Granulomatous enteritis in rainbow trout (*Oncorhynchus mykiss*) asscociated with soybean meal regardless of water dissolved oxygen level as an environmental challenge

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Running title:

Soybean meal-associated granulomatous enteritis

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Abstract

Morphological changes associated with soybean meal-induced enteritis (SBMIE) in distal intestine (DI) of rainbow trout (*Oncorhynchus mykiss*) fed soybean meal-based diet (SBM) and kept at normoxia or hypoxia created by optimal and low water flow rate, respectively, was investigated. Histopathologic evaluation revealed additional pathological features to what is commonly known about SBMIE, thus these features were also investigated. Histology and immunohistochemistry using antibodies against cytokeratin and proliferating cell nuclear antigen (PCNA) were performed. The pathological features included vacuolar degeneration of epithelial cells mainly at the base of the mucosal folds, necrosis, shedding of necrotic cells, and a granulomatous inflammation including the infiltration of enlarged, sometimes finely vacuolated or "foamy" macrophages, multi-nucleated giant cells and increased proliferation of fibroblasts. SBMIE was also associated with increased reactivity to PCNA. No acid-fast bacteria in the enlarged macrophages were revealed in sections stained with Ziehl-Neelsen, however, these cells contained Alcian blue/periodic acid-Schiff (AB-PAS) and sometimes cytokeratin-positive material, likely of epithelial/goblet cell origin. Hypoxia did not affect the pattern and the degree of morphological changes in DI. These results suggest that SBM was associated with a variant, granulomatous form of enteritis in DI of rainbow trout, although the identity of the factors inducing the delayed type hypersensitivity could not be established.

Introduction

The inclusion of soybean meal (SBM) in salmonid feed is known to adversely affect intestinal homeostasis by development of a chronic inflammation referred to as soybean meal-induced enteritis (SBMIE) (Baeverfjord & Krogdahl 1996). SBMIE is a condition which has been characterized by many publications as increased leukocyte accumulation in the subepithelial tissues, atrophy of the intestinal folds, increased number of goblet cells and changes in the morphology of the epithelial cells such as: reduced supranuclear vacuolisation (SNV), reduced height of the cells and increased cytoplasmic basophilia (van den Ingh, Krogdahl, Olli, Hendriks & Koninkx 1991; Baeverfjord & Krogdahl 1996; Bakke-McKellep, Frøystad, Lilleeng, Dapra, Refstie, Krogdahl & Landsverk 2007b; Urán, Schrama, Rombout, Taverne-Thiele, Obach, Koppe & Verreth 2009). SBMIE shares some morphological and immunological features with human models of intestinal inflammation typically inflammatory bowel disease (IBD) and celiac disease (Hisamatsu, Kanai, Mikami, Yoneno, Matsuoka & Hibi 2013; Geboes, Joossens, Prantera & Rutgeerts 2003; Dickson, Streutker & Chetty 2006). Leukocyte infiltration and proliferation into the lamina propria resulting in thickening of mucosa, villus atrophy and loss of crypts with changes in morphology of epithelial cells are typical signs of these forms of intestinal inflammation. The composition of cells infiltrating into the lamina propria differ to some extents among various types of enteritis. In addition to T cells which have been studied under SBMIE conditions in Atlantic salmon (Bakke-McKellep *et al.* 2007b; Lilleeng, Penn, Haugland, Xu, Bakke & Krogdahl 2009), population of macrophages are also involved in mucosal immune response. Macrophages are one of the main agents of innate immune system and their function is crucial to maintain tissue homeostasis. As well as in mammals, activated macrophages in fish perform phagocytic activity and produce pro-inflammatory cytokines, reactive oxygen species and nitric oxide (Forlenza, Fink, Raes & Wiegertjes 2011).

Development of SBMIE is attributed to the presence of anti-nutritional factors (ANF), however, the exact aetiology of the disease is not yet fully understood. Inclusion of SBM at as low as 20% in Atlantic salmon (*Salmo salar* L.) has been shown to induce morphological changes in the distal intestine (DI) within the first week of consumption (Urán *et al.* 2009). Rainbow trout (*Oncorhynchus mykiss*) has been suggested to be more resistant to pathological effects of SBM (Refstie, Korsøen, Storebakken, Baeverfjord, Lein & Roem 2000), however, this condition is

also evident in this specie at sufficiently high SBM inclusion levels (Romarheim, Skrede, Penn, Mydland, Krogdahl & Storebakken 2008; Mosberian-Tanha, Øverland, Landsverk, Reveco, Schrama, Roem, Agger & Mydland 2016).

Adverse environmental conditions such as hypoxia may affect the fish health and welfare as reviewed elsewhere (Wu 2002). Pathological changes may occur in intestinal tissue in response to environmental factors. For example, impaired intestinal barrier function along with morphological changes in proximate and DI (Sundh, Kvamme, Fridell, Olsen, Ellis, Taranger & Sundell 2010) and elevated mucosal neutrophil infiltration (Niklasson, Sundh, Fridell, Taranger & Sundell 2011) has been previously reported to occur in response to chronic hypoxia in Atlantic salmon. It has also been shown that the effect of diet-induced intestinal morphological changes were aggravated in Nile tilapia (*Oreochromis niloticus*) kept under hypoxic conditions (Tran-Ngoc, Dinh, Nguyen, Roem, Schrama & Verreth 2016).

The interactive effect of dietary and environmental challenge (i.e. SBM-based diet and hypoxia) on the degree and progression of SBMIE in rainbow trout was shown previously by Mosberian-Tanha *et al.* (submitted) in which additional pathological features to what has been reported on SBMIE were observed. The granulomatous response reported previously (Mosberian-Tanha *et al.* submitted) is in several aspects different from the morphological changes commonly reported in response to SBM-based diet (Romarheim *et al.* 2008; Mosberian-Tanha *et al.* 2016). The findings necessitated a further detailed investigation of the intestinal pathology.

The present study was therefore designed to 1) investigate the interactive effect of dietary and environmental challenge (i.e. SBM-base diet and hypoxia) on morphological changes in DI in rainbow trout and 2) investigate if the features were associated with hypoxia.

Materials and methods

Fish rearing and experimental procedure

The experiment was performed in accordance with the Dutch law on the use of experimental animals and approved by the ethical committee of Wageningen University (DEC: 2014006.a).

Details of diet preparation, chemical analysis and experimental design are described previously (Mosberian-Tanha *et al.* submitted). Briefly, at the start of the experiment 600 juvenile rainbow

trout with mean initial body weight (± SE) of 74.1 ± 0.3g were randomly allocated among 12 tanks (50 fish per tank). Two isoenergetic and isonitrogenous diets were formulated (Table 1); one fishmeal-based control (FM) and one containing 40% soybean meal (SBM) as experimental diet. Each diet was assigned randomly to triplicate tanks (200 L capacity) according to the treatments and fed to the fish manually twice daily throughout the experiment at 9:00 and 16:00 hours for maximally 1 hour.

The experiment was split into two periods: In period 1 the fish were adapted to FM or SBM for 28 days and were kept at normoxia by setting the water flow rate at 7.5 L min^{-1} resulting in a mean dissolved oxygen (DO) level of above 8 mg L^{-1} in the outlet (>78% saturation). Period 2 was a challenge period of 42 days where the fish were subjected to either a dietary challenge and/or exposed to hypoxia by reducing the water flow rate from 7.5 L min^{-1} to 2.25 L min^{-1} resulting in a mean DO level of below 6 mg L^{-1} in the outlet (< 55% saturation). The normoxic tanks, however, remained at the same water flow rate as used in period 1. If necessary, pure oxygen was injected into the inlet to maintain the intended DO level. The minimum DO level in the outlet, however, was maintained above 3.8 mg L^{-1} in hypoxia tanks to avoid extreme reduction in feed intake and increased mortality. At the start of period 2, the feeding level was reduced from 1.5% to 1.25% of mean biomass of 12 tanks. Water DO level is the key limiting factor when the water flow rate is reduced, however, this treatment also leads to accumulation of metabolites or fish excretions such as ammonia. To simplify nomenclature, low water flow rate is termed hypoxia (HY) and optimal water flow rate is termed normoxia (NO). Throughout the experiment the fish was reared at photoperiod of 12 L: 12 D, water temperature of 14.0±0.5˚C, pH between 7.0 and 8.0, nitrate of < 250 mg N L^{-1} and nitrite of < 0.15 mg N L^{-1}. Total ammonium nitrogen (TAN) was measured as reported previously (Mosberian-Tanha *et al.* submitted) during week five of period 2. The average TAN level was 0.14 mg N L^{-1} under hypoxic conditions and 0.06 mg N L^{-1} at normoxia. The mean of DO level (mean ±SD) in the inlet was 10.3±0.3 mg L^{-1}.

The four treatments tested in this experiment are shown in Table 2. The design of the experiment has been described previously (Mosberian-Tanha *et al.* submitted).

Sampling

During the experiment DI was sampled at days 0, 7, 14, 21 and 42 of period 2. At each time point 3 fish per tank were randomly selected, individually weighed and anesthetised by 2-phenoxy ethanol (0.25 ml L^{-1}). The anesthetised fish were then sacrificed by a blow to the head before DI tissue sampling for morphological evaluation and immunohistochemistry. DI tissue samples were dissected and cut lengthways prior to fixation in neutral buffered formalin (4% formaldehyde) for 48 hours. DI tissue samples were dehydrated in 70% ethanol and embedded in paraffin before staining by hematoxylin and eosin (H&E) and Alcian blue-Periodic acid-Schiff (AB-PAS) following standard routines.

Histological evaluation

Blinded evaluation and scoring of the following morphological parameters was carried out on each DI tissue sample:

A. Subepithelial infiltration of leukocytes: increased accumulation of leukocytes in the subepithelial area down to stratum compactum.

B. Supranuclear vacuolisation (SNV) of epithelial cells: reduced vacuolisation of the epithelial cells.

C. Atrophy (shortening) of intestinal folds.

D. Vacuolar degeneration (VD) of the epithelial cells: increased VD at the base of the intestinal folds.

E. The presence, if any, of granulomatous response and the degree of such response: increased proliferation of fibroblasts and aggregation of enlarged macrophages and multi-nucleated giant cells (MGC) along with lymphocytes in the subepithelial tissues.

Atrophy, SNV and mucosal leukocyte infiltration has been well documented in previous studies of SBMIE (Baeverfjord & Krogdahl 1996; Romarheim, Hetland, Skrede, Overland, Mydland & Landsverk 2013a). In H&E-stained sections, cells with VD and goblet cells often showed a similar morphological pattern and could be misinterpreted. Thus, AB-PAS staining was performed to detect acidic (blue) and neutral (red) mucins of goblet cells.

A score was given to each parameter which ranged from 0 (no morphological change) to 3 (severe changes) with increment of 1. Score of 1 was given to slight changes which are still assessed as

normal morphology while score 2 was given to moderate changes. For evaluation of granulomatous response score of 1 was given to the tissue containing only a few number of enlarged macrophages and/or slight increase in the number of fibroblasts. Score 2 was given to the tissue expressing increased number of fibroblast, enlarged macrophages and a few MGCs. Score 3 was given to the tissue expressing large number of foamy macrophages and increased number of MGCs. According to this protocol, at least a score of 2 should be given to parameters A, B and C to confirm incidence of a classic SBMIE.

Immunohistochemistry

Paraffin sections were placed on glass slides and air-dried for 30 min at 58°C. The sections were then deparaffinised with xylene and rehydration. The sections were autoclaved in citrate buffer (pH 6.0) for 15 min at 121°C. Endogenous peroxidase was inhibited by incubation of the tissue sections for 10 min in 3 % H_2O_2 (hydrogen peroxide) diluted in methanol. To prevent nonspecific binding of antibodies, the sections were treated with goat serum containing 5% bovine serum albumin (BSA) in a Tris buffered saline (TBS) for 20 min at room temperature. The sections were then subjected to primary antibodies and incubated for 1 h at room temperature. For PCNA detection mouse monoclonal IgG2α-κ antibody (diluted 1:25000 in 1% BSA/TBS, M0879; Dako, Norge, Oslo, Norway) and for cytokeratin detection mouse monoclonal IgG1- κ antibody (pan, clone AE1/AE3, diluted 1:50 in 1% BSA/TBS, Zymed Laboratories) was used. Sections without primary antibody incubation served as negative controls. The incubation for the proxidase-laballed secondary antibody was performed with Labelled Polymer-HRP anti-mouse (Dako, Norge, Oslo, Norway) for 30 min. All incubations were performed in a humid chamber at room temperature. The peroxidase activity was developed with a 3-amino-9-ethyl carbazole kit (Dako, Norway) for 15 min. The sections were then counterstained with Mayer's hematoxylin for 20 seconds and mounted in Aquatex mounting medium (VWR International). The sections were washed 3 times, except for the treatment with goat serum, in PBS for 5 min between each step.

Calculations and statistics

Quantification of PCNA reactivity of each DI tissue sample was measured as described elsewhere (Romarheim, Øverland, Mydland, Skrede & Landsverk 2011). Statistical analyses were performed using SAS 9.4 (SAS Institute 2012). All data were tested for normality and homogeneity by Kolmogorov-Smirnov and Bartlett tests. Data from morphological parameters

violated the normal distribution assumption after log10-transformation; and thus these data were subjected to non-parametric Kruskal-Wallis test followed by multiple pairwise comparisons (Dwass-Steel-Critchlow-Fligner) if the test was significant. PCNA reactivity score in period 1 was subjected to one-way analysis of variance (ANOVA) in GLM procedure to test the effect of diet. The effect of treatment and sampling time on PCNA reactivity score in period 2 was analysed using a two-way ANOVA in GLM procedure. Least square means comparison was used to determine which groups differed significantly in PCNA reactivity from each other. Differences were declared statistically significant if $P < 0.05$.

Results

Histopathological evaluation of the distal intestine

Histopathological examination of the tissue sections revealed presence of SBMIE in fish fed SBM-based diet (Fig. 1). Under SBMIE conditions the intestinal folds showed various degrees of atrophy. Epithelial changes were often pronounced with reduced SNV of epithelial cells. In most individuals epithelial change also included VD often progressing to evident necrosis recognized by shrinking, condensation of the chromatin and fragmentation of nucleus. Acid and neutral mucins in goblet cells were readily identified with AB-PAS staining allowing distinction between goblet cells and cells with VD (Fig. 2). The necrotic epithelial cells were extruded to the intestinal lumen resulting in denudation of the lamina propria. Apparent fusion of adjacent intestinal folds in some cases resulted in the formation of cysts filled with epithelial debris (Fig. 3). These changes were predominantly found at the base of the folds. Flattened epithelial cells covering or partly covering the lamina propria and regenerative reaction in the remaining epithelial cells was interpreted as an effort for epithelial restitution (Fig. 4). Irregular shape of the epithelial cells and their nuclei in the vicinity of these areas and the site of fusion of the intestinal folds sometimes justified the use of the term “dysplastic” changes (Fig .5). Based on our evaluation protocol, total number of 113 fish (fed SBM regardless of their environment) were diagnosed with SBMIE during the entire experimental period. Of these, 12 fish (≈10%) during the last three weeks of period 2, showed dysplastic changes in epithelium.

Granulomatous response in different degrees was evident in the lamina propria (Fig. 6). Granulomatous response included prominent macrophage aggregates. Macrophages were often

enlarged and sometimes finely vacuolated allowing the use of term “foamy macrophages” (Fig. 6). The foamy macrophages were positive for acid (blue) mucin and fewer were positive for neutral (red) mucin (Fig. 7). In addition to macrophages, infiltration of lymphocytes, eosinophilic granular cells, neutrophils and proliferation of fibroblasts were evident in the lamina propria. In individuals with marked granulomatous response, prominent presence of MGCs was also evident which, sometimes, were detected within the cysts. Ziehl–Neelsen stains were negative for acid-fast bacilli in selected sections with granulomatous response (Fig. 8).

The mean scores of morphological changes are shown in Fig. 9. These changes were characterised by reduced apical SNV, reduced height of simple and complex intestinal folds (partial atrophy), and increased number of leukocytes (e.g. lymphocytes, granulocytes and eosinophilic granular cells) in the lamina propria, the degree of VD at the base of the folds and the degree of granulomatous response. Atrophy, SNV of epithelial cells and mucosal leukocyte infiltration have been evaluated in many publications as morphological parameters associated with SBMIE (Mosberian-Tanha *et al.* 2016; Baeverfjord & Krogdahl 1996; Romarheim *et al.* 2013a). Thus these parameters are referred to as classic parameters/features of SBMIE in this paper. There was no adverse effect of hypoxia on morphological parameters in fish fed FM throughout the experiment, but exposed to hypoxia during period 2 (FMNO→FMHY). Fish fed the SBM diet throughout the experiment (SBMNO→SBMHY), developed SBMIE in the DI during period 1 and scored highest on all morphological parameters compared to fish fed the FM diet ($P<0.05$). During period 2 where this group were exposed to hypoxia, no significant change in the degree of morphological changes was observed overtime, however, granulomatous response tended to reduce at day 14 ($P=0.08$). The pattern of change in all morphological parameters was similar in the fish subjected to dietary change from FM to SBM under normoxia and hypoxia (FMNO→SBMHY and FMNO→SBMNO) during period 2. All SBM-fed fish regardless of the environment (i.e. hypoxia or normoxia), reached the same degree of change in three of the classic parameters (A, B and C) by day 7 and in VD and granulomatous response by day 14 and 21, respectively (Fig. 9).

Immunohistochemistry

Fish on a steady state SBM-based diet (SBMNO→SBMHY) showed higher degree of PCNA reactivity during period 1 than the groups fed FM-based diet (P<0.0001) and the degree of PCNA reactivity in this group remained unchanged throughout period 2. In period 2, fish fed FM-based diet but exposed to hypoxia (FMNO→FMHY) generally showed lowest degree of PCNA reactivity in the epithelium compared to other treatments (Fig. 10). The fish subjected to change from FM- to SBM-based diets under normoxia (FMNO→SBMNO) and hypoxia (FMNO→SBMHY) in period 2, showed their highest degree of PCNA reactivity at day 7 and 21 respectively, reaching the same degree as to that of the fish fed SBM throughout the experiment and exposed to hypoxia in period 2 (SBMNO-SBMHY). The degree of PCNA reactivity from day 21 onwards was not significantly different among all the fish fed SBM during period 2, regardless of their environment.

Following cytokeratin immunostaining, the epithelial cells were localised and the epithelial origin of extruded cells into the lumen was confirmed (Fig. 11). Cytokeratin reactivity was sometimes observed also within cysts (Fig. 12).

4. Discussion

The objective of the present study was to investigate if exposure to hypoxic conditions as an environmental challenge aggravates the effect of SBM on morphological changes associated with SBMIE in rainbow trout over time. Increase in morphological changes within the first week after SBM feeding is in agreement with the previous study in Atlantic salmon (Urán *et al.* 2009). In contrary to a previous study in rainbow trout (Romarheim *et al.* 2008), in the current study most of the fish fed SBM, regardless of their environment showed significant increase in change of all classic parameters of SBMIE within seven days of dietary challenge. Hypoxia did not significantly affect the degree of morphological changes in DI of SBM-fed fish. It is possible that the severe changes induced by SBM-based diet in this study concealed any additional effect of hypoxia. However, there was no adverse effect of hypoxia on morphology of DI in the fish fed FM-based diet. This may imply that hypoxia alone could not induce morphological changes in rainbow trout. This is in contrast to a study in Atlantic salmon exposed to hypoxia (50% DO saturation) reporting atrophy of DI mucosal folds (Sundh *et al.* 2010) and infiltration of

neutrophils into the mucosa of proximal intestine (Niklasson *et al.* 2011). The possible explanation is that rainbow trout may be more resistant to environmental challenges such as hypoxia than Atlantic salmon. The lack of adverse effect from hypoxia was observed for all parameters including granulomatous response and VD of epithelial cells.

This study presents a variant feature of a diet-induced enteritis and the lack of hypoxia effect on development of this form of enteritis led to the hypothesis that the pathological condition was associated with SBM diet. Although there is a clear association with the diet in the present case, the possible contribution from microbial factors cannot be excluded. T cell reactivity has shown to be a key player in the pathogenesis of SBMIE in Atlantic salmon (Bakke-McKellep *et al.* 2007b; Lilleeng *et al.* 2009), however, in the current study pronounced presence of macrophages forming MGCs were also evident under SBMIE condition in many individuals. As a result the pathological feature was characterised as granulomatous enteritis.

Regarding pathogenesis, it is likely that initial epithelial changes caused by SBM, resulted in an interruption of the intestinal mucosal barrier allowing the luminal contents including various types of microorganisms to directly interact with immune cells present in the subepithelial tissues. The demonstrated absence of acid-fast organisms in tissue macrophages, however, makes the presence of mycobacteria unlikely. However, it cannot be ruled out that other environmental factors, which could not be addressed directly in this experiment, may have contributed to the manifestation. One such factor is water temperature. In a previous experiment on rainbow trout kept at average 9 ºC (Mosberian-Tanha *et al.* 2016) inclusion of 37.5% of SBM resulted in only classic morphological changes commonly reported for SBMIE. However, in the current experiment, apart from hypoxia, water temperature was the other major environmental difference. Rainbow trout in this study was kept at relatively high temperature of 14ºC. The possible suggestion is that higher temperature used in this experiment may have been at least a contributing factor in the manifestation. Sealey, Barrows, Smith, Overturf & LaPatra (2009) reported epithelial "cystic absorptive vacuoles", fusion of intestinal folds, and change in morphology of epithelial cell nuclei in rainbow trout fed 43% SBM at 14.8 ºC temperature. Burrells, Williams, Southgate & Crampton (1999) observed increased vacuolisation of epithelial cells and extrusion of mucosal material into the intestinal lumen in rainbow trout (of 5 grams body weight) fed 80-89% SBM at 14 ºC compared to the fish fed lower doses of SBM. Increased

immune cell infiltration, however, was the only subepithelial observation made and reported by the authors. Moreover, the degree of SBMIE was found to increase at 12 ºC than that at 8 ºC in Atlantic salmon fed 20% SBM, however, without change in the form of inflammatory and tissue response (Uran, Schrama, Rombout, Obach, Jensen, Koppe & Verreth 2008). None of the above publications reported or observed a granulomatous form of enteritis. We hypothesize that the pathological effect of SBM on DI may be different at higher temperatures (at least in rainbow trout) and likely could explain the occurrence of a variant pathological manifestation, the granulomatous response. Temperature may also affect the function of immune system which has a key role in the process of inflammation. In sockeye salmon (*Oncorhynchus nerka*), change in immune response pattern and higher dependency on specific immune function has been shown to occur at higher temperatures (Alcorn, Murray & Pascho 2002).

Granulomatous enteritis involves a type 4 hypersensitivity reaction which may occur in association with chronic inflammation and is characterized by the occurrence of enlarged, activated macrophages (epithelioid cells) which sometimes are transformed into MGCs (Snyder 2016). Granulomatous inflammation typically occurs following infection with mycobacteria but may also occur in association with neoplastic diseases and intoxications (Williams & Williams 1983). Granulomatous inflammation has been reported also in fish at vaccination sites or in fish infected with mycobacteria (Koppang, Haugarvoll, Hordvik, Aune & Poppe 2005; Novotny, Halouzka, Matlova, Vavra, Bartosova, Slany & Pavlik 2010). To our knowledge, there are no reports on development of granulomatous enteritis on a SBM diet in fish. In man, a granulomatous regional enteritis of obscure etiology is associated with Crohn's disease (Lee, Maguire, Obeidat & Russell 1997). Among mammalian species, the form of granulomatous enteritis that is best defined with respect to etiology is paratuberculosis, commonly seen in ruminants (Arsenault, Maattanen, Daigle, Potter, Griebel & Napper 2014). The etiology of the disease is Mycobacterium avium spp. paratuberculosis and the bacteria are easily detected in enlarged macrophages with Ziehl Neelson staining (ref)

The enlarged macrophages containing small vacuoles, or foamy macrophages seen in the present material, likely represent activated macrophages. Such macrophages may contain various types of materials (Sagaert, Tousseyn, De Hertogh & Geboes 2012). In this study positivity of foamy macrophages for acidic and neutral mucins may imply that foamy macrophages engulfed mucins.

Foamy macrophages were mostly positive for acidic (blue) mucin which has been suggested to be an indication of mucin phagocytosis and presence of "muciphages" which are reported to occur in response to tissue injury (Sagaert *et al.* 2012). Positivity for neutral (red) mucin is an indication of a much broader array of disorders including pathogen and also mucin phagocytosis (Sagaert *et al.* 2012). These cells could be observed under various conditions such as tissue inflammation, regeneration and hyperplasia (Bejarano, Aranda-Michel & Fenoglio-Preiser 2000). Overall, these result may suggest that foamy macrophages resembled muciphages and contained mucins of endogenous origin.

It is possible that epithelial mucins were altered by the components of SBM, such as saponins, inducing a type 4 hypersensitivity. A granulomatous reaction in response to release of mucins into the subepithelial tissues has been reported in a colitis model of inflammation (Surawicz, Haggitt, Husseman & McFarland 1994).

Under SBMIE conditions increased number of goblet cells has been reported (Urán *et al.* 2009), which may be confused with vacuoles of epithelial cells. In this study, however, AB-PAS staining allowed more accurate evaluation of this pathological feature in tissue samples. VD is a feature of reversible and non-lethal cell injury which occurs as a result of fluid accumulation in the cell or swelling of the endoplasmic reticulum. If cell injury is progressive, the cell will eventually become necrotic (Kumar, Abbas, Fausto & Aster 2010). Epithelial cells with VD were mainly observed at the mucosal fold bases where cysts were also formed. Fusion of intestinal folds containing cells with VD may have formed epithelial cysts, similar to the observations made previously in rainbow trout (Sealey *et al.* 2009). Less degree of maturation at base of the folds where proliferation occurs may in turn result in increased susceptibility of epithelial cells to various harmful agents. Cytokeratin reactivity within cysts further suggests epithelial cell debris accumulation in these features.

Histopathology score of VD and granulomatous response were significantly increased after 14 and 21 days respectively in period 2 which implies that these features required more time to develop than other morphological parameters (i.e. A, B and C). This may also imply that the classic SBMIE with serious consequences on epithelial integrity may have contributed to the development of these pathological features.

Increased epithelial cells proliferation as compensatory response to cell loss under SBMIE condition has been shown previously (Bakke-McKellep, Penn, Salas, Refstie, Sperstad, Landsverk, Ringø & Krogdahl 2007a; Romarheim, Landsverk, Mydland, Skrede & Øverland 2013b) and indicates an attempt to restore tissue homeostasis. Increased proliferation as indicated in this experiment by measurement of PCNA-reactive stretch, showed a similar pattern as classic morphological changes (parameters A, B and C) and highlights the cellular proliferation in response to inflammation. Reduced SNV in the apical part of the intestinal folds under SBMIE could, at least partly, be a result of reduced maturity of the epithelium due to expanded proliferation zone. Hypoxia did not increase PCNA reactivity which implies that the tissue maintained homeostasis under this condition. The delayed increase in PCNA reactivity score in the fish challenged simultaneously to hypoxia and SBM-based diet may be an indication of a short term effect of hypoxia on cell proliferation.

Dysplastic changes (dysplasia) in areas expressing epithelial restitution and fusion of intestinal folds indicates disorder of cell proliferation in the tissue under SBMIE condition. The balance of cell death and proliferation is important to maintain tissue homeostasis. When the rate of cell proliferation exceeds that of cell death, the tissue may undergo abnormal growth with increased risk of tumorigenesis. Dysplasia is known to be associated with incomplete maturation process in new cells. Increased risk of abnormal growth of intestinal tissue towards neoplasm has been reported in human with inflammatory bowel disease (Triantafillidis, Nasioulas & Kosmidis 2009). Discrimination between regenerating epithelial cells due to inflammation and dysplastic cells could be challenging. With use of cell proliferating markers such as PCNA, some experiments have shown further expansion of proliferation zone in intestinal tissues with dysplasia (Kullmann, Fadaie, Gross, Knuchel, Bocker, Steinbach, Scholmerich & Ruschoff 1996).

Immunohistochemical detection of cytokeratin revealed epithelial origin of extruded epithelial cells explaining the cause of lamina propria denudation mainly at the tip of the intestinal folds. This indicates loss of epithelial cells and barrier damage and in turn can cause increased cell proliferation (as indicated by increased PCNA reactivity) and susceptibility of tissue to luminal contents. At the edge of denuded area, flattened epithelial cells were observed which may indicate a rapid compensatory response referred to as restitution. Restitution has been reported to occur

following severe epithelial damage in small intestine of rats (Matovelo, Sund & Landsverk 1989) and aims to cover the denuded areas and provide protection. Severe extrusion of epithelial cells has been suggested as a disturbing factor to the epithelial barrier integrity leading to inflammation (Gudipaty & Rosenblatt 2016).

In conclusion, hypoxic conditions neither induced inflammation nor aggravated the degree of SBMIE in rainbow trout. Simultaneous exposure to a SBM-based diet and hypoxia induced a delayed increase in PCNA reactivity score. Further to the commonly reported pathological features of SBMIE, additional changes such as granulomatous response and vacuolar degeneration of epithelial cells were observed. These changes were associated with more pronounced macrophage reaction. There was no indication of hypoxia effect on development of these changes. The variant pathological features reported in this study could potentially reveal new aspects of the pathogenesis of SBMIE.

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Figure legends

Fig. 1. Morphology of the distal intestine in rainbow trout (*Oncorhynchus mykiss*) stained with hematoxylin and eosin (H&E) (bars = 200 μm). A) Normal distal intestine (DI) of rainbow trout fed fish meal. Epithelial cells are regular, with a high columnar shape and contain a finely vacuolated supranuclear cytoplasm. B) DI with soybean meal-induced enteritis after 42 days of feeding a diet containing 40% soybean meal. DI shows atrophy of mucosal folds and heavy infiltration of leukocytes into the subepithelial mucosa. Epithelial cells have a darker supranuclear cytoplasm with reduced degree of supranuclear vacuolisation. The height of the epithelial cells is also reduced. Cyst-like structures at the base of the mucosal folds are formed, in part outlined by epithelial cells, containing cellular debris (arrows) (bar=200 μm).

Fig. 2. Distal intestine of rainbow trout with soybean meal-induced enteritis stained with Alcian blue - Periodic acid Schiff (AB-PAS). Vacuolar degeneration of epithelial cells (black arrows) is

identified and goblet cells containing acidic (blue) mucins are readily distinguished (blue arrows) (bar=50 μm).

Fig. 3. Immunohistochemistry using anti-cytokeratin antibody (AE1/AE3). Distal intestine of rainbow trout with soybean meal-induced enteritis. Epithelial cells, easily distinguished by their positive red reactivity, have largely disappeared from the base of the mucosal folds and replaced by cysts (arrows) containing necrotic debris (bar= 100 μm).

Fig. 4. Denudation of lamina propria due to loss of epithelial cells. Distal intestine of rainbow trout with soybean meal-induced enteritis. The section is immunostained with anti-cytokeratin antibody (AE1/AE3). In proximity to the denuded area (DN) the epithelial cells are flattening out (F), probably in an effort to cover the denuded area, an aspect of restitution. LP: lamina propria; S: shedded necrotic cells (bar=100 μm).

Fig. 5. Changes in the epithelium of rainbow trout with soybean meal-induced enteritis (SBMIE). We have chosen to use the term “dysplasia” for a particular epithelial change that were observed in 10 % of individuals with SBMIE (and sampled during the last three weeks of period 2). This change is characterised by the irregularity of the shape of the epithelial cells, their organisation and chromatin density within nuclei of the epithelial cells. In this micrograph these changes are seen at a site where intestinal folds also could be fusing (arrow) and in an adjacent with epithelium showing similar irregularities. Tissue stained with hematoxylin and eosin (H&E) (bar=100 μm).

Fig. 6. Granulomatous enteritis in rainbow trout with distal intestinal inflammation induced by soybean meal. The change was characterised by the presence of multi-nucleated giant cells (black arrows), foamy macrophages (red arrow) and increased proliferation of fibroblasts in the lamina

propria. Cysts-like structures (C) containing cell debris were also observed with or without outlining epithelial cells. Hematoxylin and eosin (H&E) (bar=50 μm).

Fig. 7. Foamy macrophages (F) in rainbow trout with granulomatous enteritis were mostly positive for acidic (blue) mucins, however, a few were also positive for neutral (red) mucins. In this micrograph cyst-like structures (C) are also observed (bar=50 μm).

Fig. 8. Distal intestinal tissue in rainbow trout with granulomatous enteritis found to be negative for Ziehl-Neelsen staining. A) Foamy macrophages and B) multi-nucleated giant cells (black arrow) did not contain acid-fast organisms. Positive controls were also included for evaluation (not shown) C: cyst-like structure. Note that there are no epithelial outlining of the cyst (bar=50 μm).

Fig. 9. Morphological evaluation of distal intestine of rainbow trout fed fish meal or soybean meal and exposed to hypoxia or normoxia for 42 days. The changes in subepithelial infiltration of leukocytes (A), supranuclear vacuolisation of the epithelial cells (B), atrophy of intestinal folds (C), vacuolar degeneration of the epithelial cells at the base of the intestinal folds (D) and the subepithelial presence and degree of granulomatous response (E) are shown. Values are means (n=9) ± standard errors represented by vertical bars. Fish was challenged with soybean meal and/or hypoxia during period 2. FM, fish meal; SBM, soybean meal; NO, normoxia; HY, hypoxia. Histopathological score of classic morphological parameters (A, B and C) in response to a soybean meal (SBM)-based diet was significantly increased at the end of period 1 (Day 0). The degree of morphological parameters remained unchanged throughout period 2 for the fish under steady-state SBM feeding. In rainbow trout subjected to change from fish meal (FM) - to SBM-based diet, regardless of water oxygen level, the score of the classic morphological parameters were significantly increased after 7 days of SBM feeding in period 2. After day 7,

there was no further change in the degree of these morphological parameters. The significant change in vacuolar degeneration of epithelial cells and granulomatous response were observed after 14 and 21 days, respectively, in period 2 in fish subjected to change from FM to SBM. After day 21, there was no further change in the degree of classic and variant morphological features among SBM-fed groups regardless of their environment.

Fig. 10. Proliferating cell nuclear antigen (PCNA) reactivity score of epithelium in the distal intestine of rainbow trout fed fish meal or soybean meal-based diets and kept at normoxia or hypoxia for 42 days. FM, fish meal; SBM, soybean meal; NO, normoxia; HY, hypoxia. Values are means (n=9) ± standard errors represented by vertical bars. PCNA reactivity score was significantly increased in response to SBM-based diet by the end of period 1 (Day 0). The score remained unchanged during period 2 in the treatment group exposed to steady state dietary challenge (i.e. SBM). In the group subjected to FM throughout the experiment, the PCNA reactivity score was significantly reduced at day 42. Change from FM to SBM without change in water oxygen level increased the score significantly after 7 days of SBM feeding and remained as high as the score observed in the group under steady state SBM challenge. Change from FM- to SBM-based diets and simultaneously a change from normoxia to hypoxia resulted in significant increase in PCNA reactivity score after 21 days in period 2. After day 21 there was no significant difference in PCNA reactivity score among all groups fed SBM-based diet, regardless of water oxygen levels.

Fig. 11. Extrusion of epithelial cells into the lumen of distal intestine of rainbow trout with soybean meal-induced enteritis. The epithelial origin of extruded material was confirmed by immunohistochemistry using antibody (AE1/AE3) directed against cytokeratins. NC: necrotic cells (bar =200 μm).

Fig. 12. Cytokeratin reactivity was occasionally found within cyst-like structures (red arrow) in distal intestine of rainbow trout with soybean meal-induced enteritis. This suggests accumulation of material of epithelial origin within these structures. Black arrow shows a multi-nucleated giant cell (bar =200 μm).

Table 1. Diet formulation and chemical composition of experimental diets fed to rainbow trout (*Oncorhynchus mykiss*) [1]

	FM	SBM
Ingredients (g kg^{-1})		
Fish meal	540.0	250.0
Soybean meal	-	400.0
Wheat flour	170.0	140.0
Rapeseed oil	100.0	120.9

Fish oil	40.0	40.0
Cellulose	143.4	30.0
Monocalciumphosphate	-	10.0
DL-methionine	-	2.5
Yttrium oxide	0.1	0.1
Vitamin/mineral premix	6.5	6.5
Proximate analysis		
Crude protein (g kg^{-1})	430.0	427.0
Crude fat (g kg^{-1})	206.0	220.0
Ash (g kg^{-1})	79.0	76.0
Gross energy (MJ kg^{-1})	23.0	23.2

FM, fishmeal; SBM, soybean meal

[1] Details of ingredients suppliers were previously reported (Mosberian-Tanha *et al.* submitted).

Table 2. Experimental design to evaluate morphological changes in the distal intestine of rainbow trout (*Oncorhynchus mykiss*) fed soybean meal and exposed to hypoxic conditions

Treatment	Period 1		Period 2	Abbreviation
1	FM at Normoxia	→	FM at Hypoxia	FMNO→FMHY
2	FM at Normoxia	→	SBM at Hypoxia	FMNO→SBMHY
3	FM at Normoxia	→	SBM at Normoxia	FMNO→SBMNO
4	SBM at Normoxia	→	SBM at Hypoxia	SBMNO→SBMHY

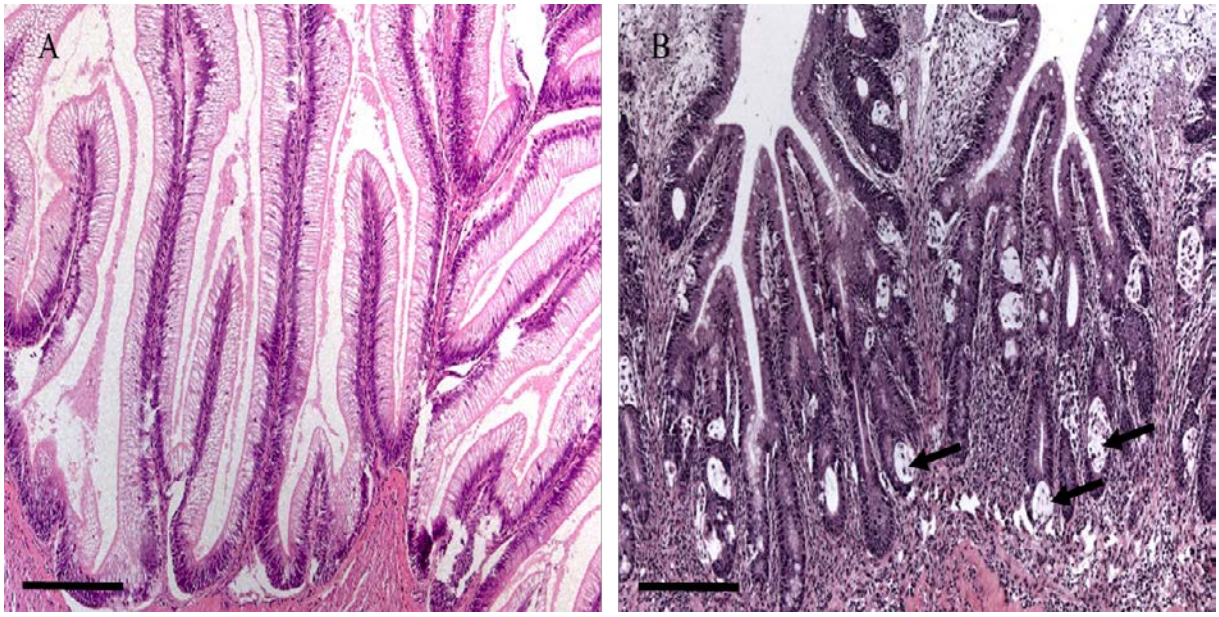
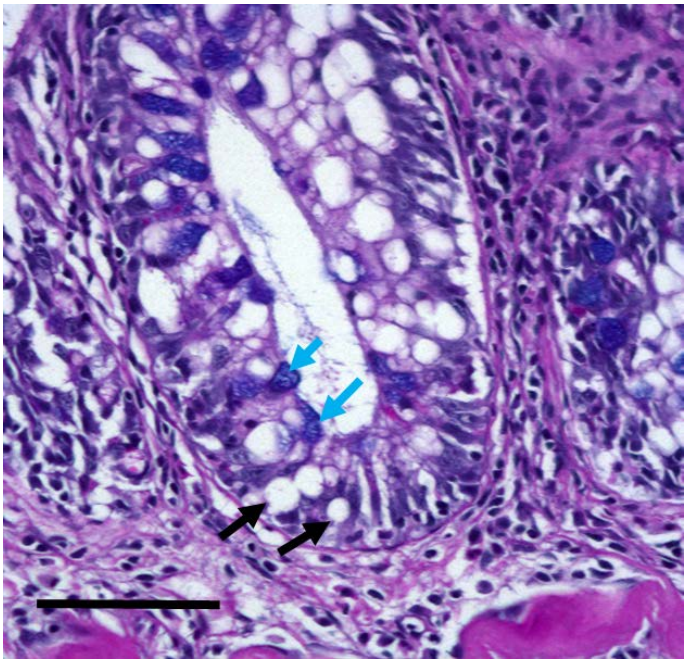


Fig. 1



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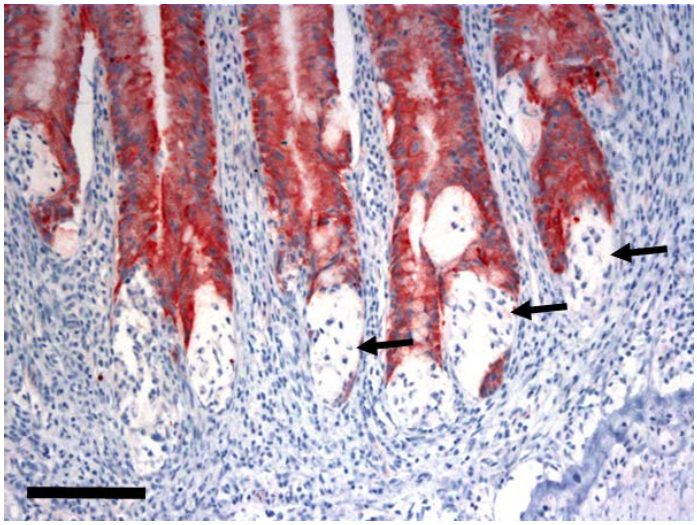

Fig. 3

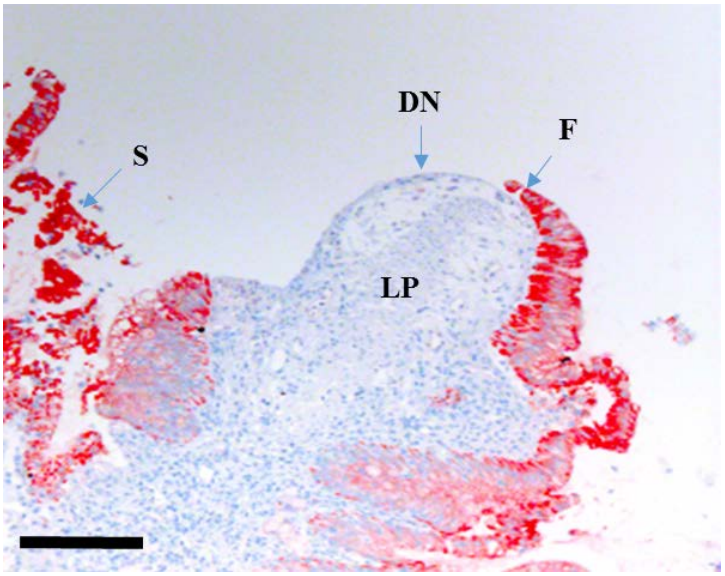

Fig. 4

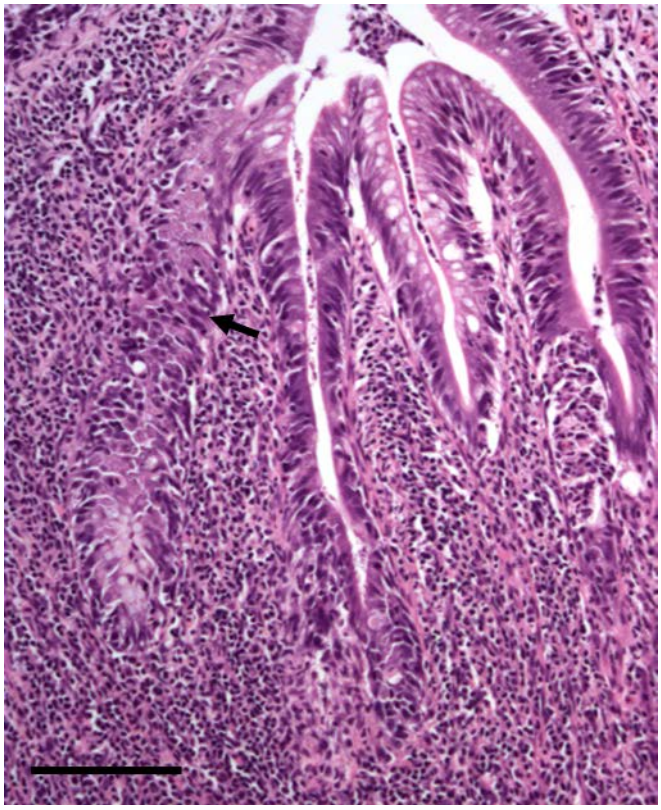

Fig. 5

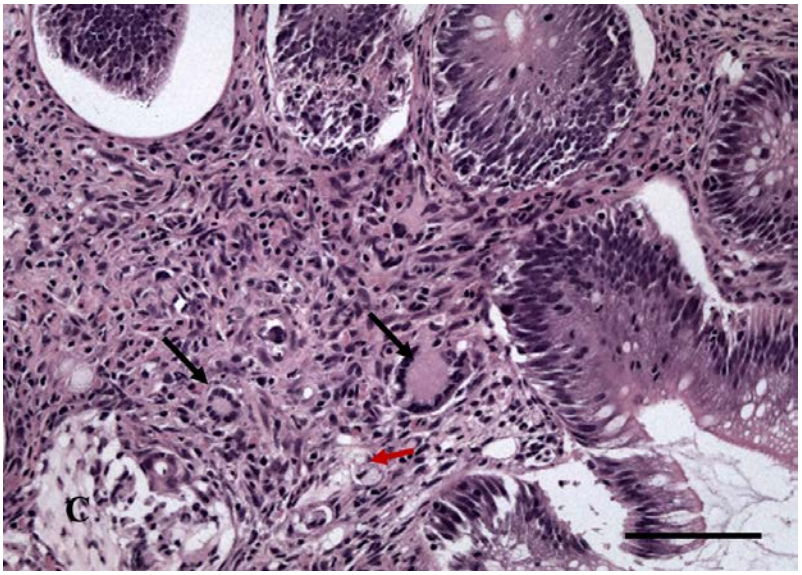

Fig. 6

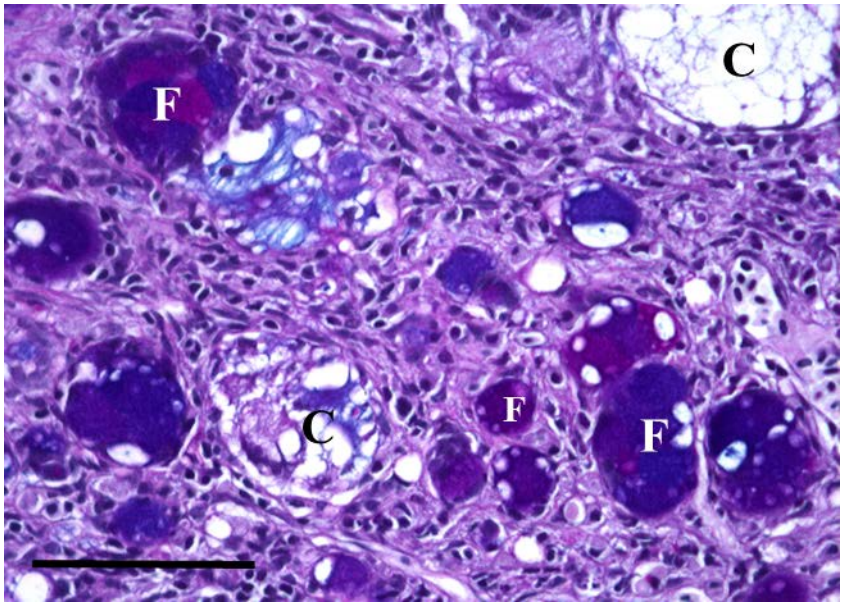

Fig. 7

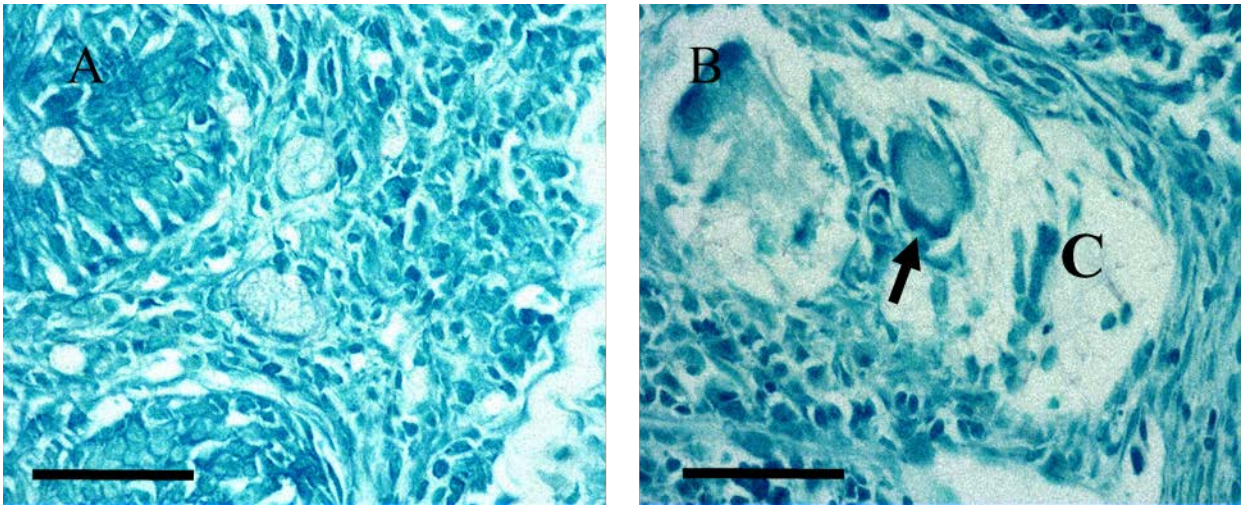

Fig. 8

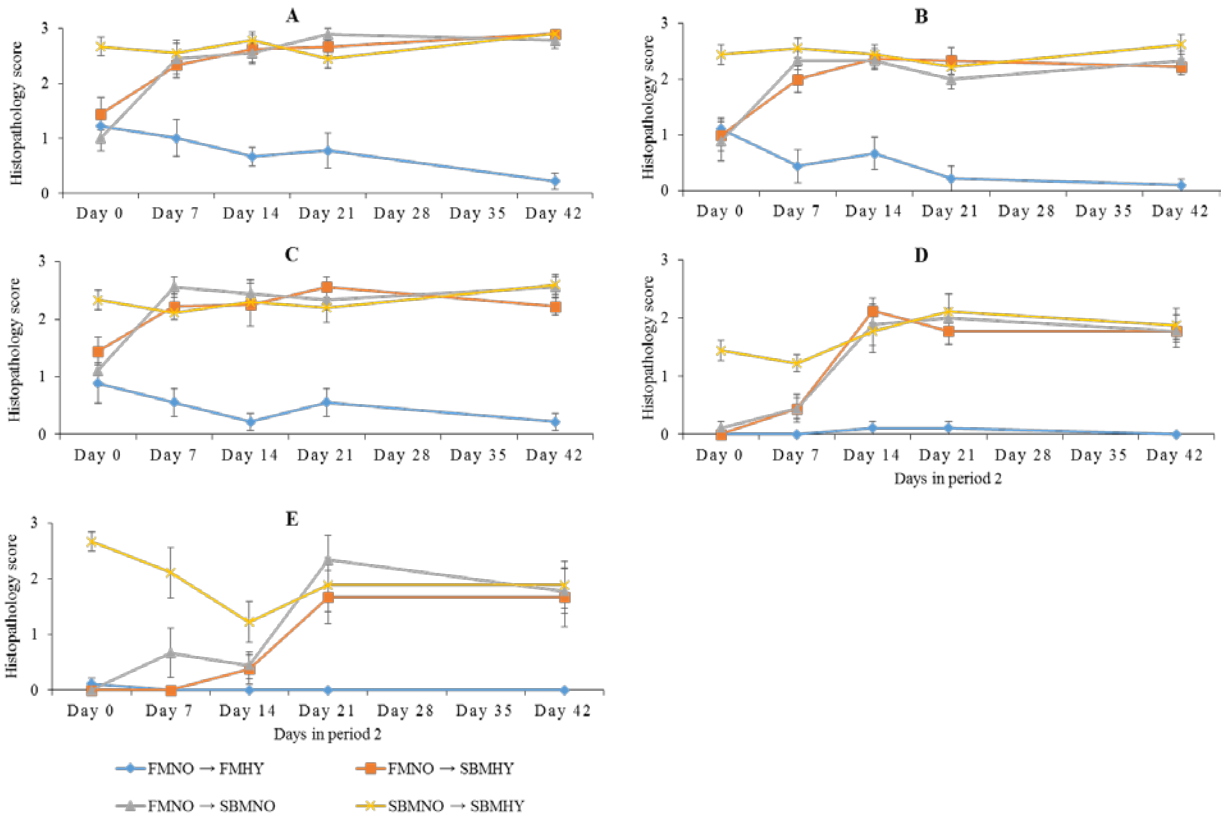
A
B
C
D
E
Histopathology score
Day 0
Day 7
Day 14
Day 21
Day 28
Day 35
Day 42
Days in period 2
FMNO → FMHY
FMNO → SBMHY
FMNO → SBMNO
SBMNO → SBMHY

Fig. 9

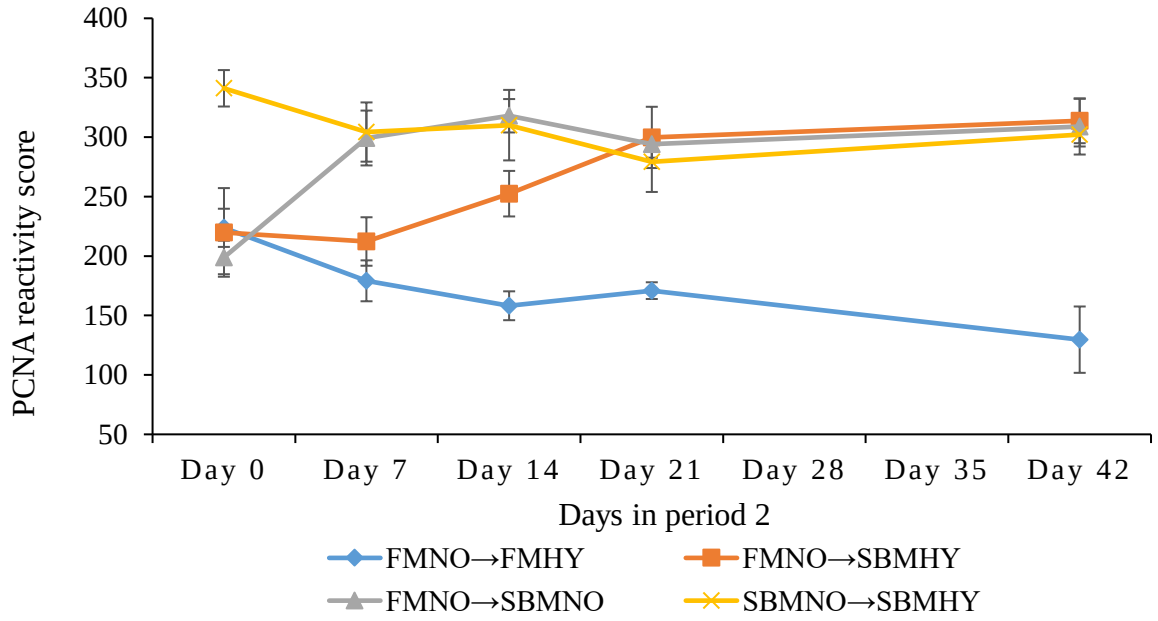
400
350
300
250
200
150
100
50
PCNA reactivity score
Day 0
Day 7
Day 14
Day 21
Day 28
Day 35
Day 42
Days in period 2
FMNO→FMHY
FMNO→SBMHY
FMNO→SBMNO
SBMNO→SBMHY

Fig. 10

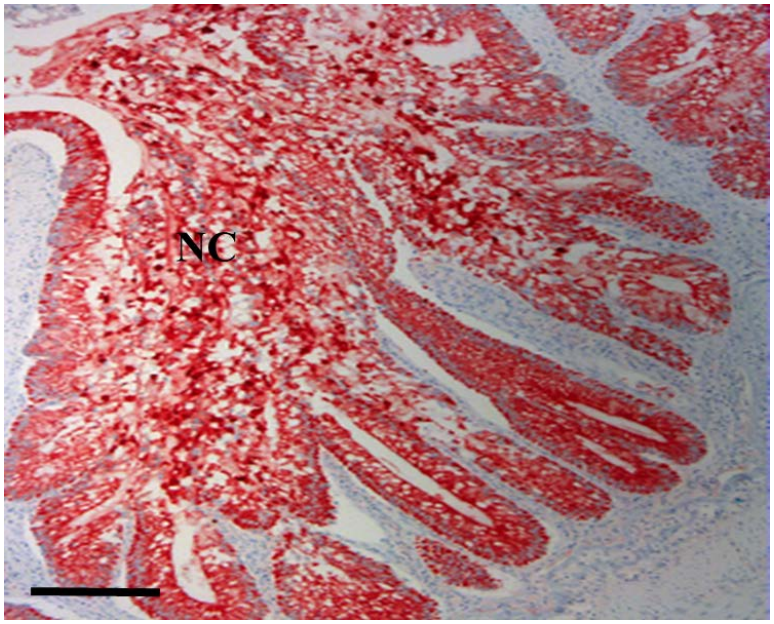

Fig. 11

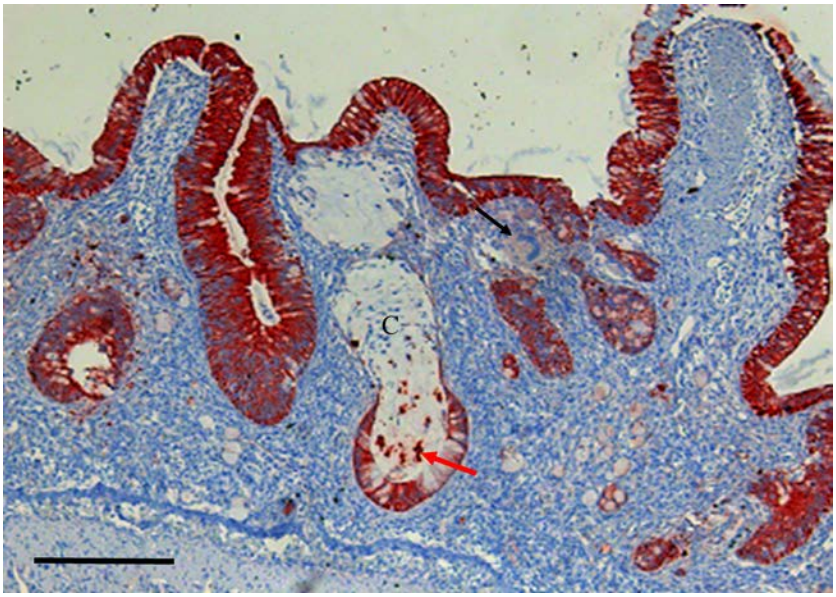

Fig. 12