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Atherogenic Diet Causes Lethal Ileo-ceco-colitis in Cyclooxygenase-2 Deficient Mice

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Abstract

Cyclooxygenases (COX) regulate a variety of inflammatory diseases, including inflammatory bowel disease (IBD). While the pathological effects of COX-1 inhibition by NSAIDs on intestinal ulceration are well established, the role of COX-2 on intestinal inflammation remains under investigation. In this paper, we report a protective role for COX-2 against diet-mediated intestinal inflammation in mice. COX-2^{-/-} mice fed an atherogenic diet or diet containing cholate, but not chow or fat alone, had a high mortality whereas COX-1^{-/-} mice and wild-type mice were unaffected by the dietary changes. Histological analysis identified the cause of death in COX-2^{-/-} mice due to severe intestinal inflammation that was surprisingly limited to the ileo-ceco-colic junction. COX-2 expression is induced in the cecum of wild-type mice fed an atherogenic diet. Our findings show that COX-2 plays an anti-inflammatory role at the ileo-ceco-colic junction in mice, and the pathology of diet-mediated intestinal inflammation in COX-2^{-/-} mice offers an excellent model system to elucidate the molecular mechanisms of intestinal inflammation.

Keywords

COX-2; bile acids; Crohn's disease; inflammatory bowel disease; intestinal inflammation

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1. Introduction

Free arachidonate released from membrane phospholipid is converted to a common precursor of eicosanoids, prostaglandin H_2 by the cyclooxygenase (COX) enzyme. Altered eicosanoid production is associated with a variety of inflammatory diseases, including colon cancer and inflammatory bowel disease [1,2]. Two COX isoforms have been described [3]. COX-1 is constitutively expressed in nearly all cells. Prolonged COX-1 inhibition by non-steroidal anti-inflammatory drugs (NSAIDs, e.g. aspirin) causes intestinal ulceration and inflammation, suggesting a physiological role for COX-1 in mucosal integrity [4,5]. The second COX isoform, COX-2, is expressed minimally in the normal intestine [6]. However, COX-2 expression is highly induced in the mucosa of *Helicobacter pylori* gastritis [7] and colitis induced by trinitrobenzene sulfonic acid [8] suggesting an important role for COX-2 in the pathology of intestinal inflammation.

During the first decade following its discovery, inhibition of COX-2 activity became a major focus for the prevention and treatment of inflammatory diseases. Indeed, COX-2-selective inhibitors, *celecoxib* and *rofecoxib*, have been used to treat colon cancer [9,10] and arthritis [11]. However, association with increased risk of cardiovascular events – heart attacks and strokes – with chronic use [12–14] have led to a reevaluation of COX-2 inhibition. Effects of diet on chronic inflammatory disorders, including atherosclerosis [15], metabolic syndrome [16], and inflammatory bowel disease [17,18] are well established. In order to determine the role of COX-2 in cardiovascular physiology Narasimha et al. utilized the mouse model of diet-induced atherosclerosis and recently reported [19] that a short term feeding (3 weeks) of an atherogenic diet (15.8% fat, 1.25% cholesterol, and 0.5% cholate) elevates serum cytokines and worsens the pro-inflammatory properties of serum lipoproteins in COX-2^{-/-} mice [19]. Based on their findings, Narasimha et al [19] reported that COX-2 plays an anti-atherogenic role in mice.

In this paper, we report a surprising finding that atherogenic diet causes a severe, specific, and lethal intestinal inflammation in COX-2^{-/-} mice. COX-2^{-/-} mice fed an atherogenic diet or diet-containing cholate, a primary endogenous bile acid, exhibited high mortality. Histological analysis revealed that these deaths were due to severe intestinal inflammation that was limited to the ileo-ceco-colic junction. Our observations suggest an anti-inflammatory role for COX-2 at the ileo-ceco-colic junction in mice.

2. Materials and Methods

2.1. Mice

COX-1^{+/+}, COX-1^{-/-}, COX-2^{+/+}, and COX-2^{-/-} 129/C57BL6J male and female mice at the age of 8 to 12 weeks were obtained from Taconic (Germantown, NY). The Chancellor's Animal Research Committee at University of California Los Angeles approved all animal protocols used in this study. Mice were fed one of four diets for 3 weeks: chow diet (Ralston Purina Mouse Chow), atherogenic diet (15.8% fat, 1.25% cholesterol, and 0.5% cholate) (Harlan Teklad, Madison, WI), cholate (1.25% cholesterol and 0.5% cholate), or fat (15.5% fat and 1.25% cholesterol).

2.2. Histology and colitis scoring of Cecum

1–2 cm of terminal ileum, the entire cecum, and 1–2 cm of ascending colon were removed *en bloc* from euthanized mice. Approximately 5 mg of apical cecal tissue was resected and immediately frozen in liquid nitrogen and stored in sterile autoclaved microcentrifuge tubes at –80°C until use. The remainder of the ileo-ceco-colonic tissue was immersion fixed in 10%

formalin for 48 hours. The fixed tissues were embedded in paraffin and 4 μ m sections cut and stained with hematoxylin and eosin (H&E).

Histologic inflammation scoring was performed according to Rath *et al* [20]. In this study, we additionally scored inflammatory cell infiltration of the basal mucosa, edema of the apical lamina propria, epithelial atypia or dysplasia, inflammatory cell infiltration of the muscularis, and necrosis of muscle fibers on a scale of 0 to 4 for each additional item. Ulcers and crypt abscesses were not scored, as they were infrequently observed in this set of experiments. To confirm the presence or absence of dysplasia, ulceration, or cryptitis in the intestinal blocks, 4 μ m H&E sections were evaluated every 100 μ m for a total of 300 μ m, or 4 slides per mouse.

2.3. Cecum RNA and protein isolation

RNA and protein were extracted from ceca of individual mice according to manufacturer's protocols (Invitrogen, Carlsbad, CA). Briefly, the apical cecal tissue was homogenized in TRIzol Reagent (Invitrogen) to isolate RNA and protein simultaneously. The concentrations of RNA and proteins were determined by spectrophotometry and Bradford reagent (Sigma, St. Louis, MO) respectively.

2.4. Western Blot

15 μ g of protein from individual mouse ceca was loaded on a 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were then immunoblotted for COX-2 (1:1000) (Cayman Chemical, Ann Arbor, MI) or β -actin (1:1000) (Cell Signaling Technology, Danvers, MA). HRP-conjugated secondary antibody (GE Healthcare, Piscataway, NJ) was used at 1:5000 and the bands were visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA). ImageJ (National Institutes of Health, Bethesda, MD) was used to analyze the digital images for semiquantitative luminescent intensity. COX-2 protein bands were normalized to β -actin protein bands.

2.5. Statistical methods

Student's t-test or ANOVA were used for comparisons of means of normally distributed data. Mann Whitney or Mann Whitney U test was used for nonparametric data distributions when t-test was not applicable. Fisher exact probability test was used for comparisons of frequencies between multiple groups. For comparisons between more than 2 groups, Fisher exact probability test or 2-way ANOVA were used as appropriate, followed by Student's t-tests or Mann Whitney tests. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Atherogenic diet containing cholate causes lethal, localized intestinal inflammation in COX-2^{-/-} mice

COX-1^{-/-}, COX-2^{-/-}, COX-1^{+/-}, and COX-2^{+/-} littermate controls were fed chow or atherogenic diet (n = 15 per group) for a planned duration of 12 weeks. Between weeks 3 and 4 of experimental diet, 10 out of 15 COX-2^{-/-} mice died unexpectedly. All of the mice in the other 7 groups survived 12 weeks of experimental diet. To determine the cause of death in the COX-2^{-/-} mice on the atherogenic diet, we placed an additional 6 mice per group (COX-1^{-/-}, COX-2^{-/-}, and wild-type littermates) on the atherogenic diet. Once again, 3 of the 6 COX-2^{-/-} mice died at 3.5 weeks on experimental diet, while the COX-1^{-/-} and wild-type littermates remained healthy. The 3 surviving COX-2^{-/-} mice were euthanized and examined for pathology. Gross and histological features of salivary glands, thymus, arteries, white fat, yellow fat, skeletal muscle, lung, duodenum, jejunum, proximal ileum, pancreas, adrenal

glands, ovary, uterus, bone, skin, central nervous system, and heart were similar to those of wild-type mice. However, adhesions between the abdominal wall and the ceco-colic junction were found in all 3 of the COX-2^{-/-} mice on atherogenic diet.

3.2. Cholate supplementation, but not fat alone, causes localized ileo-ceco-colic inflammation

To determine the component of diet responsible for the observed intestinal inflammation, COX-2^{-/-} or wild-type littermate male and female mice were fed a chow, atherogenic (fat + cholate), cholate, or fat diet for 3 weeks (n = 8 to 12 per group). We limited the experimental diet to 3 weeks in order to avoid the mortality in COX-2^{-/-} mice on atherogenic diet. All experimental groups gained weight throughout the course of the experiment (Figure 1) and remained well groomed and active without any physical abnormalities. One-quarter to one-third of COX-2^{-/-} mice fed atherogenic or cholate diet developed an indurated, tender mass in the mid-to-lower left abdomen in the 3rd week of diet. In addition, COX-2^{-/-} mice on atherogenic diet (1 female and 2 males) and cholate diet (2 female) died within 3 weeks, while none of the mice in any of the other groups died. Male mice had the same gross appearance of their intestines as females. Furthermore, 7 of 12 COX-2^{-/-} mice on atherogenic diet and 8 of 12 COX-2^{-/-} mice on cholate diet developed grossly apparent, indurated thickening of the ileo-ceco-colic junction (Figure 2), sometimes associated with intraabdominal adhesions or perforations (Figure 2 and Table 1).

3.3. COX-2 protein is elevated in the cecum of wild-type mice fed atherogenic diet

To determine the relative cecal expression of COX-2 protein, we resected 5 mg of tissue from the apical, blind end of the cecal pouch from COX-2^{-/-} or wild-type mice fed chow or atherogenic diet for 3 weeks. This portion of cecum was grossly uninflamed in all mice. However, COX-2 protein in these samples was significantly elevated in wild-type mice fed atherogenic diet compared to chow diet (Figure 3). These results suggest that diet-induced COX-2 protein expression in wild-type mice coincides with the areas of intestinal inflammation observed in COX-2^{-/-} mice.

3.4. Transmural ileo-ceco-colitis develops in COX-2^{-/-} mice fed atherogenic or cholate diet

Histologically, transmural inflammatory cell infiltration of the ileo-ceco-colic junction was observed in nearly every COX-2^{-/-} mouse on atherogenic or cholate diet, but was absent from the other groups (Figure 4). The lesions were uniformly localized to the ileo-ceco-colic junction. In affected areas the whole intestinal wall was thickened by a massive infiltration of inflammatory cells. In most cases, the lesions appeared most severe in the submucosa and muscularis propria, extending into the peritoneum. Relative to the severity of these changes, the mucosa was generally less affected. Most often, the mucosal lesions consisted of a mixed cell infiltration, more dense surrounding the bases of the crypts and becoming sparser towards the lumen, where there was often an edematous appearance of the lamina propria. In the most severe cases, epithelial glands showed cryptitis or had been replaced by dense inflammatory infiltrate. In a few cases the mucosa was eroded or ulcerated. The histological inflammation in female COX-2^{-/-} mice varied widely in severity, from nearly normal to inflammatory destruction of all layers of the intestinal wall. Male COX-2^{-/-} mice had more consistent inflammation of the ileo-ceco-colic junction with less variation but overall the same severity of inflammation. The observed lesions were qualitatively similar in atherogenic and cholate groups of COX-2^{-/-} mice. Histological colitis scores (mean ± S.D.) for COX-2^{-/-} mice on atherogenic (12 ± 8) and cholate diet (16 ± 9) were similar (p = 0.304) but were significantly elevated compared to the other experimental groups (0 ± 0, p < 0.001) (Figure 5).

3.5. Inflammatory cell infiltrate is a mixed population in the intestines of COX-2^{-/-} mice

The cellular infiltration in submucosa, muscularis and mucosa was mixed, with polymorphonuclear cells (neutrophils and eosinophils), macrophages and a predominance of lymphocytes and plasma cells (Figure 6). Fibroplasia and neovascularization were also prominent. In ulcerated areas, neutrophils predominated. All these changes extended into the serosa and sometimes formed adhesions with the peritoneal fat and the skeletal muscles of the abdominal wall.

3.6. Atherogenic diet worsens mortality compared to cholate diet

COX-2^{-/-} mice fed an atherogenic diet had a mortality of 62% (13 of 21 mice) within 4 weeks (see 3.1 above), and pathological reports indicated that the death rate would have reached 100% if the remaining mice were continued on the atherogenic diet. Moreover, we have also shown that transmural ileo-ceco-colitis develops in COX-2^{-/-} mice fed atherogenic or cholate diet for three weeks (see 3.4 above). To determine the long-term outcome of cholate feeding in COX-2^{-/-} mice, we placed 10 to 11-week-old, COX-2^{-/-} or wild-type littermate mice on chow, fat, or cholate-supplemented diet for 12 weeks (n = 8 to 11 per group). In the wild-type group, all the mice were healthy till the end of the experiment except for two mice; one chow-fed wild-type mouse was euthanized due to dental malocclusion, and another cholate-fed wild-type mouse was found dead. No deaths were observed in COX-2^{-/-} mice on chow or fat diet, whereas cholate-fed COX-2^{-/-} mice had a mortality of 27% (3 out of 11) at 4 weeks and 55% (6 out of 11) at 12 weeks. Our data suggest that although the development of transmural ileo-ceco-colitis develops similarly in COX-2^{-/-} mice fed atherogenic or cholate diet, atherogenic diet is more lethal to COX-2^{-/-} mice compared to the cholate diet..

4. Discussion

This study shows that COX-2 deficiency and lipid diet combine to induce a rapid, progressive, and lethal transmural inflammation localized to the ileo-colonic junction in mice. This surprising and dramatic inflammatory synergy produces lesions with a striking resemblance to human IBD and thus provides a novel model system to elucidate dietary modulation of intestinal inflammation.

Although majority of the focus in COX-2 research has centered on the pro-inflammatory nature of COX-2-dependent lipid mediators, it has recently become increasingly clear that COX-2 activity can also be anti-inflammatory under certain physiological conditions. Most notably COX-2 expression peaks in a biphasic manner during acute inflammation [21]. The first peak occurs within a few hours of stimulus and is associated with PGE₂ synthesis and polymorphonuclear cell infiltration. The second peak is associated with the resolution phase of inflammation. In this phase, mononuclear cell activity predominates [22], and COX-2 activity is associated with minimal prostaglandin E₂ (PGE₂) production, but pronounced production of anti-inflammatory prostanoids, particularly cyclopentenone prostaglandins that include PGD₂ and 15-deoxy-Δ¹²⁻¹⁴-prostaglandin J₂ (15d-PGJ₂) [21,22]. Inhibition of COX-2 activity in the resolution phase significantly exacerbates inflammation [21] suggesting that COX-2 plays an anti-inflammatory role.

Multiple anti-inflammatory effects of COX-2 activity are also evident in the mouse intestine. In mice treated with dextran sodium sulfate (DSS), COX-2 deficiency [23,24] or COX-2 inhibition [25] aggravates colitis. Based on the literature, three probable mechanisms could explain the beneficial effects of COX-2 activity in colitis. First, COX-2 dependent PGE₂ is suggested to be an important anti-inflammatory eicosanoid in the intestinal wall [25]. PGE₂ has been shown to reduce DSS colitis [26] and trinitrobenzene sulfonic acid colitis [27] by preserving epithelial cell proliferation [26], survival and regeneration [28]. PGE₂ mediates its

anti-inflammatory effects in mouse colitis by interacting with the EP4 receptor, since EP4 deficient mice, but not mice deficient in other eicosanoids receptors, develop severe colitis with low dose DSS treatment [28,29]. Secondly, the cyclopentenone prostaglandins synthesized in a COX-2-dependent manner may also decrease intestinal inflammation. Indeed, 15d-PGJ₂, a metabolite of PGD₂, reduces dinitrobenzene sulphonic acid-induced colitis in rats [30] and diminishes activator protein-1 dependent inflammatory gene expression [31]. Finally, COX-2 activity may affect the vascular wall by promoting angiogenesis [32] and inhibiting leukocyte adherence to mesenteric vascular endothelium [33], thereby facilitating healing of intestinal inflammatory lesions.

Our results suggest that dietary cholate is sufficient to cause intestinal inflammation in COX-2 deficient mice. Cholate is synthesized from cholesterol in the liver, then conjugated to amino acids and secreted into the intestine [34]. After reaching the increasingly bacteria-rich portions of the terminal ileum and large intestine [35], cholate is deconjugated and converted to deoxycholate by commensal microorganisms [36]. Because deoxycholate becomes part of the bile acid pool through enterohepatic circulation [36], it is likely that cholate diet results in an increased concentration of deoxycholate in the intestinal lumen. While cholate itself has minimal direct epithelial cytotoxicity [37], deoxycholate is more hydrophobic and cytotoxic [36,38–43]. This may in part account for the selective damage at this region, rather than in the jejunum and proximal ileum, where resident bacteria are present at very low levels [35]. Why the distal colon is relatively spared is less clear. One possibility is the distinct feature of the mucus layer of this region, which is much thicker and less permeant to luminal solutes. This may provide barrier protection against deoxycholate penetration and attenuate mucosal cytotoxicity in this region.

In a previous study, COX-2 deficient mice developed intestinal enteropathy when treated with indomethacin or the COX-1 selective inhibitor, SC-560 [4]. This raises the possibility that bile acids cause intestinal inflammation in COX-2^{-/-} mice by inhibiting COX-1. However, our results indicate that COX-1 activity is not inhibited in COX-2^{-/-} mice on cholate-containing diet (not shown). COX-2 deficient mice treated with COX-1 inhibitors develop upper-to-mid small intestinal ulceration and inflammation [4], whereas COX-2 deficient mice fed cholate in our study developed inflammation of the distal small intestine-to-large intestine junction without significant ulceration. Also, whereas PGE₂ levels decreased significantly in COX-2^{-/-} mice with COX-1 inhibitors [4], PGE₂ levels in our COX-2^{-/-} mice on atherogenic diet increased (reported in the accompanying paper [19]). This suggests that COX-1 activity in COX-2 deficient mice on cholate-containing diet is preserved or upregulated. Therefore, the intestinal inflammation seen in COX-2^{-/-} mice fed cholate diet is not a result of COX-1 inhibition.

We report for the first time that COX-2 deficiency is a host susceptibility trait for the development of intestinal inflammation in response to dietary cholate. Because our experimental mice are maintained on a 129 × B6 mixed background, we cannot rule out contributions from genetic segregation. In the original report that described COX-2 deficient mice a diffuse, suppurative peritonitis was reported in a minority of COX-2^{-/-} mice [44]; however, that complication is no longer observed in this strain (personal communication, R. Langenbach). The inflammation that we report here primarily affects the intestinal wall and only secondarily involves the peritoneum in the most severe cases. The location and histology is strikingly similar to the ileocolitis of human Crohn's disease, which afflicts people of various races and ethnicities and is also significantly influenced by environmental factors [45]. Our observation of a Crohn's-like disease in mice is especially significant because cholate is an endogenously synthesized bile acid and a component of intestinal luminal contents in humans and mice. Interestingly, bile acid secretion is elevated by high lipid diet in humans [46]. Furthermore, alterations of bile acids have been observed in human IBD patients [47,48]. Thus,

our observations may be even more relevant when considering dietary and pharmacologic triggers of human intestinal inflammation.

In summary, we report that COX-2^{-/-} mice fed atherogenic diet develop transmural lesions consistent with inflammatory bowel disease, localized to the ileo-ceco-colic junction, and associated with accumulation of multiple inflammatory cells. The cholate component in the atherogenic diet is primarily responsible for this intestinal inflammation, although additional high-fat content is associated with worse mortality than cholate supplementation alone. These studies reveal the presence of a local and probably immune-mediated inflammatory response resulting from the interaction of COX-2 and bile acid activity at the ileo-ceco-colic junction.

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Abbreviations

COX	cyclooxygenase enzyme
PG	prostaglandin
IBD	inflammatory bowel disease
DSS	dextran sodium sulfate
NSAIDs	non-steroidal anti-inflammatory drugs

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Figure 1. Percent difference in body weight after 3 weeks of experimental diet

Body weights of individual mice on a specific diet (n=8–12 per group) were measured after fasting overnight. Weight difference is calculated as the difference in weight between the groups on experimental diet versus their chow counterparts. Mice that died were included in this analysis. * $p < 0.05$ vs. wild-type, ** $p < 0.05$ vs. chow, Student's t-test. WT = COX-2^{+/+} (wild-type), KO = COX-2^{-/-} (knockout), Athero = atherogenic diet

Figure 2. Gross pathology of ceca from COX-2^{-/-} mice

Ceca from COX-2^{-/-} mice on chow, atherogenic, fat or cholate diet for 3 weeks were resected and contents evacuated. A perforation at the ileo-cecal junction is noted in the specimen from the mouse on atherogenic diet (arrow). PC = proximal colon, TI = terminal ileum, C = cecum

Figure 3. COX-2 expression in cecum

Total protein (15 μ g) extracted from cecum of wild-type or COX-2^{-/-} mice fed chow or atherogenic diet for 3 weeks were loaded on SDS-PAGE and immunoblotted for COX-2 or β -actin (upper panel). Cell lysate from NIH3T3 cells treated with phorbol 12-myristate 13-acetate (PMA: 1 μ M) for 4 hours was used as a positive control for COX-2. The density of COX-2 protein band was quantified by ImageJ and normalized to β -actin (lower panel). Each bar represents the average with one standard deviation. NIH = NIH3T3, WT = COX-2^{+/+} (wild-type), KO = COX-2^{-/-} (knockout), P = PMA, C = Chow, A = atherogenic diet

Figure 4. Inflammation at the ileo-ceco-colonic junction in $COX-2^{-/-}$ mice is mediated by cholate in the diet

Ceca from male and female $COX-2^{+/+}$ or $COX-2^{-/-}$ mice fed specific diets for 3 weeks were fixed and stained with H & E. All are shown at 20X magnification. Athero = atherogenic, WT = $COX-2^{+/+}$ (wild-type), KO = $COX-2^{-/-}$ (knockout)

Figure 5. Histological inflammation scores

Inflammatory scores of ceca from COX-2^{-/-} mice fed the indicated diet for 3 weeks. Scores were equal to 0 in COX-2^{-/-} mice fed chow or fat and in wild-type (COX-2^{+/+}) littermate controls fed chow, atherogenic, cholate, or high fat diets (n=8 to 12 per group). Two-way factorial ANOVA followed by Mann Whitney tests were performed for statistical analysis. $p < 0.0001$ for COX-2^{-/-} mice fed atherogenic or cholate diet vs any other group. $p = 0.304$ for atherogenic vs cholate diet in COX-2^{-/-} mice. For detailed criteria of the inflammation score, see Materials and Methods.

Figure 6. Cecal wall of a COX-2^{-/-} mouse fed atherogenic diet for 4 weeks

(A) Cecum was resected, fixed with formalin and stained with H&E (4X). um = mucosal ulceration, mm = muscularis mucosae, sm = submucosa, mp = muscularis propria, and s = peritoneum (serosa). (B) Cecal wall of a COX-2^{-/-} mouse fed an atherogenic diet is shown at 20X. Lower panel, shown at 40X: The submucosa and muscularis propria shows a predominance of plasma cells (green arrows) and macrophages (black arrow) with some eosinophils (red arrows). m = mucosa, lp = lamina propria, sm = submucosa, mm = muscularis mucosa, and mp = the two layers of the muscularis propria.

Table 1
Gross findings in COX-2^{-/-} or wild-type mice on 3 weeks of experimental diet

	Chow			Athero		Fat		Cholate	
	WT	KO	WT	WT	KO	WT	KO	WT	KO
Number of mice	8	12	12	12	12	8	12	8	12
Died < 3 weeks	0	0	0	0	1	0	0	0	2
Normal intestine	8	12*	12	12	4*	8	12*	8	2*
Ileo-ceco-colic thickening	0	0*	0	0	7***	0	0*	0	8***
Intraabdominal adhesions	0	0	0	0	3	0	0	0	4
Perforated intestine	0	0	0	0	1	0	0	0	2

* p < 0.0001 between diets (Fisher exact probability test, 2x4 contingency table)

** p < 0.05 compared to COX-2^{-/-} mice on chow or fat diet (Mann Whitney tests). WT = wild-type, KO = COX-2^{-/-} Athero = atherogenic diet