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Milk diets influence doxorubicin-induced intestinal toxicity in piglets

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Shen RL, Pontoppidan PE, Rathe M, Jiang P, Hansen CF, Buddington RK, Heegaard PM, Müller K, Sangild PT. Milk diets influence doxorubicin-induced intestinal toxicity in piglets. *Am J Physiol Gastrointest Liver Physiol* 311: G324–G333, 2016. First published July 21, 2016; doi:10.1152/ajpgi.00373.2015.—Chemotherapy-induced gastrointestinal (GI) toxicity is a common adverse effect of cancer treatment. We used preweaned piglets as models to test our hypothesis that the immunomodulatory and GI trophic effects of bovine colostrum would reduce the severity of GI complications associated with doxorubicin (DOX) treatment. Five-day-old pigs were administered DOX (1×100 mg/m^2) or an equivalent volume of saline (SAL) and either fed formula (DOX-Form, $n = 9$, or SAL-Form, $n = 7$) or bovine colostrum (DOX-Colos, $n = 9$, or SAL-Colos, $n = 7$). Pigs were euthanized 5 days after initiation of chemotherapy to assess markers of small intestinal function and inflammation. All DOX-treated animals developed diarrhea, growth deficits, and leukopenia. However, the intestines of DOX-Colos pigs had lower intestinal permeability, longer intestinal villi with higher activities of brush border enzymes, and lower tissue IL-8 levels compared with DOX-Form (all $P < 0.05$). DOX-Form pigs, but not DOX-Colos pigs, had significantly higher plasma C-reactive protein, compared with SAL-Form. Plasma citrulline was not affected by DOX treatment or diet. Thus a single dose of DOX induces intestinal toxicity in preweaned pigs and may lead to a systemic inflammatory response. The toxicity is affected by type of enteral nutrition with more pronounced GI toxicity when formula is fed compared with bovine colostrum. The results indicate that bovine colostrum may be a beneficial supplementary diet for children subjected to chemotherapy and subsequent intestinal toxicity.

chemotherapy-induced mucositis; intestinal toxicity; enteral nutrition; milk; bovine colostrum

NEW & NOTEWORTHY

In this study, preweaned pigs were used as models to study intestinal complications after doxorubicin as models for pediatric oncology patients receiving chemotherapy. This study shows that the type of enteral nutrition is an important factor that can influence the toxic responses related to doxorubicin treatment. Compared with formula, bovine colostrum nutrition provided beneficial effects against the intestinal injury, which occurred early after doxorubicin administration.

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GASTROINTESTINAL (GI) TOXICITY is a major dose-limiting adverse effect of chemotherapy with implications for both morbidity and mortality (12, 48). The associated symptoms include pain, nausea, vomiting, and diarrhea, which affect nutritional status and quality of life. These complications are related to adverse clinical outcomes, prolonged hospitalization, and need for parenteral nutrition. Due to mucosal barrier injury, there are increased risks of fever and infections, potentially leading to increasing mortality (12, 18, 48, 53). Emerging clinical strategies for GI toxicity, including drugs, probiotics, and nutritional interventions (3, 28, 65), have not been effective, and GI dysfunction often hinders adequate enteral nutrition support, which provides energy, macronutrients, and dietary bioactive factors (37, 63). Enteral nutrition may, therefore, help to reduce inflammatory conditions in the GI tract (1), and this could be important in association with chemotherapy.

While the survival of childhood cancer has improved over the last decades, it is still the leading cause of disease-related mortality in children (62). The improved survival is partly related to intensified and risk-stratified chemotherapy regimens, which have led to increased toxicity, underlining the need for better understanding and management of treatment-related complications in these patients. At the forefront is chemotherapy-induced intestinal dysfunction. The associated malnutrition and toxic complications may lead to dose reduction or treatment delays, compromising treatment efficacy. Nutrition could be particularly important for outcome in pediatric cancer patients that are still in a state of growth and organ maturation (4, 8). Studies also suggest that cancer and chemotherapy in infancy and early childhood can be more challenging to manage and may have worse outcomes compared with that in older children (40, 42, 62), potentially due to immaturity of organs such as the GI tract. Knowledge about this patient group is limited, and comparable pediatric animal models for chemotherapy-induced mucositis are relevant to provide new basic knowledge. Until now, such models have focused mainly on rodents (33), but their physiology differs markedly from humans, and their small size and immature state at birth limit their use as a translational model for infants and young children, compared with a species like the pig (39, 51). The larger size of preweaned and juvenile pigs also makes it easier to use standard clinical and medical care procedures when inducing and evaluating chemotherapy toxicity (35, 36, 45, 46, 57).

Bovine colostrum (Colos) contains many bioactive compounds, such as immunoglobulin (Ig), transforming growth

factor-β, and insulin-like growth factor I that may stimulate gut growth and function and provide mucosal protection via immunomodulatory effects and changes to the gut microbiota (44). Thus Colos may promote GI mucosal protection via antimicrobial and endotoxin-neutralizing effects, suppression of gut inflammation, and by facilitating mucosal tissue repair. In preterm pigs, enteral feeding with bovine Colos protects against necrotizing enterocolitis, a severe condition with inflammatory and ulcerative lesions in the GI tract (24). Thus bovine Colos might provide GI and systemic benefits in several disease states involving damage to the GI mucosa (44).

Recent studies have used preweaned and weaned pigs as models for chemotherapy-induced intestinal toxicity (36, 45), and these may provide animal models to study liquid diet interventions during a period of rapid growth and development, similar to pediatric patients. Liquid milk-based diets are highly digestible and easy to adjust in nutrient composition and intake for chemotherapy patients requiring tube feeding. In this study, we hypothesized that GI toxicity after treatment of preweaned pigs with doxorubicin (DOX) would be reduced by feeding bovine colostrum compared with a standard formula diet. DOX is an anthracycline, which causes DNA intercalation and is commonly used in many anticancer regimens, with multiple organ toxicities, including myelosuppression, cardiotoxicity, and GI toxicity (5, 7, 64).

MATERIAL AND METHODS

Animals, surgical preparation, clinical procedures, and chemotherapy. All animal procedures were approved by the Danish National Committee on Animal Experimentation (no. 2010/561–1760). An overview of the experimental approach is shown in Fig. 1. A total of 32 pigs (Large White × Danish Landrace) of both sexes were obtained on the 3rd day of life after having suckled their mother from birth. The pigs were brought to the research facilities where they were housed in individual cages in a facility kept at a constant temperature (~30°C) with a 12:12-h light-dark cycle. Each cage contained behavioral enrichments (toy-cloths) and allowed visual contact among individual pigs.
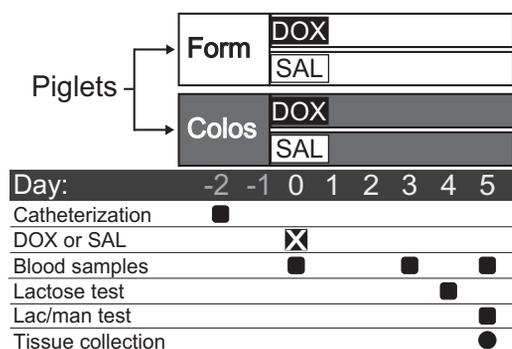

Fig. 1. Study design with enteral nutrition intervention using formula (Form) or bovine colostrum (Colos). Catheters and feeding tubes were fitted surgically 2 days before chemotherapy. Doxorubicin (DOX) or saline (SAL) was administered on *day 0*. Blood samples were collected before the treatment, *days 3* and *5*, and analyzed for hematological parameters and C-reactive protein. Digestive capacity of lactose was tested on *day 4*, and intestinal permeability was assessed with the lactulose/mannitol test (lac/man test) on *day 5* when animals were killed. Results for doxorubicin-treated pigs fed formula (DOX-Form, n = 9), saline-treated pigs fed formula (SAL-Form, n = 7), doxorubicin-treated pigs fed bovine colostrum fed (DOX-Colos, n = 9), and saline-treated pigs fed bovine colostrum (SAL-Colos, n = 7) are shown.

Shortly after arrival, the pigs were anesthetized using zoletil mix (intramuscular, 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) for placement of an orogastric feeding tube (6F Portex, Kent, UK) that was inserted through the cheek and secured with sutures. A catheter (Tygon OD 0.070 ID 0.040 Saint-Gobain Performance Plastics, France) was surgically inserted into the external jugular vein, tunneled under the skin, and exteriorized through the dorsal part of the neck and used for blood sampling and administration of DOX. The pigs were fed either bovine colostrum (Colos) or formula (Form), and within each diet group pigs received DOX chemotherapy or saline (SAL), with a randomized block design resulting in four groups with similar distribution of initial body weight, sex, and litter of origin: DOX-treated animals fed bovine Colos (DOX-Colos, n = 9) or Form (DOX-Form, n = 9), and SAL groups fed bovine Colos (SAL-Colos, n = 7) or Form (SAL-Form, n = 7). The pigs were fed every 3 h by placing 15 ml/kg of their respective diet in a feeding trough. Voluntary food intake was allowed, but, if appetite was reduced, any remaining feed was fed through the orogastric tube. The composition of the Form diet was based on commercially available clinical nutrition products, i.e., Pepdite (60 g/l; powdered enteral nutrition with nonmilk-derived low-molecular-weight peptides, essential amino acids, carbohydrate, fat, vitamins, and minerals; Nutricia, Allerød, Denmark), Lacprodan (50 g/l; whey protein; Arla Foods Ingredients, Aarhus, Denmark), Miprodan (50 g/l; casein; Arla Foods Ingredients), Calogen (50 g/l; long-chain triglyceride fat emulsion; Nutricia), Liquigen (80 g/l; medium-chain triglyceride from fractionated coconut oil; Nutricia), and Seravit-SHS (12 g/l; vitamins and trace elements; Nutricia). The macronutrient composition in the Form diet was 5,291 kJ/l energy, 96 g/l protein, 40 g/l carbohydrate, with 36 g/l maltodextrins, and 79 g/l fat. The Colos was collected from Danish dairy cows within 24 h of parturition, pooled, and spray-dried to a powder and γ-irradiated to eliminate microorganisms (Biofiber-Damino, Gesten, Denmark). The Colos powder was reconstituted in water (200 g/l) and yielded macronutrient values of 3,986 kJ/l energy, 94 g/l protein, with 28 g/l IgG, 36 g/l carbohydrate, and 48 g/l fat.

Chemotherapy. Two days after the surgery and the start of the respective feeding regimens, the pigs were administered a single intravenous dose of DOX over 30 min, corresponding to 100 mg/m² body surface area or an equivalent volume of SAL. This dose of DOX is comparable to doses used in treatment of pediatric cancers, such as acute lymphoblastic leukemia, and has been shown to induce intestinal toxicity in weaned pigs (36). Body surface area was calculated as $70 \times kg$ body $wt^{0.75}/1,000$ (10), providing ~5–5.5 mg/kg DOX for the 2.5- to 3.5-kg pigs.

Clinical assessment. Animals were monitored for clinical status, including daily measurements of body weight, temperature, and diarrhea scoring. Diarrhea was assessed by visually categorizing waste collection trays below the cages and animal staining, providing a daily score of 0 (no stools or normal stools), 1 (mild diarrhea), or 2 (severe diarrhea). Humane endpoints for euthanasia of animals before *day 5* were as described elsewhere (46).

Hematology, CRP, and in vivo markers of intestinal function. Blood samples were collected at baseline, immediately before administration of DOX, on *day 3* and on *day 5* (the time of euthanasia) for hematology and analyses of C-reactive protein (CRP) and citrulline. Hematological parameters included red blood cell (RBC), white blood cells (WBC), neutrophils (NEU), lymphocytes (LYM), and platelet (PLT) count (Advia 2120 Hematology System, Siemens, Erlangen, Germany). These parameters have previously been shown to correlate with toxicity in pigs (46). Circulating CRP is a marker of systemic inflammatory response commonly used to indicate inflammation and infection in both humans and pigs and was measured with an enzyme-linked immunosorbent assay (ELISA) using a validated method for pigs (21). Plasma citrulline is synthesized by enterocytes and is considered a marker for intestinal mass and function, as well as a marker of damage after chemotherapy (58). For detection of citrulline

(17), analytes extracted from serum samples were separated and detected by ultra-performance liquid chromatography- triple quadrupole mass spectrometry (Waters, Milford, MA). Quantification of citrulline was carried out using an analyzing software, QuanLynx (Waters).

At baseline and again on *day 4*, the pigs were fed one bolus (15 ml/kg) of a 10% lactose solution via the feeding tubes to assess the capacity for lactose digestion as a marker for intestinal function. The bolus was given 3 h after the last feed instead of the normal diet. Blood samples were collected 20 min after the bolus, and plasma galactose levels were measured spectrophotometrically (Pentra 400, Horiba ABX, Montpellier, France) using a commercial kit (55). Before death on *day 5*, animals received a bolus (15 ml/kg) of 5% lactulose and 5% mannitol solution via the feeding tube 3 h after the previous meal to assess intestinal permeability. A urine sample was collected by cystocentesis during necropsy 3–5 h after the lactulose/mannitol (lac/man) bolus. Urinary concentrations of lactulose and mannitol were determined spectrophotometrically (Pentra 400), and intestinal permeability was assessed by the ratio of lactulose to mannitol (lac/man ratio).

Necropsy and tissue collection. On *day 5*, the pigs were anesthetized using the zoletil mix before intracardial collection of a blood sample after which the pigs were euthanized by intracardial injection of sodium pentobarbital, 200 mg/kg. After death was confirmed, the abdomen was opened, and the small intestine (from the pyloric sphincter to the ileo-cecal junction) was removed quickly, placed on a cooled surface, and divided into three segments of equal length, designated proximal (Prox), middle (Mid), and distal (Dist) intestine. For each segment, two pieces of ~0.5 cm were collected and fixed in 4% neutral buffered formalin. After fixation, samples were dehydrated with ethanol, embedded in paraffin and cross-sectioned (5 μm), mounted on glass slides, and stained with hematoxylin and eosin. Additional tissue samples from each intestinal region were snap-frozen in liquid nitrogen for subsequent analyses. Finally, a 10-cm piece of each region was collected for determining the proportional weight of mucosa, relative to whole intestine. The remaining organs were collected, and their weights recorded.

Intestinal tissue structure, brush border enzymes, and proinflammatory cytokines. Villus height and crypt depth were determined as previously described (46). The proportion of the small intestine represented by mucosa was determined by scraping off the mucosal layer and drying (49). The frozen intestinal samples were processed for analyses of brush border enzymes and cytokine levels, as described elsewhere (46, 50). Briefly, the tissues were homogenized in 1% Triton X-100, and the activities of three dissacharidases (lactase, maltase, sucrase) and three peptidases (aminopeptidase A, aminopeptidase N, dipeptidylpeptidase IV) were expressed as a hydrolytic rate of 1 μmol substrate released per minute at 37°C per gram wet whole intestinal tissue. Intestinal inflammatory responses were based on cytokine levels in the Dist region and measured using the DuoSet ELISA Development kit (R&D Systems, Abingdon, UK) targeted for porcine interleukin-1β (IL-1b), interleukin-6 (IL-6), interleukin 8 (IL-8), and tumor necrosis factor-α (TNF-α), according to the manufacturer's instructions.

Intestinal proliferation, apoptosis, and structural proteins. Enterocyte proliferation was assessed by immunohistochemistry, staining for Ki-67 on sections from the Mid intestine, as described previously (60). Quantification was performed by image analysis using Visiomorph 4.6 (Visiopharm). The total area of Ki-67-positive stained nuclei were calculated and presented as a fraction of all nuclei.

Expression of PCNA was used as a marker of proliferation and that of caspase-3 as a marker of apoptosis. β-Actin and GAPDH proteins were quantified by Western blot and used as indicators of basic cytostructural and metabolic conditions, as previously described (25). Briefly, 25-mg protein extracted from the Mid intestine segments was resolved by electrophoresis and transferred onto polyvinylidene difluoride membranes. The expression of PCNA, caspase-3, and β-actin

was shown with specific antibodies (Santa Cruz, CA; and Abcam, Cambridge, UK). The protein bands on the membranes were visualized, and the band densities were detected by Image J (National Institutes of Health, Bethesda, MD). Protein loading was controlled by direct staining of the electrophoresis gels run parallel to the ones for Western blot, using methods described previously (20).

Statistical analyses. Statistical analyses were performed with the statistical software R (version 2.15.0). Weight gain was expressed as the percent change in weight, relative to the start of DOX administration. Continuous data were analyzed using mixed models (*lmer* function) with the groups as independent variables and with adjustments for sex, baseline body weight, and study round (3 rounds of pig studies with evenly distributed groups and sexes) as explanatory variables. For repeated-measures analyses, the individual pig identifier was included in the model as a random effect. Normality was assessed by means of standardized residuals and log-transformed to account for variance heterogeneity, when necessary. Adjustments for baseline values were included in the model for longitudinal data. Group comparisons were done with the *lsmeans* package with multiplicity adjustments of P values with the single-step method. For sample values lower than assay detection limit, these were assigned the lowest value of the assay range. Diarrhea score was analyzed as nonparametric data with the *nparcomp* package. P values < 0.05 were considered significant. Graphs were made with GraphPad Prism (version 5.01; GraphPad Software, La Jolla, CA). Data are presented as arithmetic means and SE of raw data, unless otherwise specified.
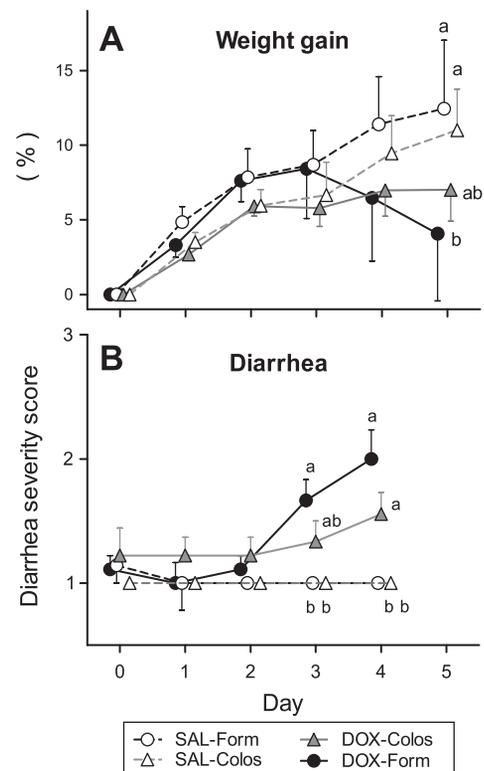

Fig. 2. Weight gain indicated as percent change relative to initial body weight before treatment on *day 0* (A) and diarrhea score (B) are presented as means ± SE. Results for doxorubicin-treated pigs fed formula (DOX-Form, $n = 9$), saline-treated pigs fed formula (SAL-Form, $n = 7$), doxorubicin-treated pigs fed bovine colostrum (DOX-Colos, $n = 9$), and saline-treated pigs fed bovine colostrum (SAL-Colos, $n = 7$) are shown. [a,b]Different superscript letters indicate significant differences among groups within the same time point ($P < 0.05$).
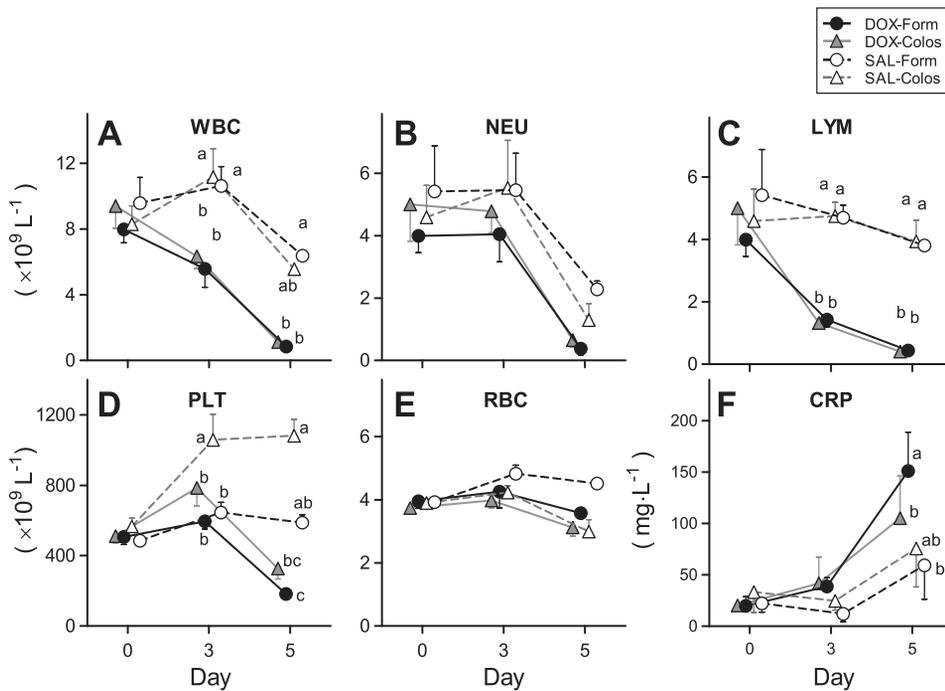
Fig. 3. Circulating levels of white blood cells (WBC; *A*), neutrophils (NEU; *B*), lymphocytes (LYM; *C*), platelets (PLT; *D*), red blood cells (RBC; *E*), and C-reactive protein (CRP; *F*) are presented as means ± SE. Results for doxorubicin-treated pigs fed formula (DOX-Form, $n = 4$–9), saline-treated pigs fed formula (SAL-Form, $n = 4$–7), doxorubicin-treated pigs fed bovine colostrum (DOX-Colos, $n = 4$–9), and saline-treated pigs fed bovine colostrum (SAL-Colos, $n = 2$–7) are shown. [a,b,c]Different superscript letters indicate significant differences among groups within the same time point ($P < 0.05$).

RESULTS

Clinical status and growth. All pigs continued to eat and remained active and clinically stable during the 5-day study period. No pigs required euthanasia according to humane endpoints before the end of the protocol. All of the pigs gained weight between *day 0*, when DOX (or SAL) was administered, and *day 2* (Fig. 2*A*). After *day 3*, weight gain decreased for both groups of DOX pigs, resulting in stable body weight of DOX-Colos pigs from *days 3–5* and weight reduction for DOX-Form pigs. The two groups of SAL pigs continued to gain weight until *day 5*. Growth on *day 5* was significantly lower in DOX-Form pigs, compared with both groups of SAL pigs ($P < 0.05$, Fig. 2*A*), whereas weight for DOX-Colos pigs was not different from that in SAL pigs. Initial weight gain (*days 0–2*) tended to be greater for both groups of Form pigs, compared with Colos pigs (Fig. 2*A*). Diarrhea was commonly observed from *day 3* after DOX administration with increasing severity thereafter (Fig. 2*B*). Diarrhea scores were similar in DOX-Form and DOX-Colos pigs. However, DOX-Form pigs had higher diarrhea score than SAL groups on *days 3* and *4*, whereas scores in DOX-Colos were not significantly elevated until *day 4*, relative to SAL pigs (Fig. 2*B*). Diarrhea in SAL pigs was restricted to one mild transient case at the start of the study period. Vomiting was observed in one DOX-Form pig on *days 4* and *5*. No effects on appetite or food intake were observed.

Blood hematology and systemic inflammatory response. Administration of DOX reduced WBC, compared with SAL on *days 3* and *5* ($P < 0.05$, Fig. 3*A*). DOX also reduced LYM values on both days, whereas NEU were not affected, and no diet effect was detected for WBC, LYM, or NEU (Fig. 3, *A–C*). On *day 5*, PLT values were lower in DOX vs. SAL pigs, as analyzed for each of the diet regimens ($P < 0.05$, Fig. 3*D*), with no differences between DOX-Colos and DOX-Form. RBC did not differ among groups (Fig. 3*E*). Longitudinal

measurements of CRP did not differ significantly among groups on *days 0* and *3*. However, on *day 5*, levels were higher for DOX-Form pigs, compared with DOX-Colos and SAL-Form pigs ($P < 0.05$, Fig. 3*F*).

Gut function markers in vivo. After 2 days of Form or Colos feeding, and before the DOX (or SAL) was administered, plasma galactose measured 20 min after a lactose bolus was lower for Form than for Colos pigs (92 ± 12 vs. 200 ± 26 μmol/l, $P < 0.01$). On *day 4*, galactose values were similar for both groups of Colos pigs, and both were higher than for the two groups of Form pigs, regardless of SAL or DOX administration (pooled means ± SE: 36.2 ± 9.7 vs. 4.4 ± 3.4 μmol/l, $P < 0.05$), indicating that diet, but not DOX treatment, affected galactose uptake capacity. Among the groups, mean gut per-
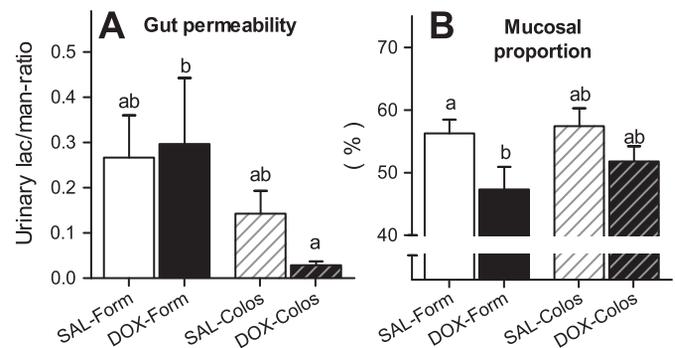

Fig. 4. Intestinal permeability measured on *day 5* as the urinary ratio of lactulose to mannitol 3–5 h after an enteral bolus of lactulose (*A*), and mannitol solution and proportion of mucosal mass relative to total intestinal mass (*B*) are presented as means ± SE. Results for doxorubicin-treated pigs fed formula (DOX-Form, $n = 4$–9), saline-treated pigs fed formula (SAL-Form, $n = 7$), doxorubicin-treated pigs fed bovine colostrum (DOX-Colos, $n = 8$–9), and saline-treated pigs fed bovine colostrum (SAL-Colos, $n = 7$) are shown. [a,b]Different superscript letters indicate significant differences among groups within the same time point ($P < 0.05$).

Table 1. *Organ weights at necropsy*

	Form		Colos	
	SAL	DOX	SAL	DOX
Stomach	7.3 ± 0.9	7.7 ± 0.7	7.5 ± 0.6	8.3 ± 0.7
Intestine	43.8 ± 1.7[b]	30.2 ± 2.3[a]	37.5 ± 1.2[a,b]	33.2 ± 2.1[a]
Colon	12.1 ± 0.7[a,b]	11.1 ± 1.6[a]	14.8 ± 1.3[b]	12.0 ± 0.7[a,b]
Liver	35.6 ± 2.0	36.0 ± 1.0	32.3 ± 0.8	32.4 ± 0.8
Lungs	19.4 ± 1.8	15.7 ± 1.3	19.5 ± 3.0	19.8 ± 1.6
Heart	7.8 ± 0.4	7.4 ± 0.5	7.6 ± 0.1	7.2 ± 0.4
Spleen	3.8 ± 0.5[b]	1.3 ± 0.2[a]	4.1 ± 0.3[b]	2.0 ± 0.2[a]
Kidneys	9.5 ± 0.7	9.1 ± 0.5	9.0 ± 0.5	8.8 ± 0.4

Values are means ± SE. Organ weights are expressed relative to body weight (g/kg). Groups are doxorubicin-treated pigs fed formula (DOX-Form, $n = 9$), saline-treated pigs fed formula (SAL-Form, $n = 7$), doxorubicin-treated pigs fed bovine colostrum (DOX-Colos, $n = 9$), and saline-treated pigs fed bovine colostrum (SAL-Colos, $n = 7$). [a,b]Different superscript letters indicate significant differences among the four groups ($P < 0.05$).

meability, as assessed by the lac/man ratio, was lowest for DOX-Colos pigs ($P < 0.05$ compared with DOX Form, Fig. 4A). Plasma citrulline did not differ significantly among groups, regardless of diet or whether DOX was administered.

Intestinal and inflammatory responses. Macroscopic signs of GI mucosal damage were not observed in any of the groups, and only one pig from the DOX-Form group had signs of edema in the colon. Only among the Form pigs did DOX treatment reduce small intestinal weight (-38%, $P < 0.05$), with less (nonsignificant) effects of DOX within the Colos pigs (Table 1). The percentage of intestinal weight represented by mucosa was significantly reduced in DOX-Form pigs, but not in DOX-Colos pigs, both compared with their respective SAL controls ($P < 0.05$, Fig. 4B). Analyzed across the two diets, the DOX treatment decreased intestinal weight and colon weight compared with SAL ($P < 0.05$), but, when analyzed for each diet separately, only the DOX-induced reduction in intestinal weight in Form-fed piglets was significant (30% reduction, $P < 0.05$, Table 1). For the weight of other internal organs, the spleen was most affected by DOX with a reduction to 30–50%, compared with SAL ($P < 0.05$), with no effect of diet (Table 1). Across SAL and DOX treatments, liver weight was significantly lower in Colos pigs compared with Form ($P < 0.05$, Table 1).

DOX-Colos pigs had the longest villi in the Mid and Dist intestinal regions compared with DOX-Form and SAL-Colos ($P < 0.05$, Fig. 5A). The DOX treatment reduced crypt depths
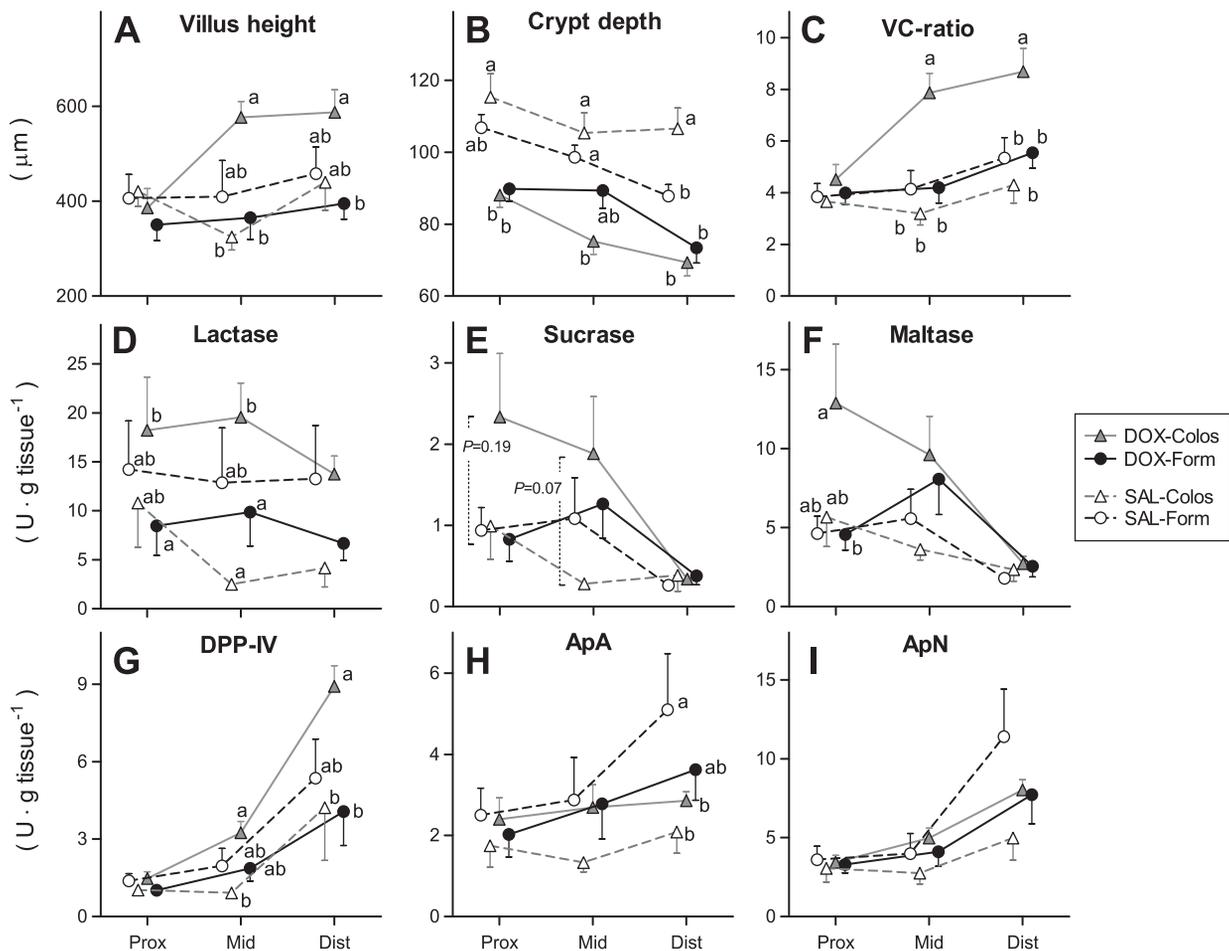

Fig. 5. Villus height (*A*), crypt depth (*B*), villus-crypt ratio (VC-ratio; *C*), and activity of lactase (*D*), sucrase (*E*), maltase (*F*), dipeptidylpeptidase IV (DPP-IV; *G*), aminopeptidase A (ApA; *H*), and aminopeptidase N (ApN; *I*) measured in the proximal (Prox), middle (Mid), and distal (Dist) regions of the small intestine, respectively (means ± SE) are shown. Results for doxorubicin-treated pigs fed formula (DOX-Form, $n = 9$), saline-treated pigs fed formula (SAL-Form, $n = 7$), doxorubicin-treated pigs fed bovine colostrum (DOX-Colos, $n = 9$), and saline-treated pigs fed bovine colostrum (SAL-Colos, $n = 7$) are shown. [a,b]Different superscript letters indicate significant differences among the four groups within the same region ($P < 0.05$).

across all three regions and across diets, indicating lowered crypt cell proliferation ($P < 0.05$ for Colos and trend for Form, Fig. 5*B*). Consequently, the villus-to-crypt ratios in the Mid and Dist regions were increased in the DOX-Colos group, compared with all the other groups ($P < 0.05$, Fig. 5*C*).

Disaccharidase activities were highest in Prox and Mid intestinal regions, while peptidase activities were highest in Mid and Dist regions (Fig. 5, *D–I*). Only for Colos pigs were the lactase, sucrose, and maltase activities higher for DOX vs. SAL pigs ($P < 0.05$, Fig. 5, *D–F*). No significant DOX effect was detected for Form pigs. Similarly, it was only for Colos pigs that dipeptidylpeptidase IV activity was higher for DOX vs. SAL pigs (Fig. 5*G*). Concentrations of the proinflammatory cytokines, IL-8 and IL-6 (Fig. 6) were lower in the Dist intestine of pigs fed Colos ($P < 0.05$ across treatments), but only for IL-8 were values for DOX-Colos lower than for DOX-Form pigs ($P < 0.05$, Fig. 6*A*). Tissue levels of IL-1β and TNF-α did not differ between diets and treatments.

Proliferation, apoptosis, and intracellular structure. Immunohistochemistry staining of Ki-67-positive cells in the intestine showed lower number of cells in DOX-Colos pigs compared with SAL-Colos pigs ($P < 0.05$), and numbers were similar to those in the DOX-Form and SAL-Form pigs, which showed no differences (Fig. 7, *A* and *G*). Yet there was a significant negative effect of chemotherapy on Ki-67-positive cells when analyzed across diets ($P < 0.001$). Western blot of PCNA revealed similar results in that values were lower in DOX-Colos than in SAL-Colos pigs ($P < 0.05$), while DOX-Form tended to show lower values than SAL-Form pigs ($P = 0.19$, Fig. 7*D*), with no significant effects of diet. Cleaved caspase-3 values were significantly lower in DOX-Form than in the other three groups, which had similar levels ($P < 0.05$ Fig. 7*B*). Uncleaved caspase-3 did not differ significantly among groups (Fig. 7*E*), although DOX increased the values, relative to SAL, as analyzed across diets ($P < 0.01$). Overall, DOX treatment decreased the β-actin and GAPDH levels, with limited effects of diet (Fig. 7, *C* and *F*).

DISCUSSION

Nutritional support is critical during treatment of pediatric cancer patients to prevent malnutrition, which decreases chemotherapy tolerance, increases toxicity and infection rates, and compromises outcome (4). Enteral nutrition formulas are preferred over total parenteral nutrition because of assumed benefits for the gut (37) and are recommended in patients with a functional GI tract. Yet there is no consensus regarding the type of enteral nutrition support for pediatric patients. Our findings indicate that the type of liquid diet support influences the response to the chemotherapeutic agent, DOX, and that a diet rich in milk bioactive factors, such as bovine colostrum, might be beneficial.

In humans, gut toxicity develops early after chemotherapy (27), and the onset of diarrhea and weight loss 3–4 days after treatment in the current study coincides with the onset of GI toxicity in older, weanling piglets after a single dose of DOX (36). Likewise, the intestinal structural response to DOX (e.g., decreased intestinal weight and amount of mucosa) is consistent with data from studies in juvenile pigs (36). On the other hand, the administration of DOX did not significantly change lactose digestive capacity, gut permeability, and tissue proinflammatory cytokines, and possibly some intestinal responses to DOX are less pronounced in preweaned vs. weaned juvenile pigs, at least on *day 5* after treatment. This may be explained by a more immature inflammatory response during early life, compared with adult patients, despite that a gut inflammatory response is thought to be a central element in the complications of chemotherapy across all patients (19, 26). Regardless, it is important to note that the present results of DOX are documented in a state of early intestinal development, and effects on a more mature intestine could be different. CRP is a systemic marker of inflammatory and infectious complications (38), and the elevated CRP levels in this study are consistent with a systemic inflammatory response after DOX treatment. Likewise, the markedly reduced spleen weight and changes in hematology after a single dose of DOX were consistent with reduced hematopoiesis.

Growing children and their GI tract are highly responsive to the type of nutrition support (6, 55). In addition to macronutrients, bovine colostrum contains numerous bioactive compounds that influence both gut maturation and systemic immunity. This includes GI growth, digestive function, mucosal immune function and inflammation, and the resident microbiota. These may have contributed to the different responses of the SAL pigs fed colostrum, compared with those fed formula. Nevertheless, the colostrum diet fed to piglets not exposed to DOX was associated with slower body weight gain, compared with pigs fed formula, and colostrum did not in this study increase the length and weight of the small intestine, villus height, crypt depth, or activity of digestive enzymes in the SAL pigs. Potentially, the most beneficial effects of colostral bioactive factors are restricted to the immediate neonatal period, consistent with the lacking effect of bovine colostrum on intestinal nutrient function in healthy adults or stable short bowel syndrome patients (13, 34, 47). Nevertheless, the observed increases in lactose digestive capacity and reduced permeability in colostrum-fed SAL pigs may suggest some intestinal benefits, relative to formula. These differences could also be related to potential direct negative effects of the chosen formula diet. In preterm pigs, detrimental effects on gut structure and function have been induced by formulas very similar to the one used in this study (43, 55), and the damaging effects were at least partly related to replacing milk lactose with
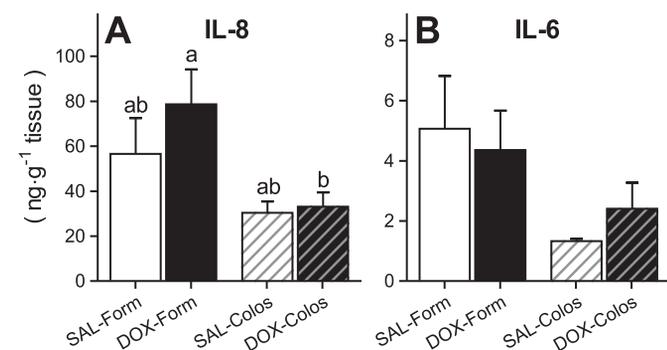

Fig. 6. Tissue levels of proinflammatory cytokines interleukin-8 (IL-8; *A*) and interleukin-6 (IL-6; *B*) in the distal region of the small intestine (means ± SE) are shown. Results for doxorubicin-treated pigs fed formula (DOX-Form, $n = 9$), saline-treated pigs fed formula (SAL-Form, $n = 7$), doxorubicin-treated pigs fed bovine colostrum (DOX-Colos, $n = 9$), and saline-treated pigs fed bovine colostrum (SAL-Colos, $n = 7$) are shown. [a,b]Different superscript letters indicate significant differences among the four groups ($P < 0.05$).
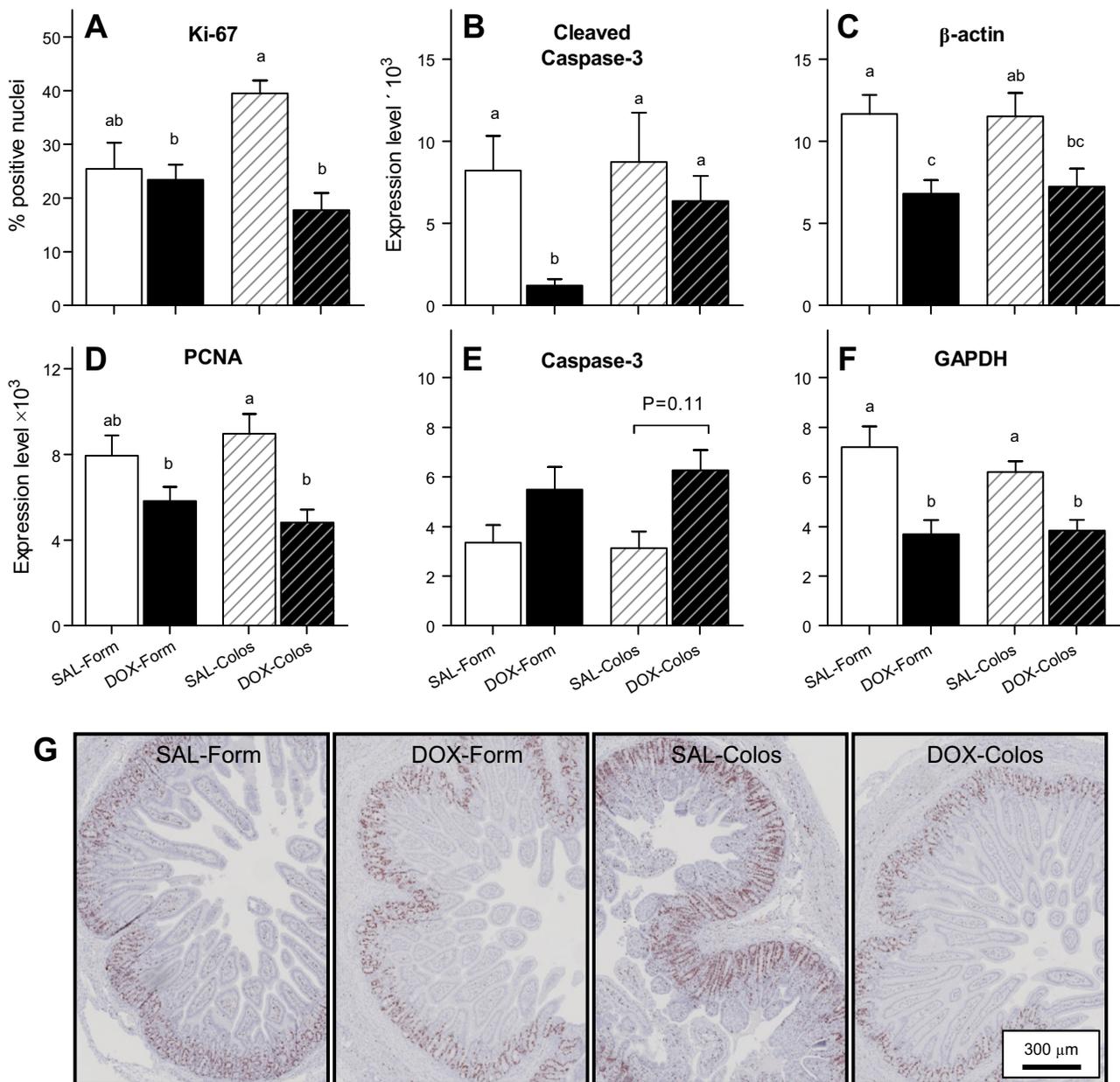
Fig. 7. Markers of proliferation, apoptosis, and intracellular structure are shown. Immunohistochemistry staining and analysis of Ki-67 (*A* and *G*) and Western blot quantification of PCNA (*D*), cleaved caspase-3 (*B*), caspase-3 (*E*), β-actin (*C*), and GAPDH (*F*) in intestinal tissue samples from the middle region (means ± SE) are shown. Results for doxorubicin-treated pigs fed formula (DOX-Form, $n = 9$), saline-treated pigs fed formula (SAL-Form, $n = 6–7$), doxorubicin-treated pigs fed bovine colostrum (DOX-Colos, $n = 8–9$), and saline-treated pigs fed bovine colostrum (SAL-Colos, $n = 6–7$) are shown. [a,b,c]Different superscript letters indicate significant differences among the four groups ($P < 0.05$).

maltodextrins (56). Such formulas only cause severe intestinal inflammation and pathology in preterm pigs, not in normal term pigs, although some intestinal functions may be negatively affected also in term pigs (55).

Bioactive factors in milk have previously been reported to ameliorate mucosal toxicity after administering chemotherapeutic agents to rats and hamsters, and there is also some evidence for beneficial effects in human chemotherapy patients (11, 14, 15, 22, 23, 44, 59). Such effects include reducing bacterial translocation across the gut mucosa in patients after abdominal surgery (9) and reducing intestinal permeability caused by nonsteroidal anti-inflammatory drugs that facilitate bacterial translocation to the mesenteric lymph nodes, liver,

spleen, and peripheral blood (29, 30). The different intestinal and systemic responses between DOX pigs fed colostrum or formula suggests that bovine colostrum may be particularly beneficial during inflammatory processes. This is consistent with the beneficial colostrum effects observed for inflammatory conditions in humans (44, 47, 52) and for the devastating GI inflammatory disease in preterm infants, necrotizing enterocolitis (24, 41, 54).

The greater mucosa proportion, villus height, lactose digestive capacity, and reduced permeability in the DOX pigs fed colostrum, relative to formula, highlight the benefits of bovine colostrum on gut structure and function during DOX chemotherapy. This is corroborated by lowered IL-8 in ileal tissue

and lower circulating CRP levels and is consistent with other results from pigs (36, 46). The higher activities of the measured brush-border enzymes and increased villus length, particularly in the DOX-Colos pigs, may indicate that colostrum stimulated intestinal regeneration after chemotherapy. Regardless, Ki-67 and PCNA analyses did not show signs of increased intestinal proliferation on *day 5* after DOX treatment, suggesting that most of the colostrum-stimulated adaptive proliferation had already occurred by this time point. In line with this, other experimental studies have demonstrated a very early onset of cellular toxicity after DOX treatment, and that intestinal responses are highly dynamic with cellular regeneration within a few days of DOX administration (16, 61). Interestingly, we found very low levels of cleaved caspase-3 in the DOX-Form group, the group with the worst clinical outcome, while DOX-Colos pigs had similar levels as SAL controls. This suggests that the caspase-3 pathway is not dominating the DOX-related cell death, at least not 5 days after DOX treatment, similar to findings in other studies (16, 31). The reduced level of cleaved caspase-3 in DOX-Form is even more evident when adjusted relative to the level of uncleaved caspase-3. This indicates that caspase-3 activation is somehow affected (66). The accumulation of uncleaved caspase-3 in the DOX groups could indicate altered expression as a response to chemotherapy. Conversely, there was a more consistent negative effect of DOX on β-actin and GAPDH protein abundance, reflecting that chemotherapy affected structural and metabolic processes of the intestinal cells (2, 32), even 5 days after treatment. These two proteins are normally considered high-abundance housekeeping proteins with fundamental functions for most cells and are often used for protein loading control when analyzing Western blots (2, 32). Such cellular protein controls are, therefore, negatively affected and, therefore, unreliable in studies of chemotherapy-induced mucositis, as also indicated in other studies (2, 32).

We have demonstrated that preweaned pigs provide a good model for studying the responses to chemotherapeutic agents during early life and for evaluating liquid diets for enteral nutrition support to address the associated toxicity and GI disturbance. Although the incidence of cancer is low among infants, the unique nature of the immature intestine and the responses to chemotherapy need to be better understood for more effective chemotherapy in this sensitive population. A limitation of our study is the use of only a single dose of DOX. Chemotherapeutic regimens typically involve multiple rounds of treatment, which, combined with other drugs, increases the toxicities. Questions remain regarding the more long-term outcomes in both animals and children (35, 57) and whether colostrum would shorten the recovery period, allowing for more frequent chemotherapy.

The findings indicate that bovine colostrum or other bioactive milk products may be beneficial to include into the enteral nutrition support during chemotherapy. DOX had more severe effects on GI parameters in Form-fed compared with Colos-fed pigs, illustrating that the intestinal response to chemotherapy is diet dependent. However, the colostrum diet was associated with relatively low body weight gains, even in control pigs, and it might be suboptimal and also unfeasible to supply bovine colostrum as the sole source of enteral nutrition for pediatric cancer patients for extended periods. Still, the present study highlights how nutritional intervention may reduce toxic responses to DOX in piglets, and, consequently, we have initi-

ated a clinical study on bovine colostrum as a diet supplement to children with leukemia receiving chemotherapy (Clinical-Trials.gov Identifier: NCT01766804).

DISCLOSURES

None of the authors have any conflicts of interest. University of Copenhagen has filed a patent application on use of bovine colostrum for human infants. P. T. Sangild is listed as sole inventor, but has declined any share of potential revenue arising from commercial exploitation of such a patent.

AUTHOR CONTRIBUTIONS

R.L.S., P.E.L.P., and P.M.H. performed experiments; R.L.S., P.E.L.P., P.J., C.F.H., and P.M.H. analyzed data; R.L.S., P.E.L.P., M.R., P.J., C.F.H., R.K.B., P.M.H., K.M., and P.T.S. interpreted results of experiments; R.L.S. prepared figures; R.L.S. drafted manuscript; R.L.S., P.E.L.P., M.R., P.J., C.F.H., R.K.B., P.M.H., K.M., and P.T.S. edited and revised manuscript; R.L.S., P.E.L.P., M.R., P.J., C.F.H., R.K.B., P.M.H., K.M., and P.T.S. approved final version of manuscript; P.E.L.P., R.K.B., K.M., and P.T.S. conception and design of research.

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