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ORIGINAL ARTICLE

Dietary glycine protects from chemotherapy-induced hepatotoxicity

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Abstract Hepatotoxic side effects of neoadjuvant chemotherapy for colorectal liver metastases increase perioperative morbidity and mortality. Glycine protects liver from injury in various animal models. Thus, this study was designed to assess its effect on liver after chemotherapy. Sprague–Dawley rats (200–220 g) were fed a synthetic diet containing 5% glycine for 5 days. Subsequently, chemotherapy (FOLFIRI: irinotecan, folinic acid and fluorouracil, or FOLFOX: oxaliplatin, folinic acid and fluorouracil) was administered at standard doses. Transaminases, histology, immunohistochemistry and in vivo microscopy were used to index liver injury, to monitor intrahepatic microperfusion and activation of Kupffer cells. Glycine significantly decreased transaminases after chemotherapy to 25–50% of control values ($p < 0.05$). Microvesicular steatosis was significantly reduced from 18.5 ± 3.4 and 57.1 ± 8.6% in controls to 9.5 ± 1.8 and 37.7 ± 4.4% after FOLFIRI and FOLFOX, respectively. Furthermore, phagocytosis of latex beads was reduced by about 50%, while leukocyte adherence in central and midzonal subacinar zones decreased to 60–80% after glycine ($p < 0.05$). Glycine significantly reduced expression of inducible nitric oxide synthase after chemotherapy, while hepatic microcirculation was increased ($p < 0.05$). This study shows for the first time that glycine reduces chemotherapy-induced liver injury. The underlying mechanisms most likely include Kupffer cells and an improved intrahepatic microperfusion.

Keywords Glycine · Chemotherapy · Kupffer cell-dependent liver injury · Steatosis · In vivo microscopy · Leukocyte–endothelium interaction

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Introduction

Since the beginning of the chemotherapy era in the 1940s, many effective drugs against malignant tumor growth have been developed with toxic side effects to the liver. Up to date, only limited efforts have been documented to protect the liver during chemotherapy for colorectal liver metastases. This is especially important since today chemotherapy is used to downsize hepatic metastases and to make curative resection possible. The introduction of new, more effective chemotherapy regimens together with today's sophisticated surgical technique enables surgeons to perform more curative resections and to increase patient survival (Kemeny 2007). Chemotherapy is designed and used for both its adjuvant and neoadjuvant application. Today, the preoperative approach is increasingly used to decrease the magnitude of resection needed, lower the rate of tumor-positive resection margins postoperatively, treat hepatic and systemic micro metastases simultaneously, downsize colorectal metastases and apply liver resections in patients

who would have been irresectable without their response to neoadjuvant chemotherapy (Zorzi et al. 2007; Welsh et al. 2007; Vauthey et al. 2006; Masi et al. 2009; Pozzo et al. 2008). Chemotherapy is regularly used in this neoadjuvant setting with good results, but complications after surgery are more common (Abdalla and Vauthey 2008; Nordlinger et al. 2008). Moreover, neoadjuvant and adjuvant chemotherapy can lead to a longer disease-free and recurrence-free survival after liver resection (Masi et al. 2009; Pozzo and Barone 2008; Nordlinger et al. 2009; Tamandl et al. 2009). There are various chemotherapy regimens available. The 5-fluorouracil (5-FU)-based chemotherapy is usually biomodulated with both folinic acid (FA) to increase its affinity for thymidinase synthase, and with either irinotecan (IRI) or oxaliplatin (OX) to further improve response rates and to prolong survival. Both combinations have been approved as standard treatment for metastatic colorectal cancer (mCRC) (Tournigand et al. 2004; Teufel et al. 2004; Goldberg et al. 2007). Although preoperative chemotherapy has been shown to be efficient and safely applied for various solid tumors (i.e., esophageal, gastric, pancreatic and colorectal cancer), recent reports on chemotherapy for mCRC clearly demonstrate its association with steatosis, liver toxicity, steatohepatitis and injury to the hepatic sinusoids (Welsh et al. 2007; Vauthey et al. 2006; Parikh et al. 2003; Khan et al. 2009). Especially after IRI, hepatic steatosis (Welsh et al. 2007) and steatohepatitis (Kemeny 2007), which can impair postoperative liver regeneration and may cause hepatic insufficiency after resection, have been identified. Further, hepatic sinusoidal dilatation is associated with OX, which may lead to significant blood loss during surgery (Kemeny 2007; Welsh et al. 2007). There is a significantly increased incidence of nonalcoholic steatohepatitis in patients after either OX or IRI compared to patients who had 5-FU alone or no neoadjuvant chemotherapy (Welsh et al. 2007). Prevention of chemotherapy-associated liver injury would lead to better liver function and thus allow a more aggressive resection. Fatty changes, which are unspecific markers of liver injury, are known to appear after chemotherapy and are associated with increased postoperative morbidity (Morris-Stiff et al. 2008). While a time interval is not needed after chemotherapy for response in terms of size reduction of the metastases, it is general practice to wait for recovery of liver specific parameters (i.e., function and enzymes).

Glycine, a simple amino acid, has been shown to be protective against hepatic injury in various models and in clinical liver transplantation (Zhong et al. 2003; Zhong et al. 1996; Wheeler et al. 1999; Luntz et al. 2005). Glycine is a strong inhibitor of resident liver macrophages and acts via a glycine-gated chloride channel, which subsequently inhibits Kupffer cell (KC)activation by a decreased calcium inflow (Wheeler et al. 2000a, b; Qu et al. 2002; Ikejima et al. 1997; Froh et al. 2002). Moreover, glycine is an essential component of glutathione and is thus needed for detoxification processes and has indirect effects as a radical scavenger (Weinberg et al. 1987; Jaeschke and Farhood 1991; Bilzer et al. 2002). The regular human diet contains about 2 g of glycine (Heresco-Levy et al. 1999); (Luntz et al. 2005). Up to 90 g glycine/day has been used in clinical trials over several weeks without any reported serious adverse effects (Heresco-Levy et al. 1999); (Luntz et al. 2005). While in humans, the normal serum level of glycine is approximately 300 μM, increasing glycine intake to such a high level increases blood levels to more than 900 μM (Heresco-Levy et al. 1999). Since its clinical application has been proven to be safe (Zhong et al. 2003; Rosse et al. 1989; Gundersen et al. 2005; D'Souza et al. 2000), patients' preconditioning with glycine before chemotherapy could reduce its hepatic toxicity preventing microvascular changes such as sinusoidal congestion and dilatation, steatosis, atrophy of hepatocytes and/or hepatic necrosis. Glycine could decrease the risk of surgery preventing hepatic injury. Thus, perioperative and postoperative complications would be decreased with better liver function.

Thus, this study has been designed to assess the effects of glycine on chemotherapy-induced liver injury.

Materials and methods

Animals

Female Sprague–Dawley rats weighing 200–220 g (Charles River, Sulzfeld, Germany) were kept in transparent polycarbonate cages at a controlled temperature of 22 ± 2°C under standard 12-h day/night rhythm and were allowed free access to standard laboratory chow (ssniff R/M-H, ssniff Spezialdiäten, Germany) and tap water.

Experimental design and treatment

Five days before the experiment, animals were distributed into two groups in a randomized, blinded fashion. Animals were fed a 5% glycine-enriched chow [modified C1000 (15% casein and 5% glycine), Altromin Spezialfutter GmbH &Co, Germany], while controls were allowed free access to chow without glycine [C1000 Kontrolldiät (20% casein), Altromin Spezialfutter GmbH &Co, Germany]. In previous studies concerning dietary glycine, a dose-dependent serum level of glycine could be shown (Heresco-Levy et al. 1999; Petzke et al. 1986). The optimal concentration of dietary glycine for hepatoprotection is 5% in various models (Stachlewitz et al. 1999; Rose et al. 1999; Wheeler et al. 2000a, b). A concentration of 5% dietary glycine has been shown to increase serum glycine

levels four- to fivefold and effectively inhibit KC activation and ameliorate liver damage under various circumstances (Stachlewitz et al. 1999; Rose et al. 1999; Wheeler et al. 2000a, b; Schemmer et al. 1999; Schemmer et al. 1998; Rivera et al. 2001). Like in many other publications on KC-dependent hepatotoxicity, control animals were fed with 5% casein as isonitrogenous control, while the experimental group received 5% glycine (Schemmer et al. 1999; Schemmer et al. 1998; Rivera et al. 2001). This is important since casein has no effect on KCs. Intravenous (i.v.) chemotherapy was started after 5 days of diet. Briefly, animals were anesthetized with Narcoren® 20 mg/kg body weight intraperitoneally (i.p.) (100 ml Narcoren includes pentobarbital-natrium 16.0 g, benzylalcohol 3.0 g, Merial GmbH, Hallbergmoos, Germany) and Ketanest® 100 mg/kg body weight intramuscularly (i.m.) (Esketaminhydrochlorid, Parke-Davis GmbH, Berlin, Germany). The depth of narcosis was controlled by intra-toe and cornea reflexes. The neck of the anesthetized animal was shaved and a vertical incision on the right side of the neck was performed. The right jugular vein (RJV) was accessed by blunt dissection of neck muscles. The distal part of RJV was ligatured using 6/0 silk (Resorba; Nürnberg, Germany). A small incision to the vessel wall was made and a polyethylene catheter (B. Braun, Melsungen AG, Germany; inside diameter (ID) = 0.4 mm, outside diameter (OD) = 0.9 mm) was introduced for infusion and fixed with two 6/0 silk ligatures. A small incision on the posterior side of the rats' neck was performed and the proximal part of the catheter was pulled through subcutaneously to the outside. Muscles and skin were closed with a running suture (4/0 Vicryl®, Ethicon, Hamburg, Germany). After introducing the catheters, all animals had free access to clean tap water and to the same chow they were fed before. Housing was provided in individual wire cages. Infusion pumps (Harvard compact infusion pump, Model 975, USA) were loaded with 5 ml syringes and connected to the catheters. All experiments were performed in accordance with institutional guidelines.

According to standard schemes, chemotherapy for mCRC was infused. At the end of infusion, blood was collected for serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) to estimate liver damage. Some rats were only observed for survival after chemotherapy. In some rats, immediately after the end of drug infusion, in vivo microscopy was performed to assess phagocytosis of KCs, leukocyte–endothelial cell interaction and hepatic microcirculation. Further, liver samples were taken at 24 h (FOLFIRI) and 36 h (FOLFOX) after the end of chemotherapy to assess liver damage. To maintain the same conditions, all experiments were carried out by the same surgeons and during the same time of the day. In total, 60 animals were used in this study (12 animals in both experimental and control groups were used for tissue and blood sampling, 8 animals per group were used for in vivo microscopy).

Chemotherapy

Two different chemotherapy regimens were investigated: FOLFIRI (IRI, FA and 5-fluorouracil) and FOLFOX (OX, FA and 5-fluorouracil). For FOLFIRI, continuous infusion was started with IRI (180 mg/m^2, Pfizer Pharma GmbH, Germany) over 30 min, followed by infusion of FA (400 mg/m^2 Pfizer Pharma GmbH, Germany) for 2 h. Prior to infusion of 5-fluorouracil (3,000 mg/m^2 Pfizer Pharma GmbH, Germany) over 10 h, a bolus (400 mg/m^2) of the latter was given (Fig. 1a). For FOLFOX, OX (130 mg/m^2, Sanofi-Aventis, Germany) was infused over9 2 h followed by FA (400 mg/m^2, Pfizer Pharma GmbH, Germany) over 2 h. Before infusion of 5-fluorouracil (2,400 mg/m^2, Pfizer Pharma GmbH, Germany) over 10 h, a bolus (400 mg/m^2) of the latter was infused (Fig. 1b). The doses were calculated according to the animal skin surface as described elsewhere (Hribaschek et al. 2006).

Enzyme assays

Serum AST and ALT levels were detected with standard enzymatic methods (Bergmeyer 1972) in blood samples collected at 0, 6, 12 and 24 h (FOLFIRI) and 0, 12, 24 and 36 h (FOLFOX) after chemotherapy.

In vivo microscopy

Immediately after chemotherapy, in vivo microscopy of liver was performed, while body temperature was kept constant at 36°C as described elsewhere (Uhlmann et al. 1999; Post et al. 1993). Briefly, an incision of the right side of the neck was performed to access the right carotid artery (RCA). Its distal part was ligatured with 6/0 silk (Resorba, Nürnberg, Germany). Through a small opening of the vessel, a polyethylene catheter (B. Braun, Melsungen AG, Germany, ID = 0.4 mm, OD = 0.9 mm) was introduced for intra-arterial injection of contrast medium, and for blood pressure and heart frequency monitoring. The catheter was connected to a monitoring system (SAMonitor 1.2., Experimental Surgery, University of Heidelberg, Germany) and the abdomen was opened by midline incision. The liver was released from its ligaments and the xiphoid was fixed upward with an Allis clamp to expose the upper abdomen. Hepatic microcirculation was observed in vivo at the upper surface of the right liver lobe with a modified inverted Leitz-Orthoplan microscope (Leica GmbH, Wetzlar, Germany) in epi-illumination technique

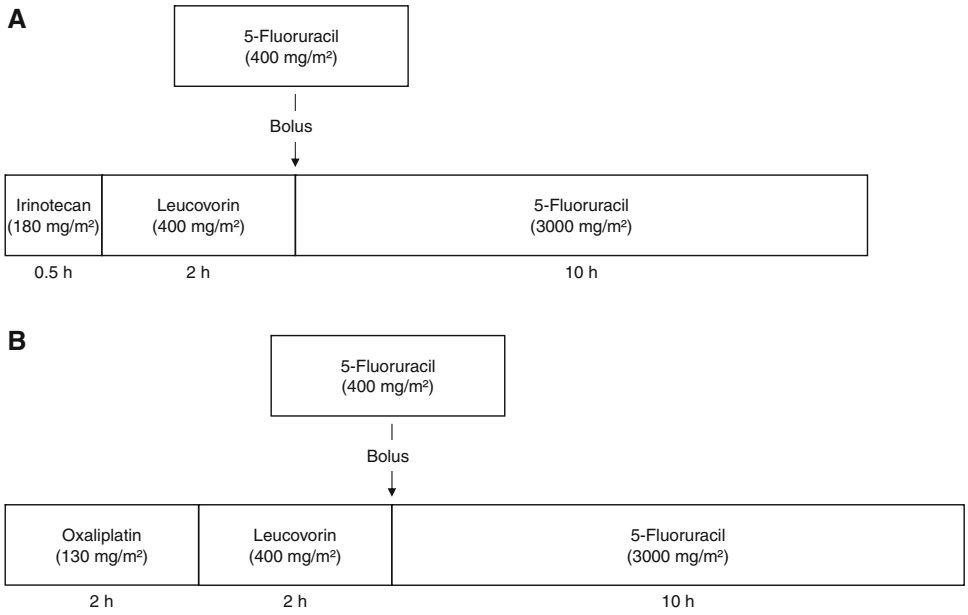

Fig. 1 Chemotherapy regimens. **a** FOLFIRI:irinotecan (180 mg/m^2) infusion was performed for 30 min, followed by folinic acid (leucovorin; 400 mg/m^2) for 2 h, 5-fluorouracil bolus (400 mg/m^2) and 5-fluorouracil infusion for 10 h (3,000 mg/m^2) **b** FOLFOX:oxaliplatin (130 mg/m^2) infusion was performed for 2 h, followed by folinic acid (leucovorin; 400 mg/m^2) infusion for 2 h, 5-fluorouracil bolus (400 mg/m^2) and 5-fluorouracil infusion for 10 h (2,400 mg/m^2)

as described previously (Post et al. 1993). Fluorescein isothiocyanate (FITC)-labeled erythrocytes (3 µmol/kg body weight; FITC Isomer 1, Sigma, Rodenmark, Germany) was used to enhance contrast for assessing sinusoidal perfusion. Rhodamine 6G (0.05 mmol/kg body weight; Sigma, Rodenmark, Germany) was given for vital staining of leukocytes (Uhlmann et al. 1999). Acinar perfusion was assessed at the end of chemotherapy infusion. Erythrocyte velocity and mobility, mostly based on manual tracing of the erythrocyte path on video images, were compared (Uhlmann et al. 1999). Red blood cell (RBC) velocity within postsinusoidal venules is described as a median value from ten measured erythrocytes speeds in each periportal, midzonal, pericentral and central vein separately in five acini of each animal. The mean value of the medians was used for comparison between groups. Further, the white blood cell (WBC)–endothelium interaction in sinusoids and postsinusoidal venules was studied. Permanent adherent leukocytes (stickers) were defined as marked cells located within blood vessels and not moving during an observation period of 20 s; they were counted separately in all five acini zones of each animal. Some rats were used to detect the phagocytotic activity of KCs. Fluorescent latex beads (3×10^8 beads/kg body weight, diameter = 1.1 µm, Polysciences Inc., USA) were infused into the RCA through the polyethylene catheter. The number of latex beads-positive KCs was counted per square millimeter within 300 s, starting 10 s after the end of injection in ten acini of each rat (Uhlmann et al. 1999). The ratio of adherent beads was quantified by counting the number of moving beads in sinusoids as a percentage of all visible (moving and adherent) beads during a period of 20 s.

Histology

Liver tissue was taken at 24 (FOLFIRI) and 36 h (FOLFOX), which was at the end of chemotherapy infusion. The livers were blood free and perfused with 0.9% NaCl solution, followed by 5% paraformaldehyde in Krebs–Henseleit bicarbonate buffer (pH 7.6). Subsequently, liver samples were processed by standard methods. Briefly, liver tissue was embedded in paraffin, sectioned at 5 µm and processed for light microscopy with hematoxylin–eosin (H&E) and periodic acid-Schiff (PAS) staining. Liver damage was estimated by an experienced pathologist in a blinded fashion. All sections were examined for grading of steatosis, inflammation and other histomorphological changes used for routine microscopic examination.

Immunohistochemistry

Staining for inducible nitric oxide synthase (iNOS) was performed using iNOS-specific antibody and the avidin–biotin method for semiquantitative evaluation. Briefly, rabbit polyclonal antibody specific to iNOS (Abcam, Cambridge, UK) was used at a dilution of 1:100. Labeling with secondary antibody and staining were performed with Vectastain ABC Peroxidase Rabbit IgG Kit and Peroxidase Substrate Kit DAB (Vector Laboratories, Burlingame, CA, USA), respectively, according to the manufacturer's instructions. Endogenous peroxidase was quenched for 30 min using 3% H_2O_2, and endogenous biotin was blocked using avidin and biotin. Hematoxylin was used for counterstaining. No unspecific staining could be detected in negative controls. iNOS expression was analyzed in at least five microscopic fields per slide at 400× magnification and

scored semiquantitatively from 0 to 3, representing negative (0), faint (1), moderate (2) and strong (3) staining, respectively.

Statistics

All results are given as mean ± standard error of the mean (SEM). For data accumulation, preparing tables and diagrams, software Excel 2003 (Microsoft Co., Portland, USA) and Sigma Plot 8.0 (SPSS Inc., Chicago, IL, USA) were used. Statistical analysis was performed using Sigma Stat 2.03 (Access Softek Inc., USA). Various groups were compared using Fisher's exact test or analysis of variance (ANOVA) and *t* test as appropriate with $p < 0.05$ selected prior to the study as the criterion for significance of differences between groups.

Results

FOLFIRI model

General data

All rats survived for 7 days after chemotherapy infusion in both glycine and control groups. Blood pressure (131.9 ± 3 mm/Hg), hematocrit (45.6 ± 2%) and body temperature (36.0 ± 0.3°C) were comparable in all groups during all experimental phases studied.

Liver injury

Serum transaminases were elevated after FOLFIRI. Twelve hours after chemotherapy, transaminases reached their maximum. Glycine decreased AST at 0, 6, 12 and 24 h after chemotherapy from 234 ± 35, 249 ± 37, 259 ± 40 and 153 ± 17 U/l in controls to 162 ± 11, 159 ± 10, 154 ± 11 and 112 ± 7 U/l, respectively ($p < 0.05$) (Fig. 2a). Further, ALT decreased from 48 ± 6, 54 ± 7, 54 ± 7 and 47 ± 6 U/l in controls to 35 ± 6, 35 ± 5, 37 ± 6 and 33 ± 5 U/l, respectively, after glycine ($p < 0.05$) (Fig. 2b).

Kupffer cell phagocytosis

At the end of chemotherapy, phagocytotic activity of KCs was measured with fluorescent latex beads as described in "Materials and methods". The percentage of latex beads inside the KCs was counted and compared between groups. Glycine significantly reduced phagocytosis of latex beads after FOLFIRI and in 19.6–73.7% (Fig. 3a) of controls as a minimum and maximum difference over time.

Leukocytes–endothelium interaction

In vivo microscopy was performed as described in "Materials and methods" and revealed a dramatic increase of leukocytes accumulated within the sinusoids in all subacinar zones after FOLFIRI (Fig. 4a). Separate analysis

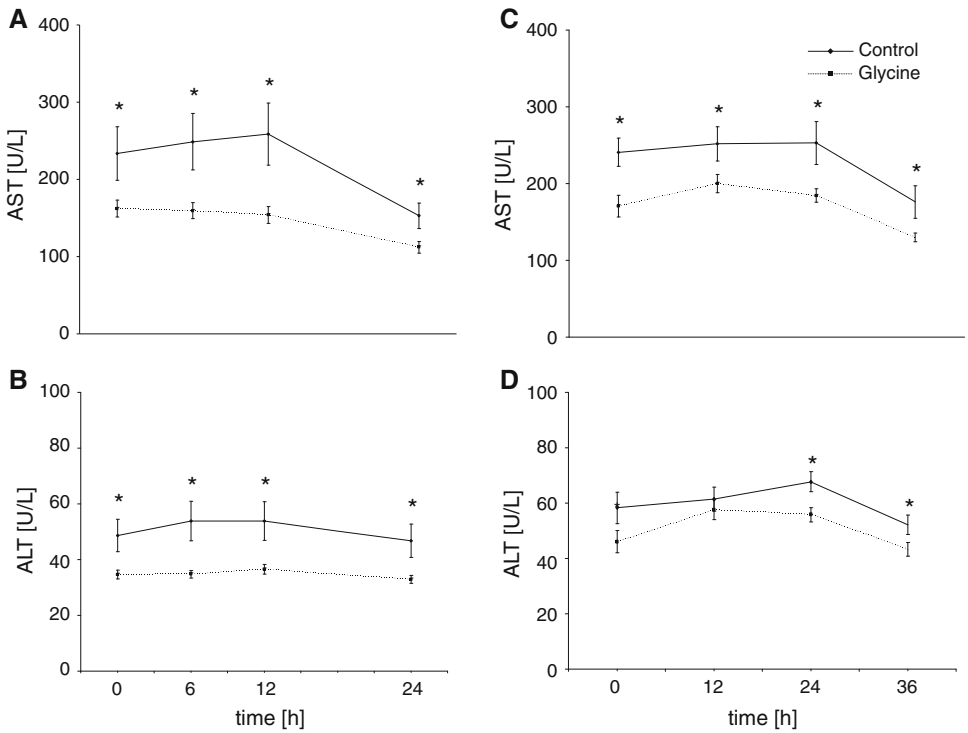

Fig. 2 Levels of serum transaminases after chemotherapy. **a** AST **b** ALT after FOLFIRI chemotherapy and **c** AST and **d** ALT after FOLFOX chemotherapy over time. Glycine significantly decreased transaminases compared to controls. Data are shown as mean ± SEM ($p < 0.05$ by one-way ANOVA, $n = 12$). *$p < 0.05$ compared to corresponding control values

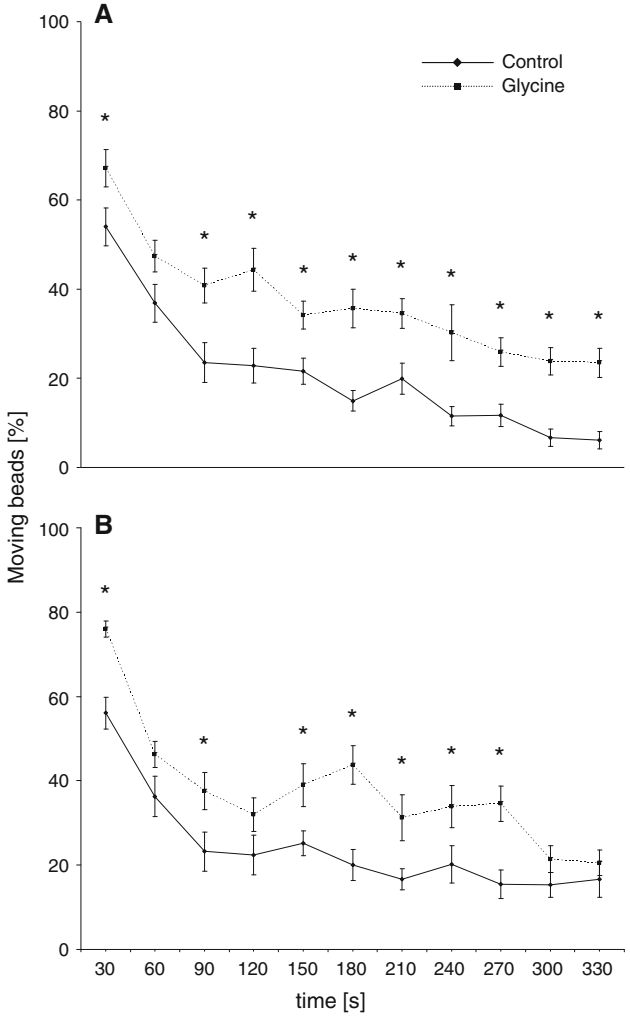

Fig. 3 Phagocytosis of latex beads by Kupffer cells. **a** FOLFIRI **b** FOLFOX. The ratio of adherent beads was quantified by counting the number of moving beads in sinusoids as the percentage of all visible (moving and adherent) beads in the field during 30 s periods as described in "Materials and methods". Glycine significantly reduced phagocytosis of KCs compared to controls. Data are shown as mean ± SEM. *$p < 0.05$ compared to corresponding control values

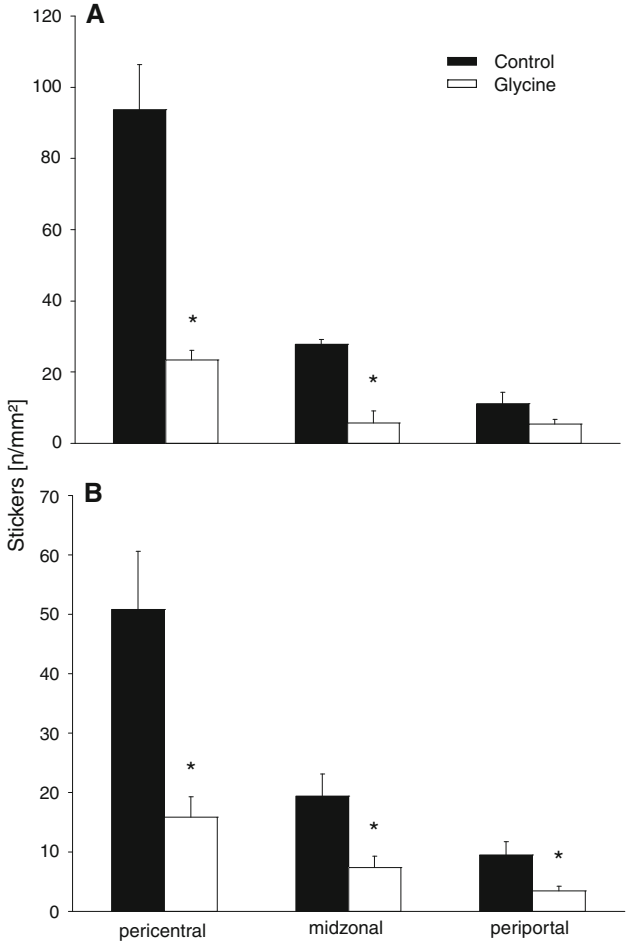

Fig. 4 Permanent leukocyte adherence in hepatic sinusoids. Acinar zones were differentiated into portal, midzonal and periportal areas to describe differences in sinusoidal perfusion. **a** FOLFIRI and **b** FOLFOX. Glycine significantly decreased leukocyte sticking after both FOLFIRI and FOLFOX. Values are mean ± SEM. *$p < 0.05$ compared to corresponding control values

within the three zones revealed a significant increase of stickers from the periportal to the pericentral zones of controls after FOLFIRI ($p < 0.05$). Glycine effectively prevented permanent adhesion, especially in the pericentral ($p = 0.006$) and midzonal ($p = 0.003$) areas (Fig. 4a).

Microcirculation

To evaluate acinar perfusion, erythrocytes velocity was measured in different zones of acini as described in "Materials and methods". While the sinusoidal diameter was not different between groups, glycine significantly increased the velocity of erythrocytes from 0.23 ± 0.01, 0.39 ± 0.01, 0.54 ± 0.02 and 0.74 ± 0.02 mm/s in periportal, midzonal, pericentral and central venous zones in controls to 0.29 ± 0.01, 0.46 ± 0.02, 0.63 ± 0.03 and 0.96 ± 0.04 mm/s, respectively, after FOLFIRI (Fig. 5a).

Histology

Microvesicular steatosis with periportal accentuation was detected in all groups studied. Controls presented accumulation of fat in 18.5 ± 3.4% of hepatocytes. After glycine, fatty changes were present in only 9.5 ± 1.8% of hepatocytes ($p = 0.028$) (Fig. 6a, b).

iNOS

Staining for iNOS was performed in tissue taken 24 h after chemotherapy as described in "Materials and methods". Controls presented an expression score for iNOS of

2.3 ± 0.16. Glycine significantly reduced the expression of iNOS in liver tissue to 1.4 ± 0.15, which was only 61% of controls ($p < 0.001$) (Fig. 7a, b).

FOLFOX model

General data

All rats survived for 7 days after chemotherapy infusion in both glycine and control groups. Blood pressure (134 ± 3 mm/Hg), hematocrit (45.6 ± 2%) and body temperature (35.6 ± 0.3°C) were comparable in all groups during all experimental phases studied.

Liver injury

Serum transaminases were elevated after FOLFOX. At 24 h after chemotherapy, transaminases reached their maximum. Glycine decreased AST at 0, 12, 24 and 36 h after chemotherapy from 241 ± 18, 252 ± 22, 253 ± 28 and 176 ± 21 U/l in controls to 171 ± 14, 200 ± 12, 184 ± 9 and 130 ± 6 U/l, respectively (Fig. 2c). Further, ALT decreased from 58 ± 6, 61 ± 4, 68 ± 4 and 52 ± 3 U/l in controls to 46 ± 4, 56 ± 4, 56 ± 3 and 42 ± 2 U/l, respectively, after glycine ($p < 0.05$) (Fig. 2d).

Kupffer cell phagocytosis

At the end of chemotherapy, phagocytotic activity of KCs was measured with fluorescent latex beads as described in "Materials and methods". The percentage of latex beads inside the KCs were compared between groups. Glycine significantly reduced phagocytosis of latex beads after FOLFOX to 26.2–55.1% (Fig. 3b) of controls, as a minimum and maximum difference over time.

Leukocytes–endothelium interaction

In vivo microscopy as described in "Materials and methods" revealed a dramatic increase of leukocytes accumulated within the sinusoids in all subacinar zones after FOLFOX (Fig. 4b). Separate analysis within the three zones also revealed a significant increase of stickers from periportal to pericentral zone in controls after FOLFOX ($p < 0.05$). Glycine effectively prevented permanent adhesion in all pericentral ($p = 0.004$), midzonal ($p = 0.01$) and periportal ($p = 0.017$) zones (Fig. 4b).

Microcirculation

Hepatic microperfusion is dependent on changes of the sinusoidal diameter, but also on sticking and rolling of leukocytes to the endothelium (Menger et al. 1992). To evaluate hepatic microperfusion, erythrocyte velocity was chosen and was found to be equivalent to sinusoidal perfusion rate (data not shown). Erythrocyte velocity was measured in different zones of acini as described in "Materials and methods". While sinusoidal diameter was not different between groups, glycine significantly increased the velocity of erythrocytes from 0.22 ± 0.003, 0.4 ± 0.01, 0.52 ± 0.01 and 0.73 ± 0.02 mm/s in periportal, midzonal, pericentral and central venous zones in controls to 0.31 ± 0.01, 0.5 ± 0.001, 0.66 ± 0.02 and 0.92 ± 0.03 mm/s, respectively after FOLFOX (Fig. 5b).

Histology

Microvesicular steatosis with periportal accentuation was detected in all groups studied. Controls presented accumulation of fat in 57.1 ± 8.6% of hepatocytes. After

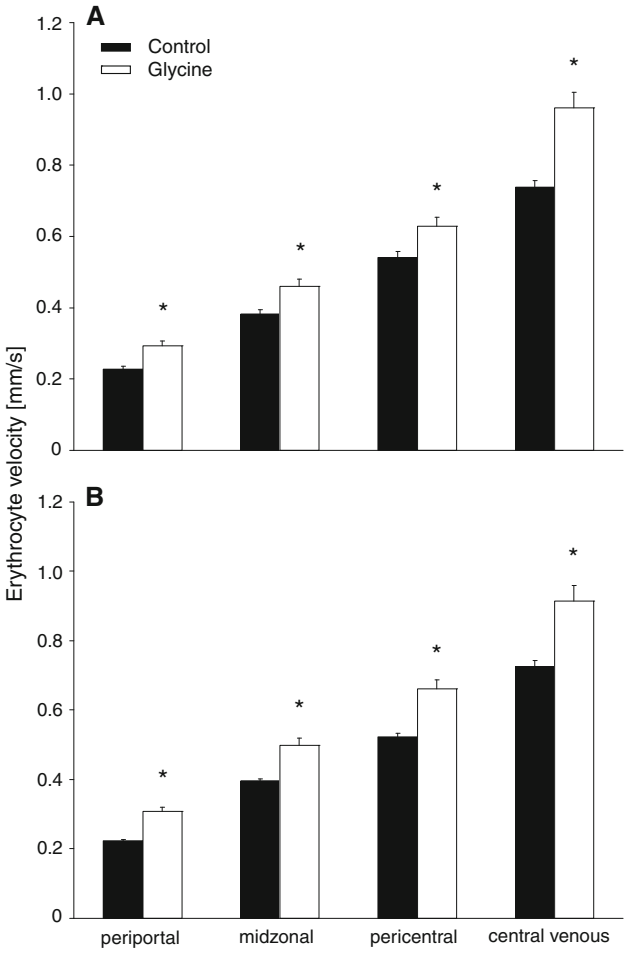

Fig. 5 Erythrocyte velocity. **a** FOLFIRI **b** FOLFOX. Glycine significantly increased the speed of erythrocytes and blood flow in sinusoids. Data are shown as mean ± SEM. *$p < 0.006$ compared to corresponding control values

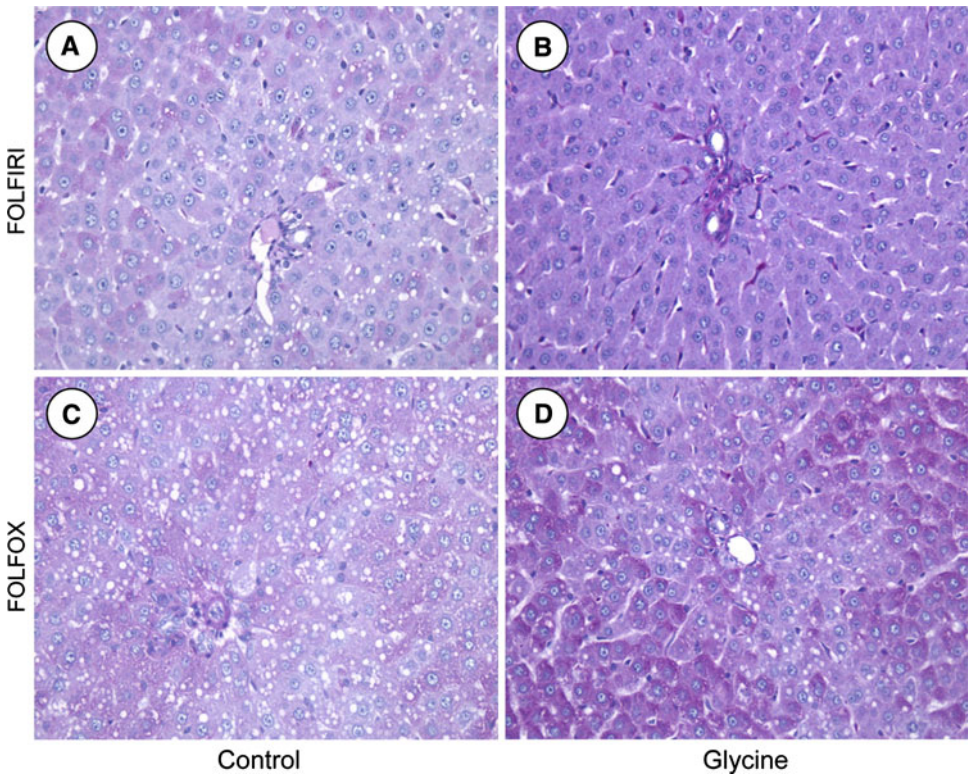

Fig. 6 Liver histology directly after chemotherapy (PAS staining, 20×). At 24 h after FOLFIRI: **a** controls **b** glycine at 36 h after FOLFOX: **c** controls **d** glycine. Glycine significantly reduced fatty accumulation in hepatocytes after both FOLFIRI and FOLFOX compared to corresponding controls

glycine, fatty changes were present in only 37.7 ± 4.4% of hepatocytes (Fig. 6c, d).

iNOS

Staining for iNOS was performed in tissue taken 36 h after chemotherapy as described in "Materials and methods". Controls presented an expression score for iNOS of 2.6 ± 0.09. Glycine significantly reduced expression of iNOS in liver tissue to 1.7 ± 0.15, which was only 65% of controls ($p < 0.001$) (Fig. 7c, d).

Discussion

Potent chemotherapeutic regimens are available for the treatment of liver metastases derived from colorectal cancer. They are used increasingly not only for palliative and adjuvant therapy, but also in a neoadjuvant setting (Zorzi et al. 2007; Welsh et al. 2007; Vauthey et al. 2006); however, their hepatotoxic side effects potentially decrease liver function even before liver resection. In the past, it has been clearly demonstrated that glycine in both experimental and clinical settings protects liver from injury caused by various toxicants and following ischemia/reperfusion (Zhong et al. 2003; Zhong et al. 1996; Wheeler et al. 1999; Luntz et al. 2005). Though taurine has been shown in various models to protect from KC-dependent injury, the therapeutic range of glycine would be much higher and, thus, clinical application would be limited due to potential side effects (Wheeler et al. 1999; Schemmer et al. 2005; Kincius et al. 2007; Guan et al. 2008; Schindler et al. 2009). Thus, this study was designed to assess its effect on liver after chemotherapy.

Indeed, glycine dramatically reduced serum AST and ALT (Fig. 2), decreased both KC activation (Fig. 3) and leukocytes–endothelium interaction (Fig. 4), and prevented steatosis of the liver (Fig. 6) after chemotherapy. Furthermore, glycine reduced iNOS expression (Fig. 7), and microcirculation was improved after chemotherapy (Fig. 5). How can this be explained?

Elevated serum transaminases, liver steatosis and decreased microcirculation are present after chemotherapy (Figs. 2, 5, 6). A glycine diet blunted all of the detrimental effects of chemotherapy well (Figs. 2, 3, 4, 5, 6, 7). Therefore, it is concluded that glycine prevents chemotherapy-induced liver injury.

The working hypothesis to explain the observed effects of chemotherapy is as follows: chemotherapy, as many drugs or toxicants, causes a hypermetabolic state and hypoxia in liver tissue, creating direct activation of KCs (Fig. 3) (Zhong et al. 2003). Activated KCs release vasoactive mediators, which impair hepatic microcirculation, thus forming a vicious circle of further hypoxia, damage and KC activation. The effect of glycine on KCs and its consequences on hepatic microperfusion and liver injury

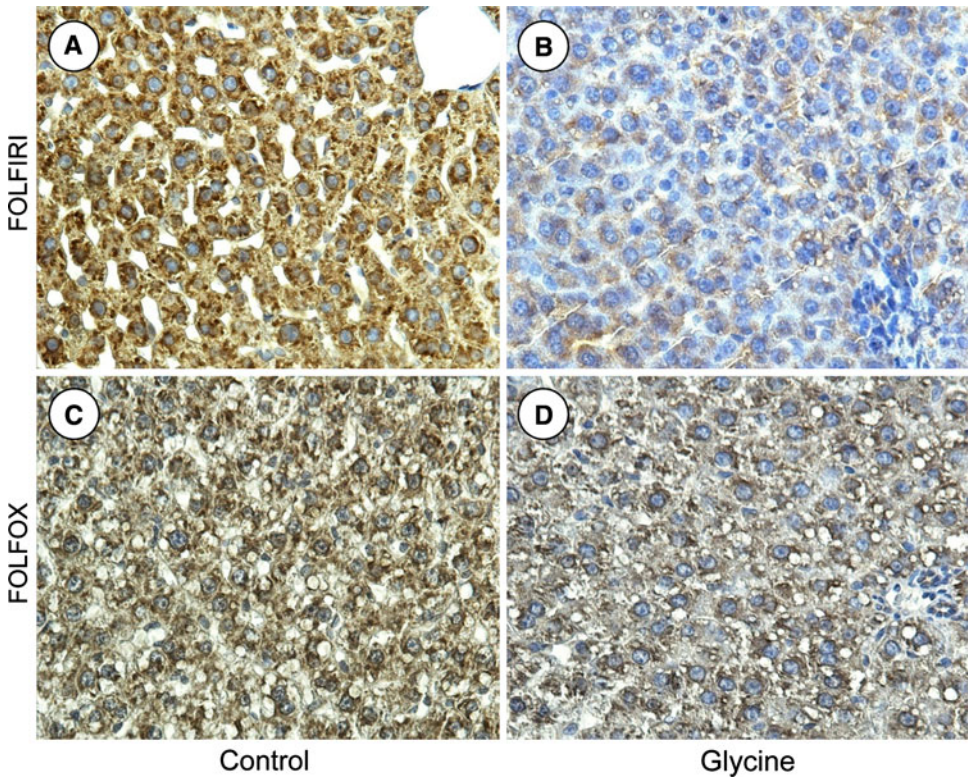

Fig. 7 iNOS expression directly after chemotherapy (400×). At 24 h after FOLFIRI: **a** controls **b** glycine. At 36 h after FOLFOX: **c** controls **d** glycine. Glycine significantly reduced iNOS expression after both FOLFIRI and FOLFOX compared to controls ($p < 0.05$)

has been extensively described in other models (Schemmer et al. 1999; Schemmer et al. 1998; Rivera et al. 2001). While KCs showed enhanced capacity for phagocytosis of latex beads after chemotherapy in controls, phagocytosis was largely reduced in glycine-treated animals after chemotherapy and KC-dependent injury to livers decreased (Schemmer et al. 2005). Since KCs could be inactivated with glycine and significantly fewer cells were latex beads positive, it can be assumed that the predominant latex beads-positive cell type was the KC in our model. Although it is not possible to directly prove with in vivo microscopy that the observed latex beads are taken up only by KCs, it has been recently demonstrated that these particles are phagocytized exclusively by KCs (Widmann et al. 1972), which was also confirmed with electron micrographs by Yano et al. (2004). In their study, latex particles of different sizes were not taken up by the sinusoidal endothelial cells of the rat liver.

Once activated, KCs impair the intrahepatic circulation by releasing vasoactive mediators (Altin and Bygrave 1988). A disturbed hepatic microcirculation most likely is involved in the development of liver injury after chemotherapy (Fig. 5). A characteristic phenomenon during the early stage after chemotherapy is the adherence of WBCs to the endothelium and their subsequent activation and release of reactive oxygen species (ROS) and various mediators. Similar to adhesive and activated WBCs, once activated KCs start producing a regulatory nuclear factor kappa B (NFκB), which in turn leads to the generation of a number of cytotoxic molecules that include ROS, nitrogen species (RNS) and various mediators involved in the development of liver injury (Schemmer et al. 2005; Younis et al. 2003; Wheeler 2003; Suzuki and Toledo-Pereyra 1994; Roberts et al. 2007; Duvnjak et al. 2007). As expected, there was an increased leukocyte–endothelial cell interaction after chemotherapy (Fig. 4). In vivo microscopy revealed that glycine improved hepatic microcirculation (Fig. 5).

NO production is an important pathway in the metabolic response to injury and inflammation. In cells expressing a high-output inducible isoform, regulation of NO synthesis occurs principally through control of iNOS transcription (Harbrecht et al. 2004). The expression of nitric oxide synthase in hepatocytes is important in the response of the liver to ROS, because NO synthesis and hepatic iNOS expression after chemotherapy are associated with a modulation of the LPS-induced hepatic injury. This finding is consistent with the work of others and demonstrates a reduction in tissue injury with modulation of iNOS activity and expression in proinflammatory states (Vos et al. 1997; Szabó et al. 1994). The reduction of iNOS expression with glycine is associated with decreased cytokines-mediated hepatic injury and decreased cytokines-mediated hepatic dysfunction (Harbrecht et al. 2004). Our data demonstrate

that glycine most likely inhibits hepatocyte nitric oxide (NO) synthesis by decreasing the expression of iNOS in response to pro-inflammatory stimuli in vivo (Fig. 7).

Only minor steatotic changes in the liver, very few infiltrating leukocytes, decreased iNOS expression and minimal disturbances of the microcirculation were identified while KC activation was almost absent after glycine, which supports the hypothesis that KCs are pivotal in chemotherapy-induced liver injury.

Data presented here, using a clinically relevant model to investigate chemotherapy and associated liver injury, demonstrates well the hepatoprotective effects of glycine in vivo.

Conclusion and clinical implication

These in vivo data indicate for the first time that dietary glycine protects liver from chemotherapy-induced injury, most likely via mechanisms including KCs. If this hepatoprotective effect of glycine can be confirmed in humans, application of glycine would be especially important in neoadjuvant regimens followed by substantial resection of liver tissue.

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Conflict of interest No conflicts of interest exist

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