Development of a pig jejunal explant culture for studying the gastrointestinal toxicity of the mycotoxin deoxynivalenol: histopathological analysis.

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Abstract

The digestive tract is a target for the mycotoxin deoxynivalenol (DON), a major cereals grain contaminant of public health concern in Europe and North America. Pig, the most sensitive species to DON toxicity, can be regarded as the most relevant animal model for studying the intestinal effects of DON.

A pig jejunal explants culture was developed to assess short-term effects of DON. In a first step, jejunal explants from 9-13 week-old and from 4-5 week-old pigs were cultured *in vitro* for up to 8 hours. Explants from younger animals were better preserved after 8 hours, as assessed by morphological scores and by villi lengths. Dose-related alteration of the jejunal tissue were observed, including shortened and coalescent villi, lysis of enterocytes, oedema. A no-effect concentration level of 1 µM was estimated (corresponding to diet contaminated with 0.3 mg DON/kg) based on morphological scores, and of 0.2 µM based on villi lengths.

In conclusion, our data indicate that pig intestinal explants represent a relevant and sensitive model to investigate the effects of food contaminants.

Keywords

Mycotoxin, Deoxinyvalenol, DON, Toxicity, Intestinal explant culture, histopathology, Food contaminants.

1. Introduction

The gastrointestinal mucosa serves as a dynamic barrier regulating uptake of nutrient and water, while excluding potential pathogens and toxicants (Oswald, 2006). Following ingestion of contaminated food or feed, intestinal epithelial cells could be exposed to a high concentration of toxicants, potentially affecting intestinal functions (Bouhet and Oswald 2005). The mycotoxin deoxynivalenol (DON), the most prevalent trichothecene mycotoxin contaminating crops in Europe and North America (Schothorst and van Egmond, 2004), is commonly detected in cereals and grains. DON is of public health concern, as it is resistant to milling, processing and heating and readily enters the food chain. Estimated daily intake in European Union countries ranged from 0.3 to 2 μg/kg body weight per day (SCOOP, 2003) and from 0.77 to 2.4 μg/kg body weight per day in Africa and Middle East (FAO/WHO Expert Committee on Food Additives, 2001).

DON causes toxic effects in humans as well as in all animal species investigated so far (Petska and Smolinski, 2005). Among animal species, pig can be regarded as the most relevant animal model for extrapolating to humans, showing the highest sensitivity to DON (Rotter et al., 1996) and a digestive physiology very similar to that of human (Kararli, 1995). Acute exposure to high doses induces radiomimetic effects including diarrhea, vomiting, leucocytose, hemorrhage/necrosis of the gastrointestinal tract. Chronic toxicity studies showed anorexia, reduced weight gain, altered nutritional efficiency, immunotoxicity, and necrosis in gastrointestinal tract and lymphoid tissues (Eriksen and Pettersson, 2004; Petska and Smolinski, 2005; Pinton et al., 2008). Human epidemiological studies from China suggest that DON causes emetic effects, diarrhea, and other gastroenteritis signs in humans (Petska and Smolinski, 2005).

Although it is a food contaminant, only scarce reports exist on the gastrointestinal effects of DON (Maresca et al., 2002; Awad et al., 2007). The Tolerable Daily Intake of 1μg/kg/d have been based on immunotoxic and general toxic effects in a two years mouse study and not on the most sensitive subchronic swine studies, because of confuding factors in the latter (Schlatter, 2004). Critical data gaps still exist regarding the potential effects of DON, and widespread human exposure needs additional research to improve capacity for assessing adverse effects of DON. Understanding the effects of DON on the intestinal epithelium is of major importance for consumer health and risk assessment.

In vitro models of intestinal mucosa have been developed for studying enteritic diseases (Girard et al., 2007) and could be used to understand the gastrointestinal effects of

DON. In the context of reducing the number of experimental animals (3Rs principles, Russel and Burch, 1959), intestinal explants represent a powerful model. Organ culture of intestinal explants allows to preserve normal histological structure *in vitro* (Nietfield et al., 1991). Large numbers of explants can be prepared from a single animal, thus reducing the number of animals required for a given study.

The aim of this work was to develop the culture of pig jejunal explants and to apply this model for studying the effect of DON on the digestive barrier, as assessed by histological lesions.

2. Materials and methods

Jejunum explants culture

Crossbreed weanling piglets of 4-5 week-old (n=5) and young pigs of 9-13 week-old (n=8) were used for explanting jejunal tissue. A 5-cm middle jejunum segment was collected in prewarmed PBS added with 200 U/mL penicillin and 200µg/mL streptomycin (Sigma, Saint-Quentin Fallavier, France). After removing external *tunica muscularis*, the jejunum was opened longitudinally and washed for 10 mn at 39°C, in culture medium supplemented with 200 U/mL penicillin and 200µg/mL streptomycin. It was dissected into 3x4 mm-pieces explants. All these operations were achieved in less than one hour after the pig's was euthanazied. In all experiments, uncultured control tissue was placed into fixative at the end of dissection time (0h).

The culture was adapted from the method of Nietfeld *et al.* (1991), as described by Girard *et al.* (2005) with minor modifications. Briefly, the pigs jejunal explants were incubated in 1ml RPMI 1640 (Sigma), prewarmed and gassed with 95%O2/5% CO2. The culture medium was supplemented as described by Girard et al. (2005), but without ascorbic acid. It contained 100 U/mL penicillin, 100µg/mL streptomycin, and 50µg/mL gentamycine (Sigma). Explants were incubated villi up in 4-well culture plates (Nunclon, Sigma) and agitated in the culture chamber by a rocking plateform. Two explants/well were incubated on 1-cm^2 biopsy sponge, in standard culture conditions, for 4 and 8 hours. Culture medium was changed after 4 hours of incubation, then every 2 hours. Fetal bovine serum came from Eurobio (les Ulis, France).

DON exposure

Explants from weanling pigs were exposed to 0, 0.2, 1, 5 µM DON (Sigma) in the culture medium for 4 hours. Preliminary cultures had shown that 10 and 30 µM DON induced necrosis of the explants after 4 h of incubation.

Morphology

For histological analysis, explants were fixed at 0h, 4h, and 8h in 10% formalin for 5 to 24 hours. After embedding in paraffin, the explants were sectionned at 2-4-µm slides parallely to the villi axis and stained by haematoxylin and eosin (H&E) using standard procedures. The resulting slides were viewed independently by two observers, at a magnification of x100.

A morphological score was designed to compare the histological lesions (Table 1). The scoring system included both morphometry and lesional informations. The following criteria were included in the score: the number of villi and the number of crypts (0 to 3 points each), the length of villi, the morphology of enterocytes, the degrees of villi coalescence and autolytic changes of the tissue (oedema, necrotic debris, apoptotic cells). Each score value was the result of 2 to 4 explants from the same pig at each incubation time and/or DON concentration. At least 4 different animals were used for each condition. In addition, the length of the villi were measured in all the explants scored and the result for each explant was the mean of all the villi measured (NIS Elements imaging software, Nikon Instruments).

Statistics

The values of scores are presented as means ± SD of independent experiments with different animals. The scores and individual endpoints were compared by ANOVA analysis after log transformation of the variables, to test the effect of incubation time and that of pigs age (SYSTAT version 10.0). If significantly different (P values ≤ 0.05), the means were compared by Dunnett's test to control values, and by Bonferroni test for multiple comparisons. DON dose-related effect after 4 h of incubation was analyzed by ANOVA followed by Dunnett's test.

3. Results and discussion

Time and age-dependant histological lesions

We first investigated the effects of (i) the age of the piglet and (ii) the duration of the culture on the histological alterations observed on jejunal explants. The explants were observed microscopically and scored from 15 (no lesion) to 0 (destroyed tissues).

Before incubation, scores values were between 13 and 15 for all the explants (Fig.1a and 1b). All the explants from weanling pigs presented a score of 15, whereas the explants from 9-13 week-old animals scored between 13 and 15. Time-dependant histological lesions were observed after 4h and 8h of incubation in all the explants (Table 2). They included flattening and coalescent villi, lysis of enterocytes, oedema in the lamina propria (Fig.1). Mitoses figures were observed in the crypt epithelium and general morphology was maintained up to 8 hours of culture. After 8h, the scores decreased to 5.5 (±2.5) and 9.2 (± 2.2) in the explants from 9-13 week-old pigs (n=8 pigs, Fig.1e) and from weanling pigs respectively (n=5 pigs; Fig.1f). These scores differed significantly ($p<0.001$). After 4 h of culture, flattening of the villi, weak coalescence, weak oedema in lamina propria, midly dilatation of the crypts by sloughed epithelial cells were observed in the slices. Mean scores were of 11.2 (±0.9) and 12.1 (±1) in explants from 9-13 week-old pigs and from 4-5 week-old ones respectively (n=8 pigs, Fig.1c; n=5 pigs, Fig.1d). These scores did not differ significantly, and were considered as acceptable for short-term testing of DON toxicity. Villi length was shown a sensitive endpoint, decreasing to less than two-third (62.5-65 %) to about one-half (46-52%) of the initial values, respectively after 4 hours and 8 hours of culture in the two age groups tested.

To the best of our knowledge, the present study is the first application of pig intestinal explants culture for toxicological studies. This technique presents two main advantages but also limitations. First, explant culture reduces the number of animals used, in the context of the 3Rs, one pig allowing multiple doses assessment. The second main advantage over culture cell is the use of a fully differenciated-three dimensional model allowing histopathology assessment. Initially described from human biopsies (Browning and Trier, 1969), intestinal explants have been developed in various species. Pig intestinal explants have been obtained from duodenum, jejunum, ileum or caecum to study enteropathogens (Heinz et al., 1987; Girard et al., 2005; Girard et al., 2007; Kik et al., 1991; Zhu et al., 1995; Williams et al., 1985). In the present study, we used the jejunum, because jejunum has been shown a target

for the mycotoxin DON *in vivo* (Pinton et al., 2009). As normal metabolism is necessary for morphological preservation, structural integrity is a valid criterion of successful culture (Heinz et al., 1987). In the present study, as in previous experiments, shorter and wider villi upon incubation were observed.

The two main limitations of the technique consist in the culture duration and the need of young pigs. The duration of culture allowing preserved morphology of the explants depends both on experimental conditions and on the age of pigs. In the present study, we observed that explants from weanling piglets were better preserved than explants from older animals, specially the length of the villi. Using explants from newborn piglets, incubation period up to 12 hours have been reached (Girard et al., 2005; Zhu et al., 1995). So younger is the animal, longer will be the culture duration : this limits the potential use of the technique for working with explants from growing-finishing pigs or sows, which are also targets for mycotoxins, as well as from pigs feds mycotoxins *in vivo*, specially chronically. The technique can be used advantageously over culture cell to investigate the digestive effects of food contaminants, as a model to protect human and animal health. Newborn animals show an immature intestinal tract, we thus preferred intestinal explants from weanling piglets to further study the effects of food contaminants.

Effect of DON exposure on morphological score and on villi length

According to the better preserved morphology in weanling piglets after 8h of incubation (Table 2), we choose to assess DON toxicity in explants from young animals after 4h of incubation. Explants were incubated with increasing concentrations of DON and quantified by scoring the histological lesions, including flattened and coalescent villi, lysis of enterocytes, oedema, apoptosis. Dose-related alterations of the jejunal epithelium and decreased villi lengths were observed after DON exposure.

High concentration of DON (10 and 30 µM) induced pronounced autolysis of the tissue (data not shown), whereas lower concentration of DON (0.2, 1 and 5 µM) induced dose-dependant histological lesions ($p<0.001$). They consisted in pyknotic nuclei in enterocytes, flattening and coalescent villi, edema and necrosis in the lamina propria showing numerous apoptotic cells at 5 µM (Fig.2). At this DON concentration, there was marked diffuse sloughing of epithelial lining from the surface of the villi and along the surface of the explants there was consistently marked coating of heterogenous material composed of secreted mucus and cellular debris. The morphological scores were of 11.9 (±1.2), 10.8 (±0.5), and 8.4 (±0.5) in control explants and explants treated with 0.2 and 1 µM DON

respectively, decreasing to 7.3 (±1.5) for explants exposed to 5 µM DON ($P<0.001$). A no-effect concentration of 1 µM was estimated, based on the morphological score (Fig. 3a). Based on the villi lengths measurements, a no-effect concentration level of 0,2 µM can be estimated (Fig.3 b).

Pig jejunal explants represent a promising model for studying the effects of DON on the digestive tract, allowing to demonstrate a dose-dependant toxicity of DON, at realistic dietary levels. As pig represents the most sensitive as well as the most relevant animal species for studying mycotoxin toxicity and shows a digestive physiology very similar to that of human (Kararli, 1995), the results should be relevant for human consumer risk assessment.

The present study showed dose-related toxicity of DON in the explants model from weanling pigs after 4h of incubation. The histopathological lesions observed in the present work, following short-term exposure to DON, are in accordance with the gastrointestinal tract necrotic lesions previously reported in animal acute and chronic toxicity studies (Petska and Smolinski, 2005). *In vivo* and *in vitro* studies suggest that DON affect intestinal epithelium integrity. *In vitro* experiments, using unrealistic high DON concentrations, showed altered absorption of nutrients: the absorptive fonctions were affected above 10 µM in intestinal human cells line HT-29 (Maresca et al., 2002), and at 10 mg/L in chicken's jejunum explants mounted in Ussing chambers (Awad et al., 2007). The pig jejunal explants culture developed in this study appears as a very sensitive model, and will allow to analyze the effects of DON on the intestinal epithelium, at relevant concentrations.

In our study, based on histopathological endpoints, the estimated no-effect concentration was 1µM DON. This *in vitro* concentration corresponds to an exposure to 0.3 mg DON/kg food, assuming that DON diluted in 1 L of gastrointestinal fluid is ingested in one meal and is 100% bioavailable (Pinton et al, 2009). This DON concentration than can easily be reached in the gut lumen after ingestion of a DON contaminated diet. Indeed, recent surveys including 11,022 cereals samples from 12 European countries indicated that 57 % of the samples were positive for DON contamination and 7 % showed levels above the regulated level of 0.75 mg/kg food (Schothorst and van Egmond, 2004). In the present study we observed intestinal damages and decreased villi lengths, after exposure to DON at the regulatory level. Considering widespread human exposure to DON *via* cereal products, the risk characterization of DON needs to include the gastrointestinal effects.

In conclusion, our data indicate that pig intestinal explants represent a sensitive model to investigate the digestive effects of DON. Intestinal explants can also contribute to improve our knowledge on plausible interactions of contaminants present simultaneously at the

intestinal level. For this purpose, in parallel to histology analysis, specific cellular responses of toxic insult such as, for example, the expression of inflammatory cytokines will need to be investigated.

Acknowledgements

We acknowledge for animals care A.M. Cossalter (INRA), for their help in histopathological techniques C. Bleuart and I. Pardo (ENVT), and F. Lyazhri (ENVT) for his help with the statistical analysis. We thank the French Ministry of Agriculture for the financial support of the Mycoxicology Unit (ENVT).

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Table 1 Endpoints used to assess histologically the explants in a morphological score (maximal score of 15 before incubation)

End-point		Score
Number of villi	0	0
	<5	1
	5-10	2
	>10	3
Number of crypts	0	0
	<5	1
	5-10	2
	>10	3
Villi lenght	0	0
	+	1
	++	2
	+++	3
Enterocytes morphology	no enterocytes	0
	+ flattened epithelium	1
	++ cuboïd epithelium	2
	+++ columnar epithelium	3
Lesions of the tissue	Lysis , necrosis	0
	Coalescence of villi, oedema, apoptosis	1
	Weak coalescence, weak oedema, weak apoptosis	2
	No lesion, slight flattening of the villi	3

Table 2 Effect of incubation time and of pig age on the morphological scores of explants. Jejunal explants obtained from 9-13 week old and from 4-5 week old pigs were culture *in vitro* for 0, 4 or 8 hours before being observed histologically and scored. For each incubation time, 2 to 4 explants from the same animal were scored. Data are mean score ± SD from 5 to 8 different animals.

Pig age (weeks)	Incubation time (hours)	**Number of villi**	**Number of crypts**[1]	**Villi length**[2]	**Enterocytes morphology**[3]	**Lesions**[3]	**Score**[2]
4-5 (n=5)	0 4 8	3 3 2,6 (0.9)	3 3 2,4 (0.8) [a]	3 2 [a] 1,5 (0.5) [b]	3 2,2 (0,7) [a] 1,4 (0.4) [b]	3 1,9 (0,4) [a] 1,3 (0.4) [b]	15 12,1 (1) 9,2 (2.2) [a]
9-13 (n=8)	0 4 8	3 2,9 (0,2) 2.0 (1.0)	3 2,7 (0,3) [b] 0,6 (1.0) [c]	3 1,7 (0,4) [c] 1,4 (0.7) [d]	2,8 (0,4) 2,1 (0,4) [a] 1,1 (0.6) [b]	2,6 (0,5) 1,8 (0,8) [a] 0,4 (0.5) [b]	14,4 (0.8) 11,2 (0.9) 5,5 (2.5) [b]

[1]Pig age effect ($P<0.05$) and incubation time effect ($P<0.001$); [2] Pig age effect ($P<0.001$) and incubation time effect ($P<0.001$); [3]Incubation time effect ($P<0.001$).

[a], [b], [c], [d] : significantly different from 0 h incubation time condition (Dunnett's test) ; two different letters show significant differences (Bonferroni test).

Figure legends

Fig.1

Morphology of jejunal explants from 9-13 week-old pigs (panels a, c and e) and from 4-5 week-old pigs (panels b, d and f) after different time of incubation. a. b. Before incubation (panels a and b); after 4h incubation (panels c and d); after 8h incubation (panels e and f). The lesions are marked with different arrows: coalescence of villi ⟶ ; lysis ⇢ ; Edema ⟶ (G x100 ; H &E staining).

Fig.2

DON-induced histological lesions. Effect of 5 µM DON on the morphology of jejunal explants after 4 hours of incubation (b), compared to a control explant after 4 hours of incubation (a): flattening and coalescent villi and marked coating of heterogenous material after DON exposure.

Fig.3

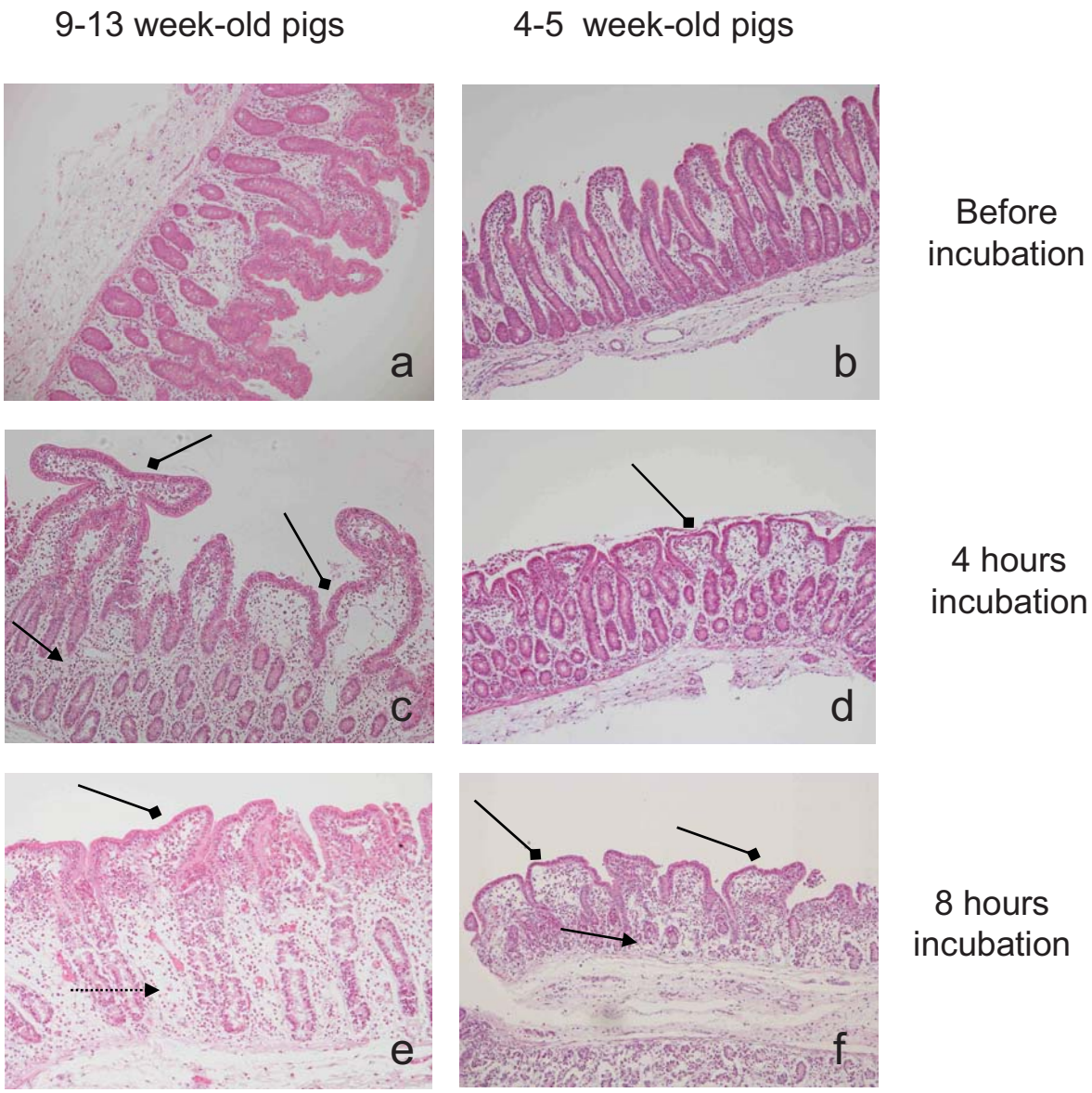
Jejunal explants obtained from 4-5 week-old pigs were cultured *in vitro* for 4 hours with different concentrations of DON before histological examination and morphological score assessment and villi length measurement. For each concentration, 2 to 4 explants from the same animal were scored. Data are mean scores ± SD from 4 to 5 different animals. ANOVA analysis was followed by Dunnett's test (*: $P<0.05$; ***: $P<0.001$).

a Effect of DON concentration on the morphological scores of explants (A.U : arbitrary unit)

b Effect of DON concentration on the villi length (µm)

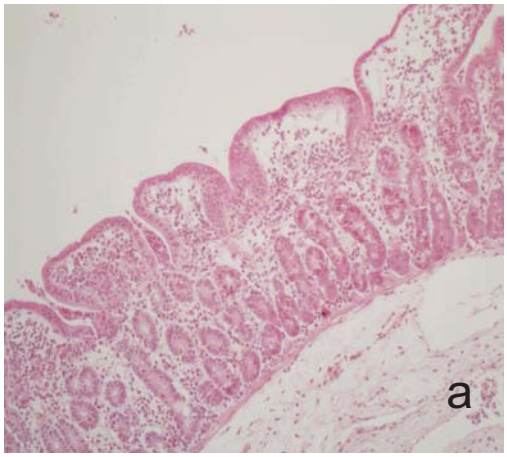
Figure(s)

Kolf-Clauw et al. Figure 1

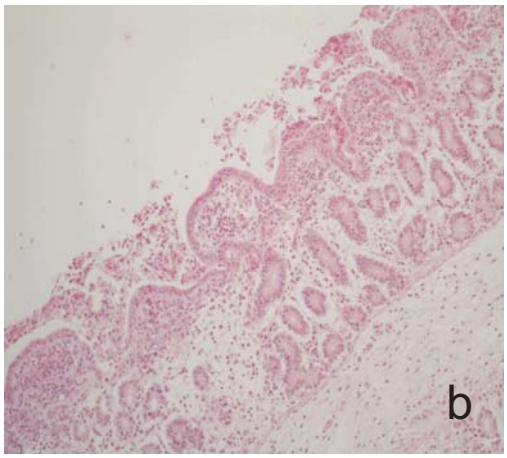


Figure(s)

Kolf-Clauw et al. Figure 2



Control explant



DON-treated explant

Figure(s)

Kolf-Clauw et al. Figure 3