Original Research

Intestinal response to myeloablative chemotherapy in piglets

Peter L Pontoppidan[1], René L Shen[1], Bodil L Petersen[2], Thomas Thymann[1], Carsten Heilmann[3], Klaus Müller[3,4] and Per T Sangild[1]

[1]Department of Nutrition, Exercise and Sports, 30 Rolighedsvej, 1958 Frederiksberg C, Denmark; [2]Department of Pathology, Roskilde Hospital, 4000 Roskilde, Denmark; [3]Pediatric Clinic, Department of Rheumatology, Rigshospitalet, Blegdamsvej 3, 2100 Copenhagen Ø, Denmark; [4]Department of Rheumatology, Institute of Inflammation Research, Rigshospitalet, Blegdamsvej 3, 2100 Copenhagen Ø, Denmark

Corresponding author: Per T Sangild. Email: psa@life.ku.dk

Abstract

Chemotherapy-induced myeloablation prior to allogeneic hematopoietic stem cell transplantation (HSCT) may be associated with severe toxicity. The current understanding of the pathophysiology of oral and gastrointestinal (GI) toxicity is largely derived from studies in rodents and very little is known from humans, especially children. We hypothesized that milk-fed piglets can be used as a clinically relevant model of GI-toxicity related to a standard conditioning chemotherapy (intravenous busulfan, Bu plus cyclophosphamide, Cy) used prior to HSCT. In study 1, dose–response relationships were investigated in three-day-old pigs (Landrace × Yorkshire × Duroc, $n = 6$). Pigs were given one of three different dose combinations of Bu and Cy (A: 4 days Bu, 2 × 1.6 mg/kg plus 2 days Cy, 60 mg/kg; B: 4 days Bu, 2 × 0.8 mg/kg plus 2 days Cy, 30 mg/kg; C: 2 days Bu at 2 × 1.6 mg/kg plus 1 day Cy, 60 mg/kg) and bone marrow was collected on day 11. Histology of bone marrow samples showed total aplasia after treatment A. Using this treatment in study 2, Bu–Cy pigs showed lowered spleen and intestinal weights and variable clinical signs of dehydration, sepsis, and pneumonia at tissue collection. Oral mucositis was evident as ulcers in the soft palate in 4/9 Bu–Cy pigs and villus height and brush-border enzyme activities were reduced, especially in the proximal intestine. There were no consistent effects on tissue cytokine levels (IL-8, IL-6, IL-1β, TNF-α) or blood chemistry values (electrolytes, liver transaminases, bilirubin, alkaline phosphatase), except that blood iron levels were higher in Bu–Cy pigs. We conclude that a myeloablative Bu–Cy regimen to piglets results in clinical signs comparable to those seen in pediatric patients subjected to myeloablative treatment prior to HSCT. Piglets may be used as a model for investigating chemotherapy-induced toxicity and dietary and medical interventions.

Keywords: Mucositis, gastrointestinal toxicity, busulfan, cyclophosphamide, pig, intestine

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Introduction

Treatment-related toxicity in the gastrointestinal (GI) tract is a major complication in relation to cytotoxic treatment, especially prior to hematopoietic stem cell transplantation (HSCT). It is thought to play an important role in the development of treatment-related complications including septicemia, organ failure, and graft versus host disease.[1,2] Accordingly, a more detailed understanding of the pathophysiology of the development of GI toxicity and the subsequent inflammatory reactions is important for the development of therapeutic interventions and prognostic markers. Clinical management of mucositis is usually restricted to oral hygiene, pain management, and the use of prophylactic antibiotics, targeting downstream effects of tissue injury and leaky intestinal barriers, rather than controlling the early events that initiate this process.[3] Preclinical animal models for chemotherapy-induced GI toxicity have been developed and this has contributed to our knowledge about the mechanisms leading to GI toxicity.[4] According to these, the development of oral and GI toxicity follows a common pathological pattern,[5] although differences in cell turnover, cell differentiation and composition of the GI microbiota may influence the sensitivity and kinetics of mucositis in each GI region.[6]

Several potential drug targets for alleviating the effects of chemotherapy-induced mucositis have been tested, such as transforming growth factor-β,[7] interleukin (IL) -11,[8] and keratinocyte growth factor (KGF),[9] but the effects have been difficult to prove due to low sample size, variable age ranges, and variations in clinical treatment regimens.[10,11] The use of pre-clinical large animal models,

such as pigs, that reflect more closely the human GI anatomy and physiology could be important for testing new treatment modalities. The piglet may be particularly relevant in pediatric research due to its similarity with infants in terms of diet, anatomy, and physiology of the GI tract.[12,13] Existing rodent models are based mainly on the use of adult or post-weaning animals and these may be less relevant for pediatric settings, including the aspects related to milk diets, organ maturation, and body growth. The local GI immune system and response to endotoxins[14] in pigs are similar to conditions in man, and drug metabolism related to enzymes of the cytochrome P450 super family[15] also appear closer to conditions in man, relative to conditions in rodents.[16] Piglets have a size that allows for a wide range of surgical procedures, longitudinal blood sampling, and nutritional modalities that easily allow enteral and parenteral nutrition options. Previous chemotherapy models in rodents have not attempted to copy the exact (and often complex) treatment regimens in humans but have usually tested single drugs administered intra-muscularly or peritoneally. Observation and quantification of clinical signs, such as vomiting and diarrhea, are also easier in pigs, relative to rodents, but until now piglets have been used seldom in mucositis research.[17]

We hypothesized that it would be feasible and relevant to use young milk-fed piglets as a model for chemotherapy-induced mucositis, using a standard myeloablative conditioning regimen for pre-transplant conditioning in relation to hematopoietic bone marrow transplantation. We first established the cytotoxic chemotherapy-dosing regimen that would result in total bone-marrow aplasia within an 11-day experimental protocol. Using this regimen, we investigated changes in clinical parameters, blood chemistry, organ dimensions and GI tract histology, brush-border enzymes, and cytokine responses to chemotherapy treatment.

Materials and methods

Animals, their preparation, and clinical care procedures

A total of 22 piglets (Landrace × Yorkshire × Duroc) from different litters and of both sexes were included and brought to the research facilities at 3 days of age, after having suckled their mother from birth. On the same day, the pigs were anesthetized using a zoletil mix (i.m. tiletamine, 0.28 mg/kg; zolazepam, 0.28 mg/kg; xylazine, 0.56 mg/kg; ketamine, 0.56 mg/kg; butorphanol, 0.11 mg/kg). During anesthesia, the pigs were fitted with an orogastric tube (6F Portex, Kent, UK) inserted through the cheek and secured with sutures. A catheter (Tygon, OD 0.070 mm, ID 0.040 mm, Saint-Gobain Performance Plastics, France) was inserted into the external jugular vein, tunneled under the skin and exteriorized on the dorsal part of the neck and used for blood sampling and administration of drugs and saline. The pigs were housed in individual cages and fed bovine colostrum at a total volume of 165 mL/kg/day divided into 11 separate feedings. Pigs were allowed to eat voluntarily from individual troughs unless appetite was reduced. In such case, the remaining volume was fed through the orogastric tube. Bovine colostrum was chosen as the enteral diet in our initial model studies because this fluid has a standardized, high-protein nutrient content with well-documented beneficial effects on intestinal structure and function in newborn pigs.[18]

The piglets received the first dose of chemotherapy or saline at the day after arrival (=day 1) and were killed for tissue collection on day 11. In parallel with the cytotoxic treatment (Table 1), pigs received supportive medication (Table 2). According to predefined humane endpoints, pigs were sacrificed earlier than day 11 if they were found to be in a clinical condition of severe discomfort. Humane endpoints were based on clinical observations evaluating their behavior, circulatory and respiratory indices (Table 3), clinical signs of pain despite analgesia treatment (enteral paracetamol, 7.5 mg/kg, and buprenorphine, 0.3 mg/kg i.v. or i.m.), or severe signs of GI toxicity with bloody feces. Pigs euthanized before day 7 were not used for further tissue analyses. All procedures were approved by the Danish Inspectorate on Animal Experimentation.

Cytotoxic treatment

The cytotoxic regimes used were all based on busulfan (Bu, Pierre Fabre Pharma, Sollentuna, Sweden) and cyclophosphamide (Cy, Baxter, Birkerød, Denmark, Table 1). The combination of Bu and Cy was chosen as it is widely used as the conditioning regime prior to HSCT.[19] Both Bu and Cy were administrated according to the manufacturers' protocol, using the jugular catheter with an infusion period of 1 h. To protect against cyclophosphamide-induced hemorrhagic cystitis, all pigs received 24 hours i.v. hyperhydration based on their body surface area (3.7 L/m^2/d) with isotonic fluids.[20] The hyperhydration period began 3 h before each Cy infusion and furosemide was added at time points 0, 3, 6, and 9 h, and uromitexan at 0, 6, and 9 h after Cy infusion (Table 2). Body surface area (BSA, m^2) was calculated using the formula (BSA = 70 × kg body weight$^{0.75}$/1000).[21]

Table 1 Cytotoxic regimens used in study 1 and study 2

Treatment	A	B	C
Busulfan[a]	4 × 1.6 mg/kg	4 × 0.8 mg/kg	2 × 1.6 mg/kg
Cyclophosphamide[a]	2 × 60 mg/kg	2 × 30 mg/kg	1 × 60 mg/kg

[a]Intravenous administration via jugular vein catheters

Study 1

Six pigs were allocated to receive three different Bu–Cy regimens that varied in doses and number of infusions (Table 1, each $n = 2$). The study was designed to investigate if a standard Bu–Cy conditioning could cause total myeloablation in piglets. The main endpoint of Study 1 was cellularity in the bone marrow, as evaluated by histology.

Study 2

Eighteen pigs were allocated to receive the Bu–Cy treatment A (Table 1, $n = 10$) or saline (controls, $n = 8$) following the 11-day protocol as in study 1. Study 2 was conducted during three different study periods with equal representation of Bu–Cy and control pigs. The two Bu–Cy pigs with treatment A from study 1 were included in the data analysis. Body weight, diarrhea, and vomiting were monitored daily. Blood samples were collected before the first dose of Bu and again at day 4, 7, 9, and 11 for complete blood count including total white blood cells (wbc), neutrophils (neu), lymphocytes (lym), red blood cells (rbc), reticulocyte percentage (ret), and platelet count (plt). Blood chemistry values (ALAT, ASAT, alkaline phosphatase, total bilirubin, GGT, creatinine, carbamide, creatine kinase, albumin, total protein, globulin, total iron, sodium, potassium, phosphate, calcium, magnesium) were measured in serum samples drawn at day 1, 7, and 11. Blood hematology and blood chemistry were analyzed using the Advia 2120 Hematology System (Siemens Germany). At day 1, 7, and 10, intestinal hexose absorptive capacity was evaluated with an oral galactose test where a bolus (15 mL/kg) of a 5% galactose solution (Sigma, St Louis, MO, USA) was given via the oro-gastric tube. Blood samples were collected at $t = 0$ min and $t = 20$ min after the bolus and plasma galactose levels were measured spectrophotometrically (Pentra 400, Horiba ABX, Montpellier, France) using a commercial kit.[22] Before euthanasia (3–5 h) at day 11, the piglets received a bolus (15 mL/kg) of a 5% lactulose (Sigma) and 5% mannitol (Sigma) solution. A urine sample was collected during tissue sampling by cystocentesis. Urinary concentrations of lactulose and mannitol were determined spectrophotometrically (Pentra 400) and intestinal permeability was expressed as the ratio of lactulose to mannitol1.[18,23]

Tissue collection

At day 11, pigs were anesthetized using the zoletil mix (see above) for collection of a blood sample by intracardia puncture, followed by euthanasia with sodium pentobarbital, 200 mg/kg. The abdomen was opened and the wet weights of all internal organs were recorded. The small intestine was divided into three segments of equal length, designated proximal, middle, and distal segments. For each segment, three pieces of 0.5 cm were collected and fixed in 4% neutral buffered formalin. After fixation, they were dehydrated with ethanol, embedded in paraffin and cross-sectioned (5 μm), mounted on glass slides, and stained with hematoxylin and eosin. Further, 1 cm pieces were snap frozen for later analyses of enzyme activities and cytokines

Table 2 Supportive drugs and fluids incl. drugs for hyperhydration

Supportive drugs	Dose and time	Administration route
Ciprofloxacine	20 mg/kg × 2 daily	Orogastric tube
Metronidazole	40 mg/kg × 3 daily	Orogastric tube
Fluconazole	0.3 mg/kg × 1 every 3rd day	Orogastric tube
Acepromazine	0.1 mg/kg × 2 daily	Orogastric tube
Clonazepam	3 mg/kg × 2 daily, day 1–4	Orogastric tube
Allopurinol	2.5 mg/kg × 2 daily, day 1–7	Orogastric tube
Hyperhydration		
Uromitexan	20% of Cy dose × 4 daily, day 5–6	Intravenous
Furosemide	6 mg/m^2 × 6 daily, day 5–6	Intravenous
Isotonic sodium bicarbonate	1450 mL/m^2 daily, day 5–6	Intravenous
Isotonic sodium chloride	860 mL/m^2 daily, day 5–6	Intravenous
Ringer's solution	575 mL/m^2 daily, day 5–6	Intravenous
Isotonic glucose potassium phosphate	870 mL/m^2 daily, day 5–6	Intravenous

Table 3 Clinical assessment of pigs in study 2

Score	0	1	2
Behavior and general activity	Normal activity, interested	Depressed, lying down but able to stand when stimulated	Not able to stand when stimulated, unresponsive
Respiratory and circulatory function	Normal skin and snout colors, warm extremities, calm breathing	Cyanosis of snout, cold extremities, calm breathing	Criteria for score 1 + tachypnea and/or retractions

levels. Finally, a 10 cm piece of the distal segment was collected for determining the proportion of wet and dry mucosa in the intestine. A piece of the sternum was collected for histology as above to assess bone marrow cellularity, expressed relative to that in a healthy three-day-old piglet. Signs of red and white cell regeneration were visually evaluated in a blinded manner by an experienced pathologist.

Intestinal morphology

Villus heights and crypt depths were determined on histology slides using an Axiophot light microscope (Carl Zeiss, Oberkochen, Germany) and ImageJ software (version 1.22 c US National Institutes of Health, Bethesda, MD, USA). At least 10 well-oriented villi and crypts from each segment were used to determine average villus height and crypt depth for each section. Mucosal proportions of the small intestine (distal segment) were determined on a 10-cm piece, slit along the length. The mucosa was scraped off, leaving the muscular layer. Samples were dried at 60°C for at least 72 h before weighing the two layers again. The proportions of dry mucosa and intestinal dry matter contents were determined.[24]

Intestinal brush border enzymes and cytokines

Frozen intestinal samples (proximal, middle, and distal) were extracted in 1% Triton X-100 (10 mL/g, tissue, Sigma-Aldrich, St. Louis, MO, USA) and homogenized (0°C, 2×25 s.). To the Triton X-100 solution for determination of cytokine levels, a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 10% protease inhibitor. Activity of disaccharidases (lactase, maltase, and sucrase) and peptidases (aminopeptidase N, ApN; dipeptidylpeptidase IV, DPPIV; aminopeptidase A, ApA) were determined, as previously described.[25] For all enzymes, a hydrolytic rate of 1 μmol substrate released per min at 37°C represents one unit of enzyme activity. Enzyme activities were expressed per gram of wet intestine (proximal, middle, and distal). Cytokine protein levels in the proximal and distal segments were determined using DuoSet ELISA Development kit (R&D Systems, Abingdon, UK) targeted against porcine IL-8, IL-6, IL-1β, and TNF-α according to manufacturer's instructions and concentrations were measured spectrophotometrically.

Statistics

Differences between groups were tested with the *t*-test for standard parametric data and with Fisher's exact test for categorical data. Longitudinal data were analyzed using linear regression analysis for mixed effects, using the mixed function in STATA (Stata, 2011; Stata Statistical Software: Release 12. College Station, TX: StataCorp LP). All parametric variables were checked for normality and distribution of residuals and they were transformed according to the Box-Cox procedure, if required. Data are shown as mean ± SEM and a probability of $p < 0.05$ was considered significant.

Results

Study 1

The three different dose regimens were tested to identify the dose that resulted in total myeloablation (see Table 1). Evaluation of the bone marrow at day 11 showed a myeloablative effect of all treatments, but only treatment A resulted in total bone marrow aplasia (Figure 1). Bone marrow from piglets receiving treatments B and C showed signs of regeneration of marrow function at the time of sampling.

Study 2, clinical observations

GI response to treatment A was then investigated in more detail and compared to saline controls. Four of the 10 Bu–Cy pigs, and none of the control pigs, were euthanized due to humane endpoints before the planned termination of the experiment on day 11. No difference in weight gain between the two groups was found. Pigs that were killed before the end of the protocol showed clinical signs of compromised circulation and respiration, and in three of the four euthanized pigs, macroscopic signs of pneumonia were detected. Macroscopic signs of pneumonia were also found in 6 of the 10 Bu–Cy pigs but in none of the eight control pigs ($P < 0.01$). The number of days with diarrhea was significantly higher in the Bu–Cy pigs than in the controls (2.9 ± 0.4 vs. 1.0 ± 0.3 days, $P < 0.01$). Likewise, ulcers in the soft palate were observed at autopsy in 44% (4/9) of the Bu–Cy pigs, but not in any pigs from the control group sacrificed after day 9 (Figure 2(a) and (b), $P < 0.05$). Relative weights of the stomach, and lungs were elevated in the Bu–Cy pigs, while spleen weight was markedly reduced (Table 4).

Haematology and blood chemistry

Bone marrow cellularity was <10% in the Bu–Cy pigs compared with 76 ± 7% in the control group ($P < 0.001$). From day 7 onwards, Bu–Cy pigs showed lower total counts of leucocytes, neutrophils, and lymphocytes than the control pigs (Figure 3). By day 11, the mean leukocyte count in the Bu–Cy pigs was $0.13 \pm 0.02 \times 10^9$ cells/L, compared with $8.4 \pm 0.7 \times 10^9$ cells/L in the control pigs ($P < 0.001$). Relative to controls, the decline in platelets and reticulocytes occurred later in the Bu–Cy group, and a significant difference between Bu–Cy and control pigs was not detected until day 9. A reduction in reticulocytes was observed in both groups during the study period. However, reticulocyte percentage at day 11 was significantly lower in the Bu–Cy pigs (0.3 ± 0.2 vs. 1.8 ± 0.6%, $P < 0.01$) compared with control pigs. By day 11, mean red blood cell count was 3.9 ± 0.4 vs. 4.8 ± 0.2 cells^9/L in the Bu–Cy and control pigs, respectively (Figure 3, $P = 0.09$).

During the study period, a decline in plasma transaminase levels (ALAT and ASAT), bilirubin, and alkaline phosphatase was observed in both groups, but at day 7 and 11, ASAT levels were higher in the Bu–Cy pigs, while alkaline phosphatase levels were lower. Total serum iron levels were significantly elevated in the Bu–Cy pigs at days 7 and 11 and tended to be higher in Bu–Cy pigs at day 7, compared

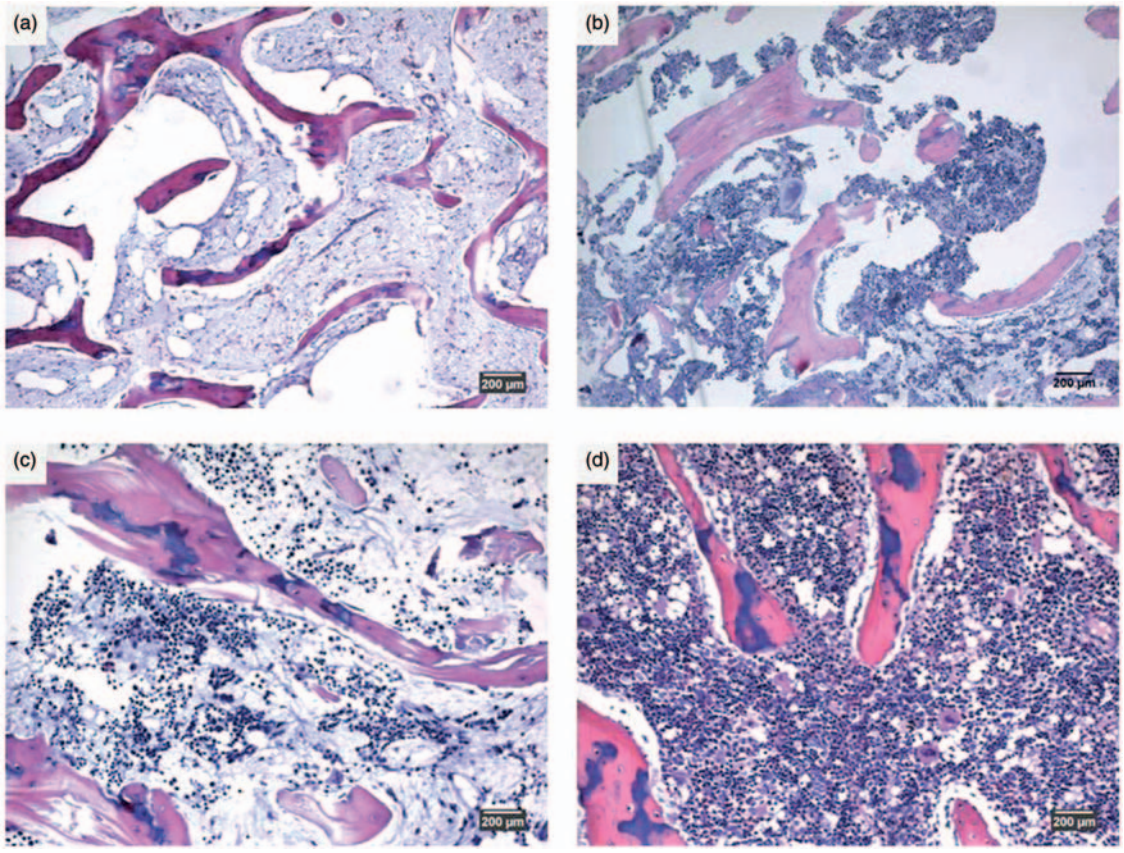

Figure 1 Histological slides of bone marrows from pigs treated with Bu–Cy. (a) treatment A, (b) treatment B, (c) treatment C, and (d) a healthy, un-treated 3 d-old pig. Magnification: ×40. (A color version of this figure is available in the online journal)

with day 11 (43.8 ± 3.6 mmol/L vs. 34.2 ± 14.6 mmol/L, $p = 0.11$, Figure 4).

Intestinal structure, function and inflammatory mediators

Relative weight (g tissue/kg body weight) of the small intestine was lower in Bu–Cy pigs, compared with controls (32.8 ± 2.3 vs. 37.2 ± 1.2 g/kg, $P < 0.001$, Table 4) but without any change in the proportion of mucosa or the relative content of water (indicating no difference in degree of edema). Relative to controls, villi were shortened especially in the proximal segment (−39%, $p < 0.001$) that also showed a tendency towards a shorter crypt depth (−14.2%, $p = 0.08$; Figure 5). Figure 2(c) and (d) show representative histological slides of the small intestine from control and Bu–Cy pigs, respectively.

Bu–Cy pigs showed 30–40% less proximal intestine ApA, ApN, and lactase activities than control pigs ($P < 0.05$), and the middle intestine also showed reduced hydrolase activities (45–60% for lactase, ApA, ApN, and DPPVI). In the distal intestine, only ApN activity was reduced by the Bu–Cy treatment (−35%, $p < 0.05$, Figure 6). No significant differences were seen for the disaccharidases, sucrase, and maltase, between groups in any segment of the small intestine. Likewise, no differences were observed in the galactose uptake capacity between the groups on days 1, 7, and 10. In the proximal intestinal segment IL-8 tended to be higher in Bu–Cy pigs, compared with controls (7187 ± 1106 vs. 5233 ± 646 pg/g, $P = 0.13$), with no difference in the distal segment. No treatment-related differences in cytokine (IL-6, IL-1β, TNF-α) concentrations were detected in the proximal or distal intestine (Table 5). The mean lactulose–mannitol ratio was six-fold higher in Bu–Cy versus control pigs (0.12 ± 0.17 vs. 0.02 ± 0.01), but due to high variation in values from Bu–Cy pigs, this difference did not reach statistical significance.

Discussion

Mucositis is an early event in the pathophysiology of treatment-related complications associated with chemotherapy. Hence, a more detailed understanding of the pathological process of mucositis may lead to more effective treatment and prophylaxis of chemotherapy-induced toxicity. Due to limited number of pediatric chemotherapy patients, and their variable ages, genetics, and clinical disease conditions, it is difficult to perform well-controlled clinical studies of the progression of tissue pathology and possible new interventions. Finally, a main limitation is the general inaccessibility of tissues that may be affected by chemotherapy. In this study, we have shown that a chemotherapy regimen that is commonly used for children prior to HSCT[19] produces a number of the clinical manifestations in piglets that are also seen in HSCT patients. In addition, we provide

baseline data on a number of tissue variables that are normally impossible to investigate in human studies.

Complete myeloablation was attained in piglets with a standard busulfan/cyclophosphamide regimen that also induced many of the clinical and laboratory changes seen in pediatric patients subjected to high dose cytotoxic chemotherapy.[26] The major complications of chemotherapy are thought to involve down-stream effects of the primary tissue injury, induced initially by the direct cytotoxic effects of chemotherapy. According to the current theory of mucositis pathology,[5] chemotherapy initially induces apoptosis and production of reactive oxygen species, as well as activation of NFκB. This leads to induction of inflammatory cytokines, further apoptosis, and tissue damage. In the subsequent amplification step, pro-inflammatory molecules act in a positive feedback loop to enhance the primary response leading to ulceration. Direct contact between proliferating pathogens, toxins, and the underlying submucosal layers facilitates the process.[27,28] In our 11-day protocol, Bu–Cy regimen produced relatively mild degrees of oral ulcers, and only mild histological intestinal damage. Intestinal damage, assessed by villus height, was most pronounced

Table 4 Organ weights and dimensions in Bu–Cy and control pigs in study 2

	Bu–Cy ($n = 7$–9)	Control ($n = 7$–8)
Body weight, day 1 (kg)	1.93 ± 0.1	1.86 ± 0.1
Body weight, day 11 (kg)	2.46 ± 0.2	2.44 ± 0.2
Daily weight gain (g/kg/day)	23.6 ± 4.4	26.9 ± 4
Stomach (g/kg)	7.0 ± 0.1*	6.2 ± 0.2
Intestine (g/kg)	32.8 ± 2.3*	37.2 ± 1.2
Dry mucosa (%)	49.5 ± 4.2	47.2 ± 2.6
Intestinal water (%)	79.7 ± 1.3	81.3 ± 0.7
Intestinal circumference (mm)	14.7 ± 0.5	16.0 ± 1.2
Colon (g/kg)	9.6 ± 1.2	12.4 ± 2.3
Liver (g/kg)	37.7 ± 1.6	34.9 ± 3.5
Spleen (g/kg)	1.8 ± 0.2*	4.3 ± 0.2
Lungs (g/kg)	31.4 ± 4.8*	20.6 ± 2.0
Heart (g/kg)	8.3 ± 0.9	7.9 ± 0.6
Kidneys (g/kg)	10.0 ± 0.4	10.4 ± 1.0

Note: Organ weights are relative to body weight of the animal at sampling time, mean ± SEM.
*Mean value in Bu–Cy pigs is different from that in control pigs ($p < 0.05$).

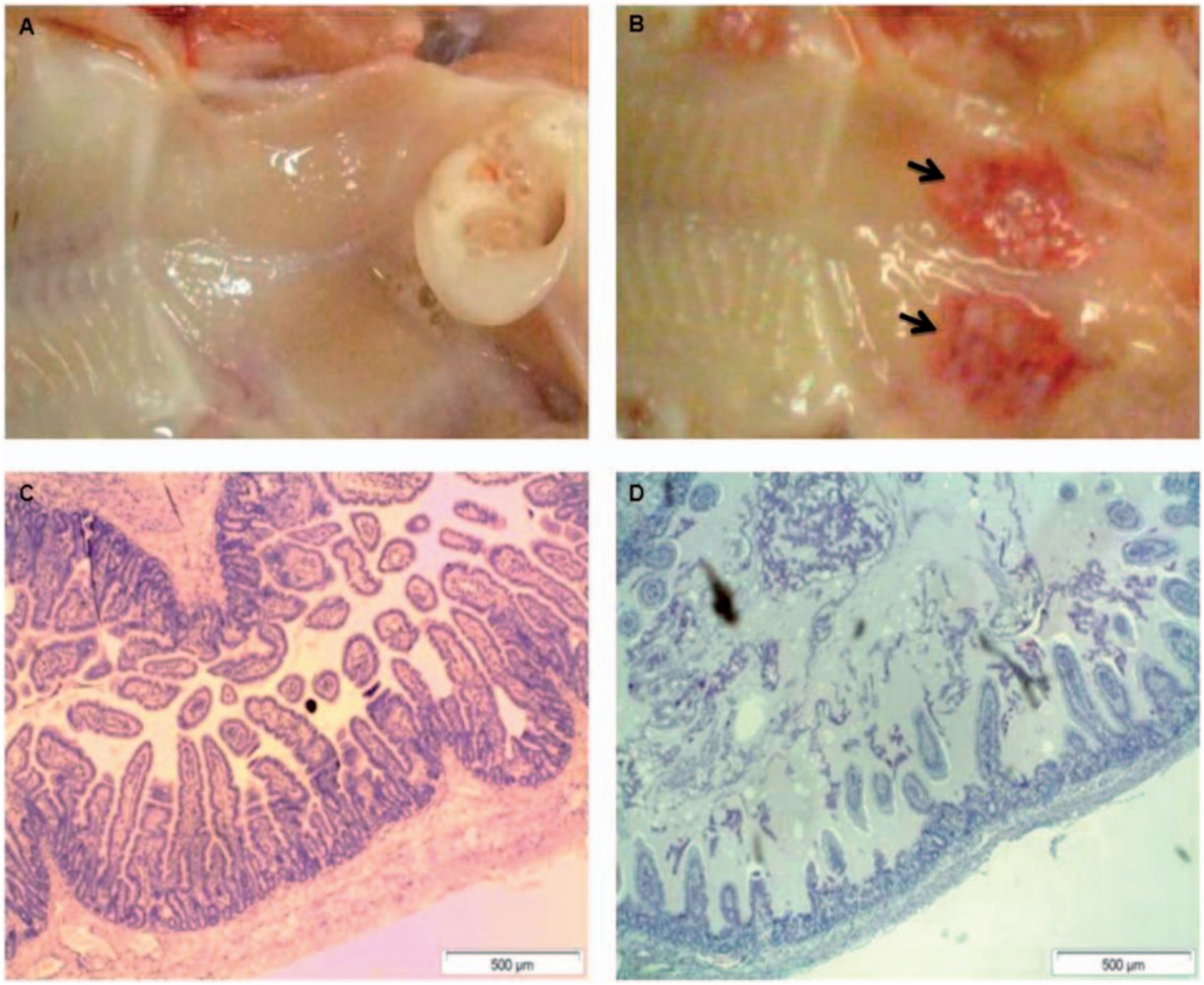

Figure 2 Photograph of oral cavity in a control (a) and Bu–Cy pig (b). Arrows indicate symmetrical lesions in the soft palate which did not cross the midline. Histological slide of control (c) and Bu–Cy pig (d), magnification ×40. (A color version of this figure is available in the online journal)

in the proximal and distal segments of the small intestine, indicating local differences in the factors influencing destruction of the mucosal structure and function. A much higher enterocyte cell turnover in the proximal intestine[29] may account for the more pronounced effects in this region. On the other hand, a higher bacterial density in the distal intestine may stimulate the damaging processes in this region.[27,28] The greater exposure of the proximal intestine to enteral diets may facilitate the possible anti-mucositic effects of certain dietary components. The proportions of dry mucosa were not affected by Bu–Cy treatment, but mucosal atrophy was evident based on reduced villus height and reduced relative small intestinal weight. This indicates that tissue destruction after cytotoxic chemotherapy is not restricted to the mucosal surfaces in the GI tract, but affects also the deeper compartments.

Our findings are in line with the clinical finding in patients receiving HSCT-relevant chemotherapy where oral mucositis is present a few days after last dose of chemotherapy.[30] Only in a few studies has small intestinal histology been evaluated in studies on adult humans.[31,32] In both studies, intestinal biopsies showed mucosal atrophy to be present two to three days after chemotherapy was administrated, and in the study of Keefe et al. villus area,

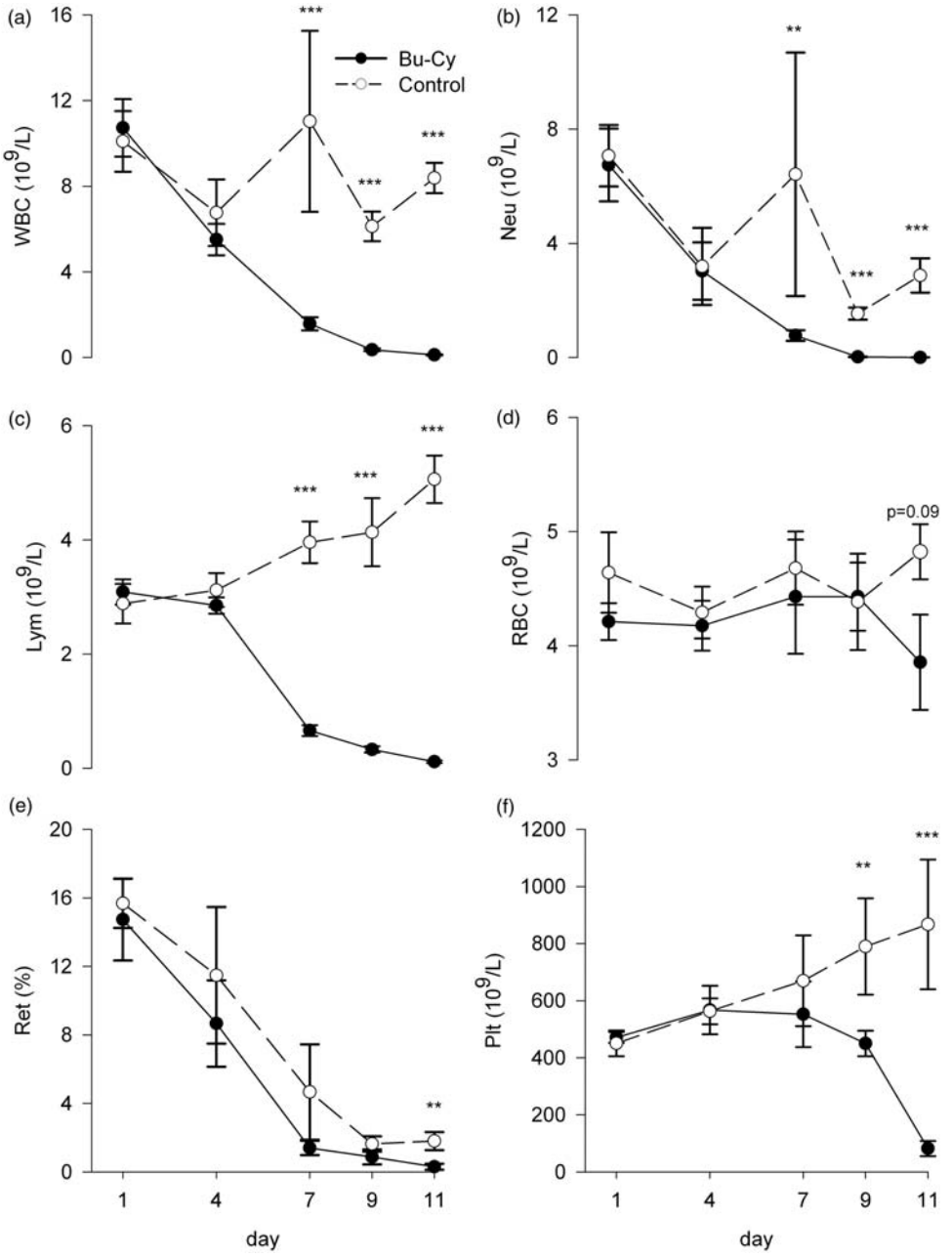

Figure 3 Circulating hematological parameters: (a) white blood cells (WBC), (b) neutrophils (Neu), (c) lymphocytes (Lym), (d) red blood cells (RBC), (e) reticulocyte percentage (Ret), and (f) platelets in Bu–Cy (solid line, black circles, $n = 10$–6) and control (broken line open circles, $n = 4$–7). Points represent mean values ± SEM (vertical bars). **, ***Mean value in Bu–Cy pigs is significantly different from that in control pigs ($P < 0.01$ and $P < 0.001$, respectively)

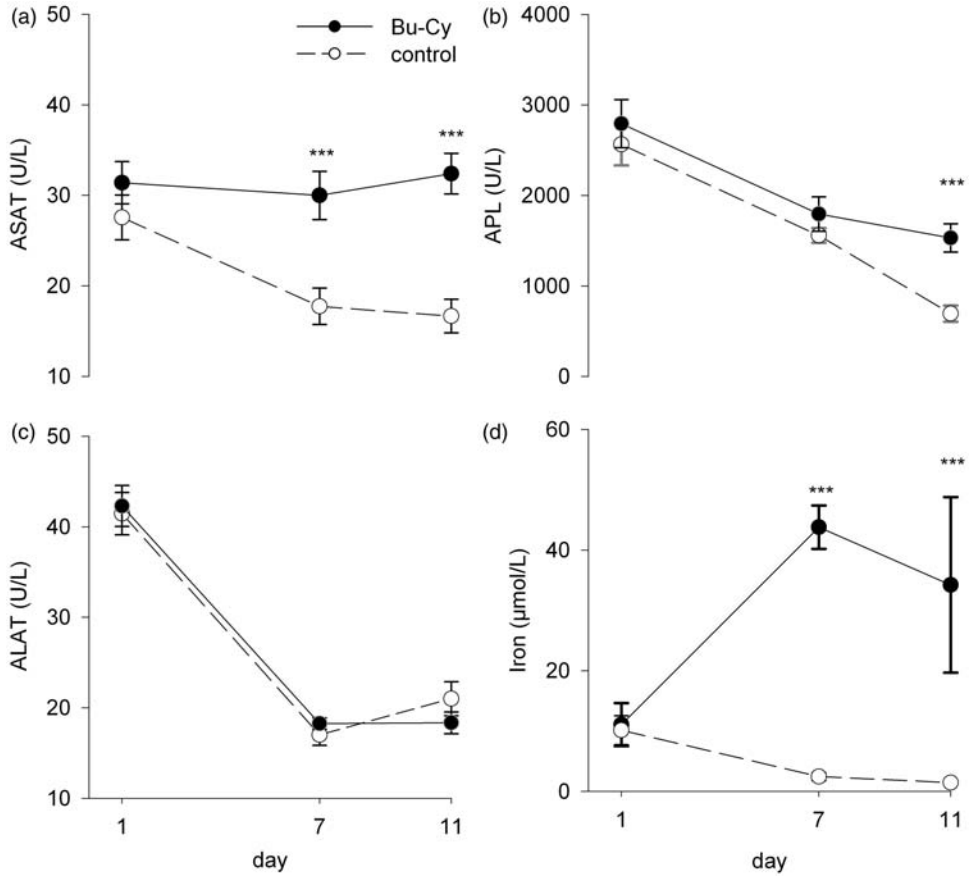

Figure 4 Selected serum biochemistry, (a) ASAT, (b) APL, (c) ALAT, and (d) total iron in Bu–Cy (solid line, black circles, $n = 5$–10) and control (broken line open circles, $n = 3$–7) during the 11-day protocol. Points represent mean values ± SEM (vertical bars). ***Mean value in Bu–Cy pigs is significantly different from that in control pigs ($P < 0.001$)

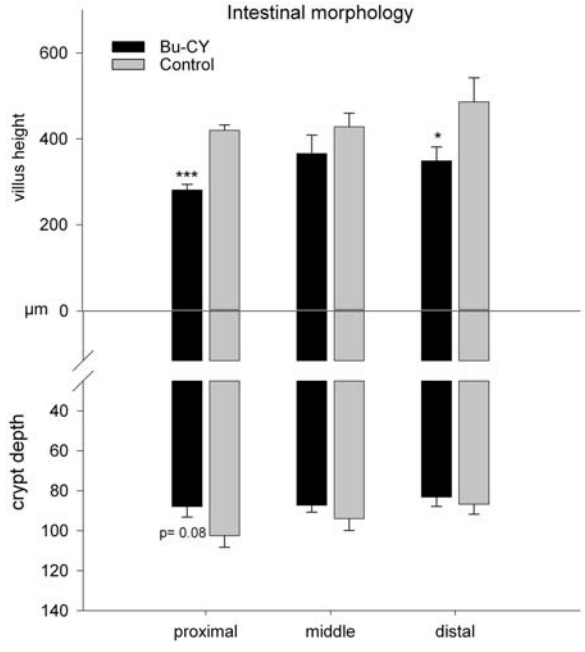

Figure 5 Villus height and crypt depth in proximal middle and distal segment of the small intestine of Bu–Cy pigs (black bars, $n = 9$) and control pigs (gray bars, $n = 8$). Points represent mean values ± SEM (vertical bars). *, ***Mean value in Bu–Cy pigs is significantly different from that in control pigs ($P < 0.05$ and $P < 0.001$, respectively)

crypt depth and mitotic index were normalized on day 16. This corresponds with findings in rodent models[33] and may explain why villus heights, and not crypt depths, were significantly affected in present study at day 11. It is indicated that the small intestine had already entered into a phase of tissue regeneration with proliferating crypt cells. Both the above studies in human adults found no evidence of reduced disaccharidase activity after chemotherapy treatment. This contrasts with our results on lactase activity and some findings in rodent models.[34] Data on GI functions, such as brush border enzyme activities, are limited from studies on children subjected to high-dose chemotherapy, but likely the effects could be more pronounced than in adults. Unlike the 5-FU model of intestinal toxicity in pigs,[34] our model does not require food restriction before administration of chemotherapy to induce mucositis in the oral cavity and intestine. This may be explained by differences between the 5-FU and Bu–Cy products as well as their length of treatment (once a week for 4 weeks for 5-FU versus daily administration of Bu–Cy for 6 days, respectively). Overall, the cytotoxic load may have been much higher in the present pig study compared with the above 5-FU study.

A clear trend towards elevated intestinal permeability, as indicated by a higher mean lactulose to mannitol ratio in

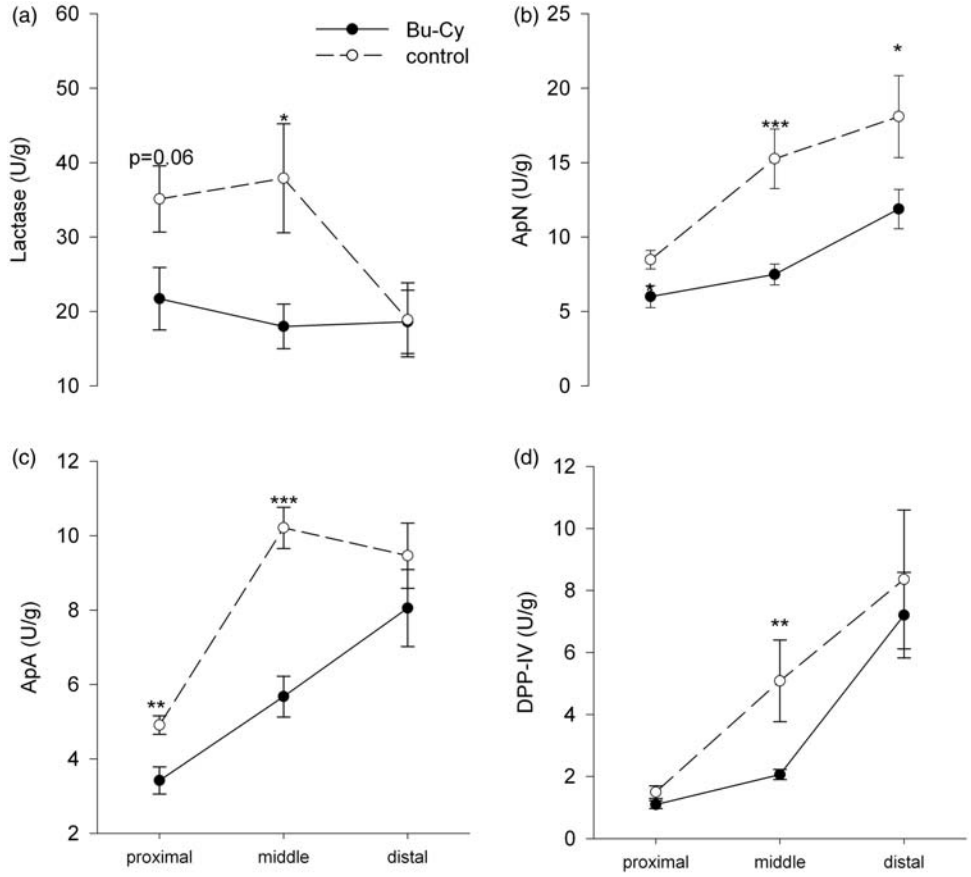

Figure 6 Brush-border enzyme activities for (a) lactase, (b) ApN, (c) ApA, and (d) DPP-IV; mean ± SEM in proximal, middle, and distal segments of the small intestine for Bu–Cy (solid line, black circles, $n = 9$) and control (broken line, open circles, $n = 8$). *, **, ***Mean value in Bu–Cy pigs is significantly different from that in control pigs ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively)

Table 5 Small intestinal cytokine levels at day 11 in Bu–Cy and control pigs in study 2

	Proximal		Distal	
	Bu–Cy ($n = 9$)	**Control ($n = 8$)**	**Bu–Cy ($n = 9$)**	**Control ($n = 8$)**
IL-8	7187 ± 1049	5233 ± 646	7788 ± 780	6631 ± 972
IL-6	115 ± 73	32 ± 23	129 ± 69	55 ± 27
IL-1β	402 ± 244	119 ± 29	200 ± 102	126 ± 53
TNF-α	69 ± 11	65 ± 121	169 ± 15	223 ± 68

Note: Values are cytokine concentration (pg/g tissue), mean ± SEM.

urine, was found in the Bu–Cy group. We did not directly investigate bacterial translocation in this study, but the Bu–Cy pigs showed clinical sings of breathing and circulatory distress, and at autopsy we observed hemorrhagic lung lesions indicating sepsis and/or pneumonia. General dehydration and direct cytotoxic effects the lung tissue are other possible contributors to the observed lung pathology. Bacterial translocation is among the factors that may explain that Bu–Cy pigs were more sensitive to infections than controls. Bu–Cy treatment impairs intestinal barrier function and may allow translocation of endotoxin and activation of the systemic inflammatory system.[30,35] GI toxicity has a significant impact on outcome and increases the risk of severe infection.[36,37] In the HSCT setting, it has been hypothesized that translocation of bacteria and toxins leading to endotoxemia during periods of GI toxicity, can trigger later development of graft versus host disease also outside of the GI tract.[38] Unexpectedly, we were not able to show any induction of a local inflammatory response in tissue extracts. However, as plasma levels of cytokines were not measured, we cannot exclude a systemic inflammatory response. Indeed, human studies have revealed release of cytokines 24–48 h after the start of chemotherapy followed by a later, secondary wave of inflammation, at the time of maximum toxicity.[39] Blood biochemistry values showed unspecific signs of tissue destruction especially indicated by the elevated total serum iron levels, consistent with the common observation that death of cells leads to release of iron into the circulation. Bilirubin levels were not elevated in the Bu–Cy group, suggesting that cholestasis was not present and that the higher values of the hepatic transaminase ASAT may be due to hepatotoxic effects directly induced by the Bu–Cy treatment, or secondary to translocation of endotoxins and bacteria from the gut.

Piglet models of chemotherapy-induced GI toxicity may be a relevant new animal platform to investigate

development of mucositis and to test new treatment strategies. The model has limitations that need careful attention. Forty percent of the Bu–Cy pigs showed clinical signs of severe infection before day 11 and were sacrificed before the pre-determined end of the protocol. The remaining Bu–Cy pigs showed macroscopic signs of pneumonia at necropsy. We cannot exclude that infections in other organs may alter the intestinal response to Bu–Cy treatment or induce intestinal damage as a consequence of septic shock. As such, the present piglet model may provide a basis for the studies of the progression and the pathology of chemotherapy-induced mucositis using Bu–Cy treatment, and may serve as a model to investigate factors that may alleviate the negative side effects of myeloablative chemotherapy. It is unclear, however, to what extent the observed clinical symptoms, and also the systemic responses and tissue responses in infant pigs, may apply for other age categories of pigs and other chemotherapy regimens. As in humans, the responses to chemotherapy in piglets are likely highly dependent on dose and product regimens, age, genetics, and the general physiological condition. In this study, we chose three-day-old piglets to mimic the responses in pediatric, milk-fed patients, but the results may be equally relevant for pediatric patients post-weaning. Piglets offer unique opportunities for the study of responses to various liquid, milk-based diets, and pediatric patients subjected to high-dose chemotherapy with severe GI toxicity are often supplied with enteral formulas based at least partly on milk products. Thus, this animal model may be relevant for a range of age groups of cancer patients receiving high-dose chemotherapy. As in other preclinical mucositis models, animals in this model were without malignancies. Interactions between healthy cells, malignant cells, and cytotoxic drugs may be important in the development of toxic reactions including inflammatory and immunological responses. We chose a fully myeloablative Bu–Cy regimen in study 2, since this cytotoxic treatment is related to severe oral and intestinal toxicity in humans, but such regimens likely also increase the sensitivity to infections. Lower dosages of Bu and Cy should be investigated for development of GI toxicity in pigs and a combination of broad spectrum antibiotics is needed in further studies. In conclusion, the present piglet model of chemotherapy-induced mucositis is feasible and may provide new insights into the development of mucositis. The model may be used to describe in more detail the age-related and organ-specific effects of various cytotoxic stimuli, as well as dietary and medical interventions that may reduce toxicity.

Author Contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. PLP, RLS, and TT conducted the experiments, BLP evaluated bone marrows, and PLP, RLS, and PTS wrote the manuscript.

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REFERENCES

1. Sonis ST. Regimen-related gastrointestinal toxicities in cancer patients. *Curr Opin Support Palliat Care* 2010;**4**:26–30
2. Silverman S Jr. Diagnosis and management of oral mucositis. *J Support Oncol* 2007;**5**:13–21
3. Bowen JM, Gibson RJ, Keefe DMK. Animal models of mucositis: Implications for therapy. *J Support Oncol* 2011;**9**:161–8
4. Keefe DM. Gastrointestinal mucositis: A new biological model. *Support Care Cancer* 2004;**12**:6–9
5. Sonis ST. The pathobiology of mucositis. *Nat Rev Cancer* 2004;**4**:277–84
6. Bowen JM, Keefe DM. New pathways for alimentary mucositis. *J Oncol* 2008; 907892.
7. Sonis ST, Lindquist L, Van Vugt A, Stewart AA, Stam K, Qu G-Y, Iwata KK, Haley JD. Prevention of chemotherapy-induced ulcerative mucositis by transforming growth factor β3. *Cancer Res* 1994;**54**:1135–8
8. Gibson RJ, Keefe DMK, Thompson FM, Clarke JM, Goland GJ, Cummins AG. Effect of interleukin-11 on ameliorating intestinal damage after methotrexate treatment of breast cancer in rats. *Digest Dis Sci* 2002;**47**:2751–7
9. Farrell CL, Bready JV, Rex KL, Chen JN, DiPalma CR, Whitcomb KL, Yin S, Hill DC, Wiemann B, Starnes CO, Havill AM, Lu Z-N, Aukerman SL, Pierce GF, Thomason A, Potten CS, Ulich TR, Lacey DL. Keratinocyte growth factor protects mice from chemotherapy and radiation-induced gastrointestinal injury and mortality. *Cancer Res* 1998;**58**:933–9
10. Keefe DM, Schubert MM, Elting LS, Sonis ST, Epstein JB, Raber-Durlacher JE, Migliorati CA, McGuire DB, Hutchins RD, Peterson DE. Updated clinical practice guidelines for the prevention and treatment of mucositis. *Cancer* 2007;**109**:820–31
11. Worthington HV, Clarkson JE, Bryan G, Furness S, Glenny AM, Littlewood A, McCabe MG, Meyer S, Khalid T. Interventions for preventing oral mucositis for patients with cancer receiving treatment. *Cochrane Database Syst Rev* 2011;**(4)**:CD000978
12. Miller ER, Ullrey DE. The pig as a model for human nutrition. *Ann Rev Nutr* 1987;**7**:361–82
13. Swindle MM, Makin A, Herron AJ, Clubb FJ, Frazier KS. Swine as models in biomedical research and toxicology testing. *Vet Pathol* 2012;**49**:344–56
14. Meurens F, Summerfield A, Nauwynck H, Saif L, Gerdts V. The pig: A model for human infectious diseases. *Trends Microbiol* 2012;**20**:50–7
15. Turpeinen M, Ghiciuc C, Opritoui M, Tursas L, Pelkonen O, Pasanen M. Predictive value of animal models for human cytochrome p450 (cyp)-mediated metabolism: A comparative study in vitro. *Xenobiotica* 2007;**37**:1367–77
16. Wernersson R, Schierup MH, Jørgensen FG, Gorodkin J, Panitz F, Staerfeldt H-H, Christensen OF, Mailund T, Hornshøj H, Klein A, Wang J, Liu B, Hu S, Dong W, Li W, Wong GKS, Yu J, Wang J, Bendixen C, Fredholm M, Brunak S, Yang H, Bolund L. Pigs in sequence space: A 0.66x coverage pig genome survey based on shotgun sequencing. *BMC Genomics* 2005;**6**:70
17. Manzano M, Bueno P, Rueda R, Ramirez-Tortosa CL, Prieto Pa, Lopez-Pedrosa JM. Intestinal toxicity induced by 5-fluorouracil in pigs: A new preclinical model. *Chemotherapy* 2007;**53**:344–55
18. Bjornvad CR, Thymann T, Deutz NE, Burrin DG, Jensen SK, Jensen BB, Molbak L, Boye M, Larsson LI, Schmidt M, Michaelsen KF, Sangild PT. Enteral feeding induces diet-dependent mucosal dysfunction, bacterial proliferation, and necrotizing enterocolitis in preterm pigs on parenteral nutrition. *Am J Physiol Gastrointest Liver Physiol* 2008;**295**:G1092–103
19. Kashyap A, Wingard J, Cagnoni P, Jones R, Tarantolo S, Hu W, Blume K, Niland J, Palmer JM, Vaughan W, Fernandez H, Champlin R, Forman S, Andersson BS. Intravenous versus oral busulfan as part of a busulfan/cyclophosphamide preparative regimen for allogeneic hematopoietic stem cell transplantation: Decreased incidence of hepatic venoocclusive

disease (hvod), hvod-related mortality, and overall 100-day mortality. *Biol Blood Marrow Transplant* 2002;**8**:493–500
20. Hows JM, Mehta A, Ward L, Woods K, Perez R, Gordon MY, Gordon-Smith EC. Comparison of mesna with forced diuresis to prevent cyclophosphamide induced haemorrhagic cystitis in marrow transplantation: A prospective randomised study. *Br J Cancer* 1984;**50**:753–6
21. Bollen PJA, Hansen AK, Rasmussen HJ, Suckow MA. *The laboratory swine*. Boca Raton: CRC Press, 2000
22. Thymann T, Burrin DG, Tappenden KA, Bjornvad CR, Jensen SK, Sangild PT. Formula-feeding reduces lactose digestive capacity in neonatal pigs. *Br J Nutr* 2006;**95**:1075–81
23. Hessels J, Snoeyink EJ, Platenkamp AJ, Voortman G, Steggink J, Eidhof HH. Assessment of intestinal permeability: Enzymatic determination of urinary mannitol, raffinose, sucrose and lactose on hitachi analyzer. *Clin Chem Lab Med* 2003;**41**:33–8
24. Sangild PT, Petersen YM, Schmidt M, Elnif J, Petersen TK, Buddington RK, Greisen G, Michaelsen KF, Burrin DG. Preterm birth affects the intestinal response to parenteral and enteral nutrition in newborn pigs. *J Nutr* 2002;**132**:2673–81
25. Sangild PT, Sjostrom H, Noren O, Fowden AL, Silver M. The prenatal development and glucocorticoid control of brush-border hydrolases in the pig small intestine. *Pediatr Res* 1995;**37**:207–12
26. Mitchell EP. Gastrointestinal toxicity of chemotherapeutic agents. *Semin Oncol* 2006;**33**:106–20
27. Sonis ST. Mucositis: The impact, biology and therapeutic opportunities of oral mucositis. *Oral Oncol* 2009;**45**:1015–20
28. van Vliet MJ, Harmsen HJM, de Bont ESJM, Tissing WJE. The role of intestinal microbiota in the development and severity of chemotherapy-induced mucositis. *PLoS Pathog* 2010;**6**:e1000879
29. Godlewski MM, Slazak P, Zabielski R, Piastowska A, Gralak MA. Quantitative study of soybean-induced changes in proliferation and programmed cell death in the intestinal mucosa of young rats. *J Physiol Pharmacol* 2006;**57**:125–33
30. van der Velden WJ, Blijlevens NM, Feuth T, Donnelly JP. Febrile mucositis in haematopoietic sct recipients. *Bone Marrow Transplant* 2009;**43**:55–60
31. Keefe DM, Brealey J, Goland GJ, Cummins AG. Chemotherapy for cancer causes apoptosis that precedes hypoplasia in crypts of the small intestine in humans. *Gut* 2000;**47**:632–7
32. Cunningham D, Morgan RJ, Mills PR, Nelson LM, Toner PG, Soukop M, McArdle CS, Russell RI. Functional and structural changes of the human proximal small intestine after cytotoxic therapy. *J Clin Pathol* 1985;**38**:265–70
33. Kissow H, Viby NE, Hartmann B, Holst JJ, Timm M, Thim L, Poulsen SS. Exogenous glucagon-like peptide-2 (glp-2) prevents chemotherapy-induced mucositis in rat small intestine. *Cancer Chemother Pharmacol* 2012;**70**:39–48
34. Fijlstra M, Rings EH, Verkade HJ, van Dijk TH, Kamps WA, Tissing WJ. Lactose maldigestion during methotrexate-induced gastrointestinal mucositis in a rat model. *Am J Physiol Gastrointest Liver Physiol* 2011;**300**:G283–91
35. Johansson JE, Ekman T. Gastro-intestinal toxicity related to bone marrow transplantation: Disruption of the intestinal barrier precedes clinical findings. *Bone Marrow Transplant* 1997;**19**:921–5
36. Al-Mousa H, Al-Shammari Z, Al-Ghonaium A, Al-Dhekri H, Al-Muhsen S, Al-Saud B, Arnaout R, Al-Seraihy A, Al-Jefri A, Al-Ahmari A, Ayas M, El-Solh H. Allogeneic stem cell transplantation using myeloablative and reduced-intensity conditioning in patients with major histocompatibility complex class ii deficiency. *Biol Blood Marrow Transplant* 2010;**16**:818–23
37. Yuen KY, Woo PC, Hui CH, Luk WK, Chen FE, Lie AK, Liang R. Unique risk factors for bacteraemia in allogeneic bone marrow transplant recipients before and after engraftment. *Bone Marrow Transplant* 1998;**21**:1137–43
38. Blijlevens NMA, Donnelly JP, Pauw BED. Mucosal barrier injury: Biology, pathology , clinical counterparts and consequences of intensive treatment for haematological malignancy: An overview. *Bone Marrow Transplant* 2000;**7**:1269–78
39. Andreassen BU, Paerregaard a, Michaelsen KF, Andersen J, Heilmann CJ, Müller K. Nutrition, anthropometry, gastrointestinal dysfunction, and circulating levels of tumour necrosis factor alpha receptor i and interleukin-1 receptor antagonist in children during stem cell transplantation. *Pediatric Transplant* 2009;**13**:182–7

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