Nutrient digestion and absorption during chemotherapy-induced intestinal mucositis in the rat
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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CONTINUOUS ENTERAL ADMINISTRATION CAN OVERCOME THE LIMITED CAPACITY TO ABSORB GLUCOSE IN RATS WITH METHOTREXATE-INDUCED GASTROINTESTINAL MUCOSITIS

Supportive Care in Cancer 2012 Sep 26; in press

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ABSTRACT

Background Patients with chemotherapy-induced gastrointestinal mucositis often suffer from weight loss. It is not well known how to enterally feed mucositis patients, potentially experiencing malabsorption. Recently, we showed in a rat model of methotrexate (MTX)-induced mucositis that intestinal absorption of glucose in trace amounts is still intact. We now determined the quantitative capacity to absorb glucose in rats with mucositis, relative to controls. Methods We administered a physiologically relevant amount of [1-\(^{13}\)C]glucose enriched glucose (meal size) as a bolus by oral gavage (2 g/kg once) or continuously by intraduodenal infusion (±1.9 g/(kg-h) for 5 h) to rats with MTX-induced mucositis and controls. Blood [1-\(^{13}\)C]glucose concentrations were determined during the experimental period. To calculate the quantitative absorptive capacity, Steele’s one-compartment model, including simultaneous intravenous infusion of [6,6-\(^{2}\)H\(_{2}\])glucose, was used. After the experiment, jejunal histology and plasma citrulline concentrations were assessed. Results MTX-induced mucositis was confirmed by a reduction in villus length and plasma citrulline (both -57%, relative to controls, \(P<0.01\)). When glucose was administered as a bolus, MTX-treated rats only absorbed 15% of administered glucose, compared with 85% in controls (medians, \(P<0.01\)). Upon continuous intraduodenal glucose infusion, the median absorptive capacity for glucose in MTX-treated rats did not differ from controls (80 versus 93% of administered glucose respectively, \(P=0.06\)). However, glucose absorption differed substantially between individual MTX-treated rats (range 21-95%), which correlated poorly with villus length (rho=0.54, \(P=0.030\)) and plasma citrulline (rho=0.56, \(P=0.024\)). Conclusion Continuous enteral administration can almost completely overcome the reduced absorptive capacity for glucose in rats with mucositis.

INTRODUCTION

Chemotherapy-induced gastrointestinal mucositis (“mucositis”) is seen in 40-100% of patients after chemotherapy [1, 2]. Pediatric as well as adult patients with mucositis often experience anorexia, nausea, diarrhea and weight loss [3]. Histologically, small intestinal villus atrophy and loss of enterocytes is seen [2]. Since accurate evaluation of mucositis via invasive intestinal biopsies is problematic, mucositis is often scored by more 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 due to their developmental stage [4]. Plasma citrulline (a nonprotein amino acid made by enterocytes [5]) has earlier been shown to be a useful marker for mucositis, and for lactose malabsorption during mucositis [4, 6, 7].
To date, there is no rational feeding strategy for mucositis patients, although nutritional support might actually improve the nutritional state, accelerate recuperation and increase survival of mucositis patients [8-11]. Enteral nutrition, which is the physiological way of feeding, is normally preferred to total parenteral nutrition (TPN) because the latter carries a high risk of infection and, upon prolonged administration, may cause liver disease [12-14]. However, when the absorptive function of the intestine is compromised, TPN offers a useful feeding alternative. Since the capacity to digest and absorb nutrients during mucositis is still largely unknown, we developed a methotrexate (MTX)-induced mucositis rat model to determine it, and to ultimately design a rational feeding strategy for mucositis patients [6]. Before, using this model, we showed that the digestion of lactose was severely reduced during mucositis. In contrast, trace amounts of glucose were absorbed normally during mucositis [6]. Therefore, we propose that enteral glucose might be a useful source of energy for mucositis patients.

Here, we aimed to compare the quantitative capacity to absorb glucose between rats with MTX-induced mucositis and saline-treated controls. We administered a physiologically relevant amount of glucose (meal size, meaning a representative percentage of average daily carbohydrate intake in controls) as a bolus by oral gavage, since intermittent bolus administration resembles the physiological situation of consuming meals [15]. We also determined absorption of a physiologically relevant amount of glucose when continuously administered by intraduodenal infusion, since continuous enteral nutrient administration has been shown to improve nutrient absorption during other forms of intestinal failure, like short bowel syndrome [13]. To address whether plasma citrulline levels could function as a surrogate parameter for the glucose absorptive capacity during MTX-induced mucositis, we related these two parameters in individual rats.

**MATERIALS AND METHODS**

**Rats and housing**

For both experiments, young male Wistar outbred rats (3-4 wk old, 45-75 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 [16], Harlan Laboratories, Madison, WI, USA) were available ad libitum unless otherwise stated. The experimental protocol, which resembled the protocol that we used previously [6], 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). [1-13C]glucose of 99% isotopic purity was purchased from Sigma Aldrich Chemie B.V. (Zwijndrecht, the Netherlands). [6,6-2H2]glucose of 98% isotopic purity was purchased from Isotec (Miamisburg, OH, USA).

Experimental procedures
The mucositis rat model. Rats (n=32, 5 wk old, 2 wk after arrival at the animal facility) were surgically equipped with permanent silicone catheters in the duodenum and/or jugular vein under isoflurane anesthesia, as described previously [17]. One week after surgery, rats (6 wk old, 180-250 g) were injected once intravenously in the tail vein with MTX (n= 20, 60 mg/kg, to induce mucositis [6]) or with saline 0.9% (n=12, controls) under isoflurane anesthesia. Intake of food (in grams, calculated by the amount of administered food minus the residual food in the cage on the next day), body weight (in grams, using a weighing scale), and diarrhea (present as watery diarrhea or completely absent, as described previously [6]) were recorded daily on clinical records at 8:00 A.M. Four days after injection, when histological and clinical symptoms of MTX-induced mucositis are most severe [6], the glucose absorption tests were performed (see below). At the end of both experiments (4h after an oral glucose bolus or 7h after starting continuous intraduodenal glucose infusion), rats were killed under isoflurane 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. Next, the abdomen was opened via a midline incision, and the small intestine was excised and flushed with ice-cold PBS. Small parts of the duodenum (proximal small intestine), jejunum (anatomical middle of the small intestine) and ileum (1/6 part proximal from the cecum) 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. An extra jejunal part (0.5 cm) for myeloperoxidase (MPO) concentrations was collected, immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

The glucose absorption tests. Similar to our previous study, where absorption of glucose in trace amounts was determined [6], rats were fasted for 9 h on the day of experiment (11:00 P.M. day 3 – 8:00 A.M. day 4). Thereafter, rats received a continuous intravenous infusion of [6,6-2H2]glucose at a rate of 12.1 μmol/(rat-h) during the whole experiment to determine the total rate of glucose appearance (RaT), as described previously [18, 19]. To determine the rate of appearance of enterally administered glucose (RaE), one hour after the start of the experiment, rats enterally received a physiologically relevant amount of glucose (meal size, meaning a representative percentage of average daily carbohydrate intake via AIN-93G in
saline-treated controls, i.e. ±12 g carbohydrates/(rat·day) ≈ ±52 g carbohydrates/(kg·day) ≈ ±2.2 g carbohydrates/(kg·h) [6, 16]). Enteral glucose (i.e. glucose monohydrate) was administered either as a bolus by oral gavage (2.0 g/kg in ±0.8 ml water as described previously [18, 19], ≈ 4% of average daily carbohydrate intake), or as continuous intraduodenal infusion (±1.3 g/(kg·h) for the first 2h, and ±1.9 g/(kg·h) for the last 5 h to reach a steady state, ≈ 85% of average hourly carbohydrate intake). An overview of the number of MTX- and saline-treated rats per glucose absorption test (glucose as a bolus or continuously) is stated in Table 1. Continuous glucose infusion rates and doses were chosen to avoid severe hyperglycemia (blood glucose concentrations >30 mmol/L) and signs of drowsiness in MTX-treated rats, which were experienced in pilot studies that we had executed earlier, upon higher doses of continuous intraduodenal glucose infusion (up to ±2.5 g/(kg·h), unpublished material). Of the administered glucose, 10% was [1-13]C glucose. Blood samples were obtained from the tail tip before the start of the experiment and at indicated time points during the experiment on filter paper to quantify the fractional contributions of [6,6-2H2]glucose and [1-13]C glucose [18, 19], and to measure blood glucose concentrations using a Lifescan EuroFlash glucose meter (Lifescan Benelux, Beerse, Belgium). Additionally, we obtained blood samples to measure plasma insulin concentrations. Blood samples were centrifuged immediately (10 min at 2,000 x g) and collected plasma was stored at -80°C until further analysis.

**Analytical methods**

**Histological assessment.** Hematoxylin and eosin (H&E) staining was performed on 3-µm-thick sections of formalin- and PFA-fixed small intestinal segments to confirm mucositis, according to standard procedures. Morphometric analysis was carried out on jejunal segments as described previously [6]. Villus length was measured manually by a blinded researcher (MF) in well-orientated sections (10 measurements per rat) from digitized images that were evaluated at 10x magnification (1 pixel = 0.397 µm) using a calibrated image analysis system (Qwin V3.0, Leica Microsystems).

**Mucosal MPO concentrations.** Mucosa of frozen jejunal sections (of rats that received glucose as a bolus) was scraped on ice to make tissue homogenates in lysis buffer (reaction tubes: Greiner Bio-One B.V., Alphen a/d Rijn, the Netherlands). Homogenates were 5-50 times diluted in dilution buffer before MPO concentrations (indicating inflammation) were quantitatively measured via a solid bound antibody against MPO as described by the manufacturer (rat MPO ELISA kit, Hycult Biotech, Uden, the Netherlands) and as done previously [6].

**Plasma citrulline concentrations.** Plasma citrulline concentrations (indicating functional enterocyte mass [5]) were measured in 30 µl plasma at room temperature by using automated ion exchange column chromatography as described previously [4, 6, 20].
Plasma insulin concentrations. Plasma insulin concentrations were measured in 5 µl plasma via a solid bound antibody against insulin as described by the manufacturer (Rat Insulin Ultrasensitive EIA, Alpco Diagnostics, Salem, NH, USA) and as done previously [6].

Intestinal [1-13C]glucose absorption. The fractional contribution of intravenously administered [6,6-2H2]glucose and enterally administered [1-13C]glucose in blood glucose was performed essentially according to Van Dijk et al. [6, 21]. All samples were analyzed by gas chromatography/mass spectrometry (GC/MS, Agilent 5957C Series GC/MSD, Agilent Technologies, Amstelveen, the Netherlands). The calculations of RaT and RaE were calculated according to Steele’s one compartment model, modified by Debodo et al. [22, 23] and Tissot et al. [24]. The absorption of bolus-administered glucose during the experimental period was expressed as the fraction of administered glucose. The average absorption of continuously administered [1-13C]glucose in steady state was expressed as the fraction of intraduodenal glucose infusion in steady state.

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 in text] or (ranges in Table 1), or first to third quartiles (Q25-Q75) in Figures 1-4, 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 the quantitative capacity to absorb glucose in a previously established rat model of MTX-induced mucositis. MTX-treated rats showed typical histological and clinical symptoms of mild to severe mucositis, in contrast to controls and as seen in previous studies by us and others [6, 25-30] (Table 1).

Most MTX-treated rats (100% of rats that received glucose as a bolus and 89% of rats that received glucose continuously) suffered from severe mucositis (i.e. villus length <300 µm and plasma citrulline concentration <30 µmol/L [6]).

Glucose absorption when administered as a bolus by oral gavage
Blood glucose and plasma insulin concentrations. From the start of intravenous [6,6-2H2]glucose infusion (-60 min) until bolus [1-13C]glucose administration (0 min), blood glucose concentrations hardly changed and were similar in controls and MTX-treated rats (Figure 1A). Upon administration of the glucose bolus, blood glucose concentrations increased only in control rats whereas it hardly changed in MTX-treated rats (maxima 10.8 versus 7.3 mmol/L respectively, after 45 versus 60 min
Intestinal glucose absorption. Upon administration of the glucose bolus, control rats showed an increase in [1-13C]glucose appearance with a maximum of 75 µmol/(kg-min) after 60 min. MTX-treated rats only showed a minor increase in [1-13C]glucose appearance (maximally 10 µmol/(kg-min) after 120 min, Figure 2A). Calculated over the experimental period, this resulted in an almost six times reduced [1-13C]glucose appearance in MTX-treated rats, compared with controls (15 versus 85% of the administered bolus respectively, P<0.01, Figure 2B). Values of individual rats for glucose absorption, villus length and plasma citrulline show that glucose absorption correlated strongly with villus length (rho=0.69, P=0.003, Figure 2C) and plasma citrulline (rho=0.90, P<0.001, Figure 2D).

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<th>Histological and clinical symptoms of mucositis in MTX treated rats</th>
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<td>An oral bolus of glucose</td>
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<td>Control (n=5)</td>
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<td>MTX (n=7)</td>
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<td>Villus length (day 4, µm)</td>
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<td>428 (382-444)</td>
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<td>403 (375-500)</td>
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<td>Citrulline (day 4, µmol/L plasma)</td>
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<td>84 (81-114)</td>
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<td>72 (51-80)</td>
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<td>MPO (day 4, ng/mg mucosa)</td>
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<td>5 (3-9)</td>
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<td>ND*</td>
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<td>9 (8-11)</td>
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<td>Body weight change (day 4, % relative to day 0)</td>
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<td>+9 (+7 to +11)</td>
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<td>+8 (+7 to +12)</td>
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<td>Presence of watery diarrhea (day 4, % of rats)</td>
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Table 1. Characteristics of rats with MTX-induced mucositis and controls used in the glucose absorption tests. Controls are saline-treated. Glucose (meal size) was administered as a bolus by oral gavage or continuously by intraduodenal infusion to determine glucose absorption. Data indicate medians of groups, and ranges are in parentheses. n no. of rats, ND not determined. * P<0.01 for controls versus MTX-treated rats

a 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 absorption tests at day 4

b Body weight change (+ indicates weight gain, - indicates weight loss) is relative to weight at day 0 (day of MTX or saline injection, see ‘Materials and Methods’)

c Diarrhea was present as watery diarrhea or completely absent (see ‘Materials and Methods’)

[1] Intestinal glucose absorption.


Figure 1. Glucose and insulin concentrations when glucose was administered as a bolus by oral gavage. Blood glucose concentrations (A) and plasma insulin concentrations (B) in controls (○, n=5) and methotrexate (MTX)-treated rats (●, n=11) before and after oral administration of the [1-13C]glucose bolus (at 0 min). Dots represent medians and p25-p75 per time point. *P<0.01 and #P<0.05 for controls versus MTX treated rats.

Figure 2. Glucose absorption when administered as a bolus by oral gavage. [1-13C]Glucose appearance (A and B) in controls (○, n=5) and methotrexate (MTX)-treated rats (●, n=11) after oral administration of the [1-13C]glucose bolus (at 0 min). Dots represent medians and p25-p75 per time point. *P<0.01 for controls versus MTX-treated rats. C and D: correlation between [1-13C]glucose absorption on the one hand and villus length (C) or plasma citrulline concentration (D) on the other hand in controls (○) and MTX-treated rats (●). Dots represent data of individual rats. Spearman correlations (r) and P values are indicated.
Glucose absorption when administered continuously by intraduodenal infusion

Blood glucose and plasma insulin concentrations. During the basal period (-60 - 0 min), blood glucose concentrations hardly changed and were similar in controls and MTX-treated rats (Figure 3A). After starting intraduodenal glucose infusion, blood glucose concentrations rose quickly in both groups to 8.7 mmol/L in MTX-treated rats and 10.1 mmol/L in controls after 30 min, and remained elevated during the experimental period. From 180 min on, blood glucose concentrations were similar in both groups. Plasma insulin concentrations were elevated during the experiment and did not differ between groups (Figure 3B).

Intestinal glucose absorption. After an initial increase, until 240 min after the start of intraduodenal glucose infusion, the rate of [1-13C]glucose appearance reached a steady state in both groups (Figure 4A). In MTX-treated rats, the average rate in steady state was not different from controls (128 [36-173] versus 138 [135-152] µmol/(kg·min) respectively, P=0.61). Similarly, the average glucose absorption in steady state did not differ significantly between controls and MTX-treated rats (93 [88-98] versus 80 [21-95]% of infused glucose respectively, P=0.06, Figure 4B), although glucose absorption varied substantially between individual MTX-treated rats (Figure 4C and D). Values of individual rats for glucose absorption, villus length and plasma citrulline show that glucose absorption correlated poorly with villus length (rho=0.54, P=0.030, Figure 4C) and plasma citrulline (rho=0.56, P=0.024, Figure 4D).
DISCUSSION

We aimed to determine the quantitative capacity to absorb glucose in rats with MTX-induced mucositis relative to that in controls. Our data indicate that continuous enteral administration can almost completely overcome the reduced absorptive capacity for glucose in rats with mucositis, although glucose absorption differs substantially between individual rats.

Glucose absorption was determined in a previously developed and characterized rat model of MTX-induced mucositis [6]. We initially administered glucose as an oral bolus to approximate the physiological situation of consuming meals. The bolus contained a physiologically relevant amount of glucose (2.0 g/kg, \( \approx 4\% \) of average daily carbohydrate intake), corresponding with the amount of glucose used for the Oral
Glucose Tolerance Test (OGTT) in rodents [18, 19] and in patients [31]. In controls, blood glucose concentrations increased with 5 mmol/L upon bolus glucose administration, and maximal blood glucose concentrations were preceded by a significant peak in plasma insulin concentration, together indicating physiological glucose absorption.

During the experimental period, 85% of the administered glucose was absorbed, as previously described by others [18, 19]. In contrast, blood glucose concentrations hardly increased upon the glucose bolus in MTX-treated rats, and there was only a small peak in plasma insulin concentration, indicating minimal glucose absorption. Indeed, during MTX-induced mucositis only 15% of the administered glucose was absorbed during the experimental period. So, although intestinal absorption of glucose in trace amounts (0.10 g/kg, ≈ 0.2% of average daily carbohydrate intake) was still intact during mucositis [6], the quantitative capacity to absorb a physiologically relevant amount of glucose during mucositis was severely reduced (when administered as a bolus). This might be explained by the reduced presence of glucose transporters sodium-dependant glucose transporter 1 (SGLT1), glucose transporter 2 (GLUT2) and glucose transporter 5 (GLUT5) during mucositis [6, 30].

We then compared absorption of a physiologically relevant amount of glucose when continuously administered by intraduodenal infusion, since continuous enteral nutrient administration has been shown to improve nutrient absorption during intestinal failure [13]. The amount of glucose infusion in steady state was set at ±1.9 g/(kg·h), ≈ 85% of average hourly carbohydrate intake. Blood glucose and plasma insulin concentrations were elevated from the start of intraduodenal infusion on, both in controls and MTX-treated rats, suggesting physiological glucose absorption during mucositis. Indeed, in steady state, similar amounts of intraduodenal infused glucose were absorbed in controls and in MTX-treated rats (medians 80 and 93% respectively). However, individual glucose absorption varied from severely reduced to completely normal between individual MTX-treated rats (range 21-95%), despite the fact that 8 out of 9 rats suffered from severe mucositis. Villus atrophy and plasma citrulline reduction in MTX-treated rats must have been caused primarily by the cytotoxic effects of MTX and not by a spontaneous reduction in food intake upon MTX-injection, as shown by others using pair-feeding of saline-treated control rats [25]. We hypothesize that the large interindividual differences in glucose absorption during mucositis can be explained by the fact that individual MTX-treated outbred rats might have been in different stages of mucositis (described by Sonis et al. [2]) during the absorption experiment. Absorption of continuously administered glucose could have been possible via glucose transporters on the recovered epithelial membrane, via residual transporters on damaged epithelial membrane [6] and/or via paracellular absorption [32]. Leakage of glucose through damaged tight junctions could also have been possible since mucositis can lead to increased gut permeability [33, 34].
Continuous administration of enteral nutrition during intestinal failure is thought to enhance enteral absorption by maximizing saturation of the (residual) carrier proteins, thereby increasing intestinal function [13]. This concept could provide an explanation for the observation that a glucose bolus is malabsorbed while continuous glucose administration seems to overcome this defect during MTX-induced mucositis.

To address whether plasma citrulline levels could function as a surrogate parameter for the glucose absorptive capacity during MTX-induced mucositis, we related these two parameters in individual rats. In the bolus experiment, glucose absorption correlated strongly (rho=0.90) with plasma citrulline, making citrulline a suitable marker for reduced absorption of bolus-administered glucose during mucositis. In the continuous infusion experiment, glucose absorption correlated poorly (rho=0.56) with plasma citrulline since half of MTX-treated rats with severe mucositis (plasma citrulline concentration <30 µmol/L) had a reduced absorption of continuously administered glucose (<40%), but the other half absorbed glucose efficiently (>80%). Citrulline is therefore excluded as a suitable marker for absorption of continuously-administered glucose during mucositis. Apparently, MTX-induced damage to enterocytes does not evenly affect different properties of the enterocyte; absorption of glucose seems less affected than the production of citrulline, or glucose absorption is earlier recovered from mucositis. This could explain why some rats absorb glucose quite well, when administered continuously, while the production of citrulline is still low.

If we extrapolate our findings in rats with (mostly severe) mucositis to the clinic, where patients show similar histological and clinical symptoms of mucositis [2], they imply that glucose should not be enterally administered as a bolus to mucositis patients. Malabsorption of bolus-administered glucose during mucositis could possibly be detected via reduced plasma citrulline concentrations or via an OGTT. In contrast, enteral glucose administration by continuous infusion could be useful for a substantial portion of mucositis patients, in order to improve their nutritional state, recuperation and survival [9-11, 35]. Moreover, enteral nutrition might accelerate intestinal recovery since intraluminal nutrients have a stimulatory effect on intestinal epithelial cells and the production of trophic hormones [15, 36, 37]. We performed the glucose absorption tests at day 4 after injection with MTX or saline, while symptoms of mucositis are actually present from day 2 until day 5 after injection with MTX [6]. We did not study whether the observed glucose malabsorption in a portion of mucositis rats is structural (present on all days during mucositis) or temporal (only present on day 4). When glucose malabsorption is structural, indeed only a portion of mucositis patients would benefit from continuous enteral glucose administration. Then, a marker that distinguishes between mucositis patients with a good or poor glucose absorptive capacity is highly desirable in order to anticipate which patients would benefit the most from continuous enteral glucose administration. However, if glucose
malabsorption is temporal, all mucositis patients might benefit from continuous enteral glucose administration during mucositis.

In conclusion, we show that continuous enteral administration can almost completely overcome the reduced absorptive capacity for glucose in rats with severe chemotherapy-induced gastrointestinal mucositis, although glucose absorption differs substantially between individual rats. Our data suggest that glucose might be an appropriate source of dietary energy for at least a substantial portion of mucositis patients, when enterally administered continuously. Future studies should focus on studying the effects of continuous enteral glucose administration in mucositis patients.

ACKNOWLEDGEMENTS

The authors wish to thank Rick Havinga, Juul Baller, Theo Boer, Angelika Jurdzinski and Pieter Klok for excellent technical assistance in our studies.

GRANTS

This study was financially supported by an unrestricted research grant from Fonds NutsOhra.

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