Nutrient digestion and absorption during chemotherapy-induced intestinal mucositis in the rat
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REDUCED ABSORPTION OF FATTY ACIDS DURING METHOTREXATE-INDUCED GASTROINTESTINAL MUCOSITIS IN THE RAT

Clinical Nutrition; in press

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ABSTRACT

Background Patients with chemotherapy-induced gastrointestinal mucositis suffer from weight loss and possibly malabsorption. Since long-chain fatty acids serve important functions in the body, we aimed to determine the intestinal capacity of fat absorption in rats with and without methotrexate-induced mucositis. Methods Four days after intravenous injection with methotrexate (60 mg/kg) or saline, rats received saturated ([U-13C]palmitic acid) and unsaturated ([U-13C]linoleic acid) fatty acids dissolved in oil, either as a single bolus by oral gavage or by continuous intraduodenal infusion. We determined plasma and liver label concentrations at specific time points. Results We confirmed methotrexate-induced mucositis by villus atrophy using microscopy. Methotrexate treatment severely reduced the appearance of [U-13C]palmitic- and [U-13C]linoleic acid in plasma and liver, compared with controls, either when administered as a bolus or continuously (all at least -63%, P<0.05). Liver [U-13C]palmitic acid appearance was higher than [U-13C]linoleic acid appearance, either when administered as a bolus (2.8-fold, P<0.01) or continuously (5.7-fold, P<0.01). Conclusions The intestinal capacity to absorb long-chain fatty acids is severely reduced in rats with methotrexate-induced mucositis. Continuous administration does not overcome this impairment. The liver takes up and/or retains mainly saturated fatty acids during mucositis.

INTRODUCTION

Gastrointestinal mucositis (further referred to as “mucositis”) is one of the most debilitating side effects of chemotherapy and radiotherapy, especially in children [1, 2]. Patients with mucositis often experience anorexia, diarrhea and weight loss [2]. Mucositis induces small intestinal villus atrophy and loss of enterocytes, suggesting loss of epithelial function [2]. However, the digestive and absorptive capacity during mucositis is still largely unknown. We earlier showed that the digestion of lactose was reduced, but that the absorption of glucose, administered in trace amounts, was still intact in a rat model of methotrexate (MTX)-induced mucositis [3]. We now aimed to determine the intestinal capacity and physiology of fat absorption during mucositis, when enterally administered in physiologically relevant amounts (meal size).

Up to now, there is still no rational feeding strategy for mucositis patients, although nutritional support might actually improve the nutritional state, accelerate recuperation and increase survival of mucositis patients [4]. It is known that long-chain fatty acids (LCFA) serve several important functions in the human body [5]. First of all, LCFA provide energy, in fact more than twice as much as carbohydrates and proteins on weight basis. Secondly, LCFA are the major constituent of cell membranes in esterified form (phospholipids, glycolipids). Thirdly, dietary LCFA are the only source of the essential fatty acids linolenic acid and linoleic acid. Finally, LCFA are particularly
needed during periods of growth and development [6]. Concerning the mode of nutrient administration, continuous rather than bolus administration of enteral nutrition is recommended during intestinal failure which is thought to enhance enteral absorption by maximizing saturation of the carrier proteins, thereby increasing intestinal function [7]. Slow continuous administration of enteral nutrition also reduces the risk of osmotic diarrhea during intestinal failure [7]. When tolerated, enteral nutrition is preferred to total parenteral nutrition because the latter carries a high risk of infection and, upon prolonged administration, may cause liver disease [7]. No data are available concerning the best mode of LCFA administration during mucositis.

Mucositis is primarily diagnosed by subjective symptoms like pain and diarrhea [2]. These symptoms correlate poorly with the severity of mucositis, especially in young children who are less capable of localizing pain and are often incontinent for feces [8]. However, plasma citrulline (a nonprotein amino acid made by enterocytes) has been shown to be a good, objective marker for mucositis [8] and even for lactose malabsorption during mucositis in the rat [3].

Here, we aimed to determine the intestinal capacity and physiology of LCFA absorption in a previously established mucositis rat model [3] when enterally administered as a bolus (exp. 1) or continuously (exp. 2).

**MATERIALS AND METHODS**

**Rats and housing**

For both experiments, male Wistar Unilever outbred rats (4 wk old, 70-85 g) were obtained from Harlan (Horst, the Netherlands). Rats were individually housed in Plexiglas cages (42.5 x 26.6 x 18.5 cm) on a layer of wood shavings under controlled temperature (21 ± 1 °C) with a relative humidity of 55 ± 10% and a 12:12-h light-dark cycle (lights on 7:00 A.M. – 7:00 P.M.). Water and purified diet (AIN-93G [9], Harlan Laboratories, Madison, WI, USA) were available ad libitum unless otherwise stated. The experimental protocol was approved by the Ethics Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen, the Netherlands.

**Materials**

MTX was obtained from Pharmachemie Holding B.V. (Haarlem, the Netherlands). [U-13C]palmitic acid was purchased from Isotec (Miamisburg, OH, USA: exp. 1) and CortecNet (Voisins-Le-Bretonneux, France: exp. 2), both of 99% isotopic purity and of identical composition. [U-13C]linoleic acid was purchased from Lans Medical (Amsterdam, the Netherlands: exp. 1) and Campro Scientific GmbH (Berlin, Germany: exp. 1), both of 99% isotopic purity and of identical composition. Different suppliers were used because of limited availability of both isotopes.
Experimental procedures

The mucositis rat model. We determined the intestinal capacity and physiology of LCFA absorption in rats with and without MTX-induced mucositis [3] by quantifying plasma, liver and fecal appearance of enterally administered [U-13C]palmitic acid and [U-13C]linoleic acid. Rats of exp. 1 (‘LCFA absorption when administered as a bolus’) did not undergo surgery, while rats of exp. 2 (‘LCFA absorption when administered continuously’) were equipped with permanent catheters in the duodenum as described previously [10]. One week later, all rats (6-7 wk old, 180-260 g) were injected once intravenously with MTX (60 mg/kg, to induce mucositis [3]) or NaCl 0.9% (controls) in the tail vein. Intake of food and water, body weight and the presence or absence of diarrhea were recorded daily around 8:00 A.M. Four days after injection, when symptoms of MTX-induced mucositis are most severe [3], the LCFA absorption tests were performed.

The LCFA absorption tests. After an overnight fast (11:00 P.M. day 3 – 8:00 A.M. day 4), rats received a physiologically relevant amount of fat (containing 20 mg ≈ 73.5 μmol [U-13C]palmitic acid, and 10 mg ≈ 33.5 μmol [U-13C]linoleic acid, per ml of oil mixture, i.e. 25% [v:v] olive oil - 75% [v:v] medium chain triacylglycerol oil), either as a single bolus by oral gavage (exp. 1: 400 μl/rat, i.e. 7.5% of the daily LCFA intake, based on earlier studies [11]) or by continuous intraduodenal infusion (exp. 2: 228 μl/rat/h for 9 h, representing a normal hourly LCFA intake, based on 20g AIN93G/rat/day [3], ≈ 2 ml/rat during the experiment). For practical reasons, fat administration was not corrected for body weight in individual rats. Blood samples (0.1 ml) were obtained before the start of enteral fat administration (0h) and afterwards (at 1, 2, 3, 4, 5 and 6 h after bolus administration or at 3, 6, 7.5 and 9 h during continuous infusion) by tail bleeding to quantify plasma appearance of [U-13C]palmitic acid and [U-13C]linoleic acid [11, 12]. Samples were centrifuged immediately (10 min at 2,000 x g) and collected plasma was stored at -80°C until further analysis.

Tissue collection. At the end of both experiments (6 h after an oral fat bolus or 9 h after starting continuous fat infusion), rats were killed under general anesthesia by obtaining a large blood sample through cardiac puncture for determination of plasma citrulline concentrations, followed by cervical dislocation. Blood samples were centrifuged immediately (10 min at 2,000 x g) and collected plasma was stored at -80°C until further analysis. Immediately after rats were killed, the abdomen was opened via a midline incision and the small intestine, large intestine and liver were quickly removed. After the small intestine was flushed with ice-cold PBS, small parts of the jejunum (anatomical middle of the small intestine) were collected for histology and fixed in formalin (1 cm) or 2% paraformaldehyde (PFA 1 cm) dissolved in PBS, dehydrated and embedded in paraffin according to standard procedures for histochemistry [3]. An extra jejunal part (0.5 cm, exp. 1) for myeloperoxidase (MPO) concentrations was collected, immediately frozen in liquid nitrogen and stored
at -80°C until further analysis. Contents from the large intestine (further referred to as “feces”) were collected and stored at -20°C until further use. The liver was freeze-clamped and stored at -80°C until further analysis.

**Analytical methods**

Hematoxylin and eosin staining of formalin- and PFA-fixed jejunal segments to assess histology, as well as their morphometric analysis, was carried out as described previously [3]. Mucosal MPO concentrations (exp. 1) and plasma citrulline concentrations were measured as described before [3].

**Plasma [U-13C]palmitic acid and [U-13C]linoleic appearance.** The quantification of enterally administered [U-13C]palmitic acid and [U-13C]linoleic acid in plasma was performed according to Oosterveer et al. [13] by gas chromatography-mass spectrometry (GC-MS, Agilent 5957C Series GC/MSD, Agilent Technologies, Amstelveen, the Netherlands). In short, C17:0 was added to the plasma as an internal standard. Then, fatty-acid Br-2,3,4,5,6-pentafluorobenzyl (PFB) derivates were made, extracted in hexane and its isotopomer patterns analyzed using GC-MS [14]. The mole percent enrichment of mass isotopomers M16 ([U-13C]palmitic acid) and M18 ([U-13C]linoleic acid), relative to their M0 isotopomers (unlabeled palmitic acid and linoleic acid), and the concentrations of these unlabeled LCFA (determined via their ratios to the added C17:0), were used to calculate the [U-13C]palmitic acid and [U-13C]linoleic acid plasma concentrations respectively. Enrichment of labeled LCFA on all time points after fat administration was corrected for baseline enrichment at 0 h. Plasma appearance of [U-13C]palmitic acid and [U-13C]linoleic acid during the experimental period was expressed as AUC of plasma [U-13C]palmitic acid and [U-13C]linoleic acid concentration respectively (0-6 h after bolus fat administration, 0-9 h during continuous fat infusion).

**Liver and fecal [U-13C]palmitic acid and [U-13C]linoleic appearance.** After frozen livers were crushed, liver homogenates were prepared in PBS and C17:0 was added as an internal standard. Then, lipids were extracted according to Bligh and Dyer and PFB derivates were made [13]. Collected feces was freeze-dried and mechanically homogenized. Subsequently, liver and fecal enrichment of [U-13C]palmitic acid and [U-13C]linoleic acid were quantified and concentrations of labeled LCFA in liver and feces were calculated as described above.

**Liver LCFA appearance versus plasma citrulline and diarrhea.** We calculated the sensitivity and specificity of plasma citrulline (<30 µmol/L, based on earlier studies [3]) and diarrhea (present as liquid diarrhea or absent, as seen before [3]) to detect reduced liver appearance of [U-13C]palmitic acid and [U-13C]linoleic acid during mucositis. Estimations of cumulative absorption of LCFA based on plasma appearance have been proven difficult [12]. Rather, we assessed the amount of LCFA present in liver at the end of the experiments (see also ‘Discussion’). Reduced liver LCFA
appearance was defined as an appearance below the fifth percentile of normal appearance, which is comparable with ≥2SD in a normally distributed population.

**Fecal/food ratio of [U-13C]palmitic acid and [U-13C]linoleic acid.** In both experiments, the relative amount of each labeled LCFA in the administered fat was 69% for [U-13C]palmitic acid (= 73.5 μmol per ml oil) and 31% for [U-13C]linoleic acid (= 33.5 μmol per ml oil). We determined the relative amount of each LCFA in the feces (GC-MS determinations), and subsequently calculated the fecal/food ratio of each LCFA, to find out if reduced LCFA absorption during mucositis could be caused by impaired micellar solubilization [15-17].

**Metabolism of [U-13C]linoleic acid to [13C18]arachidonic acid.** Linoleic acid can be metabolized into the long-chain polyunsaturated fatty acid arachidonic acid. Theoretically, MTX could affect the metabolic conversion rate of linoleic acid, either directly, or indirectly by changing the absorption efficacy of [U-13C]linoleic acid. Next to the quantification of [U-13C]palmitic acid and [U-13C]linoleic appearance, we quantified plasma and liver [13C18]arachidonic acid appearance (exp. 2, derived from metabolic conversion of absorbed [U-13C]linoleic acid [18] after 9 h of continuous fat infusion) to further determine LCFA absorption during mucositis. After C17:0 was added as an internal standard, plasma and liver PFB derivates were made, enrichment of [13C18]arachidonic acid was quantified and concentrations of labeled LCFA were calculated as described above.

**Intestinal Fat distribution.** Jejunal fat distribution was visualized to study whether intraluminal fat passes the enterocytes during mucositis, using Oil-Red-O (ORO) staining on frozen material (4-μm-thick sections, exp. 2), according to standard procedures as has been done before [19].

**Statistical analysis**
Statistical analysis was performed using the Mann-Whitney U-test (SPSS 16.0 for Windows, Chicago, IL, USA). Values represent medians and ranges (text and Table) or first to third quartiles (Figures), for the indicated number of rats (n) per group. Correlations are expressed as nonparametric Spearman correlation coefficient. P values were considered statistically significant if P<0.05.

**RESULTS**

**The mucositis rat model**
We determined intestinal LCFA absorption in a previously characterized rat model of MTX-induced mucositis. In agreement with our earlier observations, MTX-treated rats showed typical symptoms of mild to severe mucositis (i.e. villus atrophy and blunting, irregular and vacuolized enterocytes, mucosal inflammation, reduced plasma citrulline
concentrations, reduced food intake, loss of body weight and liquid diarrhea), in contrast to controls and as seen previously [3] (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>After a fat bolus</th>
<th>After continuous fat infusion</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control (n=7)</td>
<td>MTX (n=14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control (n=6)</td>
</tr>
<tr>
<td>Rat characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villus length day 4, μm</td>
<td>418 (395-467)</td>
<td>229 (109-433)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>530 (424-549)</td>
</tr>
<tr>
<td>MPO day 4, ng/mg mucosa</td>
<td>4 (3-4)</td>
<td>124 (4-250)*</td>
</tr>
<tr>
<td>Citrulline day 4, µmol/L</td>
<td>77 (66-91)</td>
<td>11 (7-59)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>61 (42-69)</td>
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<tr>
<td>Food intake day 3, g/d</td>
<td>8 (6-11)</td>
<td>1 (0-6)*</td>
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<tr>
<td></td>
<td></td>
<td>12 (9-15)</td>
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<td>Relative body weight day 4, %</td>
<td>109 (105-109)</td>
<td>96 (91-105)*</td>
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<tr>
<td></td>
<td></td>
<td>94 (88-105)*</td>
</tr>
<tr>
<td>Absolute body weight day 4, g</td>
<td>210 (190-230)</td>
<td>185 (165-220)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>205 (175-240)*</td>
</tr>
<tr>
<td>Plasma appearance^2 (AUC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[U-13C]palmitic acid, µmol/L+6h or L+9h</td>
<td>155 (116-199)</td>
<td>27 (3-153)*</td>
</tr>
<tr>
<td>[U-13C]linoleic acid, µmol/L+6h or L+9h</td>
<td>39 (35-51)</td>
<td>7 (0-36)*</td>
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<tr>
<td>Liver appearance</td>
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<td></td>
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<td>[U-13C]palmitic acid, µmol/kg</td>
<td>100 (52-124)</td>
<td>35 (13-137)*</td>
</tr>
<tr>
<td>[U-13C]linoleic acid, µmol/kg</td>
<td>15 (6-19)</td>
<td>6 (2-21)*</td>
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<tr>
<td>l-13C]arachidonic acid, µmol/kg</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fecal appearance^d</td>
<td></td>
<td></td>
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<tr>
<td>[U-13C]palmitic acid, µmol/g</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>[U-13C]palmitic acid, fecal/food ratio</td>
<td>1.4 (1.4-1.4)</td>
<td>1.4 (1.2-1.4)</td>
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<tr>
<td>[U-13C]linoleic acid, µmol/g</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>[U-13C]linoleic acid, fecal/food ratio</td>
<td>0.1 (0.0-0.2)</td>
<td>0.2 (0.0-0.4)</td>
</tr>
</tbody>
</table>

Table 1. Rat characteristics and long-chain fatty acid (LCFA) appearance after an oral fat bolus or after continuous intraduodenal fat infusion. LCFA appearance was determined in contents from the large intestine (referred to as “feces”) because of diarrhea in MTX-treated rats. Data indicate medians (ranges) of groups. #P<0.05 and *P<0.01 for control versus MTX-treated rats.

^a Food intake is shown on day 3 (8.00 A.M. – 11.00 P.M.), since rats were fasted from 11.00 P.M. on because of the LCFA absorption test at day 4

^b Body weight is relative to weight at day 0 (day of injection) which is arbitrarily set at 100%

^c Plasma appearance (AUC, area under the curve) of LCFA, 6 h after an oral fat bolus or after 9 h of continuous intraduodenal fat infusion

^d Fecal LCFA concentrations could not be determined (ND) after a fat bolus because the internal standard (i.e. C17) was not detectable
Exp. 1: LCFA absorption when administered as a bolus

Plasma \([^{13}C]palmitic\) and \([^{13}C]linoleic\) acid appearance

Figure 1A and B show that MTX treatment markedly lowered plasma concentrations of \([^{13}C]\)palmitic acid and \([^{13}C]\)linoleic acid at all time points after fat administration, compared with controls \((P<0.01)\). In MTX-treated rats, maximal concentrations of \([^{13}C]\)palmitic acid and \([^{13}C]\)linoleic acid \((6.9\) and \(1.7\) µmol/L, at 6

Figure 1. Long-chain fatty acid (LCFA) appearance after an oral fat bolus. Appearance of \([^{13}C]\)palmitic acid and \([^{13}C]\)linoleic acid in the plasma (A and B) and liver (C-F) of control (○ ---, n=7) and methotrexate (MTX)-treated rats (● – , n=14) until (plasma) or at (liver) 6 h after an oral fat bolus. C-F: plasma citrulline (C and D) versus diarrhea (E and F) as markers for liver appearance of \([^{13}C]\)palmitic acid and \([^{13}C]\)linoleic acid in control (○) and MTX-treated rats (●). Data represent medians and p25-p75 (A and B) or data of individual rats (C-F). Spearman correlations (r) and P values are indicated. The dotted horizontal line (-----) marks the cut-off of reduced LCFA appearance (i.e. <p5 of controls, C-F). *MTX differs from control, P<0.01.
and 5 h respectively) were reached with a delay, compared with controls (33.5 and 8.8 µmol/L, both at 4 h respectively). Plasma appearance of both LCFA during the experimental period was reduced to 17% in MTX-treated rats, compared with controls (AUC, P<0.01, Table 1).

Liver [U-13C]palmitic acid and [U-13C]linoleic acid appearance

Figure 1C and D and Table 1 show that liver concentrations of [U-13C]palmitic acid and [U-13C]linoleic acid were reduced to 35 and 37% respectively in MTX-treated rats, compared with controls (P<0.01). Corrected for enteral [U-13C]palmitic acid and [U-13C]linoleic acid administration (molar [U-13C]palmitic acid administration was 2.2-fold higher than molar [U-13C]linoleic acid administration), liver [U-13C]palmitic acid appearance was higher than [U-13C]linoleic acid appearance, both in MTX-treated rats (2.8-fold, P<0.01) and in controls (3.0-fold, P<0.01).

Fecal/food ratio of [U-13C]palmitic acid and [U-13C]linoleic acid

The relative amount of [U-13C]palmitic acid and [U-13C]linoleic acid in feces was similar between MTX-treated rats (medians 94 and 6% respectively) and controls (medians 98 and 2% respectively, P=0.15). The fecal/food ratio of each LCFA was also similar between MTX-treated rats and controls (Table 1).

LCFA absorption versus plasma citrulline and diarrhea

Liver appearance of [U-13C]palmitic acid and [U-13C]linoleic acid correlated with plasma citrulline (rho=0.80 and 0.76 respectively, P<0.001, Figure 1C-D) and villus length (rho=0.84 and 0.79 respectively, P<0.001, data not shown). Figure 1C-F show that the sensitivity of plasma citrulline (<30 µmol/L) to detect reduced LCFA appearance in the liver (<p5 of controls) was higher (Se 0.91 / Sp 0.80) than that of diarrhea (Se 0.72 / Sp 1.00, i.e. liquid diarrhea), although its specificity was lower.

Exp. 2: LCFA absorption when administered continuously

Plasma [U-13C]palmitic acid and [U-13C]linoleic acid appearance

Figure 2A and B show that MTX treatment caused markedly lower plasma concentrations of [U-13C]palmitic acid and [U-13C]linoleic acid at all time points during fat infusion, compared with controls (P<0.01). Maximal concentrations of [U-13C]palmitic acid and [U-13C]linoleic acid in MTX-treated rats (15.9 and 0.7 µmol/L respectively) and in controls (135.2and 8.8 µmol/L respectively) were reached after 9 h of fat infusion (no plateau reached yet). Plasma appearance of [U-13C]palmitic acid and [U-13C]linoleic acid during the experimental period was reduced to 13 and 9% respectively in MTX-treated rats, compared with controls (AUC, P<0.01, Table 1).
Liver and fecal [U-13C]palmitic acid and [U-13C]linoleic acid appearance

Figure 2C and D and Table 1 show that liver concentrations of [U-13C]palmitic acid and [U-13C]linoleic acid were reduced to 19 and 30% respectively in MTX-treated rats, compared with controls (P<0.01). Corrected for enteral [U-13C]palmitic acid and [U-13C]linoleic acid administration (molar [U-13C]palmitic acid administration was 2.2-fold higher than molar [U-13C]linoleic acid administration), liver [U-13C]palmitic acid appearance was higher than [U-13C]linoleic acid appearance, both in MTX-treated
rats (median 5.7-fold, \( P<0.01 \)) and in controls (9.0-fold, \( P<0.01 \)). Fecal concentrations of \([\text{U-}^{13}\text{C}]\)palmitic acid and \([\text{U-}^{13}\text{C}]\)linoleic acid were increased 9.1-fold and 4.4-fold respectively in MTX-treated rats, compared with controls (\( P<0.05 \), Table 1).

**Fecal/food ratio of \([\text{U-}^{13}\text{C}]\)palmitic acid and \([\text{U-}^{13}\text{C}]\)linoleic acid**

The relative amount of \([\text{U-}^{13}\text{C}]\)palmitic acid and \([\text{U-}^{13}\text{C}]\)linoleic acid in feces was comparable between MTX-treated rats (medians 94 and 6% respectively) and controls (medians 92 and 8% respectively, \( P=0.012 \)). The fecal/food ratio of both LCFA was also comparable between MTX-treated rats and controls (Table 1).

**Metabolism of \([\text{U-}^{13}\text{C}]\)linoleic acid to \([^{13}\text{C}_{18}]\)arachidonic acid**

Plasma \([^{13}\text{C}_{18}]\)arachidonic acid concentrations were extremely low in MTX-treated rats and in controls (data not shown). Absolute liver \([^{13}\text{C}_{18}]\)arachidonic acid concentrations were reduced to 32% in MTX-treated rats, compared with controls (\( P<0.01 \), Table 1). The amount of \([^{13}\text{C}_{18}]\)arachidonic acid that was present in the liver at the end of the experiment, expressed per amount of its parent compound \([\text{U-}^{13}\text{C}]\)linoleic acid, was similar in MTX-treated rats (median 5, range 3-10%) and in controls (median 5%, range 5-6%, \( P=0.75 \)).

**Intestinal fat distribution**

In controls, most enterocytes contained no fat droplets while many small fat droplets were present in the villus interstitium (Figure 3A). In contrast, some vacuolized enterocytes on remaining villus tops of MTX-treated rats contained a few fat droplets while fat droplets were completely absent in the villus interstitium (Figure 3B).

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**Figure 3. Fat distribution after continuous intraduodenal fat infusion.** Oil-Red-O (ORO) staining showing fat distribution after 9 h of continuous intraduodenal LCFA infusion in the jejunum of control (A) and methotrexate (MTX)-treated rats (B). Magnification: 20x.
**LCFA absorption versus plasma citrulline and diarrhea**

Liver appearance of [U-13C]palmitic and [U-13C]linoleic acid correlated with plasma citrulline (rho=0.84 and 0.80 respectively, P<0.001, Figure 2C-D), and appearance of [U-13C]palmitic acid with villus length (rho=0.61, P=0.012, data not shown). Figure 2C-F show that the sensitivity of plasma citrulline (<30 µmol/L) to detect reduced LCFA appearance in the liver (<p< of controls) was higher (Se ≥ 0.80 / Sp ≥ 0.83) than that of diarrhea (Se ≥ 0.50 / Sp 1.00, i.e. liquid diarrhea), although its specificity was lower.

**DISCUSSION**

We aimed to determine the intestinal capacity and physiology of LCFA absorption during mucositis. Our data indicate that the intestinal capacity to absorb LCFA is severely reduced in rats with MTX-induced mucositis. Continuous enteral administration does not overcome this impairment.

We determined LCFA absorption in a previously established rat model of MTX-induced mucositis [3] by quantifying plasma, liver and fecal appearance of enterally administered, stable isotope labeled saturated and polyunsaturated LCFA, as have been successfully used before [11, 12, 18, 22-26]. We could not use the gold standard for measuring fat absorption, i.e. the fecal fat balance [21], to determine LCFA absorption because of diarrhea, reduced food intake and non-steady state conditions during mucositis. To estimate quantitative LCFA absorption, we determined the hepatic labeled LCFA content at the end of the experiments. Theoretically, MTX could have affected liver uptake and retention of systemically available LCFA, but we do not have any indication to support this possibility: we did neither see histological differences between the livers of MTX-treated rats and controls, nor is MTX-induced liver dysfunction after short-term MTX exposure reported in the literature. For assessment of quantitative carbohydrate or amino acid absorption, dual isotope infusion (enteral and intravenous) of differentially labeled but identical nutrients can be applied: their plasma appearance can then be corrected for first pass splanchnic utilization and the flux of molecules out of the plasma compartment [27-29]. However, this methodology is not usable for LCFA absorption, since one cannot reliably reconstitute and administer LCFA in the identical physical particles (chylomicrons) and biochemical nature (acylated as triglyceride of phospholipid) in which the absorbed LCFA appear in the plasma compartment.

We initially administered LCFA as an oral bolus to approximate the physiological situation of consuming meals. Plasma and liver appearance of labeled LCFA was severely reduced during mucositis, compared with controls, indicating reduced LCFA absorption. Since all rats received the same absolute amount of fat, MTX-treated rats
received more fat per gram body weight than controls because of a lower body weight. We therefore cannot exclude that the observed LCFA acid uptake in MTX-treated rats is even overestimated. We aimed to determine the physiology of reduced LCFA absorption during mucositis. Dietary lipids undergo a number of intraluminal physicochemical alterations before lipolytic products are translocated across the enterocyte membrane [5]. After translocation, absorbed LCFA undergo reacylation and assembly into chylomicrons before they end up in mesenteric lymph and finally in the blood [15, 20]. Since we administered unesterified LCFA, we could exclude impaired emulsification or lipolysis as causes for their reduced absorption during mucositis. Impaired micellar solubilization is expected to result in a relative increase of nonabsorbed [U-13C]palmitic acid in the feces since unsaturated LCFA have appeared to be less dependent on micellization than saturated LCFA [15-17]. In MTX-treated rats, however, the fecal/food [U-13C]palmitic acid ratio was similar compared with that in controls, excluding the possibility that impaired micellar solubilization predominantly contributes to impaired LCFA absorption during mucositis. By inference, reduced LCFA absorption during mucositis seems to be mediated at the level of the enterocytes (defective translocation and/or intracellular LCFA processing), which seems plausible regarding the observed enterocyte damage and dysfunction (i.e. reduced plasma citrulline concentrations [3], which cannot merely be explained by a reduced food intake [32]). We did not study enterocyte expression of fatty acid binding proteins since they are not essential for fat absorption [30]. Others found them to be relatively resistant to chemotherapy-induced damage [31].

We then determined intestinal absorption of LCFA upon continuous administration, which is thought to improve nutrient absorption during other forms of intestinal failure [7]. Overall, the results were rather similar to those obtained after bolus administration (reduced plasma and liver LCFA appearance and increased fecal LCFA appearance during mucositis), indicating reduced LCFA absorption. Absolute liver appearance of [13C16]arachidonic acid was reduced during mucositis, compared with controls, while its relative amount compared with liver [U-13C]linoleic acid was similar, indicating that MTX does not affect the metabolic conversion rate of linoleic acid directly but indirectly by changing the absorption efficacy of [U-13C]linoleic acid. Impaired micellar solubilization due to changes in bile salt metabolism could again be excluded, since the fecal/food [U-13C]palmitic acid ratio was in the same order of magnitude in MTX-treated rats and in controls. The absence of fat droplets in the villus interstitium (draining on the lymphatic system [19]) of MTX-treated rats, in contrast to controls, was in accordance with the earlier found absorptive problems at the level of the enterocytes.

During continuous LCFA administration, the total amount of administered LCFA was about five times higher than in the bolus experiment. Accordingly, absolute plasma and liver appearance of labeled LCFA during mucositis was higher compared with
bolus administration (although their relative appearance stayed low, compared with controls). In both experiments, the fecal/food ratio of [U-13C]palmitic acid appearance was much higher than the fecal/food [U-13C]linoleic acid appearance, both in MTX-treated rats and controls, confirming the physiological lower absorption efficacy of saturated LCFA relative to unsaturated LCFA [20]. We assumed that fecal appearance of labeled LCFA was only the result of nonabsorbed enterally administered LCFA. Theoretically, excretion of labeled LCFA by the intestine via the enterohepatic loop or via enterocyte shedding, after initial absorption, could have also played a role. Of note, liver [U-13C]palmitic acid appearance was much higher than [U-13C]linoleic acid appearance, both in MTX-treated rats and controls, either after a fat bolus and after continuous fat administration. This might have been due to a high oxidation rate for [U-13C]linoleic acid after absorption [33]. Apparently, the liver has a higher uptake and/or retention of saturated than of unsaturated LCFA, under normal circumstances and during mucositis. Our findings might indicate that especially saturated LCFA are important in the diet of mucositis patients.

LCFA absorption correlated with plasma citrulline concentration and villus length, similar to previous observations on lactose absorption [3]. We also showed that plasma citrulline <30 µmol/L has a higher sensitivity (≥0.80 versus ≥0.50), but a lower specificity (≥0.8 versus 1.0), than diarrhea to detect reduced LCFA absorption during mucositis. In rats, the absence of diarrhea after chemotherapy apparently does not exclude nutrient malabsorption. Since plasma citrulline seems a valuable indicator of the functional status of the gastrointestinal tract in rats [3] as well as in humans [34], it might help to tailor the feeding strategy of mucositis patients.

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