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ORIGINAL ARTICLE

Effect of 60 and 90 days of isotretinoin treatment on the structure of the small intestine mucosa in young male Wistar rats

Bruna Fontana THOMAZINI, Mary Anne Heidi DOLDER

Department of Structural and Functional Biology, Biology Institute, State University of Campinas, Campinas – SP, Brazil

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ABSTRACT

Isotretinoin is a substance used in cases of severe acne and acne resistant to other treatments. This skin disease affects patients of all ages and can interfere with social life, especially in adolescents. The drug acts by suppressing sebaceous gland activity and creating an inhospitable environment for *Propionibacterium acne*. The integrity of the small intestine is important for correct nutrition and patient treatment. We intended to assess the small intestine structure after treatment with 5 mg/kg isotretinoin solution and after a period without the drug, which could be considered a rest period. Young male Wistar rats (n=24) were separated into 4 groups (n=6): C: water; D0: soybean oil; D5a: 5 mg/kg; D5b: 5 mg/kg for 60 days followed by 30 days of rest period. Soybean oil was used to dilute the drug and it was offered daily by gavage. The animals were euthanized and the duodenum, jejunum and ileum were collected for analysis with light and scanning electron microscopy. The treatment stimulated tissue proliferation in the jejunum and ileum but had no significant effect in the duodenum. The results also showed a modification in goblet cell frequency in the duodenum and ileum. A further finding was that some modifications disappeared during the rest period. The protocol showed that the small intestine was somewhat altered by the treatment yet no lasting damage was caused.

KEY WORDS: small intestine; microscopy; Wistar rats; histology

Introduction

Acne is a skin disorder that can affect social relations, with skin scars often leading to low self-esteem, mainly in adolescents. Isotretinoin is currently the most effective acne treatment available, with reported long-term remission rates as high as 89%. This treatment is especially indicated in cases of resistant disease, unresponsive to other therapies (Ortonne, 1997; Sieving *et al.*, 2001; Chia *et al.*, 2005; Passier *et al.*, 2006; Zane *et al.*, 2006).

The treatment usually initiates with a daily dose of 0.5mg/kg (or higher) and can be increased to 1.0 mg/kg. A low dose of isotretinoin, such as 0.15–0.40 mg/kg, has been reported to be effective with a low incidence of severe side effects. Aiming at a total dose of 120–150 mg/kg per treatment, it may last for 3–7 months depending on the daily doses used (Passier *et al.*, 2006; Akman *et al.*, 2007; Sundstrom *et al.*, 2010). About 25% of patients treated with isotretinoin have elevated triglyceride plasma levels, which in some cases may be associated with the onset of acute pancreatitis. Isotretinoin can also cause a slight decrease in plasma HDL cholesterol and increased LDL and VLDL cholesterol. Changes in serum triglycerides and cholesterol are reversible upon treatment interruption. Less frequent adverse reactions, which are reversible, include vomiting, gastrointestinal bleeding, appendicitis, gut inflammation, esophagitis, anorexia, weight loss and ulcerative colitis (Shalita *et al.*, 1983; Bigby & Stern, 1988; Diniz *et al.*, 2002; Charakida *et al.*, 2004; Akman *et al.*, 2007; Brito *et al.*, 2010) . Nonspecific symptoms include nausea, diarrhea and abdominal pain. There is also evidence that the drug may worsen the manifestations of inflammatory diseases. However, it has been administered successfully to patients with Crohn's disease and ulcerative colitis without causing discomfort (Brito Mde *et al.*, 2010) (10).

In the intestine, ingested food is converted into a small particle nutrient solution (McCarter & Chen, 1992; Barret, 2006). Isotretinoin absorption occurs in the intestine and the lymphatic system absorbs the esterified

Correspondence address:
Bruna Fontana Thomazini, PhD.
Department of Structural and Functional Biology, Biology Institute
State University of Campinas– UNICAMP
Bertrand Russel Avenue, Block N, Zeferino Vaz Campus - Barão Geraldo
13083-865, Campinas – São Paulo –Brazil
TEL.: +55 19 35216116 • E-MAIL: bruna.fth@gmail.com

chylomicron with a retinol group (Junqueira & Carneiro, 2015). Although the drug is widely used and many side effects have been described, information about the direct effect in the healthy small intestine is not available. The duodenum is the main digestion site and the jejunum and ileum seem to be the absorption sites of drugs and nutrients. Thus the aim of this study was to investigate in young male Wistar rats the structure of the duodenum, jejunum and ileum after treatment with isotretinoin and after a rest period with no exposure to this drug.

Material and methods

Experimental groups

24 male Wistar rats (*Rattus novergicus)* were randomly allocated to the following groups:

- C: control group with water;
- D0: control group with soybean oil, the vehicle where we dissolved the substance;
- D5a: 5 mg/kg solution for 60 days;
- D5b: 5 mg/kg solution for 60 days of treatment followed by 30 days drug free.

All four groups received the treatment for 60 days but the D5b group was followed up for further 30 days without any treatment in order to elucidate the conditions right after the treatment. The rats had free access to rodent food and water and the animal house had luminosity control with 12 hours of light/dark and a temperature of about 22±1 °C. We diluted the drug in soybean oil (Tsukada *et al.*, 2002) and offered it by daily gavage for 60 days. The 5 mg/kg concentration dosage was chosen as it is considered a little higher but still not a harmful dose.

Sample collection from the small intestine

After the treatment the groups C, D0 and D5a were euthanized with a mixture of 10 mg/kg of ketamin and 80 mg/kg of xylazine solution and the duodenum, jejunum and ileum were immediately sectioned and washed in saline solution. The preparations followed the usual procedure for light microscopy using Karnovsky's modified fixative (Nankervis *et al.,* 1995) which includes 4% paraformaldehyde and 2.5% glutaraldehyde solution for 48 hours. After 30 days, this same procedure was repeated for group D5b.

Light microscopy

In order to analyze the treatment effect, we chose the morphometric tool linked to stereological evaluation. After fixation, the fragments were paraffin embedded and sections with 5 µm thickness and 40 µm interval received hematoxilin-eosin staining.

Villus and crypt morphometry

All data were obtained with the software Image Pro Plus® (Media Cybernetics, version 4.5.0.29). To analyze the villus, we considered the height of 15 villi. For the Liberkühn crypt evaluation, we determined the height of 15 crypts. To determine the relation between villi and crypt height, we applied the formula (villi height/crypt height).

Mucosal morphometry

We determined the thickness of the organ wall of 10 different regions.

Absorptive surface

To find the assumed absorptive surface (AS) for the duodenum, we used the following formula (Hardin *et al.*, 1999):

AS (μm^2) = villi height (µm) × medium width at 50% of villi height (µm).

In the 5 µm thickness section we performed the combination of Alcian Blue (AB) pH 2.5 with Periodic Acid Schiff (PAS) (AB+PAS) (Alcian Blue pH 2.5-PAS®, EasyPath) to stain the goblet cells according to their mucin type. The second technique was Reticulin (Reticulina®, EasyPath) to reveal reticulin fiber distribution and structure. The third one was the Masson Trichrome Stain (Weigert´s Iron Hematoxilin Set®, Sigma-Aldrich and Masson Trichrome Stain Kit®, Sigma-Aldrich) to show muscle and connective tissue distribution.

Goblet cell evaluation

The first step was to determine the area occupied by villi and crypts. We selected ten different fields of the same samples applying the histochemical technique combining AB+PAS pH 2.5. Using the software Image Pro Plus® (Media Cybernetics, version 4.5.0.29), we counted the goblet cells considering the type of mucin revealed in the cytoplasm. The different mucins were revealed according to their dominant group. It is thus possible to assess the frequency of cells secreting basic (PAS^+, magenta color), acidic (AB^+, blue color) and a mixture thereof (AB^+PAS^+, purple color).

Scanning electron microscopy

In order to assess the surface structure, we collected fragments to be observed by scanning electron microscopy. The fragments were completely dehydrated (ascending ethanol series of 70 to 100%), after fixation with a modified Karnovsky fixative. The next steps were the critical point of drying, followed by gold sputtering.

Ethical permissions

This experiment followed the established ethical standards in accordance to the animal protection laws of Brazil. The Ethics Committee of Animal Use of the State University of Campinas (CEUA/Unicamp/ protocol #2831-1) approved the research technique.

Statistical analysis

Considering sample size, we applied the Kruskal-Wallis statistic test followed by Dunn's post test. Data are presented as mean± standard deviation. The tests considered $p<0.05$ statistically significant and were performed with Minitab® 16 program (LEAD Technologies, Inc. Charlotte, North Carolina).

Results

The treatment did not cause obvious signs of damage to the health of the rats. Gavage was performed without difficulty and, as shown in Table 1, all groups had body mass gain during the treatment.

The morphometric data indicate that the duodenum parameters did not alter with the treatment. The stereology of goblet cells showed that the effects were dosage-dependent and that the rest or recovery period was sufficient since the parameters were comparable to those of the controls. We found diminished jejunum wall thickness in the D5a group in relation to the control D0. Crypt evaluation showed that their height decreased in the recovery group D5b in relation to control DO. Considering the villus data, the treatment did not affect their height but it could have affected the assumed absorption surface because these measurements were smaller in the D5b group in relation to D5a. Stereology and morphometry of the cell types indicated that goblet

Table 1. Body weight, stereological and morphometric analysis of the duodenum, jejunum and ileum mucosa after 60 days of treatment with isotretinoin and a recovery period in young male Wistar rats.

Groups	C control with water	D0 control with soybean oil	D5a 1 mg/kg of isotretinoin	D5b 5 mg/kg of isotretinoin and a recovery period
Initial body weight (g)	192. 82±13.96	193.5±10.17	193.46±15.24	192.78±3.8
Final body weight (g)	468.43±40.15	459.44±53.34	466.68±22.54	474.25±41.73
Duodenum				
Duodenum Wall Thickness (µm)	955.47±529.22	733.07±118	761.52±168.47	679.24±122.47
Mucosal area (mm^2)	2.93±0.12	2.85±0.22	2.83±0.38	2.97±0.10
Assumed Absorption surface (mm^2)	0.043±0.011	0.046±0.01	0.05±0.0063	0.039±0.005
Villus Height (µm)	419.61±98.46	442.19±85.19	449.80±70.16	388.97±59.29
Crypt height (µm)	223.40±139.74	169.32±28.33	162.96±34.75	148.44±20.04
Villus height: Crypt height ratio	2.19±0.79	2.63±0.42	2.79±0.34	2.64±0.37
Goblet cells- Units/mm^2	559.80±134.54^{ab}	657.83±5.45^{a}	426.48±62.44^{b}	663.99±163.81^{a}
Goblet cells- PAS^+/mm^2 (%)	97.30±1.17^{a}	98.96±0.27^{ab}	99.39±0.35^{b}	99.10±0.24^{ab}
Goblet cells- PAS^+AB^+/mm^2 (%)	2.70±1.17^{a}	1.04±0.27^{ab}	0.61±0.35^{b}	0.90±0.24^{ab}
Jejunum				
Jejunum Wall Thickness (µm)	734.69±40.26^{ab}	717.88±82.68^{b}	803.28±71.50^{ab}	760.91±56.81^{a}
Mucosal area (mm^2)	3.12±0.31	2.97±0.28	2.87±0.26	2.89±0.36
Assumed Absorption surface (mm^2)	0.49±0.07^{ab}	0.46±0.12^{ab}	0.55±0.063^{b}	0.45±0.09^{a}
Villus Height (µm)	468.00±21.22	442.38±80.42	502.61±28.14	475.49±46.85
Crypt height (µm)	176.73±18.24^{ab}	180.95±29.98^{a}	176.98±13.91^{ab}	155.29±9.38^{b}
Villus height:Crypt height ratio	2.67±0.25	2.53±0.72	2.85±0.25	3.08±0.40
Goblet cells - Units/mm^2	62.46±13.02^{a}	76.01±1.60^{ab}	70.35±10.09^{ab}	81.74±6.51^{b}
Goblet cells - PAS^+/mm^2 (%)	99.18±0.46	99.54±0.20	99.66±0.32	99.27±0.23
Goblet cells - PAS^+AB^+/mm^2(%) (%)	0.81±0.46	0.46±0.20	0.34±0.33	0.73±0.23
Ileum				
Ileum Wall Thickness(µm)	580.05±68.33^{b}	618.25±76.57^{ab}	583.54±42.53^{a}	585.65±41.75^{a}
Mucosal área (mm^2)	0.25±0.01^{a}	0.24±0.06^{ab}	0.28±0.02^{ab}	0.29±0.03^{b}
Assumed Absorption surface (mm^2)	0.30±0.04	0.30±0.06	0.32±0.06	0.27±0.03
Villus Height (µm)	333.89±7.51^{ab}	313.65±30.98^{a}	308.31±27.95^{ab}	296.88±25.04^{b}
Crypt height (µm)	145.47±19.39^{a}	137.61±19.87^{a}	171.69±11.26^{b}	156.31±12.41^{ab}
Villus height:Crypt height ratio	2.33±0.27^{a}	2.30±0.28^{ab}	1.80±0.11^{b}	1.91±0.18^{b}
Goblet cells - Units/mm^2	236..8±19.35^{a}	234.52±61.06^{a}	192.8±21.80^{b}	245.4±9.35^{a}
Goblet cells - PAS^+/mm^2 (%)	4.20±1.86^{a}	1.73±0.96^{a}	0.60±0.39^{b}	1.41±0.19^{ab}
Goblet cells - PAS^+AB^+/mm^2(%) (%)	98.2±0.76^{a}	99.2±0.45^{a}	99.7±0.18^{b}	99.42±0.07^{ab}

Mean ± standard deviation. Averages in the same row followed by different letter differ by the Kruskal-Wallis test followed by Dunn post test at a 5% significance level.

cell frequency increased in group D5b in relation to C, while the frequency of subtypes showed no statistical difference among the groups (Table 1 and Figure 1).

Observing all data obtained for the ileum segment, we found indication of tissue recovery as well as indications of direct effect of the treatment. The crypt height was higher in the D5a group in relation to control groups. On the other hand, villus height was higher in the D5b group in relation to the control, indicating that even after the end of the treatment, the drug, which is dose dependent, was

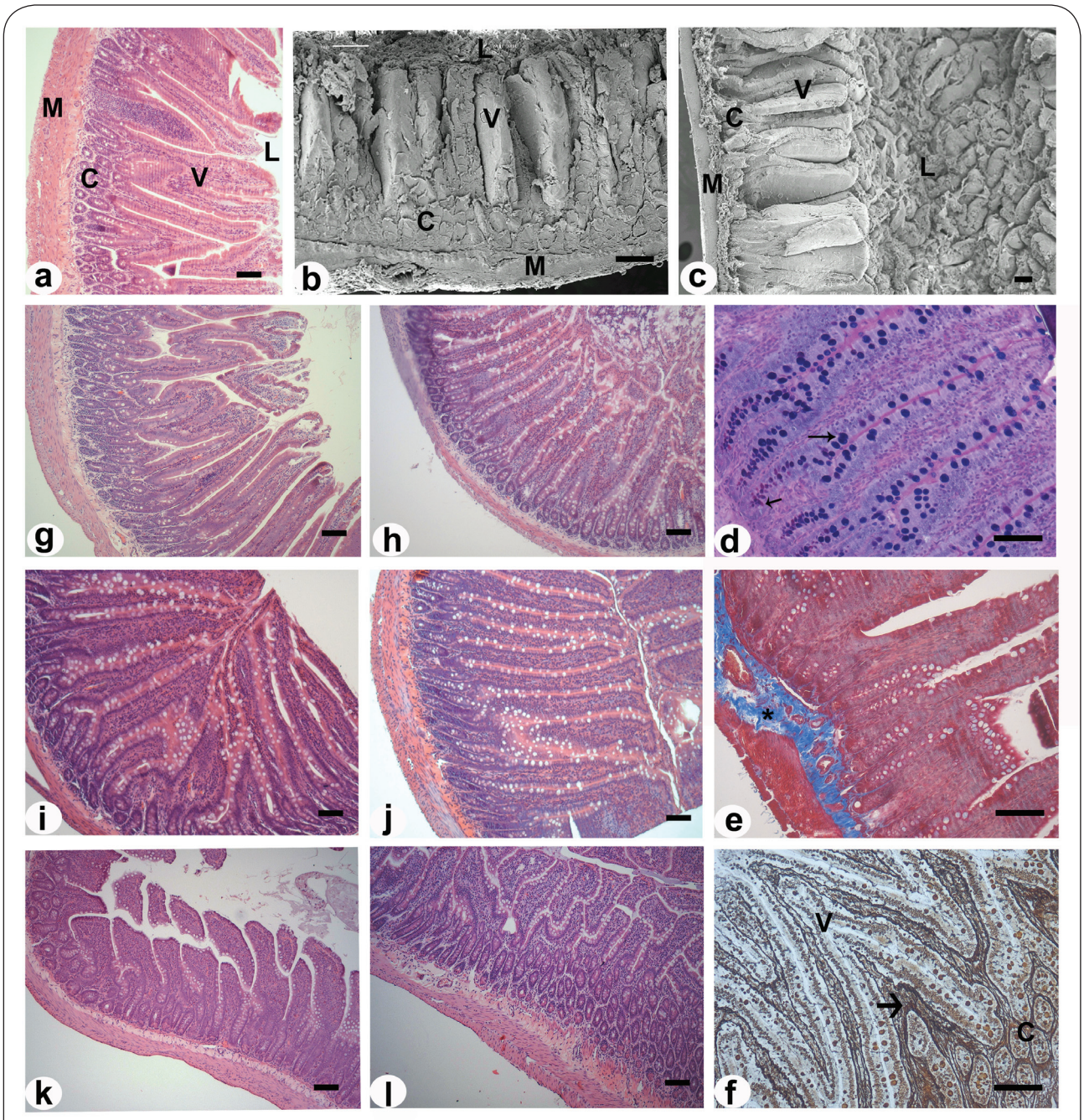

Figure 1. Light microscopy and scanning electron microscopy images of duodenum, jejunum and ileum mucosa. **a–f**: images of duodenum in control group (Group C). Image ***a*** shows the expected structure in light microscopy. V: villus; C: crypt; M: muscle; L: lumen. This image was chosen as a model to show the structure of the two regions, jejunum and ileum. Images ***b*** and ***c***: duodenum in control group obtained using scanning electron microscopy. V: villus; C: crypt; M: muscle; L: lumen. We chose this image as a model to show the structure found in the other two regions, jejunum and ileum. Image ***d***: using the histochemical technique of Alcian Blue pH 2.5 combined with PAS. In purple (large arrow), the goblet cells secreting neutral mucin and in pink (small arrow) the goblet cells secreting basic mucin. ***e***: Masson´s Trichrome technique. The connective tissue is stained in blue (*) and the normal structure of epithelium and muscle in red. ***f***: results after Reticulin technique. The arrow indicates reticulin fibers, seen throughout the connective tissue, providing support for the organ. ***g***: duodenum in group D5a; ***h***: duodenum in group D5b; ***i***: jejunum in group D5a; ***j***: jejunum in group D5b; ***k***: ileum in group D5a; ***l***: ileum in group D5b. Images ***a***, ***g–l***: hematoxilin-eosin staining; Bar: 100 µm.

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still active until its complete elimination, interfering with this parameter. For the goblet cells, we also found some indication of recovery in the D5b group after treatment and a drug-free period. We found no AB+goblet cells. The frequency of PAS+goblet cells was smaller in the D5a group in relation to both controls. Further, the frequency of AB+PAS+goblet cells was higher in the D5a group in relation to both controls. Since we found no difference in the D5b group in relation to D5a or control groups, we can assume that this represents a recuperation. The mucosa area considered in this analysis was higher in the D5b group in relation to C. The total number of goblet cells was smaller in the D5a group than in D5b and higher in relation to the C group.

Goblet cells occurred throughout the mucosa, more concentrated at the base of the crypt and villus and diminishing in the direction to the apex. Cells secreting acidic mucins (AB^+) were not found and secretory cells with basic mucins (PAS^+) were more frequent in the crypts, reducing in number as the villi were reached, where they occurred rarely or not at all. Regarding secreting Goblet cells with mucin composed of a mixture of basic and acidic mucin (AB^+PAS^+), these were infrequent in the crypts and increased to predominate in the villi. This pattern applied to all groups, indicating that the proposed treatment did not change the basic distribution of secretory cell types.

Reticulin histochemistry is based on silver impregnation of collagen fibers. The fibers were found throughout the connective tissue, forming a framework. This distribution was consistent for the different groups (Figure 1). In the samples stained with Masson's Trichrome technique, the connective tissue appeared in blue. All groups showed the same distribution pattern of connective and muscle tissue labeling.

Scanning electron microscopy performed for small samples of the different groups showed the expected morphology, already described in the literature. We found villi extending into the lumen and intact absorptive epithelium with microvilli on the surface. The crypt below the villi was observed and connective tissue was found just below this tissue. The muscle layer seals the wall of the intestinal segment. This pattern occurred in all groups.

Discussion

Many publications can be found concerning the role of isotretinoin in cancer and its effects on the nervous system of patients (Hardin *et al.*, 1999; Cisneros *et al.*, 2005; Ferguson *et al.*, 2005; Bremner & McCaffery, 2008), but its direct effects on the structure of the gastrointestinal tract is rarely discussed (Thomazini & Dolder, 2017). The exposure to isotretinoin did not cause any obvious signs of damage to the rat's health although there was a trend to reduced food and water intake. In a previous study (Cisneros *et al.*, 2005), the authors observed a reduction in food intake linked to consumption of isotretinoin. In our study, since the consumption of food and water was reduced in both groups receiving the drug and in the control groups, this reduction cannot be directly linked to the consumption of the drug, but it does follow a trend already suggested in the literature (Cisneros *et al.*, 2005; Bremner & McCaffery, 2008).

Intestinal mucosa should provide appropriate morphological and functional characteristics, since the absorption processes are dependent on epithelium integrity. Numerous infectious or noninfectious agents can damage the intestinal mucosa and compromise the digestive processes. Inflammatory bowel disease is attributed to the use of some substances, including isotretinoin (O'Reilly *et al.*, 2006; Passier *et al.*, 2006; Reddy *et al.*, 2006). Treatment with the substance could cause inflammatory diseases, but these symptoms disappeared at the end of the medication period (Passier *et al.*, 2006).

The gastrointestinal tract has primarily a mechanical function. The overall rating of each histological section of the small intestine regions stained with hematoxylin-eosin revealed the general morphology corresponding to that already described in the literature (Lu *et al.*, 2005; Barret, 2006; Shale *et al.*, 2009). The absorptive capacity of the gut is initially proportional to the number of villi present (Kierszenbaum & Tres, 2011). A desirable ratio describes crypts that are shallower when compared to the villi and this situation is found in all groups. The optimum ratio of villus height: crypt height varies, and the accepted ratios are 3:1 to 5:1, but ratios of 2:1, 1.82:1 and 1:1 have also been found and are accepted as normal (Pelicano *et al.,* 2003). In this study, the average found for the groups followed the trend of approximately 2:1, in agreement with descriptions proposed in the literature.

Crypts consist predominantly of increased goblet cells, Paneth cells in the base, enteroendocrine cells along the structure and some enterocytes. In the villi, the enterocytes with some goblet cells predominate. We found a normalization of goblet cell frequency after treatment interruption. This result is in accordance with the alteration found for D5a in the duodenum and jejunum.

Mucus is viscoelastic, gel-like and its primary function is to protect the mucosal cell surface from acids and peptidases. In addition, it serves as a lubricant for the passage of solids and as a barrier to antigens, bacteria and viruses (Shirazi *et al.*, 2000; Walker & Talley, 2011; Chawla *et al.*, 2013; Kim & Khan, 2013). The diet and the use of drugs can alter the distribution and frequency of goblet cells and it is related to the maintenance of bacterial translocation and microbiota (Frankel *et al.*, 1995; Deplancke & Gaskins, 2001; Azevedo *et al.,* 2007; Vieira-Lopes *et al.,* 2014). The modifications concerning goblet cells were concentrated in the duodenum and ileum and the results are interesting, considering the typical goblet cell type in both regions. In the duodenum the tendency of increase in PAS+ cell during the treatment and a decrease of the same cell after treatment interruption indicates a potential of bolus hydration, linked to this type of mucin. In the ileum, the opposite situation was observed, with a decrease of PAS+ cell during the treatment and an increase after this phase. In the ileum, we had an increase in PAS+AB+ goblet cells. This result indicates a potential of microbiota

maintained by PAS+AB+goblet cell secretion (Frankel *et al.*, 1995; Deplancke & Gaskins, 2001; Azevedo *et al.,* 2007; Vieira-Lopes *et al.,* 2014). The difference found in these proportions is related to the treatment, considering the reversal observed after its interruption, but the alteration is not sufficient to cause loss of the small intestine function. Considering the three segments, duodenum, jejunum and ileum, we observed that compared to the duodenum, the jejunum and ileum were the two regions most sensitive to treatment with isotretinoin, with the ileum being the most strongly affected. The activity potential seems not to be modified, although in the jejunum we observed an increased wall thickness during the treatment and its reduction after the interruption. In the ileum we observed a tendency of continuing increase of the wall thickness throughout the experimental period.

The evaluation of the reticulin fibers and connective tissue distribution showed the distribution following the expected structure, as described in the literature (Lu *et al.*, 2005; Barret, 2006), with fibers throughout the submucosa, and particularly in the connective tissue surrounding the villi and crypts.

Conclusions

We hypothesized that the jejunum and ileum are the most sensitive regions for this protocol with retinoid treatment, since they are the main absorption sites for these substances. Goblet cell type frequency was altered as an adaptive effect of this protocol. In general, the segments showed some adaptive condition for the treatment and no signs of damage to the small intestine were found with this protocol.

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