Postoperative Hyperoxia (60%) Worsens Hepatic Injury in Mice

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ABSTRACT

Background: Liver damage by ischemia and reperfusion injury is a risk factor for morbidity and mortality after liver surgery. Postoperative oxygen treatment is routinely applied in the postanesthesia and intensive care unit after liver surgery. The risks of aggravating the injury by increasing inspiratory oxygen from 21 to 60% in the postoperative period were investigated in mice.

Methods: Parameters of liver injury were compared after induction of hepatic ischemia–reperfusion injury, by clamping the left liver lobe for 45 min, and reperfusion for 24 h either under normoxic (21% oxygen) or hyperoxic (60% oxygen) conditions (n = 22 per group). The extent of tissue injury and oxidative responses was analyzed in the presence or absence of polymorphonuclear leukocytes, functional Kupffer cells, and the p47phox unit of the nicotinamide adenine dinucleotide phosphate oxidase (n = 6 to 11 per group).

Results: Compared with postoperative normoxic conditions, hyperoxia increased cell damage (glutamate-pyruvate transaminase: 1,870 [±968 SD] *vs.* 60% 2,981 [±1,038 SD], 21 *vs.* 60% oxygen, in U/l as mean ± SD; $P < 0.01$), liver weights (341 ± 52 *vs.* 383 ± 44, 21 *vs.* 60% oxygen, in mg as mean ± SD; $P = 0.02$), damage scores (1.9 ± 0.8 *vs.* 3.1 ± 1.0, 21 *vs.* 60% oxygen, score as mean ± SD; $P = 0.02$), and reactive oxygen species (15.0 ± 12.0 *vs.* 30.4 ± 19.2, 21 *vs.* 60% oxygen, in μmol/l as mean ± SD; $P < 0.05$). The aggravation of the tissue damaging effects as a result of hyperoxia was not seen in mice with depletions of polymorphonuclear leukocytes or Kupffer cells, or with nonfunctioning nicotinamide adenine dinucleotide phosphate oxidase.

Conclusion: Liver injury after ischemia was significantly aggravated by hyperoxia as a consequence of immune cell-mediated oxidative burst. Further studies are needed to elucidate whether routine delivery of high inspirational oxygen concentrations postoperatively should be limited. **(Anesthesiology 2014; 121:1217-25)**

A CRITICAL intracellular oxygen pressure of 1 to 5 mmHg is needed for cellular respiration by enabling mitochondria to produce adenosine triphosphate.[1–3] Inspiration of 21% oxygen at sea level leads to 20 ml/dl of oxygen content in arterial blood of healthy adults. Under normal physiologic conditions, 25% of oxygen is used averagely (central venous blood content: 15 ml/dl), resulting in a decrease in arterial oxygen pressure from 75 to 120 to 40 mmHg in mixed-venous blood.[4] This physiologic excess can be interpreted as a "buffer" to avoid cellular hypoxia during conditions of reduced oxygen delivery, like malperfusion.

Oxygen delivery to the spontaneously breathing patient with elevated fraction of inspired oxygen (up to 0.6) is routinely used postoperatively as standard procedure and as a safety measure to reduce the risks of hypoxemia due to cardiopulmonary complications or hemorrhage. Furthermore, prolonged delivery of oxygen is recommended after some types of surgery, such as upper abdominal surgery,

What We Already Know about This Topic

- There are variable results regarding the benefit or harm from postoperative hyperoxia treatments

What This Article Tells Us That Is New

- In anesthetized mice subjected to ischemia–reperfusion injury of their livers, the provision of 60% inspired oxygen postoperatively led to significantly more cell-driven (polymorphonuclear and Kupffer cells) induced tissue injury
- Elimination of either polymorphonuclear or Kupffer cells abolished the oxygen-induced damage

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). The first two authors contributed equally to this study.

Submitted for publication November 8, 2013. Accepted for publication July 31, 2014. From the University Hospital of the Ludwig-Maximilians-University, Department of Anesthesiology, Klinikum Grosshadern, Munich, Germany (Q.Z., A.M., I.K., A.C.); National Institute on Minority Health and Health Disparities, National Institutes of Health, Division of Intramural Research, Bethesda, Maryland (S.H.J.); National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland (A.O., D.L., J.M.W., M.S., A.C.); New England Inflammation and Tissue Protection Institute Consortium at Northeastern University, Boston, Massachusetts (A.O., D.L., M.S.); Mouse Imaging Facility, National Institute of Neurological Disorder and Stroke, National Institutes of Health, Bethesda, Maryland (B.K.); and Department of Anaesthesiology and Intensive Care, Medical Faculty of Mannheim, University of Heidelberg, Mannheim, Germany (M.T.).

 Anesthesiology 2014; 121:1217-25

for example.[5] However given the physiologic buffer in tissue arterial oxygen pressures and the fact that increasing evidence indicates that mitochondrial respiration cannot be increased by more than 16 to 40%,[6] the routine use of oxygen may not be needed, and may even be harmful. Indeed, the latest guidelines of the European Resuscitation Council suggest titrating oxygen supply to achieve oxygen saturations (Spo_2) between 94 and 98% after recovery of spontaneous circulation following cardiac arrest to limit the potentially damaging effects of oxygen in the context of a generalized ischemia–reperfusion injury (IRI).[7] The use of inspired oxygen concentrations up to 50 to 60% (closed system, 6 l/min) postoperatively may be excessive and may aggravate tissue damage *via* the increased production of reactive oxygen species (ROS) and immune cell activation. As IRI seems to be mostly susceptible to oxygen-derived aggravation of tissue damage, the authors selected a murine model of hepatic ischemia and reperfusion to test for the effects of elevated postoperative oxygenation. This murine model mirrors the impact of IRI as a consequence of temporary ligation of hepatoduodenal ligament during extended liver surgery in patients. This procedure is known as the Pringle maneuver. The aim of the Pringle maneuver is the control of the blood flow to the liver to reduce the risk of excessive intraoperative blood loss during surgical treatment of liver diseases (primary carcinomas, metastasis), or in the course of liver transplantation. However, a consequence of the Pringle maneuver is IRI, as a result of a cascade of metabolic and inflammatory steps. IRI in this context results in tissue edema and malperfusion, hepatocellular dysfunction, and inflammation which can ultimately lead to liver dysfunction and in worst cases to organ failure.[8–13]

We hypothesized that elevated inspirational oxygen content (60% oxygen) aggravates hepatic IRI in conjunction with circulating or resting innate immune cells. The experimental protocol in mice was therefore designed to compare the influence of increasing oxygen concentrations (21 *vs.* 60% oxygen) on cell-driven liver tissue damaging processes. Specifically, the roles of polymorphonuclear (PMN) leukocytes, Kupffer cells, and of the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase in hyperoxia-driven IRI with increased ROS and coincident hepatocellular damage were systematically examined.

Materials and Methods

Animals

The experiments were conducted in accordance to the "U.S. Government Principles For The Utilization And Care Of Vertebrate Animals Used in Testing, Research And Training"* and the German local committee for animal welfare at University of Munich, approved in protocol nr. 55.2-1-54-2531-60-08. Mice were maintained under specific pathogen-free conditions at the animal care facilities. All mice used were on C57BL/6 background. NADPH p47 knockout (KO) mice were backcrossed at least 10 times. Phenotypic comparisons were made between KO mice with the corresponding littermates (wild-type [WT]). All mice used in the experiments were age-matched males of 9 to 12 weeks. The experiments were repeated at least two times. The selection to which group the mice were assigned was done in an alternate fashion and mice were taken out from the cage arbitrarily.

Hepatic Ischemia and Reperfusion

The induction of anesthesia was realized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (4 mg/kg) and maintained by repeated boluses of ketamine. During surgery, mice spontaneously breathed room air at all times. The Spo_2 of their blood was measured using intermittent animal "paw-pulse-oximetry." In addition, femoral or carotid arterial blood was sampled in selective experiments to quantify the arterial oxygen pressure (po_2) and to confirm the normoxemic conditions during anesthesia and surgical intervention (Spo_2: 92 to 94%, arterial po_2: 78 to 82 mmHg [Radiometer ABL 77 Series blood gas analyser; Diamond Diagnostics, Holliston, MA]). Spontaneous breathing of the mice during surgery was monitored by the experimenter who was on-site all the time.

After midline laparotomy, liver ischemia was realized by clamping the left lower lobe with a microvascular clip (Fine Science Tools FST, Inc., Foster City, CA), resulting in perfusion stop in at least one-third of total liver parenchyma. Thereafter, the abdomen was closed temporarily along the incision by small clamps. After 45 min of ischemia, reperfusion was established by removal of the clamp after a second laparotomy. The peritoneum and skin were then closed. Normothermic conditions were established using a homoeothermic blanket and temperature control system at a preset target value of 37°C and use of a rectal thermometer. The heating pad temperature was automatically adjusted to maintain the desired body temperature ±0.3°C (Stoelting Co., Wood Dale, IL). In addition, liver temperature between the liver lobes was taken intermittently by a temperature probe and showed similar values.

Animals were placed for postoperative recovery on a water heating pad at 37°C for 1 h. For compensation of fluid losses, animals received heparinized saline (20 μl/g body weight) subcutaneously, after completion of surgery. Heparin was added to the saline (heparin 5 IU/ml) to limit the risk of coagulation/thrombosis at this site of clamping. Postoperative monitoring occurred at a minimum of every 30 min in the first 6 h of surgery and thereafter in intervals of a few hours. This included intermittent rectal temperature at 4 and 24 h assessments to verify the normothermic conditions of the mice.

* Available at: http://oacu.od.nih.gov/regs/USGovtPrncpl.htm. Accessed August 6, 2014.

One hour postoperatively mice were either treated with inspirational oxygen concentrations of 21 or 60% for the following 24-h reperfusion period. In selected experiments, mice were subjected to an intermediate oxygen concentration of 30% instead of 60% oxygen to test for gradual responses to oxygen. The mean arterial p_{O_2} as shown in complementary experiments for 30 or 60% oxygenation were 125 or 230 mmHg, respectively. The correct F_{IO_2} during all experiments was confirmed intermittently using an oxygen-analyzer (Billups-Rothenberg, San Diego, CA).

Hepatocellular Damage Assessments by Liver Cell Integrity Markers from Blood

Blood was collected from the carotid artery to determine serum activities of glutamate-pyruvate transaminase and glutamate-oxalacetate transaminase. Serum analyses were performed with an automated analyzer (Synchron LX20 Clinical System; Beckman Coulter, Brea, CA).[14]

Liver Tissue Damage Assessments by Histology Scoring, In Vivo *Micro-computed Tomography, and Determination of Liver Lobe Weights*

After 24 h of reperfusion and following euthanasia under deep sedation, the abdominal sutures where opened and the left liver lobe was excised. Liver samples for histology analyses were prepared and stained by hematoxylin and eosin according to standard protocols. Stained tissue slices were analyzed in duplicates or triplicates in a blinded fashion by an experienced veterinary pathologist (J.M.W.). The liver sections were quantified with respect to hepatocellular degeneration, defined as the presence of hepatocyte paleness and swelling, and loss of glycogen compared with normal adjacent hepatocytes. The degree of tissue damage was scored on a scale from 0 to 4 with zero defining no damage or degeneration, and 1, 2, 3, or 4 showing degeneration of less than 25% of the liver section, of 25 to 50% of the liver section, of 50 to 75% of the liver section, and of more than 75% of the section, respectively. To yield the highest possible evidence on the damage of the liver, thereby avoiding any potential bias through the preparation of the liver, additional and confirmatory experiments were included using *in vivo* micro-computed tomography (CT) imaging as described before.[15] Mice underwent live micro-CT imaging to verify *in vivo* the histology results and the extent of the liver tissue damage *in situ* as function of oxygenation. The same time points were used as for all other experiments and data were analyzed in a fully blinded fashion (see fig. 1 and table 1, Supplemental Digital Content, http://links.lww.com/ALN/B93). Quantification of left liver lobe weights as a marker of tissue edema was performed as previously described.[16] As for the histological analyses, the left liver lobe was taken from the euthanized animals and weighed on a precision balance.

Oxidative Burst Measurement

H_2O_2 Production. Spontaneous H_2O_2 (hydrogen peroxide) production was determined by the oxidation of dihydrorhodamine to fluorescent rhodamine. Incubation of cells occurred in tubes placed in a tempered water bath for 30 min at 37°C. Reaction was stopped by addition of ice-cold Hank's Balanced Salt Solution and the cells were washed, pelleted, and placed on ice. Thereafter, cells were incubated with PerCP-anti-mouse Ly6G (Gr-1) (15 min, 3.3 μg/ml, 4°C) for identification of granulocytes. After washing with 2-(4-hydroxyphenylazo)benzoic acid and resuspension in Hank's Balanced Salt Solution, cell suspensions were analyzed in flow cytometry and H_2O_2 production was assessed by the fluorescence intensity of rhodamine on the first fluorescence detector using CellQuest software (Becton Dickinson, San Diego, CA).

Peroxides in Plasma. The plasma concentrations of total peroxides were measured with an oxystat kit (Biomedica, Vienna, Austria). With this assay, a direct proportionality between circulating ROS is measured indirectly by using a reaction between them and tetramethylbenzidine in a biological sample. After addition of a stop solution, a photometrical analysis at 450 nm detects the concentration of ROS. The intraassay coefficient of variation is—as referred to the manufacturer of assay—3.1% at a peroxide mean concentration of 221 μmol/l, SD = 6.9 μmol/l.

Protocols Targeting Sources of Oxygen Radical Formation

1. Pretreatment by antigranulocyte antibody 1 (AntiGr-1): AntiGr-1 antibodies were used for depletion of granulocytes from mice. AntiGr-1 (Becton Dickinson, Franklin Lakes, NJ) was injected intraperitoneally 1 h before starting the surgical procedures. Efficiency of depletion was confirmed by cell blood counts. Control animals received the same volume of the vehicle solution only.[17]
2. Pretreatment by gadolinium chloride ($GdCl_3$): Animals were pretreated with $GdCl_3$ (Sigma, St. Louis, MO) by injection of a standard dose of 10 mg/kg, dissolved in acidic 0.9% saline solution intraperitoneally at 24 h before definitive surgical treatment.[18,19] Control animals received the same volume of the vehicle solution only.
3. KO of cytosolic components of the NADPH-oxidase (p47phox): The generation of recombinant mice was realized as described by Jackson *et al.*[20] Animals underwent the same liver ischemia–reperfusion protocol as described above in Materials and Methods (see "Hepatic ischemia and reperfusion," second paragraph).

Statistics

A calculated number of experimental cases with the primary endpoint of glutamate-pyruvate transaminase changes were performed beforehand. Accordingly, sample size calculation was calculated expecting a change of difference to be detected (in means) of 1,000, the expected SD was 700. An estimated sample size of six mice was calculated to be

sufficient for an alpha = 0.05 and a power of 0.8 (two-sided sample size estimations, SigmaPlot [San Jose, CA]). For the calculations of the overall mouse number to be applied for the ethical/animal review boards, the number per group was counted to be maximum eight per condition/experiment taking into account to compensate for potential animal loss as a consequence of possible complications during surgical procedures and/or during induction of anesthesia. The number of mice per experiment is described in the legends of the figures (according to different legends of the figures). The variations in the number of cases for the different analyses result from the fact that (1) not under all conditions an optimal match of age and weight of the animals was achievable for six or higher (per group, especially for the KO). Also, (2) complications occurred during surgery and the total number might have reduced, respectively, as it was impossible to replace the lost mice instantly by new mice on the same day due to animal facility regulation. Moreover, (3) we used many repeats but it was not possible to equally increase all n by sharing the blood and organs, as it needed to be selected for the different aspects (histology, liver weight, bleeding out for cell functional test, CT, *etc.*). Data were all normally distributed as assessed by the Kolmogorov–Smirnov test.

Data were tested by paired *t* test for detection of in-between differences and are presented as means and SD. *P* values less than 0.05 were considered to be statistically significant. All statistical analyses were performed by IBM SPSS 20 program (IBM, Armonk, NY).

Results

Effect of Postoperative Oxygenation on Liver Damage

Hyperoxic Treatment and Liver Damage. After 45 min of ischemia, hyperoxic treatment (60 *vs.* 21% oxygen in inspired air) during a 24-h period of reperfusion resulted in a significant ($P < 0.01$) increase in liver enzyme activities of glutamate-pyruvate transaminase and glutamate-oxalacetate transaminase by 45 and 48%, respectively (fig. 1). An increase was also observed in mice exposed to 30% oxygen though not reaching the level of two-sided significance ($P = 0.059$; see table 1, Supplemental Digital Content, http://links.lww.com/ALN/B93).

Histologic Damage Score after IRI. The comparison of histologic damage score of mice livers following ischemia and reperfusion showed significantly more than 30% higher damage scores in the 60% oxygen group as compared with 21% of oxygen group (fig. 2). *In vivo* micro-CT indicated a significant increase in the volume of nonviable liver tissue by a third after exposition to 60% oxygen compared with exposition to 21% oxygen (n = 5 per group; see fig. 1 and table 2, Supplemental Digital Content 1, http://links.lww.com/ALN/B93).

Weights of Liver Tissue after IRI. The weights of the left liver lobes were significantly higher in the 60% oxygen group following ischemia and reperfusion (fig. 3).

Levels of ROS after IRI. The ROS (H_2O_2) production by granulocytes was significantly higher by almost 50% in the hyperoxia group (60% oxygen) as compared with normoxia group (21% oxygen) after ischemia and reperfusion. The serum levels of ROS (in µmol/l) were significantly higher and doubled in the 60% oxygen-treated group (figs. 4 and 5).

Sham-operated Animals. Sham-operated animals showed no significant differences between normoxic and hyperoxic treatment for the liver enzymes, histology, liver weights, and ROS, respectively (data not shown).

Modulation of the Effectors of Oxygen-induced Hepatic Damage

Effects of Granulocyte Depletion. Pretreatment of mice with AntiGr-1 is resulting in the depletion of granulocytes. Hyperoxic treatment (60% oxygen) did not result in an increase in liver enzymes in the blood (fig. 6). No significant differences between 21 and 60% oxygenation were seen also for the histological scores and left liver lobe weights in the granulocyte-depleted mice (figs. 2 and 3).

Effects of Kupffer Cell Depletion. Pretreatment of mice with $GdCl_3$ resulted in depletion of Kupffer cells. Low levels of liver enzymes were seen in both the 21 and 60% groups (no significant difference between groups) in Kupffer cell-depleted mice following ischemia and reperfusion (fig. 7). No significant differences between 21 and 60% oxygenation were seen for the histologic scores (fig. 2) or left liver lobe weights in Kupffer cell-depleted mice (fig. 3).

p47phox KO (Nonfunctional NADPH-oxidase). The KO of the p47phox unit of the NADPH-oxidase—responsible for production of ROS—resulted in no significant increase

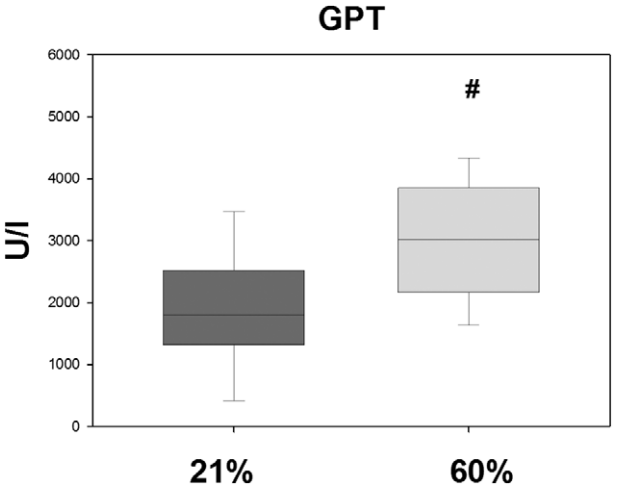

Fig. 1. Glutamate-pyruvate-transaminase (GPT) blood concentrations after 45 min of ischemia and 24 h of reperfusion, either under normoxic (21%) or hyperoxic (60%) conditions. #Significance of difference. GPT (U/L): 21% O_2 *versus* 60% O_2: 1,870 (±968) *versus* 2,981 (±1038); $P < 0.01$; n = 22. Glutamate-oxalacetat-transaminase (IU/l) (not illustrated): 21% O_2 *versus* 60% O_2: 1,587 (±1030) *versus* 2,301 (±871); $P < 0.02$; n = 22. All data displayed as medians, 75 + 25% quantile, maximum, and minimum. All written data are shown as mean value ± SD.

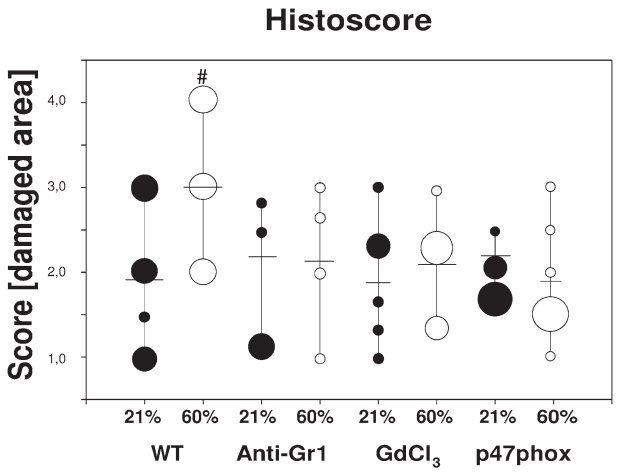

Fig. 2. Individual histological liver damage scores as assessed from liver samples taken after 45 min of ischemia and 24 h of reperfusion, either under normoxic (21%) or hyperoxic (60%) conditions. The experiments were performed in either wild-type (WT) and not pharmacologically pretreated mice (WT; n = 7; #*P* = 0.03), after granulocyte depletion (AntiGr-1; n = 4; *P* = 0.53), after Kupffer cell depletion ($GdCl_3$; n = 6; *P* = 0.69) and in animals with nonfunctional nicotinamide adenine dinucleotide phosphate oxidase (p47phox knock out; n = 6; *P* = 0.43), respectively. All given score values range between 1 (areas of tissue damaged <25%) and 4 (areas of tissue damaged >75%). #Significance of difference. The number of data points that are overlying each other are indicated by *larger circles* (= larger number of data points). Data also displayed as mean (*horizontal line* in each scatter group).

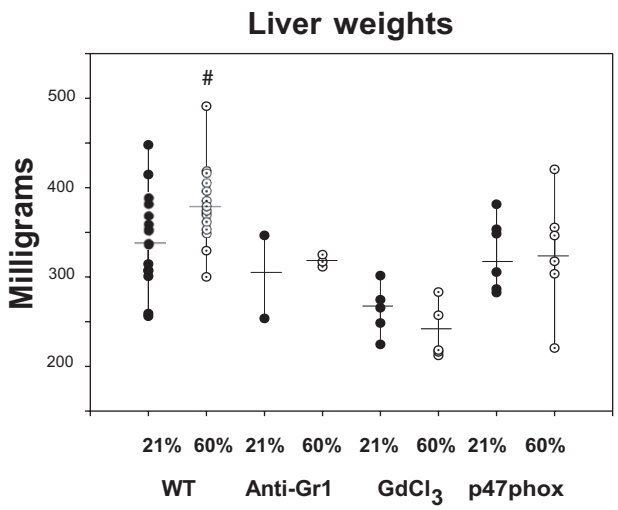

Fig. 3. Comparison of the liver weights after 45 min of ischemia and 24 h of reperfusion, either under normoxic (21%) or hyperoxic (60%) conditions. The results display the wild-type (WT) and not pharmacologically pretreated group (WT; n = 16; #*P* = 0.02), the granulocyte-depleted (AntiGr-1; n = 2–3) or Kupffer cell-depleted group ($GdCl_3$; n = 5; *P* = 0.22) and mice with nonfunctional nicotinamide adenine dinucleotide phosphate oxidase (p47phox knockout; n = 6; *P* = 0.97) as scatter plots. # Significance of difference. Sham-operated control mice (data not shown in the table): liver weights 21 *versus* 60% O_2: 293 ± 11 (n = 5, mean value ± SD, n.s.) *versus* 294 ± 13 (n = 7, mean value ± SD, n.s.). All given values were measured in milligrams (mg, mean value displayed as *horizontal line* in each scatter group).

in liver cell damage in the 60% oxygen-treated mice after ischemia and reperfusion (fig. 8). Damage scores and liver weights following ischemia and reperfusion were comparable between 21 and 60% oxygen-treated mice (figs. 2 and 3).

Discussion

Ischemia and reperfusion injury is a well-known problem in the surgical treatment of liver diseases, such as resection of metastasis and liver transplantation. The avoidance of excessive blood loss often requires the clamping of blood vessels (Pringle maneuver), with the unavoidable consequence of ischemia.[11,21,22] Ischemia results in tissue hypoxemia, a reduction of the cells' energy charge and subsequent changes in cell homeostasis, and renders the tissue very susceptible to further damage. During reperfusion, PMN and Kupffer cells become activated, resulting in the release of ROS and causing consequent tissue damage.[23–26]

Historically, oxygen has been viewed as a safe tool, which was administered in high concentrations during critical situations and postoperatively, with the intention of creating a hyperoxic microenvironment to ensure adequate and safe oxygen supply to all organs.[27,28] Today however, the liberal supply of oxygen is questioned.[29] For example, the return of spontaneous circulation after cardiac arrest and resuscitation represents an important example of a generalized IRI. Latest international recommendations suggest administering oxygen only to a measured Spo_2 between 94 and 98%. This suggestion is based on the potentially damaging effects of hyperoxygenation in the reperfusion period.[7] One of the key elements for the creation of an inflammatory microenvironment is the generation of ROS. The crucial element for the generation of "respiratory burst," mainly consisting of ROS, is oxygen.[30,31] The wide clinical usage of high oxygen concentrations in inspired air without a specific cause has now more and more to be questioned.[29,32]

Based on the pathology of the IRI, we questioned whether higher inspiratory oxygen concentrations can further affect the extent of the tissue damaging process in the prolonged postoperative reperfusion period.[33–35] We compared in a mouse liver IRI model the effects of elevated oxygen concentrations in inspired air (60% oxygen) during a 24-h reperfusion period after 45 min of ischemia of the left liver lobe in mice. Normoxic breathing (21% oxygen) in the same period was applied in the reference experiments and at 30% to test for gradual effects in selective experiments. All indicators of hepatocellular damage, either blood serum transaminases (glutamate-pyruvate transaminase and glutamate-oxalacetate transaminase), histology damage scores, *in vivo* visualized liver damage using micro-CT, and increased liver weights provided evidence, that postoperative oxygenation has aggravated the IRI. Moreover, cell-derived ROS formation from innate immune cells increased as a function

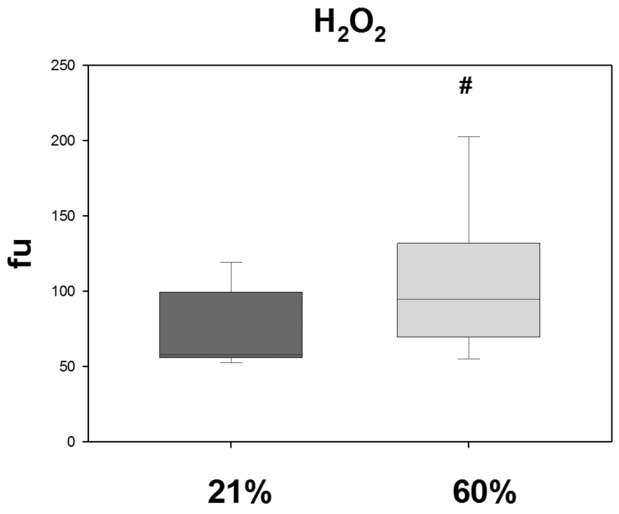

Fig. 4. Reactive oxygen species production. The measurements of H_2O_2 (hydrogen peroxide) with dihydrorhodamine and flow cytometry after normoxic treatment (21%) showed significant lower values than after hyperoxic treatment (60%): 76.0 (±30) *versus* 111.1 (±55.6) $P < 0.02$, n = 18–19; measured in relative fluorescence units (fu). #Significance of difference. Sham-operated animals showed no significant differences between normoxic and hyperoxic treatment (not shown, dihydrorhodamine method, in fu): 21% O_2 *versus* 60% O_2: 60.7 (±8.2) *versus* 57.4 (±4.4); n.s.; n = 5–6. All data are displayed as median, 75 + 25% quantile, maximum, and minimum. All written data are shown as mean value ± SD.

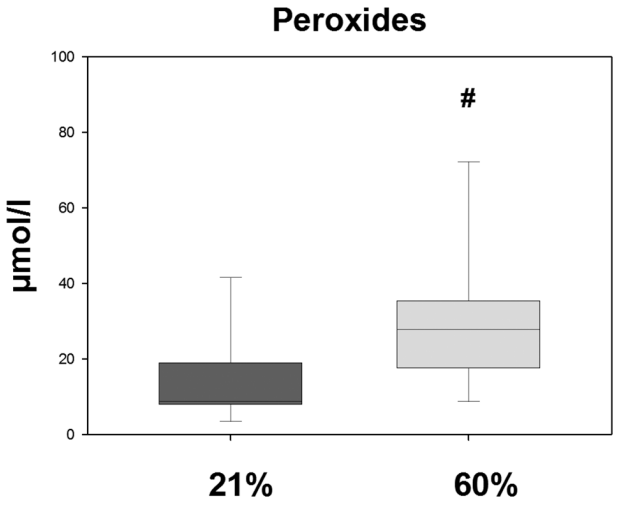

Fig. 5. Reactive oxygen species levels (peroxides) in blood after 45 min of ischemia and 24 h of reperfusion, either under normoxic (21%) or hyperoxic (60%) treatment (in µmol/l, 21 *vs.* 60%): 15.0 (±12.0) *versus* 30.4 (±19.2); #$P < 0.05$; n = 10. All data displayed as median, 75 + 25% quantile, maximum and minimum. All written data are shown as mean value ± SD.

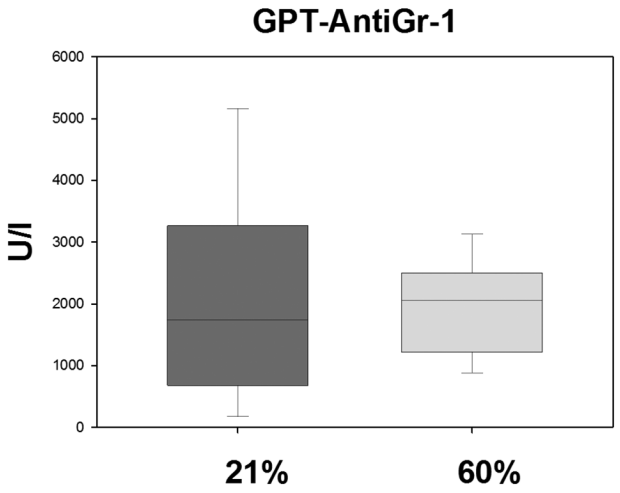

Fig. 6. Glutamate-pyruvate-transaminase (GPT) after 45 min of ischemia and 24 h of reperfusion in absence of granulocytes (AntiGr-1), either under normoxic (21%) or hyperoxic (60%) conditions: GPT (IU/L): 2,158 (±1642) *versus* 1,991 (±775); n.s.; n = 9–11. Glutamate-oxalacetat-transaminase (IU/L) (not illustrated): 21% O_2 *versus* 60% O_2: 1,427 (± 605) *versus* 1,192 (±690); n.s.; n = 5–6). All data displayed as medians, 75 + 25% quantile, maximum and minimum. All written data as mean value ± SD.

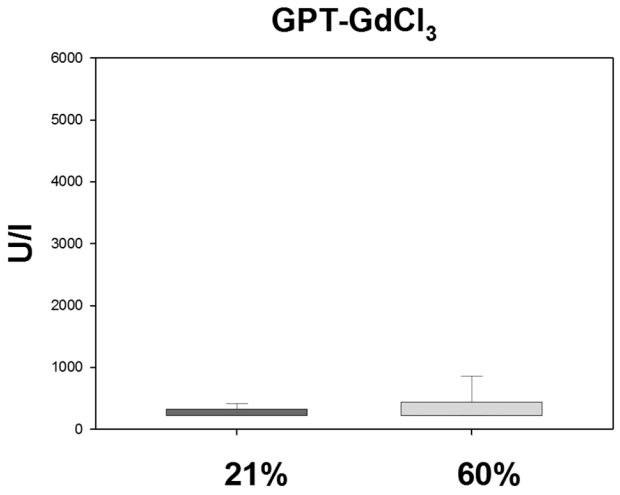

Fig. 7. Glutamate-pyruvate-transaminase (GPT) after 45 min of ischemia and 24 h of reperfusion in absence of monocytes (gadolinium chloride [$GdCl_3$]), either under normoxic (21%) or hyperoxic (60%) conditions of left liver lobe of mice. GPT (IU/L): 286 (±81.6) *versus* 360 (±256.7); n.s.; n = 6. Also glutamate-oxalacetat-transaminase levels (not illustrated) were comparable and not significantly different: (IU/l) 21% O_2 *versus* 60% O_2: 1,120 (±338) *versus* 973 (± 464); n.s., n = 6. All data displayed as medians, 75 + 25% quantile, maximum and minimum. All written data as mean value ± SD.

of oxygenation while this was not seen in sham-treated animals which accordingly showed no increase in liver damage markers which were in the reported normal range.[36] To identify some of the key immune cell populations mediating the oxygen-related damage, 60% inspiratory oxygen concentration was chosen and immune cell depletion experiments were performed. One of the central drivers of the IRI are PMN[31,37,38] and the Kupffer cells.[39,40] Their main functions are mainly exhibited during the earlier phase of reperfusion by phagocytosis, and the production of ROS, chemokines, and cytokines.[38,41] The elimination of either

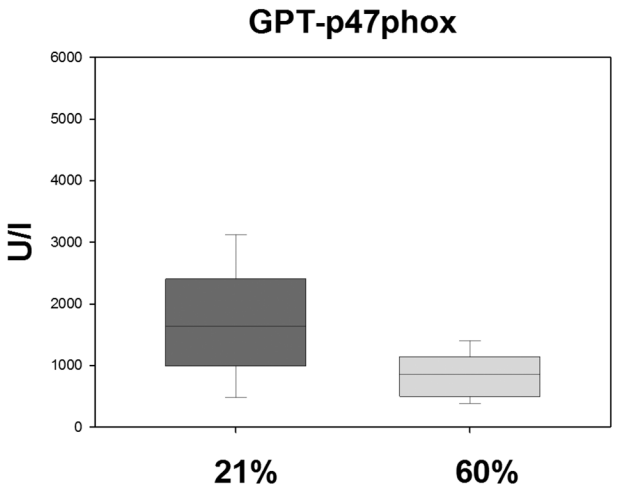

Fig. 8. Glutamate-pyruvate-transaminase (GPT) blood concentrations after 45 min of ischemia and 24 h of reperfusion as quantified in mice with nonfunctional nicotinamide adenine dinucleotide phosphate oxidase (p47phox), either under normoxic (21%) or hyperoxic (60%) conditions of left liver lobe of mice: GPT (IU/L): 21 *versus* 60%: 1,732 (±895) *versus* 840 (±351); n.s.; n = 10–11. Glutamate-oxalacetat-transaminase values (measured in [IU/l], data not illustrated) differed non-significantly: 60% O_2 *versus* 21% O_2: 2,011 (±1,897) *versus* 1,668 (±978); n.s.; n = 10–11. All data displayed as median, 75 + 25% quantile, maximum and minimum. All written data are shown as mean value ± SD.

PMN or Kupffer cells entirely abolished the oxygen-induced damage. Moreover, the comparison of 21 *versus* 60% oxygen in sham-operated mice showed no oxygen-mediated aggravating effect, confirming that oxygenation is not toxic *per se*,[42,43] but becomes harmful only after IRI and only in the presence of PMN and Kupffer cells. Furthermore, the lack of significant damage in the absence of the main source of ROS, the cells' NADPH-oxidase,[41] supports the fact that the effects of 60% oxygen are mediated through cell-derived ROS formation. Overall, these results indicate that PMN cells, Kupffer cells, and the NADPH-oxidase system play a central role in the pathology of iatrogenic oxygen-enhanced tissue damage in liver IRI.

Limitations and Considerations

This study addresses the effects of oxygenation after liver ischemia and reperfusion at oxygen concentrations similar to those applied routinely in the postanesthesia care units. However, some important animal model-related aspects, such as the anesthesia and fluid regimens, on surrogate markers and the postoperative oxygen protocols should be taken into account in the interpretation of the data.

Anesthesia. In general, ketamine/xylazine anesthesia and isofluran vapor anesthesia have both been widely applied for surgical procedures in murine models and are also recommended by ethical boards as the regimen of choice. The choice of the anesthetic drugs is of relevance as increasing evidence describes the preconditioning potential of inhaled anesthetics and intravenous anesthetics such as propofol.[44] Ketamine/xylazine is not as much known for inducing protective effects in IRI.[45,46] Although ketamine/xylazine anesthesia is not used in humans, it was the preferred regimen to avoid potential confounding interactions, for example, due to the potential of variable preconditioning effects when volatile anesthetics would have been used.[44] By using this approach, it was accepted that a possible lack of preconditioning effects of ketamine/xylazine *may* have resulted in more tissue damage in all experiments.

Fluid Management. The use of heparinized saline in the postoperative course was based on the rationale that clamping and unclamping of the liver lobe vessels induces a small trauma to the vessels (controlled for in the sham-operated mice). Protective effects may have resulted from the heparin application as some evidence points to a lower degree of IRI in lung and heart tissues due to therapeutic anticoagulation with heparin after ischemia–reperfusion in rats.[47] Whether this is also true for hepatic tissue in mice is not known. As the use of saline and heparin occurred in all experiments in a standardized fashion, variations due to irregular thrombosis at the liver clamping site were unlikely.

Biochemical and Histological Markers. The analyses included biochemical and histological markers of liver tissue injury as the primary endpoints, rather than death, as death is not characteristic of this model and mice would have survived and recovered from the left liver lobe ischemia and reperfusion. Although histology (complemented by micro-CT) shows visible damage, surrogate markers such as serum transaminases are indirect measures of hepatocyte injury. Nevertheless, they are well established and routinely used clinical markers of liver damage and liver failure. Monitoring of serum transaminases is recommended for the diagnosis and prognosis of acute liver failure in humans.[48] Also for quantifying the severity of liver damage following IRI,[10,16,22–26,49] the dynamics of the organ insult, and the potential organ recovery, liver enzyme quantifications in blood reflect a very suitable tool.

Postoperative Oxygenation. Although patients vary in their baseline physiological statuses, for example, because of age and comorbidities, such conditions could not be addressed in this murine model. Similar to postoperative conditions in nonventilated patients, mice were spontaneously breathing air with variable oxygen concentrations up to 60%. At an F_{IO_2} of 0.6, a strong and significant aggravation of the IRI was observed, while at a lower postoperative oxygenation at a F_{IO_2} 0.3, this aggravation was not significant ($P = 0.059$). Because the application of higher F_{IO_2} concentrations, including a F_{IO_2} of 1.0 is not a routinely applied procedure in the spontaneously breathing postoperative care patient, the authors limited oxygen delivery concentration to 60%. This was further based on the rationale to avoid any potential impact of direct oxygen toxicity when using 80 or 100% of it. However and by far exceeding a routine application in the postanesthesia care unit, hyperbaric hyperoxygenation

regimens might be of interest to be tested in the future as they may increase the benefits of direct oxygen delivery to the damaged tissue and might compensate for cell-mediated damage as hyperbaric oxygenation is usually exceeding an arterial oxygen partial pressures of 2,000 mmHg and has previously been shown to be beneficial to decrease inflammation and the damage related to it.[42,43]

In summary, the authors demonstrate that moderate oxygenation with 60% oxygen for 24 h significantly amplified the ischemia- and reperfusion-induced liver tissue damage in a mouse model. This effect is mediated through circulating and resting myelopoetic cells and involves cell-derived oxygen radical formation. These results indicate that the consequences of the widely used liberal oxygen regimens in the perioperative phase must be further investigated. Higher oxygenation may worsen postoperative outcome, in particular the extent of liver injury and risk of liver failure in patients after ischemic liver surgery.

Acknowledgments

The authors thank staff of the National Institutes of Health Clinical Centre (Bethesda, Maryland) and of the animal facilities in Bethesda and Munich, as well as of the laboratory team of the Department of Anesthesiology (University of Munich, Munich, Germany) for the quantification of liver enzymes and efficient support during the experiments, respectively. The authors also thank Martina Choukèr, M.D. (Munich, Germany), Heidi Unkle (Rockville, Maryland), and Daryl Despres and Daniel Schimel (Mouse Imaging Facility, National Institute of Neurological Disorder and Stroke, National Institutes of Health, Bethesda, Maryland). The authors are also very thankful to Alex Paddy Salam, M.D. (Guy's and St. Thomas' National Health Service Trust, London, United Kingdom), who helped to improve the article in the revision process, and want to express their highest appreciation to William Paul, M.D. (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland), and to Bernhard Zwißler, M.D. (University Hospital of the Ludwig-Maximilians-University, Klinikum Grosshadern, Munich, Germany), for their continuous support.

This research work was supported, in part, by the Intramural Research Program and a John E. Fogarty research grant awarded to Dr. Choukèr by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland. Financial support was also awarded by the medical faculty of the Ludwig-Maximilians-University through the facilitation program for research and education of the Ludwig-Maximilians-University, Munich, Germany (to Dr. Martignoni). In-house support was provided by the Department of Anesthesiology, Ludwig-Maximilian-University of Munich, Munich, Germany (to Drs. Zangl, Martignoni, and Choukèr).

Competing Interests

The authors declare no competing interests.

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