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Steroid administration before partial hepatectomy with temporary inflow occlusion does not influence cyclin D1 and Ki-67 related liver regeneration

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Abstract *Background and aims:* If temporary inflow occlusion is required during liver resection, the postoperative course might be complicated by ischaemia–reperfusion injury. Steroids protect against ischaemia–reperfusion injury; however, due to its anti-proliferative character concerns exist on its use on liver regeneration after resection. We investigated the effects of methylprednisolone on hepatocyte proliferation after partial hepatectomy with temporary inflow occlusion. *Patients and methods:* Prior to surgery, one group of Wistar rats received methylprednisolone, while a second group served as non-treated controls. Ischaemia–reperfusion injury was indicated by AST, ALT, and GLDH at 6 h after surgery. Immunohistochemistry tools were used to determine the mitotic index and Ki-67 expression, while cyclin D1 expression characterized the proliferative activity on days 1, 4, 7, and 10. *Results:* The post-ischaemic liver enzyme release had significantly decreased in the methylprednisolone group, while expression of cyclin D1, percentage of Ki-67-positive cells, and mitotic cell index were comparable in both groups. Similar results were found for bilirubin and albumin and for weight of proliferating liver. *Conclusion:* Although steroid administration significantly reduced ischaemia–reperfusion-associated tissue injury, it has no apparent effects on hepatic regeneration. Thus, steroids could be recommended if a temporary liver ischaemia is required during surgery, in order to reduce complications caused by severe ischaemia-related organ dysfunction.

Keywords Liver regeneration · Partial hepatectomy · Methylprednisolone

Introduction

In cases of excessive intraoperative bleeding, liver surgery may sometimes require temporary inflow occlusion to reduce the need for blood transfusion, representing an independent predictor of decreased short-term and long-term patient outcome [1, 2]. It is well recognized that the associated warm organ ischaemia can result in severe ischaemia–reperfusion (IR) injury, which is an important source of postoperative organ dysfunction or even liver failure [3].

The underlying mechanisms leading to IR-related organ injury are still under intensive investigation [3–5]. In 1975, Santiago-Delpin et al. first reported that steroid pretreatment reduced reperfusion injury after warm liver ischaemia in rabbits, as well as in humans, when adopting the experimental set-up in clinical liver trauma surgery [6]. In the same line of evidence it was demonstrated that steroid treatment reduced IR-related tissue injury in postischaemic liver tissue by suppression of inflammatory cytokine liberation such as that of interleukin 6 (IL-6) [7, 8] or the inhibition of calpain-μ activation [9].

Although recent studies confirmed the protective effects of steroids, such as that of methylprednisolone (MP) towards IR-related liver injury [10–12], there is still major concern about the use of steroids in hepatic regeneration, due to their anti-proliferative character [13]. The mechanisms that lead to this adverse effect of steroids are still not clear, but it is well established that steroids suppress secretion of inflammatory cytokines such as tumour necrosis factor (TNF) and IL-6 [7, 8, 11, 14]. Most interesting is that both cytokines are also well known to play a pivotal role as initiators of liver regeneration to trigger liver regeneration following partial hepatectomy by priming the remnant hepatocytes for division by increasing the sensitivity and responsiveness to growth factors [15–17].

If these observations are taken together, we have an apparent paradox: steroids seem to reduce the regenerative capacity of remnant liver tissue, which would represent an undesirable effect of a treatment given to increase cell function and viability of liver tissue, ischaemically injured during partial hepatectomy. In order to clarify the role of steroids for liver regeneration we investigated the effects of MP on hepatic proliferation in regenerating liver tissue following partial (70%) hepatectomy with temporary inflow occlusion in rats.

Materials and methods

Animals and surgical procedure

The experimental design was reviewed and approved by the local government (Senator für Gesundheit und Soziales, Berlin), and carried out according to the European Union regulations for animal experiments and the *Guide for the Care and Use of Laboratory Animals* [DHEW publication no. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205].

Male Wistar rats weighing between 230 g and 270 g were starved overnight but were given free access to tap water. Under isoflurane/oxygen inhalative anaesthesia, the animals were placed on a heating pad in a supine position. After midline laparotomy, we accomplished hepatic ischaemia by using microvascular clips, which were placed on the hepatoduodenal ligament. Reflow was initiated after 30 min of hepatic ischaemia by removal of the clamp. Before hepatic ischaemia, animals in the steroid group received methylprednisolone (MP, Hoechst, Frankfurt, Germany), intravenously, 3 min before ischaemia at a dose of 30 mg/kg BW according to the protocol of Santiago-Delpin et al. [6]. A second group was not pretreated and served as non-treated (ischaemic) controls. During ischaemia, partial hepatectomy was performed by resection of the left and median lobes of the liver, according to the method of Higgins and Anderson [18]. The operation was performed between 09:00 h and 12:00 h. Postoperatively, animals were allowed food and water ad libitum.

At postoperative days 1, 4, 7, and 10, the animals were exsanguinated, and blood and liver samples were stored for enzymatic, biochemical and histological analyses (*n*=8 each day and group). The residual liver lobes were removed and weighed. Growth of residual liver lobes was assessed by the following equation: weight of residual liver lobes/body weight × 100 (vol%) [10].

Measurement of hepatocellular damage and postoperative liver function

We measured the extent of hepatocellular damage at 6 h after surgery by enzymatic determination of AST, ALT, and GLDH plasma levels, using commercially available reaction kits (Roche Diagnostics, Mannheim, Germany). In the same way, serum bilirubin and albumin concentrations were measured at days 1, 4, 7, and 10 after surgery for analysis of postoperative liver function. The measurements were performed at the Institute of Laboratory Medicine, Charité, Campus Virchow Klinikum, Universitätsmedizin Berlin.

Measurement of hepatocyte proliferation (Ki-67-positive cells, mitotic index, cyclin D1 protein expression)

The labelling index of MIB-5, a novel antibody reacting with the equivalent Ki-67 protein, to detect all active parts of the cell cycle, was determined immunohistochemically by an indirect enzyme-linked antibody method [19]. The proliferative capacity was expressed in terms of labelling indices, determined as the number of labelled hepatocytes per 1,000 hepatocyte nuclei (high-power field; ×200). Data are expressed as the mean percentage of Ki-67-positive cells. We evaluated the number of mitotic hepatocytes (mitotic index) by counting 2,000 cells in haematoxylin–eosin-stained tissue sections. Data are given as the mean number of mitotic cells per 2,000 hepatocytes.

Cyclin D1 expression was visualized by Western Blot analysis and analysed by densitometry. Protein concentrations were determined with a commercially available test based on the Lowry reaction (Sigma, Taufkirchen, Germany). Proteins were separated on a 12.5% SDS polyacrylamide gel and then transferred onto nitrocellulose membranes [20]. Non-specific binding sites were blocked by 5% non-fat milk solution dissolved in PBS/Tween-20 at 4°C. Membranes were then washed and incubated with a mouse caspase 3/32 monoclonal antibody (1:1,000 dilution, BD Transduction, Heidelberg, Germany), followed by an incubation step with a horseradish-peroxidase-conjugated anti-mouse antibody (1:2,000 dilution, BD Transduction) at room temperature for 1 h. Membranes were then washed again (PBS/Tween-20), and the immune complexes were visualized with a chemiluminescence detection system (ECL; Amersham, Freiburg, Germany). Equal loading of total protein was verified with a commercially available antibody against β-actin (AC-15, Sigma, Taufkirchen, Germany) [21].

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). After proving the assumption of normality and equal variance across both groups, we assessed differences between groups, using analysis of variance (overall differences) followed by the appropriate post hoc method (pairwise multiple comparisons). In all instances, $P<0.05$ was considered statistically significant.

Results

Animal recovery and postoperative liver function

All animals survived the operating procedure, and no death occurred until the end of the observation period. As depicted in Table 1, the extent of partial hepatectomy was comparable between both groups, reaching 6.7±0.8 g and 5.9±0.7 g, respectively, of resected liver tissue.

Table 1 Relation of resected liver and body weight indicating comparable liver mass reduction after partial hepatectomy (70%) in non-treated (control) and MP-treated animals. Serum liver enzyme levels (AST, ALT, GLDH) at 6 h after surgery, demonstrating the diminished IR injury following MP-treatment. *NS* not significant

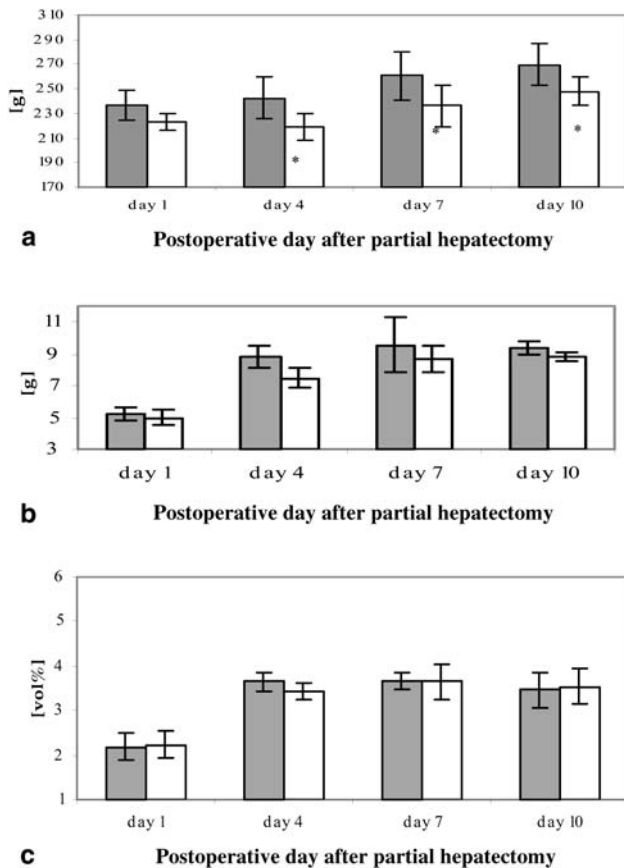| Parameter	Control	MP	Significance
Body weight (g)	253.7±14	242.5±8	NS
Weight of resected liver mass (g)	6.7±0.8	5.9±0.7	NS
Weight of resected liver/body weight (vol%)	2.6±0.4	2.4±0.6	NS
AST at 6 h (U/l)	467±46	287±116	0.033
ALT at 6 h (U/l)	552±177	288±129	0.028
GLDH at 6 h (U/l)	128±48	46±29	0.021

Fig. 1 Postoperative regenerative parameter in non-treated (*grey bars*) and MP-treated (*white bars*) animals following partial hepatectomy (70%). **a** Body weight, **b** weight of remnant liver, **c** weight of remnant liver/body weight; *P<0.05
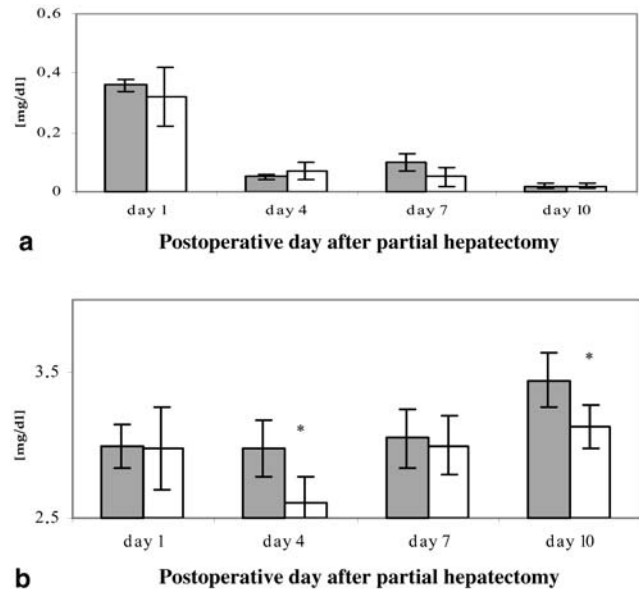

Fig. 2 Serum levels of **a** bilirubin and **b** albumin as a measurement of postoperative liver function following partial hepatectomy (70%) in non-treated (*grey bars*) and MP-treated (*white bars*) animals; *P<0.05

er lobes showed no statistical differences at these days (Fig. 1b). Moreover, with regard to the growth of the remnant liver, given as weight of remnant liver per body weight, no significant differences were observed between either group during the whole observation period (Fig. 1c), indicating comparable liver proliferation in both groups.

The postoperative serum bilirubin was elevated on day 1 in both groups and had decreased almost to baseline levels by day 4 (Fig. 2a). Statistical analysis revealed no significant differences between either groups. Measurement of serum albumin revealed significantly lower levels on day 4 and day 10 in the MP-treated group (*P<0.05*), while albumin secretion on day 1 and day 7 was comparable in both groups (Fig. 2b).

Hepatocellular damage

As depicted in Table 1, the postischaemic rise in AST and ALT levels was significantly reduced in MP-treated animals at 6 h after surgery, compared with that of non-treated animals (*P<0.05*). Mitochondrial damage, measured by the rise of serum GLDH, was also significantly reduced (*P<0.05*) after MP treatment.

Hepatocyte proliferation (Ki-67-positive cells, mitotic index, cyclin D1 protein expression)

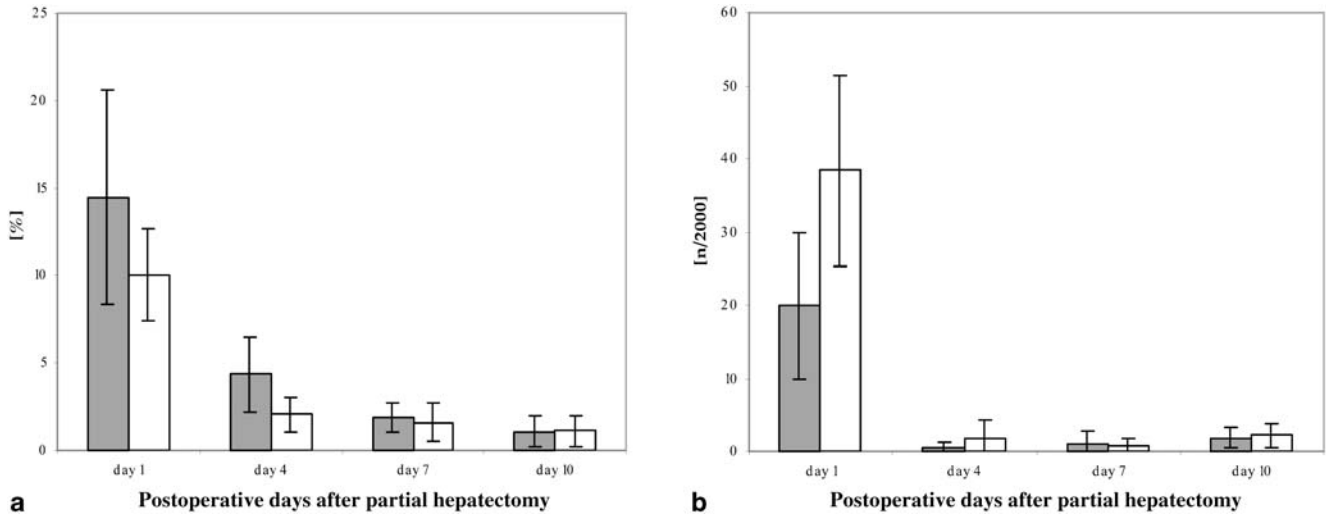The highest percentage of Ki-67-positive cells was found on day 1 after surgery, without any statistical difference

Following surgery, we observed a decrease in rat body weight in both study groups when compared with preoperative weight. As depicted in Fig. 1a, the postoperative body weight of MP-treated rats was significantly lower at days 4, 7, and 10 than that of non-treated animals (*P<0.05*), whereas a comparison of the weight of the remnant liv-

Fig. 3 a Ki-67-positive cells and **b** mitotic cells in regenerating livers after partial hepatectomy (70%) in non-treated (*grey bars*) and MP-treated (*white bars*) animals, indicating no impairment of the postoperative proliferative capacity following MP-treatment. Indirect enzyme-linked antibody measurement of Ki-67 protein, immunohistochemically determined as the number of labelled hepatocytes per 1,000 hepatocyte nuclei (high-power field; ×200), given as mean percentage of Ki-67-positive cells. Mitotic cells were measured in haematoxylin–eosin-stained tissue sections and given as mean number of mitotic cells per 2,000 hepatocytes
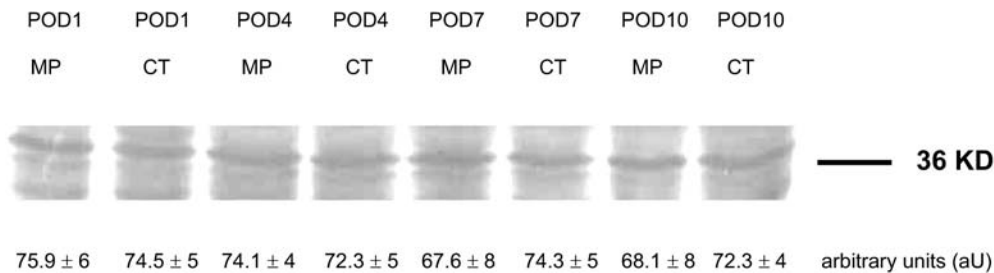

Fig. 4 Expression of cyclin D1 (Western blot analysis, 36 KD and densitometry) in regenerating livers in non-treated and MP-treated animals, demonstrating no differences between either group with regard to the postoperative proliferative activity in the remnant liver tissue after partial hepatectomy (70%). Densitometric analysis is given as difference from equal b-actin loading (arbitrary units). *MP* methylprednisolone group, *CT* control group, *POD* postoperative day

between either group, reaching 14.46±0.8% in non-treated animals and 10.06±2.6% in MP-treated animals (Fig. 3a). The percentage of Ki-67-positive cells continuously decreased in both groups during the time period investigated. Similar results regarding the hepatocyte mitotic index were observed. Following an initial marked increase of hepatocyte mitosis on day 1 after surgery, the number of mitotic cells had clearly disappeared by day 4 after partial hepatectomy (Fig. 3b). Again, no statistical differences were found between either group during the early phase of hepatocyte proliferation. With regard to the expression of cyclin D1, which is an indicator for DNA replication and mitosis [15], no differences occurred between MP-treated and non-treated animals. As shown in Fig. 4, both groups equally expressed cyclin D1 at the time points investigated, as measured by Western blot analysis and densitometry.

Discussion

Liver surgery has markedly developed within recent years, and results are continuously improving. Nevertheless, the main concerns in surgical practice still consist of avoiding excessive intraoperative bleeding and blood transfusion, as long as both factors have clearly been proven to be associated with reduced short-term and long-term clinical outcome [1, 2]. Therefore, temporary inflow occlusion, the so-called Pringle manouevre, might sometimes become necessary during liver resection. However, as a consequence of organ hypoxia, IR injury may develop, resulting in severe organ dysfunction or even lethal organ failure [3–5].

Several strategies to reduce hepatic IR injury have been reported, although the underlying mechanisms are still unclear [3, 4]. In this context, several investigators, in-

Table 2 In vivo studies on steroid administration during partial hepatectomy with or without temporary organ ischaemia (*i.v.* intravenously, *i.p.* intraperitoneally, *i.m.* intramuscular, *CLD* chronic liver disease, *n.a.* not analysed)

Author	Substance	Dosage in mg/kg BW	Adminis-tration	Species	Hepatocyte proliferation	Interleukin-6	Hepatic injury
Without liver ischaemia							
Nagy et al. [14]	Dexamethasone	2	i.p.	Rat	⇓	⇓ Gen expression	n.a.
Nagy et al. [14]	Dexamethasone	0.2	i.p.	Rat	⇓	⇓ Gen expression	n.a.
Nagy et al. [14]	Dexamethasone	0.1	i.p.	Rat	⇓	⇓ Gen expression	n.a.
Nadal [24]	Hydrocortisone	6.25	i.p.	Rat	⇓	n.a.	n.a.
Nadal [24]	Hydrocortisone	1.25	i.p.	Rat	⇑	n.a.	n.a.
Kim et al. [25]	Methylprednisolone	8	i.v.	Rat	⇓	n.a.	⇔
Fujioka et al. [10]	Methylprednisolone	3	i.p.	Rat	⇔	⇓ Serum levels	⇓
Azzarone et al. [26]	Methylprednisolone	1	i.m.	Rat	⇑	n.a.	n.a.
Shimada et al. [7]	Methylprednisolone	30+2×1 g[a]	i.v.	Human	n.a.	⇓ Serum levels	⇔
With liver ischaemia							
Glanemann et al. [12]	Methylprednisolone	30	i.v.	Rat	⇔	n.a.	⇓
Muratore et al. [11]	Methylprednisolone	30	i.v.	Human	n.a.	⇓ Serum levels	⇓ in CLD
Yamashita et al. [8]	Methylprednisolone	7	i.v.	Human	n.a.	⇓ Serum levels	⇔

[a] One gramme immediately after surgery and again at postoperative day 1

cluding our own group, have demonstrated that the administration of MP provides strong protective effects [6–12, 14, 22, 23]. One way in which steroids act is by the suppression of transcription factors such as nuclear factor kB (NFkB), thereby reducing the expression of dependent inflammatory genes such as ICAM-1, E-selectin, or various inflammatory cytokines including TNF, IL-1, and IL-6 [7, 8, 10, 11, 14, 23]. In the same line of evidence we have recently shown that MP may also reduce the apoptotic activity in postischaemic liver tissue [12]. Thus, we believe that steroid treatment is justified for clinical application in order to reduce IR-related hepatocellular damage and to increase cell function and viability, unless adequate regeneration is absolutely mandatory for successful patient recovery. However, due to the anti-proliferative character of steroids, concerns exist about the safe use of glucocorticoids in resected livers, which is usually characterized by massive hepatocyte proliferation after surgery [15]. Liver regeneration is a multi-step process, in which TNF and IL-6 are important initiators of liver regeneration, priming remnant hepatocytes for cell division [15–17]. However, as mentioned above, several reports have well documented steroid-dependent suppression of these pro-inflammatory cytokines [7–9, 11]. Overall, the question arises as to whether the beneficial effects of steroids on IR injury may be compensated in resected, proliferating livers by steroid-dependent inhibition of regeneration.

The aim of our study was to clarify the beneficial and/or detrimental role of steroids in a clinically relevant, experimental model of partial (70%) hepatectomy. According to our results, we observed no impairment of postoperative hepatocyte proliferation by previous steroid administration. While MP treatment significantly reduced the hepatic IR injury, as measured by the postischaemic rise in liver enzymes, parameters on hepatocyte proliferation (Ki-67, mitotic index, cyclin D1) were comparable between steroid-treated and non-treated livers. Indeed, we observed lower postoperative body weight in MP-treated rats at day 4 and day 7 than in non-treated animals, and we also measured significantly lower serum albumin levels in this group. Speculatively, this difference might be due to steroid-related postoperative fluid disturbances; however, we have no explanation for this observation, and our data will not further elucidate this issue. Nevertheless, we strongly believe that this difference was not due to an impairment of hepatocyte proliferation, so long as statistical analysis did not reveal significant differences regarding the weight of the remnant liver lobes at these days, indicating comparable liver proliferation postoperatively. In consequence, we believe that parameters such as body weight and/or liver weight itself do not ideally reflect postoperative liver growth. From our point of view, the ratio of weight of remnant liver to body weight represents a more reliable parameter of postoperative liver growth. Thus, based on our results, we believe that the concerns raised against the anti-proliferative effects of steroids are no longer justified.

This situation might change, if glucocorticoids are given in different substances and/or dosages (Table 2). With respect to hydrocortisone, Nadal et al. reported dose-related effects of hydrocortisone on hepatocyte proliferation in rats [24]. They measured high doses (6.25 mg/kg BW) and low doses (1.25 mg/kg BW) of hydrocortisone on hepatic regeneration following different levels of proliferation (suckling rats, adult rats, and 2/3 hepatectomized rats). They indeed observed that doses in the therapeutic range or close to physiological values did not inhibit, but on the contrary, stimulated, hepatocyte proliferation, especially during acute inflammation and early regeneration following 2/3 hepatectomy [24]. Contrary to this observation, Nagy et al. revealed a dose-independent inhibition of hepatocyte proliferation after steroid treatment, when assessing dexamethasone doses of 0.1, 0.2, and 2 mg/kg BW in resected rats [14]. Regarding MP, Kim et al. ob-

served that MP was antimitotic in rats, if it was administered at a dose of 8 mg/kg BW [25], while Fujioka et al. revealed no differences in hepatic regeneration when using 3 mg/kg BW [10]. Moreover, Azzarone et al. even reported an augmentation of liver regeneration in intact animals that underwent 70% partial hepatectomy compared to their controls, when MP was given in a dose of 1 mg/kg BW [26]. Overall, one might suggest that the lower the dose, the better the effect on hepatocytes in terms of proliferation (apart from dexamethasone). However, we are aware that different steroids (such as hydrocortisone, dexamethasone, or MP) may also have different anti-inflammatory and anti-proliferative properties, which makes appropriate comparison more difficult.

In all above-mentioned studies, the experimental protocol consisted of partial hepatectomy in rats, but without warm ischaemia of the remnant liver lobes. With respect to the literature, neither a decrease nor an increase in hepatocyte proliferation was reported when analysed in oxygen-deprived liver injury following partial hepatectomy, regardless of the steroid dose given to protect from IR-related cellular injury. Muratore et al. performed 53 liver resections, for which 25 patients were randomized for MP administration at a dose of 30 mg/kg BW and compared with 28 patients without steroid protection. The authors observed significantly lower IL-6 serum levels in the steroid group, but patients in both groups recovered equally following surgery, and no differences in morbidity or mortality were observed [11]. In the same way, 17 patients who received 500 mg of MP 2 h prior to hepatic resection recovered similarly, as did those 16 patients without steroid protection [8]. Unfortunately, the authors gave no detailed information about the exact body weight relation to MP. However, their patients would have received approximately 7 mg/kg BW (at an estimated 70 kg per person). Thus, it would be of great interest to establish whether lower doses of MP might benefit patient outcome following liver resection including temporary inflow occlusion; however, relevant information regarding this issue is currently still unavailable.

As a result of this observation, one might suggest that intact hepatocytes obviously respond in a dose-dependent manner to steroid protection, contrary to ischaemically injured hepatocytes that are protected, regardless of the MP dosage. In addition, hepatocyte proliferation may, perhaps, also be induced through pathways that are independent of TNF or IL-6 expression, so long as adequate proliferation takes part despite reduced IL-6 serum levels [7, 8, 10, 11]. However, further studies are required to elucidate this topic.

In conclusion, in our experimental study, steroid administration significantly reduced the associated IR injury, without affecting hepatocyte proliferation following liver resection. Despite steroid-dependent suppression of IL-6, TNF, or NFkB expression, we and others have clearly shown that MP administration before liver regeneration did not affect hepatocyte proliferation, either in the early or in the later postoperative period.

Thus, we recommend steroid administration in clinical liver resections that require temporary inflow occlusion in order to reduce complications caused by severe IR-related organ dysfunction. However, further studies are mandatory to clarify whether steroids may also have a dose-related effect on hepatocyte proliferation in ischaemically injured livers.

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