Chapter 2

Effect of oral insulin on severity and recovery of methotrexate-induced gastrointestinal mucositis in the rat

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Introduction: Gastrointestinal (GI) mucositis is a severe side effect of chemo- and radiotherapy. Oral insulin has been suggested as possible intestinal growth factor and possible intervention for GI mucositis. We aimed to determine the effect of oral insulin on the severity and recovery of mucositis in a methotrexate (MTX)-induced GI mucositis rat model.

Methods: Male Wistar rats (n=24) received a single injection of 60 mg/kg MTX iv at day 0. From day -3 oral insulin was added to the drinking water. Group MTX received normal drinking water, group MTX+INS0.5 received 0.5 U/ml insulin and group MTX+INS1 received 1 U/ml insulin in drinking water. The severity of mucositis was determined by intake, bodyweight, illness and plasma citrulline. In the recovery phase the function of the gut was tested with an oral glucose tolerance test, and villus and crypt length of the small intestine were measured.

Results: MTX induced mucositis in all three groups and oral insulin did not cause a change in the severity of mucositis, with comparable bodyweight, food intake and water intake. Oral insulin did not alter the enterocyte mass, determined with plasma citrulline. The glucose level after bolus was higher in the MTX group compared to MTX+INS1 group (p<0.05). Histology was not significant different between all groups.

Conclusions: Oral insulin does not alter the severity or the acceleration of recovery of mucositis. Therefore, we conclude that it is not useful to further study oral insulin as possible intervention to prevent or treat chemotherapy induced GI mucositis.
INTRODUCTION

Gastrointestinal mucositis, further referred to as ‘mucositis’, is one of the most severe side effects of chemotherapy and radiotherapy [1,2]. The frequency of side effects has increased due to more intensive treatment protocols in cancer therapy. Mucositis has an estimated incidence of 40-100%, depending on treatment and patient related factors [2,3]. In children it is particularly high due to high doses of chemotherapy. Children with acute myeloid leukemia experience mucositis in 55% of the chemotherapy cycles [4]. Patients suffer from abdominal pain, vomiting, diarrhea, decreased weight and increased risk of developing an infection [3,5]. Eventually, mucositis can cause a reduction of the dosage, a delay or even discontinuation of the next chemotherapy course [2,3]. Unfortunately, although several interventions have been studied, to date there is no treatment, giving supportive care a primary role during mucositis in the pediatric oncology [3,5].

Currently, the understanding of mucositis is based on the five phase model of oral mucositis; although the precise mechanism of mucositis is not completely elucidated [1,6]. Stem cells are situated in the crypts and give rise to daughter cells continuously [7]. The entire intestinal epithelium is renewed every 3-5 days, and is therefore particularly vulnerable for chemotherapeutic agents [8-10]. From previous studies we know that nutrient digestion and absorption is decreased during mucositis [11-14]. Intestine specific growth factors cause mucosal proliferation and might be of great value for the protection of the intestinal stem cell during chemotherapy, and thereby for the maintenance of nutrient digestion and absorption. Furthermore, growth factors could accelerate the intestinal recovery.

Although the intestine is not the primary target of insulin and normally insulin is administered subcutaneously, oral insulin is an intervention with possible positive effects on the growth in the intestine [15]. From several studies in animal models concerning different intestinal diseases, a small dosage of oral insulin in the drinking water has been shown to cause an increase in villus length, enterocyte proliferation, decreased apoptosis, and a faster intestinal recovery [16-20]. These positive results show that oral insulin is a possible intervention to decrease the severity of mucositis and possibly accelerates recovery. Oral insulin has been studied when administered after chemotherapy, with positive effects on histological features [21]. However, it is unknown what the effect is if administration of oral insulin starts prior to chemotherapy. Moreover, it is unknown what the effect is on the intestinal absorptive function, in order to know if oral insulin can be of clinical relevance in the pediatric cancer patients suffering from mucositis. Therefore, we aimed to determine the effect of oral insulin on the severity and recovery in a previously established methotrexate (MTX)-induced gastrointestinal mucositis rat model.
MATERIAL AND METHODS

Animals and housing
Male Wistar outbred rats (4-5 weeks old, 82-108 gram) were obtained from Charles River (Sulzfeld, Germany). They were individually housed with a humidified temperature of 21°C and a 12:12 hours light-dark cycle from 7:00AM - 7:00PM. AIN93G diet and water were available ad libitum unless otherwise stated. The study protocol was approved by the Ethics committee for Animal experiments, Faculty of Medical Sciences, University of Groningen, Groningen, The Netherlands.

Materials
Methotrexate was obtained from Pharmachemie Holding B.V. (Haarlem, the Netherlands). AIN93G diet was obtained from Research Diet Services (Wijk bij Duurstede, the Netherlands). Insulin, Novorapid, was obtained from Novo Nordisk B.V. (Alphen aan den Rijn, the Netherlands).

Experimental methods
We performed a pilot experiment (male Wistar rats, n=9) with 0, 1 and 3 U/ml insulin in drinking water, to find an optimal dosage of insulin in our mucositis rat model. In the pilot experiment 1 U/ml insulin had an optimal effect, a higher dose of 3 U/ml insulin did not improve any parameter (data not shown). Based on the pilot experiment we decided to perform the experiment with three MTX groups.

Male Wistar rats were, after an acclimation period of 1 week, randomized into four groups; Group MTX (n=8) received 60 mg/kg MTX + normal drinking water. Group MTX+INS0.5 (n=8) received 60 mg/kg MTX + 0.5 U/ml insulin in drinking water. Group MTX+INS1 (n=8) received 60 mg/kg MTX + 1 U/ml insulin in drinking water. Moreover, a control group (n=5) was included, receiving no MTX but NaCl 0.9% iv, to show normal intake, relative bodyweight and histology.

At day 0 the rats received MTX 60 mg/kg (n=24) or NaCl 0.9% (n=5) intravenously (iv) in the dorsal penile vein under isoflurane anesthesia. The insulin was added to the drinking water from day -3 till day 6. The drinking water was freshly prepared every 48 hours, as done before [16]. Daily measurements of food intake, water intake, bodyweight, illness (bad fur, red nose, decreased activity) and diarrhea were documented. Plasma glucose levels were daily monitored in all MTX groups by blood sampling from the tail tip. To study the severity of mucositis sequentially, plasma citrulline was measured in blood samples obtained via the tail tip at day -3, at day 0 before MTX injection, and every 48 hours after MTX injection, as a marker for the enterocyte mass [4,22-24]. At day 6, to clinically evaluate the function of the gut in the recovery phase, the MTX rats were fasted for four hours and received a bolus mealsize oral glucose (2 g/kg, 30% glucose (glucosemonohydrate) in 1-2 ml Phosphate Buffered Saline (PBS) as described previously [13,25]) by oral gavage under light isoflurane anesthesia. Blood samples were obtained via the tail tip by
blood spot technique, at indicated time points, after 10, 20, 30, 40, 50, 60, 75, 90, 105 and 120 minutes. Blood glucose was measured using a LifeScan EuroFlash glucose meter (Lifescan Benelux, Beerse, Belgium). Three hours after the glucose bolus, the rats were euthanized under isoflurane anesthesia by opening the abdomen, obtaining blood from the vena cava inferior followed by cervical dislocation. Rats from the control group were euthanized at day 4 ($n=3$) and at day 10 ($n=2$). Blood samples were centrifuged 10 minutes at 2,000 g and stored at -20°C until further analysis. The small intestine was excised, divided in three parts (duodenum, jejunum and ileum), flushed with PBS and smaller parts (1 cm) were fixed in formalin, dehydrated and embedded in paraffin according to standard procedures for histology.

Analytical methods

Plasma citrulline concentration
Plasma citrulline concentration was measured in 30 µl plasma at room temperature using automated ion column chromatography, as described before [11,26,27].

Histology
Hematoxylin and eosin staining (H&E) was performed on 3 µm-thick sections of formalin-fixed duodenal, jejunal and ileal segments, as described previously [11]. H&E slides were scanned with the Aperio Scanscope for digitized images (Aperio Technologies, Vista, CA, USA). Villus and crypt length were blindly measured manually in well-orientated sections from digitized images, 10 measurements per rat evaluated at 10x magnification by using Aperio Imagescope software (Aperio Technologies).

Statistical analysis
Statistical analysis was done using SPSS for Windows version 22.0 (SPSS Inc., Chicago, IL, USA). Data represent medians and ranges in text, interquartile ranges in figures. Data was analyzed using Kruskall Wallis with Dunn-Bonferroni post hoc pairwise comparison test. A $p$ value < 0.05 was considered statistically significant.

RESULTS

Mucositis rat model
MTX induced mucositis in all three groups, with a decreased food intake, decreased water intake, bodyweight loss, and illness (bad fur, red nose and decreased activity). The overall incidence of diarrhea was non-significantly increased in the MTX+INS1 group, 75% ($p=0.124$), compared to 37.5% and 25% in MTX and MTX+INS0.5 group, respectively. One rat from the MTX group was
too ill and had to be terminated at day 6, based on humane endpoints, prior to the glucose tolerance test. All other rats developed mucositis and survived until the experiment ended on day 6.

**Bodyweight**

**Figure 1a** shows the relative bodyweight. The bodyweight was comparable in all groups before MTX injection. In comparison with controls, the relative bodyweight started to decrease from day 2 onwards, comparable in all MTX groups. At day 6 the relative bodyweight from the MTX and MTX+INS0.5 group started to recover, in contrast to the MTX+INS1 group which was the lowest, although there was no significant difference between the groups. Insulin did not cause any difference in relative bodyweight between the MTX groups.

**Intake**

**Figure 1b** shows the food intake per day. There was a decrease in food intake from day 2 after MTX injection in all groups, in comparison with controls, and an increase in food intake at day 5. There was no significant difference between MTX+INS0.5 and MTX+INS1 group and between the MTX and MTX+INS0.5 group. **Figure 1c** shows the water intake per day. The water intake was comparable in the healthy rats before MTX injection; therefore, the addition of insulin to the drinking water did not influence the amount of water intake. In comparison with controls the water intake decreased to the lowest level at day 4 and recovered at day 5, comparable in all MTX groups; oral insulin did not cause a significant difference between the groups.
Citrulline

Figure 2 shows the plasma citrulline level in healthy rats before insulin in the drinking water, before MTX injection, and after MTX injection. Oral insulin did not cause a difference in citrulline levels before MTX injection between groups. Citrulline levels decreased to the lowest level at day 4, compared to controls, and were comparable in all MTX groups, no effect of oral insulin at day 2 and 4. The citrulline level started to recover at day 6; again oral insulin did not influence the plasma citrulline level, which is comparable in all MTX groups at day 6.

Oral glucose tolerance test

Figure 3 shows the oral glucose tolerance test. The glucose level after a bolus meal size glucose was significantly higher in MTX group after 10 min (p<0.05), after 20 min (p<0.05), after 30 min (p<0.05), after 75 min (p<0.05), and after 105 min (p<0.05) compared to the MTX+INS1 group. The area under the curve (AUC) was significantly higher in the MTX group compared to the MXT-INS1 group. However, the most severely ill rat from the MTX group was terminated prior to the glucose test due to humane endpoints. There was no significant difference between the

Figure 2. Plasma citrulline. Plasma citrulline measured before insulin in drinking water, before MTX injection, and 2, 4, and 6 days after MTX injection. Data shows median and interquartile ranges. No significant differences. The dashed line shows median and interquartile ranges of controls from day 0 till day 6 (n=5, at day 6 n=2).

Figure 3. Oral glucose tolerance test. At day 6, after 4 hours fasting, a bolus glucose administered via oral gavage. A. Blood glucose levels after bolus, over time. B. Area under the curve of the blood glucose levels. MTX group n=7, one rat had to be terminated due to humane endpoints, before the glucose test. Data shows median and interquartile ranges. * p<0.05 MTX vs MTX+INS1.
MTX and MTX+INS0.5 group and no significant difference between the MTX+INS0.5 and the MTX+INS1 group.

**Histology**

*Figure 4* shows the jejunal villus and crypt length at day 6, which were not significantly different between all MTX groups, showing crypt hyperplasia in all MTX groups in comparison with controls. In duodenum there was also no significant difference between the MTX, MTX-INS0.5 and MTX-INS1 group in villus length (524.5 µm, 200-628; 437, 147-550; and 506.5, 78-623; respectively) and crypt length (292 µm, 169-314; 226, 160-279; and 234.5, 219-309; respectively). Furthermore, in the ileum there was no significant difference between the MTX, MTX+INS0.5 and MTX+INS1 group in villus length (377.5 µm, 322-425, 363, 296-518; and 448.5, 296-489; respectively) and crypt length (232 µm, 138-279, 234, 158-292; and 235, 162-322; respectively).

**Glucose level**

The glucose levels before MTX injection were comparable in all three MTX groups, as shown in *figure S1*. During the days of severe mucositis, the glucose level in plasma decreased in all groups probably due to the diminished food intake. At day 6 the glucose level in the MTX-INS1 group was significantly lower compared with the MTX-INS0.5 (p<0.05). There was no difference between the MTX-INS1 and MTX group, and between the MTX-INS0.5 and MTX group.
DISCUSSION

This study aimed to determine the effect of oral insulin on the severity and recovery in a previously established MTX-induced gastrointestinal mucositis rat model, in order to know if oral insulin might be of value for the pediatric cancer patients suffering from mucositis. Our data indicate that oral insulin did not influence the severity of MTX-induced gastrointestinal mucositis. Furthermore, the results suggest that oral insulin did not improve the intestinal absorption of glucose in the recovery phase, and did not accelerate the recovery after MTX-induced gastrointestinal mucositis.

The intestine is not the primary target of insulin and normally it is administered subcutaneously. Moreover, oral insulin is broken down in the acidic environment in the stomach and by digestive enzymes in the small intestine [21]. However, insulin receptors have been found on luminal and basolateral membrane of enterocytes [15]. Although the exact working mechanism has not yet been cleared, oral insulin has possible gut trophic effects. Oral insulin has been shown in animal models concerning different intestinal diseases, to increase mucosal weight, villus height, proliferation of enterocytes and gut growth, whereas apoptosis decreased [16-20]. In all these studies they administered 1 U/ml insulin in drinking water, comparable with the dosage we administered in our study. However, in healthy control rats oral insulin had no effect and in a diabetes animal model oral insulin decreased proliferation of epithelial cells [28]. These different local effects of oral insulin were explained by a possible difference between rapidly proliferating mucosa, for example after bowel resection or intestinal injury, and normal proliferation mucosa in healthy and diabetic rats [28].

In this study we measured plasma citrulline sequentially as marker for the enterocyte mass, which correlates with the severity of mucositis clinically, and with villus length in our mucositis rat model as shown previously [4,11,22-24,29,30]. Our data shows that oral insulin did not influence the enterocyte mass in healthy mucosa, during mucositis, or in the recovery phase. Therefore, this suggests that oral insulin does not alter the enterocyte mass, regardless of the proliferation state in this mucositis rat model.

Our results concerning the clinical parameters are in agreement with Sukhotnik et al., in which oral insulin also had no effect on bodyweight in a mucositis rat model [21]. However, the villus and crypt length were not altered in our study, in contrast to Sukhotnik et al.[21]. There are important differences between the studies. First, in our study we used a much higher MTX dosage, which is more comparable to the clinical practice in pediatric oncology, and another route of administration; therefore, we have a much more severe mucositis rat model. Second, Sukhotnik et al., only added oral insulin to the drinking water 72 hours after MTX injection, therefore the termination date and thereby the timing of the determination of histology is different, consequently, the studies are not directly comparable. Moreover, Sukhotnik et al., showed positive effects on histological features, but it was not determined if this had any effect on the
function of the intestine [21]. We know from previous studies that nutrient digestion and absorption is decreased during mucositis [11-14]. Therefore, in order to know if oral insulin might have any clinical relevance in improving the absorption of nutrients, we performed a function test of the gut, the glucose tolerance test at day 6, in the recovery phase. Our results did not show an increased glucose level after a glucose bolus in the insulin groups. This test suggests that the absorptive function of the small intestine in the recovery phase is not improved by oral insulin. However, there are some limitations to our study. First, it is possible that oral insulin has gut trophic effects other than in GI mucositis; we did not investigate this specifically, since this was not the purpose of the study. However, we can conclude that oral insulin did not affect the enterocyte mass in this mucositis rat model, regardless of the proliferation state, which is the clinically relevant question. Second, we do not know if starting the administration 3 days before MTX injection is long enough to alter the severity of mucositis. We can not exclude the possibility that oral insulin might be effective when the administration starts earlier, however we do not expect any difference since the enterocyte mass was not influenced by oral insulin.

In summary, this study determined the effect of oral insulin, started prior to MTX injection and continued after MTX injection, on the enterocyte mass and on the absorptive function in the recovery phase of mucositis. We have administered insulin in two concentrations in the drinking water, which did not improve the clinical parameters, histology, plasma citrulline, or glucose absorption. Our data suggest that oral insulin in drinking water does not influence the enterocyte mass in healthy rats. Furthermore, oral insulin does not alter the severity or the acceleration of recovery of mucositis. This suggests that oral insulin has limited clinical relevance in the prevention or treatment of mucositis in the pediatric cancer patients. Therefore, we conclude that it is not useful to further study oral insulin as possible intervention to prevent or treat chemotherapy induced gastrointestinal mucositis. Other interventions should be studied to prevent and/or treat this clinically very important side effect.

Acknowledgments

The authors wish to thank Angelika Jurdzinski for technical assistance in our studies.
REFERENCES


SUPPLEMENTAL FIGURES

Figure S1. Blood glucose levels. Blood glucose levels measured every morning before injection and/or blood sample. Data shows median and interquartile ranges. * p<0.05 MTX+INS0.5 vs MTX+INS1.