anti-CD163-dex and high dose dexamethasone groups. Alanine aminotransferase and alkaline phosphatase were significantly higher in the high dose dexamethasone group compared to controls after 24 h' reperfusion.

Conclusions: We show that pharmacological preconditioning with anti-CD163-dex and high dose dexamethasone reduces the number of apoptotic cells following ischemia/reperfusion injury.

1. Introduction

The level of intraoperative blood loss and the transfusion requirement are factors known to be associated with the degree of morbidity and mortality following liver resection [1,2]. Moreover, these factors are important determinants of the morbidity, mortality and other postoperative complications and the risk of tumor recurrence [3–9]. Several surgical procedures have been used during liver resection in an attempt to reduce excessive perioperative bleeding [10]. The most common is Pringle's maneuver, using inflow occlusion by portal triad clamping [11,12]. Lack of oxygen in the tissue leads to cell damage [13]. This can lead to important clinical problems including liver dysfunction and liver failure [14].

Ischemia/reperfusion injury (IRI) in the liver results from direct cellular damage caused by hypoxia and by the associated inflammatory tissue response, that results in further tissue damage and dysfunction [15,16]. The ischemic insult results in metabolic disturbances, caused by lack of oxygen, ATP-depletion and glycogen consumption [17].

An excessive inflammatory response during reperfusion plays an essential role determining the degree of IRI [14]. IRI occurs in two distinct phases after reperfusion, an early phase within the first 2 h and a later phase after 6–48 h [18]. In the early phase, reactive oxygen species (ROS) mainly produced and released from Kupffer cells aggravate the ischemic injuries [19,20]. In addition, an increased production of cytokines and chemokines can be detected. Signaling from ROS, cytokines and chemokines, recruits neutrophil granulocytes to the hepatic sinusoids, resulting in both local and systemic inflammation [21,22]. Late phase IRI is dominated by neutrophil infiltration, microcirculatory disturbance, and further damage to the liver [23–25].

Surgical intervention, pharmacological agents and gene therapy have been investigated as preventative strategies against IRI [26]. Pharmacological studies based on the pathophysiology of IRI have focused on the use of corticosteroids and antioxidants. Steroids induce an anti-inflammatory effect by providing negative feedback on immunological stimulation, stabilizing cell membrane integrity by inhibition of lysosome peroxidation and through regulation of gene expression pathways [27,28]. Corticosteroids seem to have a positive impact on the extent of IRI in the liver through an anti-apoptotic effect [29–33].

A possible pharmacological method to reduce IRI could be a newly developed antibody drug conjugate (ADC) targeting dexamethasone to the receptor CD163 expressed on the surface macrophages and monocytes, anti-CD163-dexamethasone [34–36]. The primary function of CD163 is as a scavenger receptor for the hemoglobin-haptoglobin (Hb–Hp) complex formed after the lysis of red blood cells [37]. Kupffer cells are liver-resident macrophages that contribute to activation of the immune system by releasing cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin 1 and 6 (IL-1 and IL-6) and by the production of ROS [38]. Targeting the CD163 receptor in macrophages highly increases the anti-inflammatory potency of dexamethasone. This has been tested in a rat and porcine model of inflammation after administration of lipopolysaccharides [34,36]. In these studies anti-CD163-dex at low dose was comparable to the anti-inflammatory effect of high dose free dexamethasone.

Given that Kupffer cell activation may result in inappropriate immune system up-regulation crucial to the pathogenesis of IRI, we hypothesized that anti-CD163-dex at a lower dose than free dexamethasone, might protect the liver from ischemic damage, thus preventing some of the well-known adverse effects of high dose glucocorticoids. We investigated this in the rat liver.

2. Methods

2.1. Experimental design

All experimental protocols used in this study were approved by the Danish Animal Experiments Inspectorate according to license number 2009/561-1644 and followed the rules of the "Guide for the Care and use of Laboratory Animals" published by the National Institutes of Health. In the experiment, we used 36 male Wistar rats (Taconic, Eiby, Denmark) with an average weight of 225 g (range 195 g–301 g). The rats were housed in standard animal cages in an environmental- and temperature-controlled facility, at 23 °C and with a 12-h light–dark cycle. They were given free access to water and standard food (Altrombin).

Anesthesia was achieved using a mixture of oxygen (2.0 l/minute) and nitrous oxide (0.5 l/minute) containing 4.5% sevoflurane (forene; Abbott Laboratories, UK), blown through an induction chamber. During tail vein injection and surgery, anesthesia was maintained with 3% sevoflurane in the same mixture of oxygen and nitrous oxide, administrated through a mask covering the nose of the rat.

The animals were randomly divided into four groups receiving, respectively, anti-CD163-dex, high dose dexamethasone (HDD), low dose dexamethasone (LDD) or PBS intravenous through the tail vein 16–18 h before liver ischemia. During surgery, the rat was in a supine position on a temperature-controlled heated pad to maintain the core temperature at 37 °C. A transverse abdominal incision was made right under the rib cage. Under a dissecting microscope, all the hepatic ligaments were resected to achieve abruption of the collateral blood supply. In addition, the hepatoduodenal ligament was identified. A partial hepatic ischemia model was used to prevent mesenteric congestion by allowing blood flow through the right liver lobe [39].

Portal triad clamping was performed with a micro vascular clamp after the bifurcature of the right lobe, interrupting the portal triad flow to the left and median lobes. Discoloration of the lobes was used as a positive marker indicating hepatic ischemia. The abdomen was temporarily closed and the respiratory rate was measured every 10th minute to avoid major loss of fluid and bradypnea. As analgesics, the animals were given a subcutaneous injected of carprofen 5 mg/kg (Rimadyl; Pfizer Animal Health, Exton, USA) and given intravenous isotonic saline 1 ml. After 30 min of ischemia, a blood sample was collected through the lateral tail vein to monitor the acid-base status and blood lactate concentration.

After 60 min of ischemia, the clamp was removed and the blood flow was re-established to the median and left lobes, verified by the return of the normal reddish color of the liver. The abdomen was closed in two layers, the fascia-layer with continuous suture and the skin-layer with single knots. After 30 min of reperfusion, a blood sample was collected. No antibiotics were administrated during the study period.

After 24 h of reperfusion the animals were re-anesthetized as described above. The abdomen and thoracic cavity were opened, and cardiac cannulation was performed to collect blood. Subsequently, the animals were euthanized by an intra cardiac injection of 1 ml pentobarbital 400 mg/ml. To ensure rapid fixation of the liver, the animal was perfused with phosphate-buffered 4% formaldehyde for 10 min through a cardiac cannula. The left portion of the median lobe was then resected and preserved in the same fixative for an additional 24 h, after which the lobe was embedded in paraffin.

2.2. ADC synthesis

Anti-CD163-dexamethasone (AD) was produced by conjugating dexamethasone hemisuccinate-NHS to primary amino groups of the rat CD163 antibody Ed-2 (ABD Serotec, Düsseldorf, Germany) as described [34].

Dexamethasone phosphate (Sigma–Aldrich, Broendby, Denmark) dissolved in PBS and PBS-buffer (Invitrogen, Taastrup, Denmark) was filtered and stored as AD, the study was conducted blinded [34].

2.3. Stereological sampling

The formaldehyde-fixed left median lobe was cut into 2 mm thick parallel slabs using a tissue slicer. All slabs were placed horizontally with the same side down in a cage before paraffin embedding allowing correct estimation [40]. A 2 μm section was cut from each paraffin embedded block, creating a set of systematic, uniform, random sampling (SURS) sections for analysis.

2.4. Immunohistochemistry

Apoptotic cells were identified by immunohistochemical staining for caspase 3. This cytosolic molecule is an effector protein, which causes DNA fragmentation and cell death [41]. Immunohistochemical staining was performed on 2.5 μm paraffin slides using a Benchmark XT automatic stainer (Ventana Medical Systems, Roche Group, AZ, USA). The primary antibody used in this study was Asp175 (clone5A1E, 1:80 dilution; Cell Signaling Technology, Glostrup, Denmark) against the activated form of caspase 3. Bound antibody was detected using the UltraView DAB detection kit (DAKO, Glostrup, Denmark). Sections were counterstained with hematoxoxylin.

2.5. Stereological quantification of IRI

All sections were blinded to the investigator and analyzed using a microscope (Olympus BX-50) modified for stereology, with a motorized stage (Prior H138 with controller H29, Cambridge, UK) and a digital camera (Olympus camera DP72) connected to a PC with newCAST 3.6.5.0 software (Visiopharm, Hørsholm, Denmark.) The same investigator analyzed all sections. Intra-observer variance of stereological data was tested by re-evaluating multiple randomly chosen livers as described below.

2.6. Volume estimation

Volumes were estimated in hematoxylin and eosin (H&E)-stained SURS sections by the Cavalieri method [42]. A point grid was placed randomly over the H&E section using the software, and the areas of the entire section and of the necrotic tissue were estimated, as described [43]. To estimate the volume the following formula was used:

$$V = t \cdot \frac{a}{p} \cdot \sum_{t=1}^{n} Pi \tag{1}$$

t was the distance between each sampled section (2 mm = 2000 μm); (a/p) the area covered by each test point; Σ Pi was the number of points counted.

The necrotic volume ratio (NVR) was analyzed for each liver sample. NVR was calculated as:

$$NVR = \frac{Vnecrotic}{Vtotal} \cdot 100\% \tag{2}$$

Results were adjusted for volume shrinkage as described [43].

2.7. Caspase 3 quantitation

Two-dimensional quantitation of caspase 3-positive cell profiles was performed on SURS sections from each animal as described [42]. Caspase 3-positive cell profiles were estimated by counting the number of positive cells within an unbiased counting frame. An average of 150 randomly selected, non-overlapping fields of view, were analyzed in each animal. The fraction of caspase 3-positive cell profiles (QA) was calculated using the following formula:

$$QAviable = \frac{\sum Q}{\sum A} = \frac{\sum Q(Casp3)}{a(mm^2) \cdot \sum P \cdot fviable};$$
$$fviable = 1 - \frac{aNecrotic}{aTotal} \tag{3}$$

in which Q is the number of caspase 3-positive cell profiles; a is the area of the counting frame; and P is the number of counting frames evaluated; *fviable* is the fraction of non-necrotic liver tissue used to correct the reference area. The counting rules used are described in Fig. 1b.

2.8. Liver parameters

Blood samples obtained after 30 min of reperfusion and from the cardiac cannulation at 24 h of reperfusion were processed and stored at −80 °C until analysis. Alanine aminotransferase (ALT), alkaline phosphatase (AP), bilirubin (BR), gamma-glutamyl transferase (GGT) and haptoglobin (HP) were measured using Roche Hitachi Cobas 6000 (Roche diagnostics, Mannheim, Germany).

2.9. Inflammatory mediators

Plasma IL-1, IL-6 and TNF were measured using a specific rat IL-1 (eBioscience San Diego, CA, USA), IL-6 (RayBiotech, Norcross, GA, USA), and TNF immunoassays (Invitrogen, Carlsbad, CA, USA). Rat acute phase protein α-2-macroglobulin was measured using a specific ELISA kit (Immunology Consultants Laboratory, Portland, OR, USA). All of the assays used in this study have previously been validated for use in rats and were performed as described by manufacturer's instructions.

2.10. Statistical analysis

Statistical analyses were performed using SPSS 19 (SPSS Inc., Chicago, Illinois, USA). All data are expressed as mean (CI). Data were tested for normal distribution and analyzed by ANOVA. For comparison of the two groups, normal distributed data were tested with Student's t-test. Nonparametric data were tested using Kruskal–Wallis test, followed by the Wilcoxon Mann–Whitney Rank sum test. P-values <0.05 were considered significant.

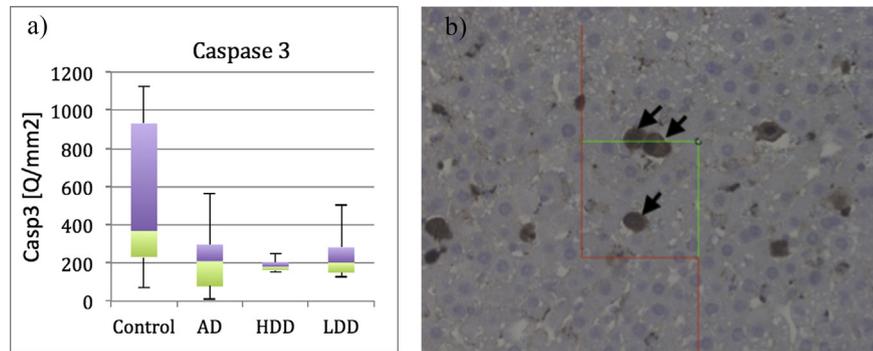The reproducibility of the stereological data was tested by re-

Fig. 1. *Quantification of caspase 3 positive cell profiles.* **a)** Box plot of apoptotic cell profiles/mm^2 liver after 1 h of liver ischemia and 24 h of reperfusion. Horizontal markers indicate median values of the groups. Apoptosis was significantly lower in the HDD (p = 0.026) and the anti-CD163-dex (p = 0.047) groups compared with the control group. **b)** A typical study section. Cell profiles were counted if they stained positive for caspase 3, if the nucleus of the cell was visible and if the cell profile was inside the counting, touched the green inclusion lines and did not touch the red exclusion lines. Cell profiles counted are marked with a black arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
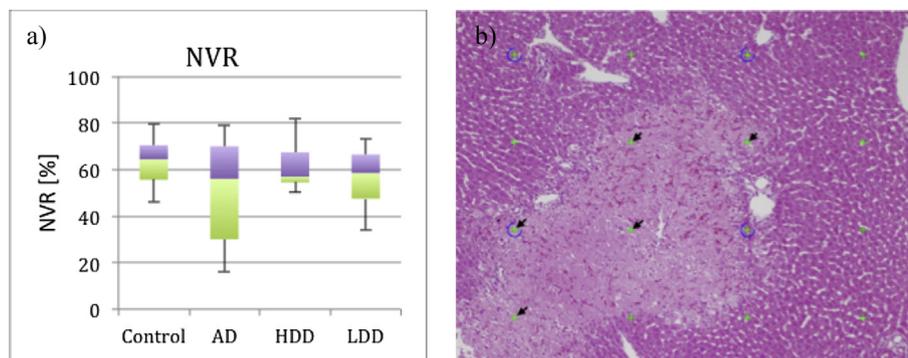

Fig. 2. *Quantification of necrotic areas.* **a)** Box plot of liver damage after 1 h of liver ischemia and 24 h of reperfusion. Horizontal markers indicate median values of the groups. There were no significant differences in the NVR between the groups. **b)** An example of a H&E stained study section. Areas of necrosis to be counted are marked with a black arrow.

evaluation of all sections from respectively 11 and 8 randomly chosen livers from the two analyses. Data from the first (A1) and the second evaluations (A2) were compared using Wilcoxon Mann—Whitney Rank sum test. The variability between analysis was assessed by difference-average as described by Bland and Altman plot [44].

3. Results

Four animals were excluded because of insufficient clamping of the blood supply to the liver evaluated by ALT values of less than 3000 U/L. One animal was excluded due to portal vein thrombosis.

3.1. Stereology

3.1.1. Apoptosis

After 24 h of reperfusion, the fraction of apoptotic cell profiles in the non-necrotic tissue were 530 cell profiles/mm^2 (240—821) in the control group, 219 cell profiles/mm^2 (83—354) in the anti-CD163-dex group, 188 cell profiles/mm^2 (158—218) in the HDD group, and 235 cell profiles/mm^2 (129—362) in the LDD group. Apoptosis was significantly lower in the HDD (p = 0.03) and the anti-CD163-dex (p = 0.05) groups compared with the control group. The mean number of apoptotic cell profiles was lower in the LDD group (p = 0.06) compared with the control group, but the difference was not statistically significant (Fig. 1).

3.1.2. Necrotic volume and volume ratio (NVR)

After 24 h of reperfusion, the average Vnecrotic was 600 (505—827) in the control group, 498 (161—801) in the anti-CD163-dex group, 626 (438—832) in the HDD group, and 625 (282—1247) in the LDD group.

The average Vtotal was 970 (768—1205) in the control group, 991 (853—1146) in the anti-CD163-dex group, 1021 (886—1328) in the HDD group, and 1087 (814—1745) in the LDD group.

After 24 h of reperfusion, the average NVR could thus be calculated to 63% (55—71) in the control group, 51% (33—68) in the anti-CD163-dex group, 62% (52—71) in the HDD group, and 56% (45—68) in the LDD group. We found no significant differences between the groups (Fig. 2).

3.2. Biochemistry

3.2.1. Liver parameters

As illustrated in Table 1, the post ischemic rise of ALT was significantly higher in the HDD group compared with the control group after 24 h of reperfusion. However, when testing for multiple groups (ANOVA) there was no significant difference between groups. After 30 min of reperfusion, we found no difference in ALT between groups. After 24 h of reperfusion, the mean AP level was significantly higher in the HDD group compared with the control group, and the mean level of HP was significantly higher in the anti-CD163-dex and HDD groups (Table 1).

Table 1
The table shows an average of the liver parameters measured with confidence intervals.

		Control (n = 10)	Anti-CD163-dex (n = 9)	HDD (n = 8)	LDD (n = 8)
ALT [U/l] (30 min)	Mean	1853	1730	2258	2496
	95% confidence interval for mean	(1438–2268)	(1259–2201)	(1703–2812)	(1584–3409)
ALT [U/l] (24 h)	Mean (10^3)	8.96	7.90	12.2*	9.2
	95% confidence interval for mean (10^3)	(6.63–11.3)	(4.40–11.4)	(10.1–14.2)	(5.60–12.8)
AP [U/l] (30 min)	Mean	218	251	287	268
	95% confidence interval for mean	(196–239)	(213–288)	(212–362)	(186–351)
AP [U/l] (24 h)	Mean	376	393	577*	366
	95% confidence interval for mean	(315–437)	(300–487)	(442-711	(300–432)
BR [μmol/l] (30 min)	Mean	1.63	1.26	1.49	1.56
	95% confidence interval for mean	(1.28–1.98)	(0.91–1.61)	(0.91–2.06)	(1.09–2.04)
BR [μmol/l] (24 h)	Mean	6.69	5.19	6.24	5.76
	95% confidence interval for mean	(3.58–9.80)	(2.862–7.516)	(4.26–8.21)	(3.22–8.31)
GGT [U/l] (30 min)	Mean	1.12	0.52	0.85	0.61
	95% confidence interval for mean	(0.21–2.03)	(0.07–0.98)	(−0.13–1.83)	(0.19–1.04)
GGT [U/l] (24 h)	Mean	8.89	5.90	7.85	5.45
	95% confidence interval for mean	(5.05–12.73)	(2.59–9.21)	(6.15–9.55)	(3.31–7.59)
HP [g/l] (30 min)	Mean	0.111	0.158*	0.334*	0.139
	95% confidence interval for mean	(0.09–0.13)	(0.12–0.20)	(0.299–0.368)	(0.111–0.167)
HP [g/l] (24 h)	Mean	0.161	0.244	0.218	0.204
	95% confidence interval for mean	(0.067–0.256)	(0.132–0.356)	(0.107–0.329)	(0.106–0.303)

* indicates a significant difference compared with the control group ($P < 0.05$).

3.2.2. Inflammatory markers

As shown in Table 2, we found no difference in the post-ischemic rise in IL-1. The average level of α-2-macroglobulin was lower in the anti-CD163-dex group compared with the control group, but this was not significant (p = 0.063). After 30 min of reperfusion, levels of TNF-α and IL-6 were close to the detection limit in all groups (Table 2).

4. Discussion

We investigated the effect of pharmacologic conditioning with HDD, LDD and anti-CD163-dex on IRI. We show that conditioning with anti-CD163-dex and HDD significantly decreased the number of apoptotic cell profiles after 24 h of reperfusion, compared with controls, but was without effect in relation to the amount of necrosis. Our results suggest that both HDD and anti-CD163-dex, at a 50 times lower net dexamethasone dose, protected the rat liver against IRI, as shown by a reduction in apoptotsis. That HDD protected against IRI is in agreement with studies by Glanemann et al. and Saidi et al. who showed that rats pre-treated with systemic methylprednisolone (MP) 2 min before hepatic ischemia had fewer apoptotic cells than controls [29,33]. However, the present study is the first to show a reduction in the apoptotic cell count after administration of macrophage-targeted corticosteroids. Since the majority of resident macrophages are found in the liver as Kupffer cells, the greater part of anti-CD163-dex activity is likely to be directed against these [45]. Thus, we can speculate that corticosteroids administered as anti-CD163-dex might have the same beneficial effects on apoptosis after IRI in the liver as systemic corticosteroids, but without their side effects [46].

We found no significant differences with regard to the amount of necrosis when comparing anti-CD163-dex, HDD, LDD and control groups. This is in contrast to Wang et al. [30], who found a reduction in cell damage after IRI when conditioning with prednisolone, 30 min before 60 min of partial hepatic ischemia. However, these authors assessed both necrotic and apoptotic cells in their histological studies, using a semi-quantitative method with several pitfalls, as discussed later. In contrast, Seleri et al. found that IRI was aggravated in rats when conditioning with intravenous corticosteroids 1 h before 60 min of partial hepatic ischemia [47]. Again, liver cell damage was assessed in this paper by semi-quantitative methods, which might explain some of the discrepancies compared with our study.

ALT and aspartate transaminase (AST) have been used as biochemical markers of liver cell damage in many studies [29,47]. We found no differences in ALT levels after 30 min of reperfusion between groups. However, after 24 h of reperfusion, ALT levels were significantly higher in the HHD group compared with the control group. We did not show any differences with regard to necrosis between groups, and in addition there were fewer apoptotic cells in the HDD group. This apparent paradox can most likely be explained by side effects of systemic corticosteroids in high doses on the liver as demonstrated by Sileri P et al. [45]. AP was significantly higher in the HDD group compared with the control group after 24 h. This might of course be a coincidence. Alternatively, it might be explained by a possible damaging effect of corticosteroids in high systemic doses on the biliary epithelium, similar to the one demonstrated by Seleri et al. on hepatocytes. We cannot decide this for certain, since we did not separately investigate the degree of necrosis or apoptosis in the biliary epithelium.

HP is produced by hepatocytes and is an acute phase protein elevated in inflammatory conditions [48]. HP levels after 30 min of reperfusion were significantly higher in the anti-CD163-dex and HDD groups compared with the control group. No difference was

Table 2
Interleukin-1 and α-2-macroglobulin: Averages are shown for measurements of IL-1 after 30 min of reperfusion and α-2-macroglobulin after 24 h of reperfusion with confidence intervals. There were no significant differences compared with the control group.

		Control (n = 10)	Anti-CD163-dex (n = 9)	HDD (n = 8)	LDD (n = 8)
IL-1 [pg/mL] (30 min)	Mean	670	898	761	923
	95% confidence interval for mean	(252–1089)	(485–1310)	(−68–1590)	(−320–923)
α-2-macroglobulin [mg/ml] (24 h)	Mean	1.17	0.81	1.25	1.05
	95% confidence interval for mean	(0.87–1.48)	(0.53–1.09	(0.65–1.84)	(0.50–1.60)

found after 24 h. We did not investigate the amount of inflammation in these experiments and, therefore cannot decide on this matter. IRI is known to initiate a strong inflammatory response, while in contrast corticosteroids have anti-inflammatory properties [27,28]. However, corticosteroids are also known to increase HP levels, which might explain this apparent paradox [49].

Kupffer cells produce cytokines, important mediators of the inflammatory response [21,50,51]. We measured cytokines after 30 min. Il-6 and TNF-α were close to the detection limit and there were no differences in IL-1 between groups. Other studies have shown a fall in TNF-α levels when preconditioning with prednisolone 2 h after reperfusion [31], and a decrease in IL-6 3 h after reperfusion when preconditioning with MP [33]. One possible explanation for not detecting higher levels of IL-6 and TNF-α in our analysis could be that samples were taken early in the inflammatory response, i.e. after 30 min.

The exact mechanism of corticoid action on hepatic IRI is unknown. Steroids in general induce an anti-inflammatory effect by providing negative feedback on immunological stimulation, stabilizing cell membrane integrity by inhibition of lysosome peroxidation and through regulation of gene expression pathways [27,28]. Figuaro at al. were the first to suggest that corticosteroid had a protective effect on IRI in their study from 1975 [52]. They suggested that this protective mechanism might be mediated by cell membrane protection, either through stabilization of the lysosomal membrane or by the inhibition of circulating toxic substances excreted from the liver during ischemia [52]. In the kidneys, dexamethasone has been shown to up-regulate Bcl-xL, down-regulate ischemia-induced Bax, and inhibit activation of caspase-9 and caspase-3, thereby reducing both apoptosis and necrosis. Dexamethasone has also been shown to decrease the number of infiltrating neutrophils and lower ICAM-1 [53]. However, Wang et al. suggested that prednisolone appeared to reduce IRI in the liver by suppressing cytokine release [31] and this might be the mechanism by which anti-CD163-dex acts in the present study. Although we could not detect any major rise in cytokines in the present study at the time of blood sampling, it is likely that this may be one way by which corticosteroids protect against IRI in the liver.

Clinical studies have shown a beneficial effect of systemic corticosteroid conditioning before liver surgery [32]. Moreover, Kotsch et al. [54] showed that MP therapy improved outcome after deceased donor liver transplantation, reducing inflammation in the donor organ. In liver transplantation, the degree of apoptosis is an important marker of outcome [55,56]. An anti-apoptotic effect of treatment with anti-CD163-dex might also improve the outcome in liver transplantation, opening the way for further therapeutic opportunities. Thus, if the severity of IRI can be substantially reduced, it might be possible to split the donor liver between more recipients.

In our study, rats were treated with a single dose of anti-CD163-dex, resulting in a reduction in the number of apoptotic cells. It is possible, that administering several doses of the conjugate before liver ischemia could enhance this protective effect, as well as timing of treatment could perhaps be optimized. One could also speculate that anti-CD163-dex might be a potential treatment of autoimmune liver disorders including primary biliary cirrhosis, autoimmune hepatitis, primary sclerosing cholangitis and IgG4-related liver diseases. In this way one might avoid many of the complications seen after long-term high-dose corticosteroid therapy. These considerations need further research.

A major strength of our study was that we used stereological quantification in a blinded and randomized fashion. This is in marked contrast to previous studies that have used a variety of semi-quantitative methods to assess IRI. Moreover, an intra-observer analysis found no significant differences in the study

results. In addition, our study was limited by lack of data concerning the inflammatory markers due to timing of blood sampling. However, with the experimental model used, it was not possible to obtain more than one adequate blood sample before euthanisation.

In conclusion, our study shows that pharmacological conditioning with anti-CD163-dex and HDD reduces the number of apoptotic cells following IRI compared with controls. We found no differences with regard to the degree of necrosis. Our findings do not allow us to decide whether or not the anti-CD163-dex effect on IRI was caused by inhibition of the inflammatory response.

Ethical approval

Financial support

Author contribution

Lin Nanna Okholm Møller: Data collection, data analysis, writing, approval.

Anders Riegels Knudsen: Study design, data collection, data analysis, writing, approval.

Kasper Jarlhelt Andersen: Study design, data analysis, writing, approval.

Jens Randel Nyengaard: Study design, data analysis, writing, approval.

Stephen Hamilton-Dutoit: Study design, writing, approval.

Elise Marie Okholm Møller: Data collection, writing, approval.

Pia Svendsen: Data collection, data analysis, writing, approval.

Holger Jon Møller: Study design, data analysis, writing, approval.

Søren Kragh Moestrup: Study design, writing, approval.

Jonas Heilskov Graversen: Study design, data analysis, writing, approval.

Frank Viborg Mortensen: Study design, data analysis, writing, approval.

Conflict of interest statement

Holger Jon Møller, Søren Kragh Moestrup and Jonas Heilskov Graversen own shares in Affinicon, which holds IP protecting the use of CD163 drug targeting.

Acknowledgments

References

[1] J. Belghiti, R. Noun, R. Malafosse, P. Jagot, A. Sauvanet, F. Pierangeli, et al., Continuous versus intermittent portal triad clamping for liver resection: a controlled study, Ann. Surg. 229 (3) (1999) 369–375.

[2] W.R. Jarnagin, M. Gonen, Y. Fong, R.P. DeMatteo, L. Ben-Porat, S. Little, et al., Improvement in perioperative outcome after hepatic resection: analysis of 1,803 consecutive cases over the past decade, Ann. Surg. 236 (4) (2002) 397–406 discussion-7.

[3] K.R. Stephenson, S.M. Steinberg, K.S. Hughes, J.T. Vetto, P.H. Sugarbaker, A.E. Chang, Perioperative blood transfusions are associated with decreased time to recurrence and decreased survival after resection of colorectal liver metastases, Ann. Surg. 208 (6) (1988) 679–687.

[4] J. Yamamoto, T. Kosuge, T. Takayama, K. Shimada, S. Yamasaki, H. Ozaki, et al., Perioperative blood transfusion promotes recurrence of hepatocellular carcinoma after hepatectomy, Surgery 115 (3) (1994) 303–309.

[5] S. Ibrahim, C.L. Chen, C.C. Lin, C.H. Yang, C.C. Wang, S.H. Wang, et al., Intraoperative blood loss is a risk factor for complications in donors after living donor hepatectomy, Liver Transpl. 12 (6) (2006) 950–957.

[6] Y. Yoshimura, S. Kubo, K. Shirata, K. Hirohashi, H. Tanaka, T. Shuto, et al., Risk factors for postoperative delirium after liver resection for hepatocellular carcinoma, World J. Surg. 28 (10) (2004) 982–986.

[7] D.A. Kooby, J. Stockman, L. Ben-Porat, M. Gonen, W.R. Jarnagin, R.P. Dematteo, et al., Influence of transfusions on perioperative and long-term outcome in patients following hepatic resection for colorectal metastases, Ann. Surg. 237 (6) (2003) 860–869 discussion 9–70.

[8] L.S. Jensen, E. Puho, L. Pedersen, F.V. Mortensen, H.T. Sorensen, Long-term survival after colorectal surgery associated with buffy-coat-poor and leucocyte-depleted blood transfusion: a follow-up study, Lancet 365 (9460) (2005) 681–682.

[9] D.M. Nagorney, J.A. van Heerden, D.M. Ilstrup, M.A. Adson, Primary hepatic malignancy: surgical management and determinants of survival, Surgery 106 (4) (1989) 740–748 discussion 8–9.

[10] T.M. van Gulik, W. de Graaf, S. Dinant, O.R. Busch, D.J. Gouma, Vascular occlusion techniques during liver resection, Dig. Surg. 24 (4) (2007) 274–281.

[11] J.D. van der Bilt, D.P. Livestro, A. Borren, R. van Hillegersberg, I.H. Borel Rinkes, European survey on the application of vascular clamping in liver surgery, Dig. Surg. 24 (6) (2007) 423–435.

[12] J.H.V. Pringle, Notes on the arrest of hepatic hemorrhage due to trauma, Ann. Surg. 48 (4) (1908) 541–549.

[13] F. Serracino-Inglott, N.A. Habib, R.T. Mathie, Hepatic ischemia-reperfusion injury, Am. J. Surg. 181 (2) (2001) 160–166.

[14] H. Jaeschke, Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning, Am. J. Physiol. Gastrointest. Liver Physiol. 284 (1) (2003) G15–G26.

[15] N.C. Teoh, G.C. Farrell, Hepatic ischemia reperfusion injury: pathogenic mechanisms and basis for hepatoprotection, J. Gastroenterol. Hepatol. 18 (8) (2003) 891–902.

[16] Y. Zhai, R.W. Busuttil, J.W. Kupiec-Weglinski, Liver ischemia and reperfusion injury: new insights into mechanisms of innate-adaptive immune-mediated tissue inflammation, Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg. 11 (8) (2011) 1563–1569.

[17] H. Malhi, G.J. Gores, J.J. Lemasters, Apoptosis and necrosis in the liver: a tale of two deaths? Hepatology 43 (2 Suppl. 1) (2006) S31–S44.

[18] J.R. Klune, A. Tsung, Molecular biology of liver ischemia/reperfusion injury: established mechanisms and recent advancements, Surg. Clin. N. Am. 90 (4) (2010) 665–677.

[19] H. Jaeschke, Role of reactive oxygen species in hepatic ischemia-reperfusion injury and preconditioning, J. Invest. Surg. 16 (3) (2003) 127–140.

[20] H. Jaeschke, C.V. Smith, J.R. Mitchell, Reactive oxygen species during ischemia-reflow injury in isolated perfused rat liver, J. Clin. Invest. 81 (4) (1988) 1240–1246.

[21] G.A. Wanner, W. Ertel, P. Muller, Y. Hofer, R. Leiderer, M.D. Menger, et al., Liver ischemia and reperfusion induces a systemic inflammatory response through Kupffer cell activation, Shock 5 (1) (1996) 34–40.

[22] L.M. Colletti, S.L. Kunkel, A. Walz, M.D. Burdick, R.G. Kunkel, C.A. Wilke, et al., Chemokine expression during hepatic ischemia/reperfusion-induced lung injury in the rat. The role of epithelial neutrophil activating protein, J. Clin. Invest. 95 (1) (1995) 134–141.

[23] P.A. Clavien, P.R. Harvey, J.R. Sanabria, R. Cywes, G.A. Levy, S.M. Strasberg, Lymphocyte adherence in the reperfused rat liver: mechanisms and effects, Hepatology 17 (1) (1993) 131–142.

[24] H. Jaeschke, Mechanisms of liver injury. II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions, Am. J. Physiol. Gastrointest. Liver Physiol. 290 (6) (2006) G1083–G1088.

[25] M. Goto, Y. Takei, S. Kawano, K. Nagano, S. Tsuji, E. Masuda, et al., Endothelin-1 is involved in the pathogenesis of ischemia/reperfusion liver injury by hepatic microcirculatory disturbances, Hepatology 19 (3) (1994) 675–681.

[26] N. Selzner, H. Rudiger, R. Graf, P.A. Clavien, Protective strategies against ischemic injury of the liver, Gastroenterology 125 (3) (2003) 917–936.

[27] N. Auphan, J.A. DiDonato, C. Rosette, A. Helmberg, M. Karin, Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis, Science 270 (5234) (1995) 286–290.

[28] M.W. Whitehouse, Anti-inflammatory glucocorticoid drugs: reflections after 60 years, Inflammopharmacology 19 (1) (2011) 1–19.

[29] M. Glanemann, R. Strenziok, R. Kuntze, S. Munchow, N. Dikopoulos, F. Lippek, et al., Ischemic preconditioning and methylprednisolone both equally reduce hepatic ischemia/reperfusion injury, Surgery 135 (2) (2004) 203–214.

[30] M. Wang, F. Shen, L.H. Shi, T. Xi, X.F. Li, X. Chen, et al., Protective effect of prednisolone on ischemia-induced liver injury in rats, World J. Gastroenterol. 14 (27) (2008) 4332–4337.

[31] M. Wang, M. Sakon, K. Umeshita, M. Okuyama, K. Shiozaki, H. Nagano, et al., Prednisolone suppresses ischemia-reperfusion injury of the rat liver by reducing cytokine production and calpain mu activation, J. Hepatol. 34 (2) (2001) 278–283.

[32] L.A. Orci, C. Toso, G. Mentha, P. Morel, P.E. Majno, Systematic review and meta-analysis of the effect of perioperative steroids on ischaemia-reperfusion injury and surgical stress response in patients undergoing liver resection, Br. J. Surg. 100 (5) (2013) 600–609.

[33] R.F. Saidi, J. Chang, S. Verb, S. Brooks, I. Nalbantoglu, V. Adsay, et al., The effect of methylprednisolone on warm ischemia-reperfusion injury in the liver, Am. J. Surg. 193 (3) (2007) 345–347 discussion 7–8.

[34] J.H. Graversen, P. Svendsen, F. Dagnaes-Hansen, J. Dal, G. Anton, A. Etzerodt, et al., Targeting the hemoglobin scavenger receptor CD163 in macrophages highly increases the anti-inflammatory potency of dexamethasone, Mol. Ther. 20 (8) (2012) 1550–1558.

[35] H.J. Moller, Soluble CD163, Scand. J. Clin. Lab. Invest. 72 (1) (2012) 1–13.

[36] A. Granfeldt, C.L. Hvas, J.H. Graversen, P.A. Christensen, M.D. Petersen, G. Anton, et al., Targeting dexamethasone to macrophages in a porcine endotoxemic model, Crit. Care Med. 41 (11) (2013) e309–18.

[37] M. Kristiansen, J.H. Graversen, C. Jacobsen, O. Sonne, H.J. Hoffman, S.K. Law, et al., Identification of the haemoglobin scavenger receptor, Nature 409 (6817) (2001) 198–201.

[38] H. Jaeschke, A. Farhood, Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver, Am. J. Phys. 260 (3 Pt 1) (1991) G355–G362.

[39] S. Suzuki, S. Nakamura, T. Sakaguchi, H. Mitsuoka, Y. Tsuchiya, Y. Kojima, et al., Pathophysiological appraisal of a rat model of total hepatic ischemia with an extracorporeal portosystemic shunt, J. Surg. Res. 80 (1) (1998) 22–27.

[40] C. Muhlfeld, J.R. Nyengaard, T.M. Mayhew, A review of state-of-the-art stereology for better quantitative 3D morphology in cardiac research, Cardiovasc. Pathol. 19 (2) (2010) 65–82.

[41] D.W. Nicholson, A. Ali, N.A. Thornberry, J.P. Vaillancourt, C.K. Ding, M. Gallant, et al., Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis, Nature 376 (6535) (1995) 37–43.

[42] H.J. Gundersen, T.F. Bendtsen, L. Korbo, N. Marcussen, A. Moller, K. Nielsen, et al., Some new, simple and efficient stereological methods and their use in pathological research and diagnosis, APMIS Acta Pathologica Microbiol. Immunol. Scand. 96 (5) (1988) 379–394.

[43] A.R. Knudsen, A.S. Kannerup, H. Gronbaek, S.H. Dutoit, J.R. Nyengaard, P. Funch-Jensen, et al., Quantitative histological assessment of hepatic ischemia-reperfusion injuries following ischemic pre- and post-conditioning in the rat liver, J. Surg. Res. 180 (1) (2013) e11–20.

[44] J.M. Bland, D.G. Altman, Statistical methods for assessing agreement between two methods of clinical measurement, Lancet 1 (8476) (1986) 307–310.

[45] M.M. Diesselhoff-den Dulk, R.W. Crofton, R. van Furth, Origin and kinetics of Kupffer cells during an acute inflammatory response, Immunology 37 (1) (1979) 7–14.

[46] T. Rhen, J.A. Cidlowski, Antiinflammatory action of glucocorticoids–new mechanisms for old drugs, N. Engl. J. Med. 353 (16) (2005) 1711–1723.

[47] P. Sileri, S. Schena, J. Fukada, C. Rastellini, J. Pirenne, E. Benedetti, et al., Corticosteroids enhance hepatic injury following ischemia-reperfusion, Transplant. Proc. 33 (7–8) (2001) 3712.

[48] C. Gabay, I. Kushner, Acute-phase proteins and other systemic responses to inflammation, N. Engl. J. Med. 340 (6) (1999) 448–454.

[49] S. Marinkovic, H. Baumann, Structure, hormonal regulation, and identification of the interleukin-6- and dexamethasone-responsive element of the rat haptoglobin gene, Mol. Cell. Biol. 10 (4) (1990) 1573–1583.

[50] L.M. Colletti, A. Cortis, N. Lukacs, S.L. Kunkel, M. Green, R.M. Strieter, Tumor necrosis factor up-regulates intercellular adhesion molecule 1, which is important in the neutrophil-dependent lung and liver injury associated with hepatic ischemia and reperfusion in the rat, Shock 10 (3) (1998) 182–191.

[51] L.M. Colletti, D.G. Remick, G.D. Burtch, S.L. Kunkel, R.M. Strieter, D.A. Campbell Jr., Role of tumor necrosis factor-alpha in the pathophysiologic alterations after hepatic ischemia/reperfusion injury in the rat, J. Clin. Invest. 85 (6) (1990) 1936–1943.

[52] I. Figueroa, E.A. Santiago-Delpin, Steroid protection of the liver during experimental eschemia, Surg. Gynecol. Obstet. 140 (3) (1975) 368–370.

[53] S. Kumar, D.A. Allen, J.E. Kieswich, N.S. Patel, S. Harwood, E. Mazzon, et al., Dexamethasone ameliorates renal ischemia-reperfusion injury, J. Am. Soc. Nephrol. JASN 20 (11) (2009) 2412–2425.

[54] K. Kotsch, F. Ulrich, A. Reutzel-Selke, A. Pascher, W. Faber, P. Warnick, et al., Methylprednisolone therapy in deceased donors reduces inflammation in the donor liver and improves outcome after liver transplantation: a prospective randomized controlled trial, Ann. Surg. 248 (6) (2008) 1042–1050.

[55] W. Gao, R.C. Bentley, J.F. Madden, P.A. Clavien, Apoptosis of sinusoidal endothelial cells is a critical mechanism of preservation injury in rat liver transplantation, Hepatology 27 (6) (1998) 1652–1660.

[56] G. Borghi-Scoazec, J.Y. Scoazec, F. Durand, J. Bernuau, J. Belghiti, G. Feldmann, et al., Apoptosis after ischemia-reperfusion in human liver allografts, Liver Transplant. Surg. Off. Publ. Am. Assoc. Study Liver Dis. Int. Liver Transplant. Soc. 3 (4) (1997) 407–415.