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Gross Morphometry, Histomorphometry, and Immunohistochemistry Confirm Early and Persistent Jejunal Crypt Hyperplasia in Poult with Enteritis and Depressed Growth

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SUMMARY.

Phosphorylated histone 3 (PH3) and cleaved caspase 3 (CCASP3) were used to detect proliferating and apoptotic cells, respectively, in the jejunums of female sibling poult, with and without enteritis and depressed growth, from hatch to day 35. Poult that developed enteritis and depressed growth (SIB flock) were raised on a commercial farm in eastern North Carolina, whereas poult with normal growth and no enteritis (TAU flock) were raised in the Teaching Animal Unit at North Carolina State University College of Veterinary Medicine. Beginning on day 5 through day 35 and at processing, TAU poult were significantly heavier than SIB poult. Jejunal weights, relative jejunal weights, and jejunal densities were greater in SIB poult from day 10 through 35. Jejunal efficiency (body weight /jejunal length) was higher in TAU poult at day 5 and days 10 through 35. Mucosal thickness was greater in SIB poult between days 7 and 21 but greater in TAU poult at days 28 and 35. From day 7 to 35, villus-to-crypt ratios were higher for TAU poult and lower for SIB poult because hyperplastic crypts formed a greater percentage of the mucosa in SIB poult. By day 7, PH3- and CCASP3-positive cells were increased in SIB poult, showing that mucosal changes resulted from combined crypt epithelial hyperplasia and increased apoptosis of villous enterocytes. Findings in this study confirm that enteritis, in the absence of clinical signs, and depressed growth in turkey poult begins by day 7, can be identified microscopically, persists for at least 35 days, is associated with lower processing weights, and has a profound negative effect on turkey growth.

Keywords

turkey; poult enteritis; immunohistochemistry; histomorphometry; gross morphometry; crypt hyperplasia; growth; gut health

Enteritis in poult is described in various syndromes, including poult enteritis complex (5), stunting syndrome (2), poult malabsorption syndrome (37), light turkey syndrome (30),

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transmissible enteritis (1), poult enteritis mortality syndrome (4,22), spiking mortality (6), enteric disease in poults (12), turkey enteritis (27), and big bird–little bird syndrome (47). Collectively, these are all lumped together under the global term of poult enteritis complex (25). Estimates of annual losses of \$300 million to \$400 million were based on growth depression of 10%–15% in affected poults (7).

Viruses isolated from poults with enteritis include turkey astrovirus (3,34,35,36,42), coronavirus (10,21,23), enterovirus-like virus (25), parvovirus (49) picornavirus (12), reovirus (27), rotavirus (9,23), small round virus (41), torovirus (39), and others (11). Viruses associated with poult enteritis also have been demonstrated by electron microscopy (48). Bacteria, notably enteropathogenic *Escherichia coli*, are associated with some of these syndromes in the poult enteritis complex (24). The multifactorial pathogenesis of poult enteritis with emphasis on viruses as initiators and the role of bacteria and/or protozoa including cryptosporidia and coccidia as well as management and nutrition are reviewed (33).

Comparison of the gut virome in commercially raised poults (termed SIB for this study) with the virome in poults from the same breeder flocks, hatched in the same hatchery, but raised in comparative isolation in the Teaching Animal Unit (TAU) at the North Carolina College of Veterinary Medicine showed differences in several enteric picornavirus sequences (12). The availability of similar paired SIB-TAU poults provided an opportunity to repeat earlier studies (unpublished results) using body weight and gross morphometry to show differences between poults raised in these two different environments. Availability of immunohistochemistry and histomorphometry used in the study of enteric disease in horses and porcine models provided additional tools for evaluation of enteric disease in turkey poults (17,18,19,20).

Using gross morphology, histomorphology, and immunohistochemistry, crypt hyperplasia with increased numbers of proliferating and apoptotic cells in the jejunums from SIB poults occurring by day 7 and persisting through day 35 is described.

MATERIALS AND METHODS

Poults and flock management.

Sibling female poults from the same breeder flock were hatched together and divided into two flocks. One flock ($n = 2000$) was placed in the Teaching Animal Unit (TAU) at North Carolina State University, College of Veterinary Medicine, in Raleigh, North Carolina. The sibling (SIB) flock ($n = 8000$) was placed in one section of a brooder house on a commercial farm in eastern North Carolina. Poults in both flocks received the same formulated feed from the same mill and were provided free access to water. SIB flocks were raised using commercial turkey production methods, whereas TAU poults were raised in relative isolation, intensively managed with lower stocking density, and used as a teaching flock. Protocols for animal care were approved by the Institutional Animal Care and Use Committee of North Carolina State University.

Sample collection.

Samples were obtained from 6 poult on the day of hatch and from 6 poult in each flock on days 3, 5, 7, 10, 12, 14, 21, 28, and 35 ($n = 114$) to determine body weights and jejunal weights and lengths and to collect jejunal segments for histomorphometry and immunohistochemistry. Preprocessing body weights were obtained on days 72 and 73 for the TAU and SIB flocks, respectively. On sampling days, approximately 60 poult in each flock were caught in a catch pen and weighed individually. Until 6 poult were obtained, every third poult was selected for sampling when it was weighed and euthanized by cervical dislocation. The jejunum (small intestine between the hepatoduodenal ligament and attachment of ileocecal ligaments) was removed, weighed (g), and measured (cm). Jejunal density (jejunal weight/jejunal length), relative jejunal weight (jejunal weight/body weight $\times 100$), and jejunal efficiency (body weight/jejunal length) were calculated. A 2–3 cm section of jejunum proximal to Meckel's diverticulum was opened longitudinally, stapled to cardboard, and placed into 10% neutral buffered formalin. After 24 hr fixation, jejunal sections were transferred to 70% ethanol and stored at 4° C until processed for histomorphology and immunohistochemistry.

Histomorphometry.

Paraffin embedded tissue was sectioned at $\sim 5 \mu\text{m}$ and stained with hematoxylin and eosin. Villous height and crypt depth were measured (CellSens™, ver. 1.11, Olympus Corp., www.olympus-sis.com) for 8–10 paired regions. Inappropriately angled or poorly fixed samples were excluded. Mucosal thickness and villus-to-crypt ratio (V:C) were calculated.

Immunohistochemistry.

Jejunal sections ($\sim 5 \mu\text{m}$) were mounted on positively charged glass slides, deparaffinized, and rehydrated. Heat-induced epitope retrieval was done by placing slides into citrate Target Retrieval Solution™ (DakoCytomation, Glostrup, Denmark) for 30 sec at 119° C followed by 90° C for 10 sec in a Pascal pressure chamber (DakoCytomation). Slides were cooled to room temperature and moved to a DakoAutostainerPlus™ (DakoCytomation) after which they were incubated in a peptide-blocking agent, Background Buster™ (Innovex Biosciences, Richmond, CA), for 30 min. Primary antibodies were applied to tissue sections diluted in Common Antibody Diluent™ (BioGenex, Fremont, CA). For staining of phosphorylated histone 3 (PH3), α -phospho-histone H3 (PH3, rabbit, EMD Millipore, Darmstadt, Germany) was diluted 1:500. Cleaved caspase 3 (CCASP3) was detected using α -cleaved caspase 3 (CCASP3, rabbit, Cell Signaling Technology, Inc., Danvers, MA) diluted 1:400. Primary antibody incubation was done for 30 minutes at room temperature. Subsequently, slides were incubated for 30 min in a 1:1 dilution of ImmPRESS™ (peroxidase) polymer anti-rabbit IgG reagent (Vector Laboratories, Burlingame, CA) followed by 30 min in ImmPact DAB peroxidase (HPR) substrate solution™ (Vector Laboratories). To identify nonspecific binding, Rabbit Super Sensitive Negative Control™ (BioGenex) was applied to tissue sections.

PH3-positive enterocytes were counted in 100 crypts. For CCASP3, the number of positive enterocytes in 30 crypt-villus pairs was determined. If a full set of 100 crypts or 30 crypt-villus pairs could not be evaluated, that sample was eliminated from the study. Except for

samples obtained on the day- of hatch and day 3, which were not counted, at least 4 jejunal sections were analyzed for each sampling.

Statistical analyses.

The Kruskal-Wallis test (JMP™, Pro 13.0.0, SAS Institute Inc., Cary, NC) was used to determine significance for all data except preprocessing body weights at 72 and 73 days, which were analyzed using a *t*-test to compare means because of their normal distribution. Significance was set at $p = 0.05$.

RESULTS

Gross morphometry.

Beginning on day 5 through day 35, sampled TAU poultts were significantly heavier than sampled SIB poultts. At day 35, sampled SIB poultts weighed 29.2% less than sampled TAU poultts (1707 g vs. 1208 g) (Fig. 1A). Prior to processing, weights of turkeys in the TAU flock were still significantly greater than those in the SIB flock (6398 g vs. 5426 g; 15.2%).

Jejunal weights were similar in poultts from both flocks until day 10. Median jejunal weight increased 1.1 g in TAU poultts from 6.8 g at day 7 to 7.9 g at day 10, whereas median jejunal weights in SIB poultts increased 2.9 g from a median of 8.6 g at day 7 to 11.5 g at day 10. Jejunal weights remained significantly heavier in SIB poultts compared to TAU poultts between days 10, 14, 21, and 35, except for day 28 (Fig. 1B). Relative jejunal weight was higher in TAU poultts at day 3 (4.8% compared to 3.8%), but higher in SIB poultts at days 10, 14, 21, and 35 (Fig. 2A). Relative median weight of the jejunum in TAU poultts at day 35 was 2.02% compared to 4.62% in SIB poultts.

Jejunal length was similar in both TAU and SIB poultts except for day 14 when the median length in SIB poultts was 84 cm compared to 69 cm for TAU poultts (Fig. 2B). Jejunal density was higher in TAU poultts at day 3 but higher in SIB poultts at days 10, 14, 21, and 35 (Fig. 2C). Median jejunum density for SIB poultts at day 35 was 0.467 g/cm compared to 0.306 g/cm for TAU poultts. Day 28 values were not statistically different, although median jejunum density in TAU poultts was higher (0.572 g/cm) compared to SIB poultts (0.491 g/cm).

Jejunal efficiency was higher in TAU poultts at days 10, 14, 21, 28, and 35 (Fig. 2D). At day 35, 1 g of jejunum supported 16 g of body weight in TAU poultts compared to 11.2 g of body weight in SIB poultts.

Gross enteric lesions observed in the SIB flock were watery intestinal contents in 1 of 6 poultts each at days 3 and 5, congested mesenteric blood vessels in 1 of 6 poultts at day 7, and mild catarrhal enteritis in 3 of 6 poultts at day 10. Gross lesions of enteritis were not observed in TAU poultts. Clinical signs of enteritis were not observed in either TAU or SIB poultts.

Histomorphometry.

Mucosal thickness was greater in SIB poult at days 7, 12, 14, and 21 but lower at days 28 and 35 compared to TAU poult (Fig. 3A).

Villous height increased from 367 μm at day 3 to 845 μm (478 μm increase) at day 35 in SIB poult and from 369 μm at day 3 to 1105 μm (736 μm increase) at day 35 in TAU poult. Villous height was greater in TAU poult at days 10, 12, 21, 28, and 35 with SIB poult higher only at day 14 (Fig. 3B). Median crypt depth at day 3 was 84.7 μm in SIB poult and 81 μm in TAU poult. Crypt depth was significantly higher in SIB poult at days 7 through 35 with the median for SIB poult at day 35 being 404 μm compared to 184 μm in TAU poult (Fig. 3C). V:Cs were higher in TAU poult from day 7 through 35 (Fig. 3D). Sections of H&E-stained jejunums from 7-day-old TAU and SIB poult show relationships of crypts to villi at successively higher magnifications (Fig. 4A–F). Increased crypt depth due to proliferating cells is shown in a 7-day-old SIB poult (Fig. 4F). Crypt hyperplasia was characterized by disruption of normal crypt architecture, bulging of epithelial cells into the lumen, crowding of enlarged epithelial cells, karyomegaly of crypt epithelial cells, prominent nucleoli, and increased mitotic figures.

No other histologic lesions were found.

Immunohistochemistry.

Proliferating PH3-positive cells occurred in the crypts (Fig. 4G–L) while CCASP3-positive enterocytes undergoing apoptosis were located on tips and upper parts of villi (Fig. 4M–R). SIB poult had more proliferating PH3-positive cells than TAU poult from day 7 through days 21 and 35 (Fig. 5A). CCASP3-positive cells were increased in SIB poult compared to TAU poult at days 7 through 12 and days 21 through 35 (Fig. 5B).

DISCUSSION

Crypt hyperplasia and increased apoptosis were identified by immunohistochemistry in SIB poult as early as day 7 and was the first evidence of intestinal disease in the poult. Histomorphometry correlated with immunohistochemistry, which showed decreased jejunal V:Cs at day 7. Crypt hyperplasia, apoptosis, and decreased V:C in SIB poult persisted until at least day 35. Gross morphometry, while useful for assessing enteric health, did not show differences between SIB and TAU poult until day 10. Discovery of early and persistent jejunal lesions is important for designing and implementing management and treatment interventions to reduce economic losses from enteritis in poult. The jejunum was chosen because this region has a major role in the absorption of nutrients (14).

SIB poult met several criteria for poult enteritis including reduction in body weight, increased jejunal weight, relative jejunal weight, and jejunal density, reduced jejunal efficiency, and decreased V:C (7,33). It was not an objective of this study to investigate the possible causes of these jejunal lesions, but confirmation of differences in the jejunums and time course of these changes between SIB and TAU flocks supports this TAU-SIB model as an important one for the study of enteric disease in turkeys. A study using a previous SIB-

TAU model documented differences in the enteric picornavirus virome of SIB poult (11), but those procedures were not applied in this study.

In this study, dysregulation of the relationship between crypt and villous epithelial cells occurred in SIB poult with enteritis. Proliferation of crypt epithelial cells normally correlates with the number of enterocytes that move up the sides of villi and are eventually extruded at villous tips. Increasing crypt cells coupled with decreasing enterocyte loss results in increased villous length. In contrast to TAU poult, this relationship was not maintained in SIB poult with enteritis. Marked hyperplasia of crypt epithelium was not matched by increased length of villi, which resulted in villi remaining short or even decreasing in length (villous atrophy). Physical changes in villi resulted in decreased villous area and decreased ability of affected poult to absorb nutrients. These changes are not etiology specific. It is also possible that enterocytes being lost prematurely as indicated by increased apoptosis of enterocytes were also functionally deficient in addition to being decreased numerically. Decreased mucosal absorption is indicated by the lower jejunal efficiencies in SIB poult compared to TAU poult. Increases in jejunal length and weight in SIB poult resulted from an attempt to compensate for lost nutrient absorption and inflammation respectively. Decreased growth rate paralleled changes in mucosal morphology. Lower body weights of SIB turkeys just prior to processing indicate the functionally impaired nutrient uptake when the birds were younger remained incompletely compensated to market. Some compensatory growth occurred as evidenced by the improvement in relative body weight differences between the TAU and SIB poult at day 35 (29.2%) and preprocessing (15.2%). However, as the birds grew, absolute weight differences continued to increase until there was an average difference of 972 g (2.14 lb) at preprocessing. At a current average value of US\$0.98 (USDA Turkey Market News Report 64:40; October 6, 2017), losses from weight alone would approximate US\$2.12/bird.

Differences between TAU and SIB poult are consistent throughout the 35-day experimental period except for day 28. Jejunal weight, relative jejunal weight, and jejunal density were increased in TAU poult at day 28 and were not significantly different from values in SIB poult at the same age. Reasons for this are unknown, as there were no clinical signs of enteric or other disease in the TAU poult at any time interval. Jejunal efficiency remained higher in TAU than SIB poult at day 28. V:Cs were lower in TAU poult but still significantly higher than SIB poult. PH3-positive cells were increased in TAU poult, suggesting some stimulus for cell proliferation, but CCASP3-positive cells were not increased. The impact at day 28 in TAU poult did not affect poult at day 35 as no lesions were identified by gross morphometry, histomorphometry, or immunohistochemistry in the TAU poult. It is possible the TAU flock experienced mild transient enteritis at this time, but the cause is unknown.

In chickens, crypts are rudimentary at hatch but are well organized by day 2 with a peak in number of crypts per villi by day 3 (16). Villous height in poult measured immediately post-hatch through day 11 showed a significant increase in villous height. Crypt depth increased, but not significantly after day 4 (26). Crypts begin to form at hatch and are recognizable within several days post-hatch (45). We were able to measure crypt depth and calculate V:Cs from hatch until day 35 when the study was concluded. TAU and SIB poult

did not differ significantly in V:Cs until day 7 when crypts became hyperplastic and occupied a greater percentage of the mucosa.

Growth and development of the small intestine in chicks and poults peak within days 6–10 post-hatch. Enterocyte proliferation occurs throughout villi in the pre-hatch and immediate post-hatch period but rapidly shifts to localized proliferation within the crypts (28). A continuing increase in the difference between villous height and crypt depth occurs between days 1 and 3 post-hatch (28). Gross morphometry, histomorphometry, and measurement of mucosal enzyme activities in poults from hatch through day 12 showed intestinal weight increased to a peak at day 6, villous length increased rapidly, and crypt depth increased but at a slower rate than villous height (46). In our findings, jejunal weight increased from day 3 to 35, but, except for the 28-day value, relative jejunal weights progressively declined. A marked rise in jejunal efficiency occurred after day 10 in TAU poults. The V:C in TAU poults from day 0 through 35 remained relatively constant.

We do not know if TAU poults had access to feed after placement before SIB poults. Early access to feed has a positive effect on increasing body weight and increasing relative intestinal weight by day 2 post-hatch (31). Intestinal development is slower when access to first feed is delayed (44). The lack of significant differences in body weight, jejunal weight, and jejunal efficiency between TAU and SIB poults at day 3 suggests no early access to feed effects. TAU poults did have significantly higher jejunal density and relative jejunal weight at day 3 than SIB poults; thus an effect of early access to feed as a factor influencing our results cannot be ruled out.

Expression of PH3 and CCASP3 using immunohistochemistry is reported in avian tissues including PH3 expression in turkey (38) and chicken erythrocyte nuclei (43), PH3 expression in a study on male meiosis in finches (13), PH3 expression in the brain of Bobwhite quail (8), PH3 and CCASP3 expression in an investigation of the cell cycle in the proliferative zone of chick embryo retinas (32), CCASP3 for detection of apoptotic cells in reovirus infected chick embryo fibroblasts (40), *Mycoplasma synoviae* infection of chicken chondrocytes (15), and apoptosis in the early development of the bursa of Fabricius (29). In this study, PH3-positive cells in crypts correlated with hyperplasia of crypt enterocytes. Apoptotic cells as detected by CCASP3-positive staining were comparable to patterns observed in a porcine model (20).

Comparison of jejunal changes using multiple techniques as described in this study clearly show SIB poults have lesions detectable by day 7 that persist through day 35. A significant loss of body weight amounting to approximately 15% was associated with early enteritis in the SIB poults and persistence intestinal lesions. Immunohistochemistry and histomorphometry detected lesions by day 7 compared to detection at day 10 by gross morphometry. All methods detected jejunal lesions in the absence of clinical signs of enteritis. These observations are critical in guiding efforts to control enteric disease in poults. Interventions undertaken by the time clinical signs appear or growth depression is noted are likely to be too late. Based on this study's findings, measures to prevent enteritis in young poults must be done *in ovo* or in the first few days of life to avert the millions of dollars in industry losses associated with decreased body weight.

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Abbreviations:

CCASP3	cleaved caspase 3
H&E	hematoxylin and eosin
PH3	phosphorylated histone 3
SIB	commercial poult
TAU	Teaching Animal Unit
V:C	villus-to-crypt ratio

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Fig. 1.

Poult (A) and jejunal (B) weights for sampled SIB and TAU poults from day 0 to 35. Box plots show minimum, first quartile, median, third quartile, and maximum values.

Significantly different values ($p < 0.05$) are enclosed in rectangles. (A) TAU poults at 5, 10, 12, and day 21 to 35 were significantly heavier than SIB poults. (B) Except for day 28, jejunal weights of SIB poults were significantly heavier than TAU poults between days 10 and 35. Differences in poult and jejunal weights were greatest at day 35.

Fig. 2.

Relative jejunal weight (A), jejunal length (B), jejunal density (C), and jejunal efficiency (D) for sampled SIB and TAU poult groups from day 0 to 35. Box plots show minimum, first quartile, median, third quartile, and maximum values. Significantly different values ($p < 0.05$) are enclosed in rectangles. (A) Relative jejunal weight was greater in TAU poult groups at day 3 and greater in SIB poult groups at days 10 to 35, except for day 28 when the relative jejunal weight was high in TAU poult groups. (B) Jejunal length was similar in both SIB and TAU poult groups except for day 14 when the jejunum of SIB poult groups was significantly longer. (C) At day 3, jejunal density was greater in TAU poult groups. Jejunal density was significantly greater in SIB poult groups at days 10 to 35, except for day 28. Median values at day 28 were similar for both SIB and TAU poult groups. (D) Jejunal efficiency was significantly greater in TAU poult groups at day 5 and days 10 to 35.

Fig. 3.

Median mucosal thickness (A), villous height (B), crypt depth (C), and V:C (D) in sampled SIB and TAU poult groups from day 0 to 35. Box plots show minimum, first quartile, median, third quartile, and maximum values. Significantly different values ($p < 0.05$) are enclosed in rectangles. (A) Compared to TAU poult groups, mucosal thickness was significantly greater in SIB poult groups at days 7 and 12 to 21, but significantly lower at days 28 and 35. (B) Villous height was greater in SIB poult groups at day 14, but greater in TAU poult groups at days 10, 12, and 21 to 35. (C) Crypt depth was greater in SIB poult groups at days 7 to 35. (D) V:C ratios were greater in TAU poult groups at days 7 to 35.

Fig. 4.

Day 7 TAU and SIB poult, jejunums. (A–C, TAU poult, H&E). (A) Normal villi, crypts, and portion of muscularis; (B) relationship of crypts to base of villi; (C) organized arrangement of crypt enterocytes with occasional mitotic figures. (D–F, SIB poult, H&E, compare with A–C). (D) Mild to moderate crypt hyperplasia; (E) early crypt hyperplasia with dysplasia of crypt enterocytes; (F) hyperplasia and dysplasia of crypt enterocytes. (G–I, TAU poult, PH3). (G) PH3-positive cells in the crypts, brown staining at the tips of villi is not specific; (H) cells positive for PH3 expression in the crypts; (I) higher magnification showing PH3-positive cells in crypts. (J–L, SIB poult, PH3, compare with G–I). (J) PH3-positive cells in the crypts; (K) PH3-positive cells in the crypts; (L) higher magnification showing PH3-positive cells in crypts. (M–O, TAU poult, CCASP3). (M) Relatively few positive cells at villous tips; (N) CCASP3-positive cell at the villous tip; (O) higher magnification showing CCASP3-positive cell at the villous tip. Compare with the number of CCASP3-positive cells in the SIB poult. (P–R, SIB poult, CCASP3, compare with M–O). (P) CCASP3-positive cells in upper third of villi; (Q) CCASP3-positive cells along upper third of villus; (R) higher magnification showing detail of CCASP3-positive cells. Bars, low magnification = 50 μ m, two higher magnifications both = 5 μ m.

Fig. 5.

Numbers of immunopositive cells expressing PH3 (A) and CCASP3 (B) in SIB and TAU poult groups. Box plots show minimum, first quartile, median, third quartile, and maximum values. Significantly different values ($p < 0.05$) are enclosed in rectangles. (A) The number of PH3-positive cells was significantly higher in SIB poult groups from day 7 through 35, except for day 28 when the number of PH3-positive cells was also high in TAU poult groups. (B) Compared to TAU poult groups, SIB poult groups had greater numbers of CCASP3-positive cells from day 7 to 35, except for day 14. TAU poult groups had consistently low numbers of CCASP3-positive cells at all sampling times.