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ABSTRACT

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 gastrointestinal trophic effects of bovine colostrum would reduce the severity of GI complications associated with doxorubicin treatment. Five-day-old pigs were administered doxorubicin (DOX, 1 x 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 five 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 (CRP), compared with SAL-Form. Plasma citrulline was not affected by DOX treatment or diet. Thus, a single dose of doxorubicin 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.

Keywords: Chemotherapy-induced mucositis, intestinal toxicity, enteral nutrition, milk, bovine colostrum

NEW AND NOTEWORTHY

In this study, pre-weaned 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.

INTRODUCTION

Gastrointestinal (GI) toxicity is a major dose-limiting adverse effect of chemotherapy with implications for both morbidity and mortality (11, 48). The associated symptoms include pain, nausea, vomiting, and diarrhea 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 (11, 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 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 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 differ 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 make it easier to use standard clinical and medical care procedures when inducing and evaluating chemotherapy toxicity (35, 36, 45, 46, 59).

Bovine colostrum contains many bioactive compounds, such as immunoglobulin (Ig), transforming growth factor β (TGF-β) and insulin-like growth factor 1 (IGF-1) that may stimulate gut growth and function and provide mucosal protection via immunomodulatory effects and changes to the gut microbiota (44). Thus, colostrum 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 colostrum protects against necrotizing enterocolitis (NEC), a severe condition with inflammatory and ulcerative lesions in the GI tract (24). Thus, bovine colostrum might provide gastrointestinal 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. Doxorubicin (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 third 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 (~30^0 C) with a 12 h light/12 h dark cycle. Each cage contained behavioral enrichments (toy-cloths) and allowed visual contact among individual pigs.

Shortly after arrival, the pigs were anesthetized using 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) 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 colostrum (DOX-Colos, $n = 9$) or formula (DOX-Form, $n = 9$), and saline groups fed bovine colostrum (SAL-Colos, $n = 7$) or formula (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 non-milk 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 5291 kJ/L (energy), 96 g/L (protein), 40 g/L (carbohydrate, with 36 g/L maltodextrins) and 79 g/L (fat). The colostrum 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 3986 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 (i.v.) dose of DOX over 30 min, corresponding to 100 mg/m^2 body surface area or an equivalent volume of SAL. This dose of DOX is comparable with doses used in treatment of pediatric cancers such as acute lymphoblastic leukemia (ALL) and has been shown to induce intestinal toxicity in weaned pigs (36). Body surface area was calculated as 70 × kg body weight $^{0.75}$ /1000 (9), providing approximately 5-5.5 mg/kg DOX for the 2.5-3.5 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 prior to d 5 were as described elsewhere (46).

Hematology, CRP, and in vivo markers of intestinal function

Blood samples were collected at baseline, immediately prior to administration of DOX, on d 3 and on d 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 (60). 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, USA). Quantification of citrulline was carried out using an analysing 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 sacrifice on d 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 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 d 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, respectively. 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

184 Villus height and crypt depth were determined as previously described (46). The proportion of
185 the small intestine represented by mucosa was determined by scraping off the mucosal layer and drying
186 (49). The frozen intestinal samples were processed for analyses of brush border enzymes and cytokine
187 levels as described elsewhere (46, 50). Briefly, the tissues were homogenized in 1% Triton X-100 and
188 the activities of three dissacharidases (lactase, maltase, sucrase) and three peptidases (aminopeptidase
189 A, ApA; aminopeptidase N, ApN; dipeptidylpeptidase IV, DPP-IV) were expressed as a hydrolytic rate
190 of 1 μmol substrate released per min at 37 ^{o}C per g wet whole intestinal tissue. Intestinal inflammatory
191 responses were based on cytokine levels in the Dist region, and measured using the DuoSet ELISA
192 Development kit (R&D Systems, Abingdon, UK) targeted for porcine interleukin-1β (IL-1b),
193 interleukin-6 (IL-6), interleukin 8 (IL-8), and tumor necrosis factor α (TNF-α), according to the
194 manufacturer's instructions.
195
196 *Intestinal proliferation, apoptosis, and structural proteins.*
197 Enterocyte proliferation was assessed by immunohistochemistry, staining for Ki-67 on sections
198 from the Mid intestine, as described previously (58). Quantification was performed by image analysis
199 using Visiomorph 4.6 (Visiopharm, Copenhagen). The total area of Ki 67-positive stained nuclei were
200 calculated and presented as a fraction of all nuclei.
201 Expression of PCNA was used as a marker of proliferation and that of Caspase-3 as a marker of
202 apoptosis. β-actin and GAPDH proteins were quantified by Western blot and uused as indicators of
203 basic cytostructural and metabolic conditions, as previously described (25). Briefly, 25 mg protein
204 extracted from the mid intestine segments was resolved by electrophoresis and transferred onto PVDF
205 membranes. The expression of PCNA, caspase-3 and β-actin were shown with specific antibodies
206 (Santa Cruz, CA, USA, and Abcam, Cambridge, UK). The protein bands on the membranes were

visualized and the band densities were detected by Image J (NIH, MD, USA). 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 percentage 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 genders) 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, USA). Data are presented as arithmetic mean and SEM of raw data unless otherwise specified.

RESULTS

Clinical status and growth

All pigs continued to eat and remained active and clinically stable during the 5 d study period. No pigs required euthanasia according to humane endpoints prior to the end of the protocol. All of the pigs gained weight between d 0, when DOX (or saline) was administered, and d 2 (Fig. 2A). After d 3, weight gain decreased for both groups of DOX pigs, resulting in stable body weight of DOX-Colos pigs from d 3-5 and weight reduction for DOX-Form pigs. The two groups of SAL pigs continued to gain weight until d 5. Growth on d 5 was significantly lower in DOX-Form pigs, compared with both groups of SAL pigs ($P < 0.05$, Fig. 2A), whereas weight for DOX-Colos pigs was not different from that in SAL pigs. Initial weight gain (d 0-2) tended to be greater for both groups of Form pigs, compared with Colos pigs (Fig. 2A). Diarrhea was commonly observed from d 3 after DOX administration with increasing severity thereafter (Fig. 2B). Diarrhea scores were similar in DOX-Form and DOX-Colos pigs. However, DOX-Form pigs had higher diarrhea score than SAL groups on d 3 and d 4 whereas scores in DOX-Colos were not significantly elevated until d 4, relative to SAL pigs (Fig. 2B). 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 d 4 and d 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 d 3 and 5 ($P < 0.05$, Fig. 3A). 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. 3A-C). On d 5, PLT values were lower in DOX versus SAL pigs, as analyzed for each of the diet regimens ($P < 0.05$, Fig. 3D), with no differences between DOX-Colos and DOX-Form. RBC did not differ among groups (Fig. 3E). Longitudinal measurements of

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

Gut function markers in vivo

After two days of Form or Colos feeding, and before the DOX (or saline) was administered, plasma galactose measured 20 min after a lactose bolus was lower for Form than for Colos pigs (92 ± 12 versus 200 ± 26 μmol/L, respectively, $P<0.01$). On d 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 ± SEM: 36.2 ± 9.7 versus 4.4 ± 3.4 μmol/L, respectively, $P < 0.05$), indicating that diet, but not DOX treatment, affected galactose uptake capacity. Among the groups, mean gut permeability, 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 (non-significant) 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 to their respective saline 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 formula-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 middle and distal intestinal regions compared with DOX-Form and SAL-Colos ($P<0.05$, Fig. 5A). The DOX treatment reduced crypt depths across all three regions and across diets, indicating lowered crypt cell proliferation ($P < 0.05$ for Colos and trend for Form, Fig. 5B). Consequently, the villus to crypt ratios in the middle and distal regions were increased in the DOX-Colos group, compared with all the other groups ($P < 0.05$, Fig. 5C).

Disaccharidase activities were highest in Prox and Mid intestinal regions, while peptidase activities were highest in Mid and Dist regions (Fig. 5D-I). Only for Colos pigs were the lactase, sucrase and maltase activities higher for DOX versus SAL pigs ($P < 0.05$, Fig. 5D-F). No significant DOX effect was detected for Form pigs. Similarly, it was only for Colos pigs that DPP-IV activity was higher for DOX versus SAL pigs (Fig. 5G). Concentrations of the proinflammatory cytokines, IL-8 and IL-6 (Fig. 6) were lower in the Dist intestine of pigs fed colostrum ($P < 0.05$ across treatments), but only for IL-8 were values for DOX-Colos lower than for DOX-Form pigs ($P < 0.05$, Fig. 6A). 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. 7A, G). Yet, there was a

297 significant negative effect of chemotherapy on Ki-67 positive cells when analyzed across diets
298 (P<0.001). Western blot of PCNA revealed similar results in that values were lower in DOX-Colos
299 than in SAL-Colos pigs (P<0.05), while DOX-Form tended to show lower values than SAL-Form pigs
300 (P=0.19, Fig 7D), with no significant effects of diet. Cleaved caspase-3 values were significantly lower
301 in DOX-Form than in the other three groups which had similar levels (P<0.05 Fig. 7B). Uncleaved
302 Caspase-3 did not differ significantly among groups (Fig. 7E), although DOX increased the values,
303 relative to SAL, as analyzed across diets (P<0.01). Overall, DOX treatment decreased the β-actin and
304 GAPDH levels, with limited effects of diet, Fig. 7C and 7F).
305
306 **DISCUSSION**
307 Nutritional support is critical during treatment of pediatric cancer patients to prevent
308 malnutrition which decreases chemotherapy tolerance, increases toxicity and infection rates, and
309 compromises outcome (4). Enteral nutrition formulas are preferred over total parenteral nutrition
310 because of assumed benefits for the gut (37) and are recommended in patients with a functional
311 gastrointestinal tract. Yet, there is no consensus regarding the type of enteral nutrition support for
312 pediatric patients. Our findings indicate that the type of liquid diet support influences the response to
313 the chemotherapeutic agent, doxorubicin, and that a diet rich in milk bioactive factors, such as bovine
314 colostrum, might be beneficial.
315 In humans, gut toxicity develops early after chemotherapy (27) and the onset of diarrhea and
316 weight loss 3-4 days after treatment in the current study coincides with the onset of GI toxicity in older,
317 weanling piglets after a single dose of doxorubicin (36). Likewise, the intestinal structural response to
318 DOX (e.g. decreased intestinal weight and amount of mucosa) is consistent with data from studies in
319 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 versus 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 (12, 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 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 (10, 13, 15, 22, 23, 44, 57). Such effects include reducing bacterial translocation across the gut mucosa in patients after abdominal surgery (14) and reducing intestinal permeability caused by non-steroidal 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

366 proliferation had already occurred by this time point. In line with this, other experimental studies have
367 demonstrated a very early onset of cellular toxicity after DOX treatment and that intestinal responses
368 are highly dynamic with cellular regeneration within few days of DOX administration (16, 61).
369 Interestingly, we found very low levels of Cleaved Caspase-3 in the DOX-Form group, the group with
370 the worst clinical outcome, while DOX-Colos pigs had similar levels as saline controls. This suggests
371 that the Caspase-3 pathway is not dominating the DOX-related cell death, at least not 5 d after DOX
372 treatment similar to findings in other studies (16, 31). The reduced level of cleaved Caspase-3 in DOX-
373 Form is even more evident when adjusted relative to the level of uncleaved Caspase-3. This indicates
374 that Caspase-3 activation is somehow affected (66). The accumulation of uncleaved Caspase-3 in the
375 DOX groups could indicate altered expression as a response to chemotherapy. Conversely, there was a
376 more consistent negative effect of DOX on β-actin and GAPDH protein abundance, reflecting that
377 chemotherapy affected structural and metabolic processes of the intestinal cells (2, 32), even 5 d after
378 treatment. These two proteins are normally considered high-abundance housekeeping proteins with
379 fundamental functions for most cells and are often used for protein loading control when analyzing
380 Western blots (2, 32). Such cellular protein controls are therefore negatively affected and therefore
381 unreliable in studies of chemotherapy-induced mucositis, as also indicated in other studies (2, 32).
382 We have demonstrated that preweaned pigs provide a good model for studying the responses to
383 chemotherapeutic agents during early life and for evaluating liquid diets for enteral nutrition support to
384 address the associated toxicity and GI disturbance. Although the incidence of cancer is low among
385 infants, the unique nature of the immature intestine and the responses to chemotherapy need to be
386 better understood for more effective chemotherapy in this sensitive population. A limitation of our
387 study is the use of only a single dose of doxorubicin. Chemotherapeutic regimens typically involve
388 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, 59) 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 initiated a clinical study on bovine colostrum as a diet supplement to children with leukemia receiving chemotherapy (ClinicalTrials.gov Identifier: NCT01766804).

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DISCLOSURES

None of the authors have any conflicts of interest. Biofiber-Damino provided the bovine colostrum. University of Copenhagen has filed a patent application on use of bovine colostrum for pediatric patients. Per Sangild is listed as sole inventor but has declined any share of potential revenue arising from commercial exploitation of such a patent.

AUTHOR CONTRIBUTIONS

Experiments were conceived and designed by P.E.L.P., R.L.S., K.M., R.K.B., and P.T.S. Data were collected and analyzed by R.L.S., P.E.L.P., P.J., C.F.H., and P.M.H.H. The manuscript was drafted by R.L.S, M.R., R.K.B. and P.T.S. All authors interpreted data, critically revised the manuscript for content, approved the version for submission, and agreed to be accountable for all aspects of the work to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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FIGURE CAPTIONS

Fig. 1. Study design with enteral nutrition intervention using formula (Form) or bovine colostrum (Colos). Catheters and feeding tubes were fitted surgically two days prior to chemotherapy. Doxorubicin (DOX) or saline (SAL) was administered on d 0. Blood samples were collected prior to the treatment, d 3 and 5, and analyzed for hematologic parameters and C-reactive protein. Digestive capacity of lactose was tested on d 4 and intestinal permeability was assessed with the lactulose/mannitol-test (lac/man test) on d 5 when animals were sacrificed. 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, $n = 7$).

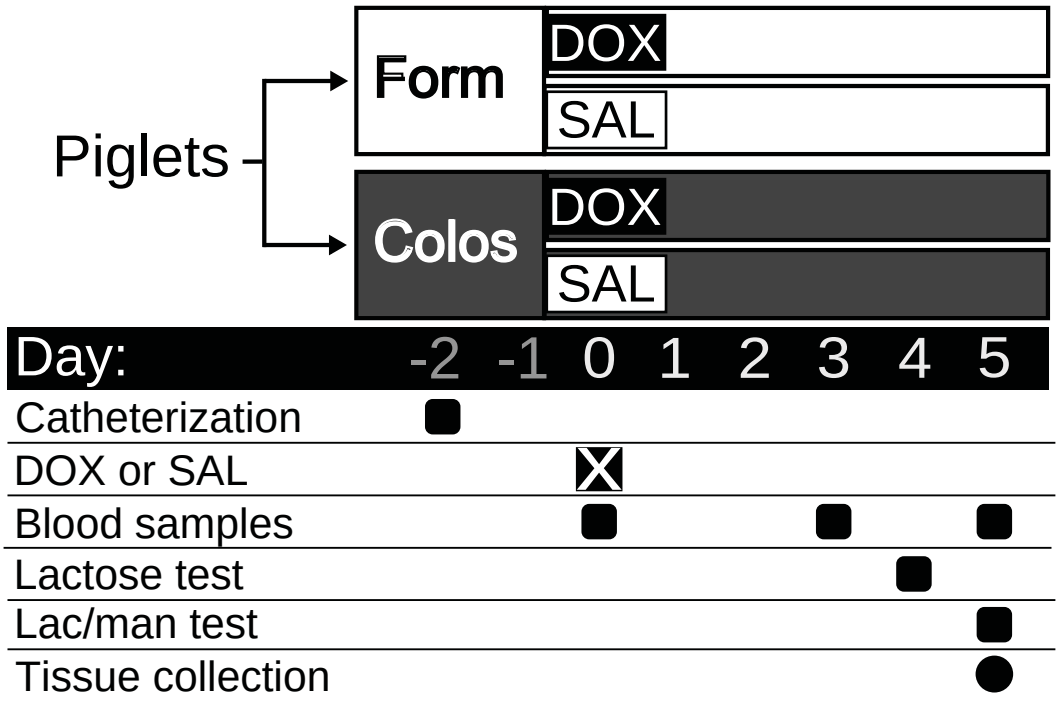
Fig. 2. Weight gain (A) indicated as percent change relative to initial body weight prior to treatment on d 0 and diarrhea score (B) presented as mean ± SEM. Doxorubicin-treated pigs fed formula (DOX-Form, $n = 9$), saline-treated pigs fed formula (SAL-Form, $n = 7$), doxorubicin-treated pigs fed bovine colostrum (DOX-Colos, $n = 9$), and saline-treated pigs fed bovine colostrum (SAL-Colos, $n = 7$). 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) presented as means ± SEM. 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-

631 treated pigs fed bovine colostrum (SAL-Colos, n = 2-7). Different superscript letters indicate
632 significant differences among groups within the same time point ($P < 0.05$).
633
634 Fig. 4. Intestinal permeability (A) measured on d 5 as the urinary ratio of lactulose to mannitol 3-5 h
635 after an enteral bolus of lactulose and mannitol solution and proportion of mucosal mass relative to
636 total intestinal mass (B) presented as means ± SEM. Doxorubicin-treated pigs fed formula (DOX-Form,
637 n = 4-9), saline-treated pigs fed formula (SAL-Form, n = 7), doxorubicin-treated pigs fed bovine
638 colostrum (DOX-Colos, n = 8-9), and saline-treated pigs fed bovine colostrum (SAL-Colos, n = 7).
639 Different superscript letters indicate significant differences among groups within the same time point
640 ($P < 0.05$).
641
642 Fig. 5. Villus height (A), crypt depth (B), villus-crypt ratio (VC-ratio, C), and activity of lactase (D),
643 sucrase (E), maltase (F), DPP-IV (G), ApA (H), and ApN (I) measured in the proximal (Prox), middle
644 (Mid), and distal (Dist) regions of the small intestine, respectively (mean ± SEM). Doxorubicin-treated
645 pigs fed formula (DOX-Form, n = 9), saline-treated pigs fed formula (SAL-Form, n = 7), doxorubicin-
646 treated pigs fed bovine colostrum (DOX-Colos, n = 9), and saline-treated pigs fed bovine colostrum
647 (SAL-Colos, n = 7). Different superscript letters indicate significant differences among the four groups
648 within the same region ($P < 0.05$).
649
650 Fig. 6. Tissue levels of proinflammatory cytokines interleukin-8 (IL-8, A) and interleukin-6 (IL-6, B)
651 in the distal region of the small intestine (mean ± SEM). Doxorubicin-treated pigs fed formula (DOX-
652 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, $n = 7$). Different superscript letters indicate significant differences among the four groups ($P < 0.05$).

Fig. 7. Markers of proliferation, apoptosis, and intracellular structure. Immunohistochemistry staining and analysis of Ki-67 (A, 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 (mean ± SEM). 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). Different superscript letters indicate significant differences among the four groups ($P < 0.05$).


Piglets
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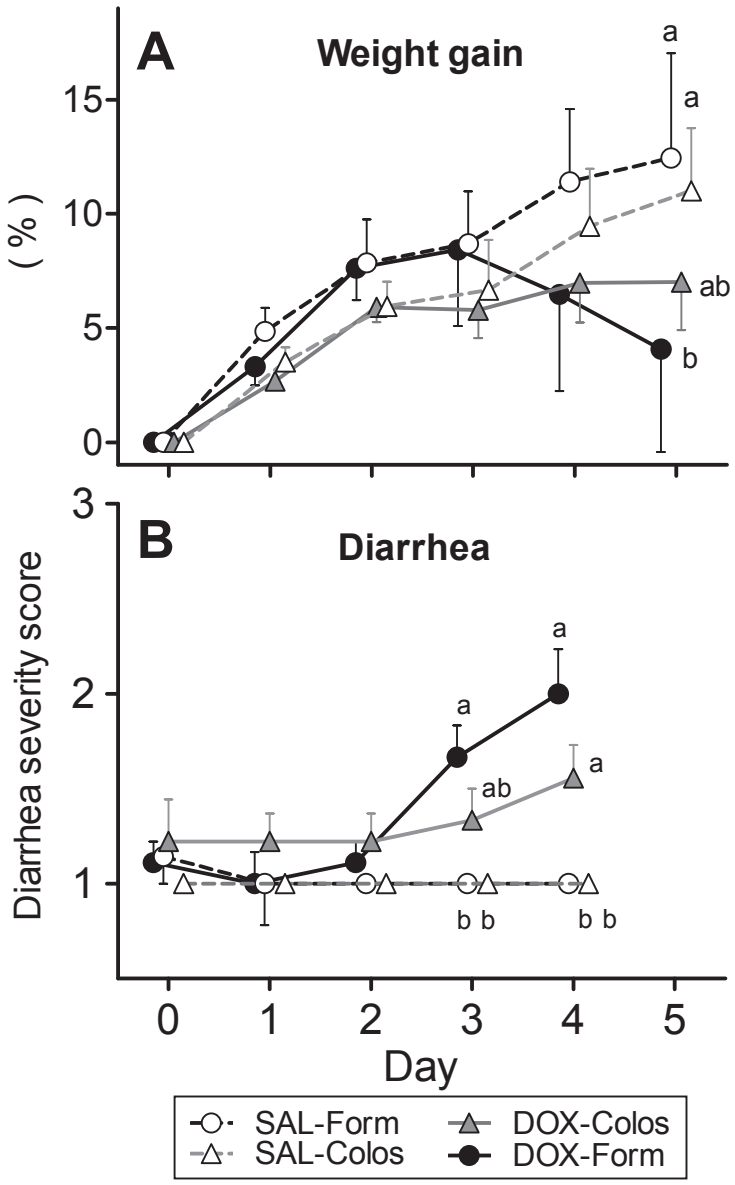
Lac/man test
Tissue collection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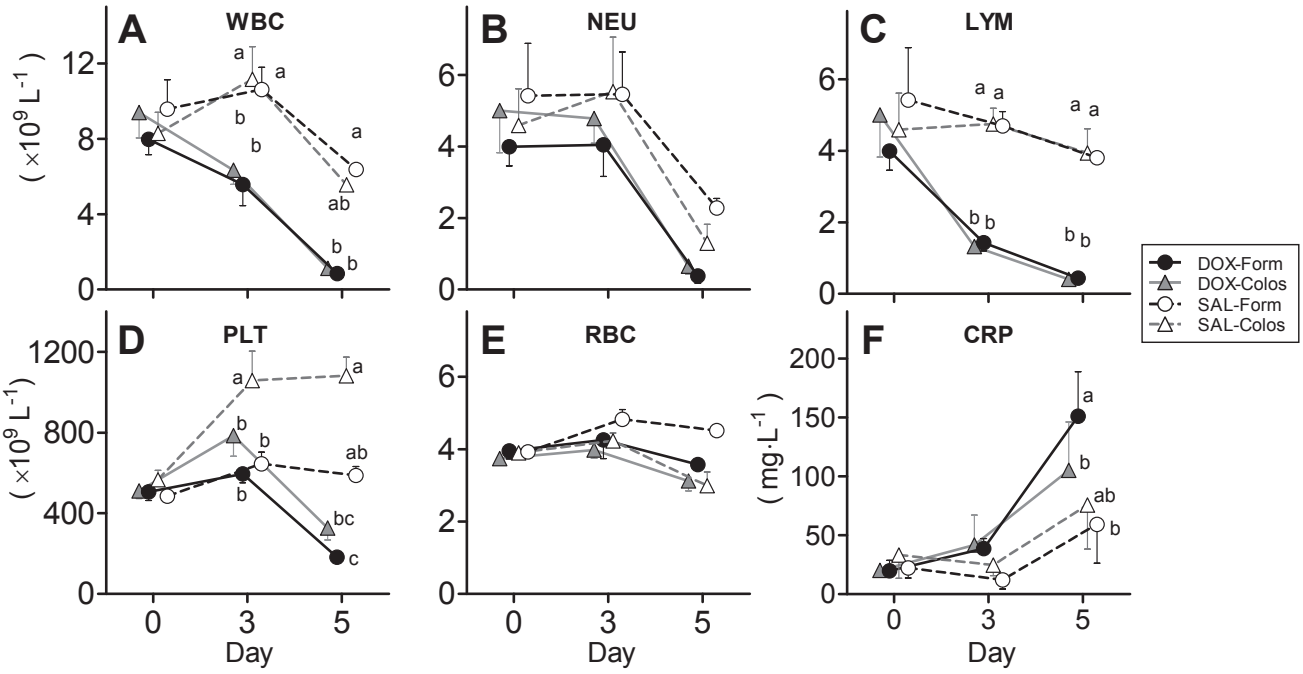
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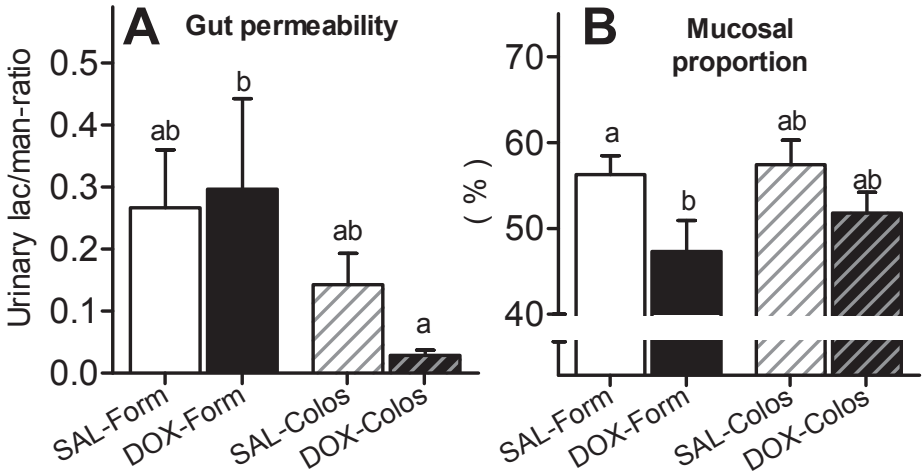
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Gut permeability
Urinary lac/man-ratio
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0.3
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SAL-Form
DOX-Form
SAL-Colos
DOX-Colos
B
Mucosal proportion
(%)
70
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50
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SAL-Form
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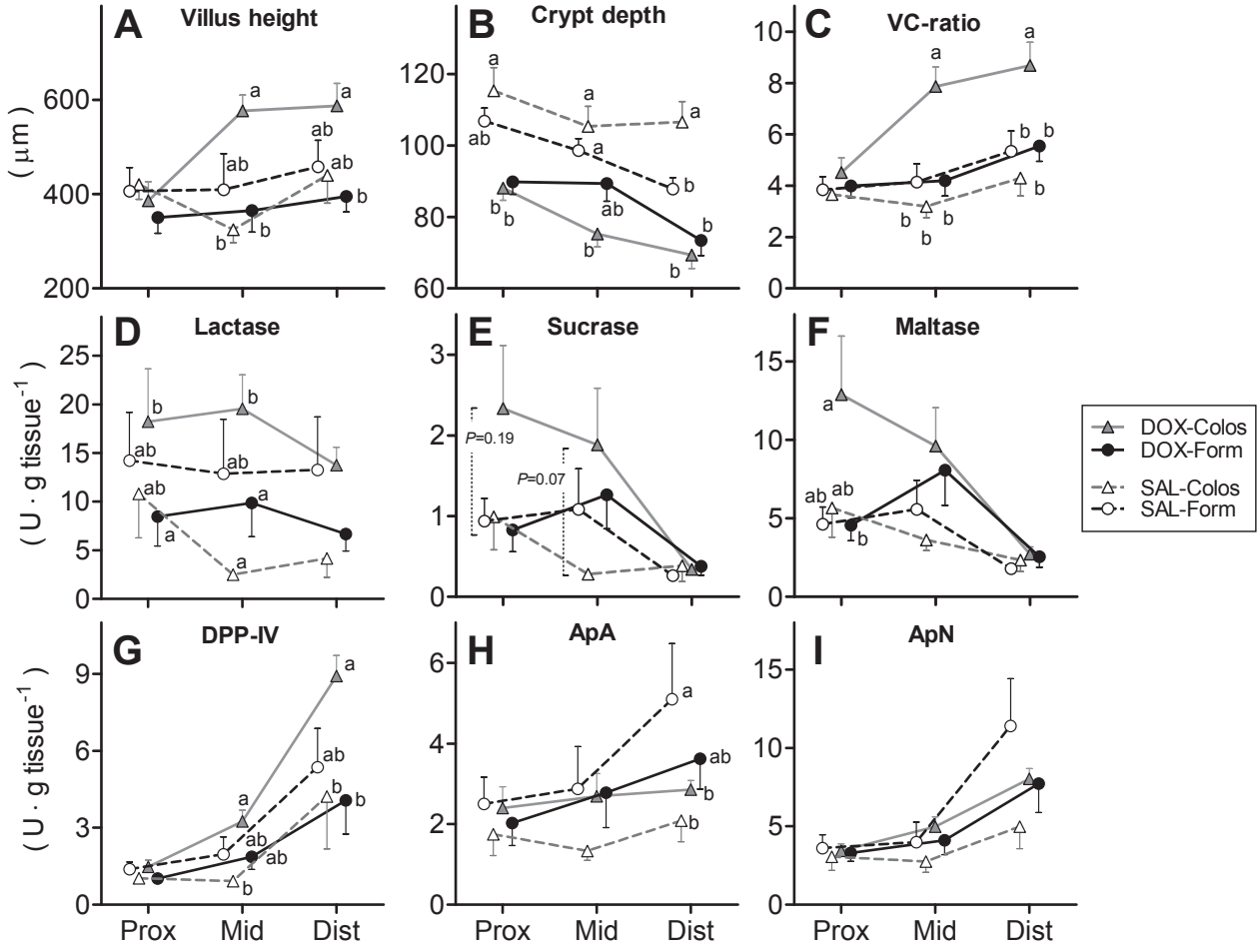
DOX-Colos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Villus height
(µm)
B
Crypt depth
C
VC-ratio
D
Lactase
(U · g tissue-1)
E
Sucrase
P=0.19
P=0.07
F
Maltase
G
DPP-IV
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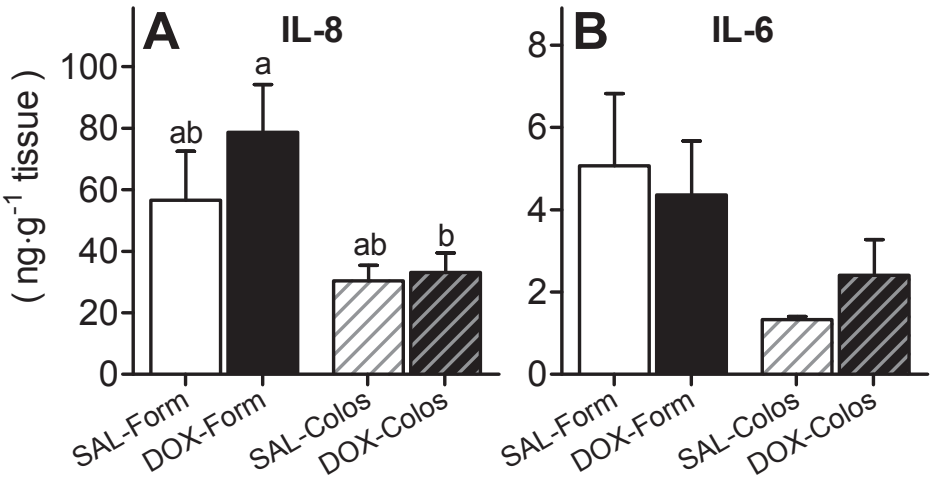
DOX-Form
SAL-Colos
SAL-Form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IL-8
(ng·g-1 tissue)
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80
60
40
20
0
ab
a
ab
b
SAL-Form
DOX-Form
SAL-Colos
DOX-Colos
B
IL-6
8
6
4
2
0
SAL-Form
DOX-Form
SAL-Colos
DOX-Colos

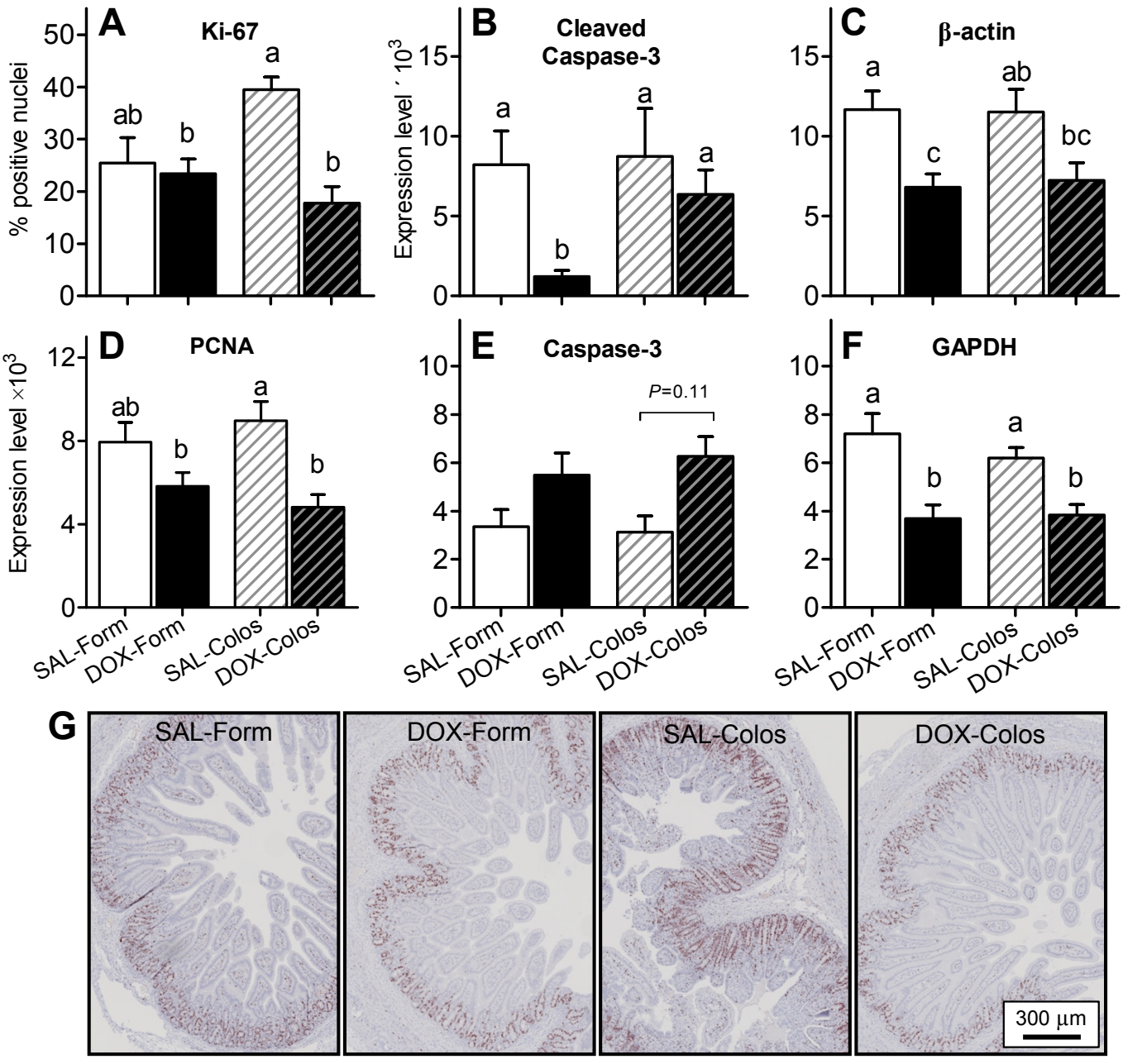
A
Ki-67
% positive nuclei
50
40
30
20
10
0
ab
b
a
b
B
Cleaved Caspase-3
Expression level ´ 10^3
15
10
5
0
a
b
a
a
C
β-actin
15
10
5
0
a
c
ab
bc
D
PCNA
Expression level ×10^3
12
8
4
0
ab
b
a
b
E
Caspase-3
P=0.11
10
8
6
4
2
0
F
GAPDH
10
8
6
4
2
0
a
b
a
b
SAL-Form
DOX-Form
SAL-Colos
DOX-Colos
G
SAL-Form
DOX-Form
SAL-Colos
DOX-Colos
300 µm

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 [ab]	33.2 ± 2.1 [a]
Colon	12.1 ± 0.7 [ab]	11.1 ± 1.6 [a]	14.8 ± 1.3 [b]	12.0 ± 0.7 [ab]
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

Organ weights expressed relative to body weight (g/kg, mean ± SEM). 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, $n = 7$). Different superscript letters indicate significant differences among the four groups ($P < 0.05$).