In a Methotrexate-Induced Model of Intestinal Mucositis, Olmesartan Reduced Inflammation and Induced Enteropathy Characterized by Severe Diarrhea, Weight Loss, and Reduced Sucrose Activity

Aurigena Antunes de Araújo,*a Pedro Brito Borba,b Fernando Henrique Destefani de Souza,c Anália Cristina Nogueira,c Tais Suassuna Saldanha,c Thayse Emanuele Franklin Araújo,c Aldemara Ingrid da Silva,d and Raimundo Fernandes de Araújo Júniord

aDepartment of Biophysics and Pharmacology, Federal University of Rio Grande Norte (UFRN), Post Graduation Program Public Health/Post Graduation Program in Pharmaceutical Science; Natal 59078–970, RN, Brazil; bDepartment of Biophysics and Pharmacology, UFRN; Natal 59078–970, RN, Brazil; cDepartment of Medicine, UFRN; Natal 59078–970, RN, Brazil; and dDepartment of Morphology, Post Graduation Program Health Science/Post Graduation Program in Functional and Structural Biology, UFRN; Natal 59078–970, RN, Brazil.

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The aim of this study was to evaluate the effect of olmesartan (OLME), an angiotensin II receptor antagonist, on an intestinal mucositis model. Briefly, daily intraperitoneal (i.p) injections of methotrexate (MTX) 7 mg/kg were administered to rats on 3 consecutive days. A subset of these rats was also pretreated with oral administration of OLME (0.5, 1.0, or 5.0 mg/kg) or vehicle as a control 30 min prior to MTX injection. Body weight, feces scoring, and death were recorded daily. On day 4, the rats were killed, and intestinal tissues were assayed for levels of interleukin (IL)-1β and tumor necrosis factor (TNF)-α, myeloperoxidase and sucrase activity, and histopathological findings. A significant reduction in body weight was observed in the MTX+1.0 mg/kg OLME group (p<0.01). The feces scores for the MTX+0.5 mg/kg OLME and MTX+5.0 mg/kg OLME groups were also significantly higher (p<0.001). Sucrose activity was reduced in all groups treated with OLME (p<0.05). Treatment with MTX+OLM at all doses resulted in reduced inflammatory infiltration, ulcerations, vasodilation, and hemorrhagic areas (p<0.05), as well as reduced concentrations of myeloperoxidase (p<0.001). The IL-1β and TNF-α levels were decreased in the MTX+OLM 5.0 mg/kg (p<0.01 and p<0.05, respectively) compared with the MTX-alone group. Overall, antiinflammatory activity was observed in rats with MTX-induced intestinal mucositis that were administered OLME. However, further studies are needed to elucidate the adverse effects of OLME.

Key words intestinal mucositis model; olmesartan; inflammation

It has been reported that 40% of cancer patients develop intestinal mucositis during a standard cancer chemotherapy regimen, while almost 100% of patients who receive high dose treatments are affected.1 Mucositis represents a dose-limiting toxicity of therapy and it currently affects approximately 500000 patients annually worldwide.2 The entire alimentary tract (mouth to anus) is affected, which leads to clinical symptoms that derive from ulcerations, and these include abdominal pain, nausea, vomiting, bloating, diarrhea, constipation, and subsequent weight loss.3 Furthermore, these complications can lead to longer hospitalisations and increasing health care costs.4

Methotrexate (MTX) is a structural analogue of folic acid and is widely used in the treatment of leukaemia and other malignancies. Tissues with high rates of proliferation are affected.5 MTX affects gut tissues, gastrointestinal mucositis develops. Correspondingly, approximately 60% of cancer patients that receive a chemotherapy treatment that includes MTX experience diarrhea.5

The currently accepted hypothesis for the development of alimentary mucositis (AM) suggests there are five intertwined phases: (1) initiation, (2) up-regulation and generation of messenger signals, (3) signal amplification, (4) ulceration, and (5) healing.6 Data from both animal and human studies support this hypothesis.7–9 Furthermore, these same phases can lead to damage of the gastrointestinal tract, including cell death, which leads to villous atrophy and crypt ablation in the small intestine. This can affect the activity of hydrolases, enzymes which participate in the metabolism of carbohydrates and are present at the border brush of enterocytes.10

There are a limited number of instruments available for the assessment of gastrointestinal mucositis. Moreover, these scales typically measure indirect outcomes of mucosal injury, including diarrhea.11 To reduce tissue injury and to enhance proliferative repair for chemotherapy-induced epithelial cell damage, several studies have aimed at identifying new agents and evaluating substances that have other indications. Antihypertensive angiotensin II inhibitors are a class of drugs with a low adverse event profile.12 In particular, olmesartan (OLME) is an angiotensin II type 1 receptor (AT1) blocker that inhibits levels of tumor necrosis factor (TNF)-α and interleukin (IL)-1β, thereby mediating anti-inflammatory activity.13 In the present study, the effects of OLME on a MTX-induced gastrointestinal mucositis model established in rats were examined.

MATERIALS AND METHODS

Chemicals MTX was purchased from Libbs Pharmaceuticals Ltd. (São Paulo, Brazil). Olmesartan medoxomil (Benicar, 20 mg) was obtained from Daiichi Sankyo Brazil Farmacêutica LTDA (São Paulo, Brazil). O-Dianisine was purchased from Sigma (São Paulo, Brazil).

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Animals  Albino, male Wistar rats (180–330 g; Biotério Department of Biophysical and Pharmacology) were maintained in a temperature- and humidity-controlled environment with a 12-h light/dark cycle (lights on at 6 a.m.). Food and water were available ad libitum. The National Institutes of Health Guidelines for the Care and Use of Laboratory Animals were followed. All efforts were made to minimise the number of animals used and their degree of suffering. The experimental methods used were approved by the Animal Ethics and Use Committee (CEUA) of the Federal University of Rio Grande Norte (UFRN) (Approval number: 016/2013).

Induction of Experimental Intestinal Mucositis  Forty two rats were randomly divided into eight groups. The MTX positive control group received normal saline orally for 3 d, and these were also followed by an i.p. injection of MTX (7 mg/kg) (n=6). The negative control group received normal saline orally for 3 d, followed by daily i.p. injections of saline (n=6). Three groups received oral OLME at concentrations of 0.5, 1, or 5 mg/kg (n=6 per group), respectively, by gastric gavage daily for 3 d. Thirty minutes after each administration of OLME, an i.p. injection of saline was performed. The last three groups received oral OLME at concentrations of 0.5, 1, and 5 mg/kg (n=4 per group), respectively, by gastric gavage daily for 3 d, and these were also followed by an i.p. injection of MTX (7 mg/kg) 30 min after the administration of OLME (OLME+MTX groups). The animals that died during the experiment in the groups were replaced for complete the sample size.

Animals were subsequently anesthetised on the fourth day, and blood samples were obtained for biochemical analyses. The small intestines of the rats were then resected, and intestine tissues were collected (as described below) and immersed in 10% buffered formalin for histopathological analysis. Intestine tissues were also frozen at −80°C for subsequent analyses of cytokines (IL-1β and TNF-α), myeloperoxidase (MPO) levels and sucrose activity.

Clinical Analysis  Animal weight, faeces scoring, and death were recorded daily. Faeces scoring was based on observations regarding the appearance of stools and perianal staining of the mouse coat. Consequently, all of the mice were allocated into individual cages. Diarrhoea was scored as normal (1), mild (slightly wet stool without staining of the coat) (2), moderate (wet and unformed stool with moderate perianal staining of the coat) (3), or severe (watery stool with severe staining of the coat around the anus) (4).16,17

Tissue Collection  From each sacrificed animal, three tissue samples, each 4 cm in length, were collected from the small intestine: 1) one comprising the duodenum in a sample region immediately after the gastric mucosa of the jejunum, 2) one being a sample completely surrounded by mesentery—priority tissue samples in the presence of inflammation with signs of erythema, and 3) a sample of ileum involving the ileoceleal junction. The entire tissue collection process was performed on ice. Approximately 4 cm of each sample was frozen at −70°C to later measure levels of MPO and cytokines (IL-1β and TNF-α) while the remaining samples were each homogenized in 1.5 mL phosphate buffered saline (PBS) using a portable homogenizer (Ultra80-i Ultra Stirrer, São Paulo, Brazil). Following centrifugation, 1 mL aliquots of each supernatant were stored at −70°C until invertebrate activity was assayed.

Sucrose Activity  To assay invertase activity, the 3,5-dinitrosalicylic acid (DNS) method was used.18 To generate a standard curve, a stock solution of 1 g/L glucose was prepared, and 1 mL, 0.8 mL, 0.6 mL, 0.4 mL, and 0.2 mL aliquots of this solution were added to five tubes, followed by the addition of 0 mL, 0.2 mL, 0.4 mL, 0.6 mL, and 0.8 mL distilled water to achieve the following concentrations of glucose: 1 g/L, 0.8 g/L, 0.6 g/L, 0.4 g/L, and 0.2 g/L. In addition, a sixth tube contained 1 mL distilled water. DNS (3,5-dinitro-2-hydroxybenzoic acid, Sigma-Aldrich, São Paulo, Brazil) reagent (0.5 mL) was added to each test tube. The tubes were heated in a 100°C water bath for 5 min, and then were transferred to a cold water bath. Distilled water (8.5 mL) was added to each tube (total volume, 10 mL), and the percent transmittance values at 540 nm were recorded.

Myeloperoxidase (MPO) Assay  The extent of neutrophil accumulation in the intestinal samples was measured by assaying MPO activity. Briefly, intestinal mucositis five samples (per group per segment of duodenum, jejunum, and ileum) were harvested as described above and were stored at −70°C. Upon thawing of these samples on ice, the samples were homogenized and centrifuged (2000×g for 20 min). MPO activity was determined by a colorimetric method described previously.19 The results are reported as units of MPO per nmol per g of tissue.

Cytokines Assay (IL-1β and TNF-α)  The intestinal tissue samples (duodenum, one sample per group; jejunum, three samples per group; ileum, one sample per group) were stored at −70°C until use. The tissue was homogenised and processed as described by Safieh-Garabedian et al.16 Levels of IL-1β (detection range: 62.5–4000 pg/mL; sensitivity or lower limit of detection (LLD): 12.5 pg/mL of recombinant mouse IL-1β), and TNF-α (detection range: 62.5–4000 pg/mL; sensitivity or LLD: 50 ng/mL of recombinant mouse TNF-α) in the intestinal samples were determined with a commercial ELISA kit (R&D Systems, Minneapolis, MN, U.S.A.), as described previously.17 All samples were within the wavelength used in UV-VIS spectrophotometry (absorbance measured at 490 nm).

Histopathology  The small intestines (duodenum, jejunum and ileum) were excised quickly and washed with cold isotonic saline. Each segment was weighed and cut longitudinally. Three sections of small intestine (five animals per group) were analysed. The specimens were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. Sections of 5 μm thickness were obtained for haematoxylin–eosin staining (H&E) and examined by light microscopy (40×, Olympus BX50, Morphology Department/UFRN). The parameters of inflammatory cell infiltration, vasodilatation, and the presence of haemorrhagic areas, oedema, ulcerations and abscesses were determined in a single-blind fashion and graded as follows: Score 1—normal epithelium and connective tissue without vasodilatation; absence of or discreet cellular infiltration; and absence of haemorrhagic areas, ulcerations or abscesses; Score 2—discreet vasodilatation and areas of re-epithelialisation; discreet inflammatory infiltration with mononuclear prevalence; and absence of haemorrhagic areas, oedema, ulcerations or abscesses; Score 3—moderate vasodilatation, areas of hydropic epithelial degeneration; inflammatory infiltration with neutrophil prevalence; and presence of haemorrhagic areas, oedema and eventual ulcerations, and absence of abscesses; and Score 4—severe vasodilatation; inflammatory infiltration with neutrophil prevalence; and presence of haemorrhagic areas, oedema, ulcerations, and abscesses.18
Statistical Analysis Data analysis was performed using GraphPad Prism software (version 5.01, GraphPad Software Inc., San Diego, CA, U.S.A.). When appropriate, one-way ANOVA was performed followed by Bonferroni’s post hoc test. For the parameters that were monitored daily, a repeated-measures ANOVA was performed. A p-value less than 0.05 was considered significant.

RESULTS

Weight Loss and Death On the first day of the experiment, the mean weight of each group was similar. However, a significant decrease in the mean weight of the animals treated with OLME (1 mg/kg) was observed on the second, third, and fourth days of the treatment period (p<0.01), Fig. 1. In addition, one and two of the rats of the MTX group (MTX + MTX-OLME 5) and the OLME (1 mg/kg) group died, respectively.

Faeces Scoring The negative control group received a normal faeces aspect score of 1.3 (1.3–1.3). The fecal score for the MTX group was 2 (1.3–1.7), with moderate diarrhoea observed. In the group where there was no association between MTX and OLME, the highest faeces score was recorded, 2.67 (2.33–4). In the OLME (5 mg/kg) group, the diarrhoea was observed to worsen significantly during the day (2 rats; p<0.01). For the group treated with OLME, half of the animals exhibited mild diarrhoea by the fourth day, and the rats that received a dose of 0.5 mg/kg OLME exhibited moderate diarrhoea, Fig. 2.

Sucrose Activity A statistically significant reduction in sucrase activity was detected in the tissues obtained from the animals treated with OLME (1 mg/kg) and in the tissues from the animals treated with MTX and OLME at all dose levels (p<0.05). However, a statistical analysis showed that the sucrase levels for the MTX group did not statistically differ from the sucrase levels of the negative control group, Fig. 3.

MPO Assays MPO levels were assayed to confirm the effects of OLME on the inflammatory process. Significantly lower levels of MPO were detected in the duodenum and jejunum samples collected from the rats treated with OLME alone, and from the rats treated with MTX+OLME (p<0.001), Fig. 4.

Effect of Treatment on Levels of IL-1β and TNF-α Inflammatory Activity MTX elevated levels of IL-1β (p<0.05) and TNF-α (p<0.05) than the Negative control. The IL-1β and TNF-α levels were decreased in the MTX-OLME 5 mg/kg (p<0.01 and p<0.05). Levels of IL-1β were significantly decreased in the MTX-OLME 0.5 mg/kg and MTX-OLME 1 mg/kg (p<0.05) groups, compared to the MTX group (Fig. 5).

Histopathological Analysis MTX-treated animals histologically exhibited significant loss of crypt architecture and signs of crypt remodelling, severe villous epithelial atrophy, degeneration and shortening of the villus, and polymorphonuclear leukocyte infiltration in the lamina propria. Histological damage was initially assessed in the duodenum, jejunum and ileum with a semi-quantitative score. Intestinal damage was reduced in animals treated with MTX-OLME 5 mg/kg in the duodenum, ileum (p<0.001, Figs. 6m, o, 7) and jejunum (p<0.0001, Figs. 6n, 7) compared to animals that received mg/kg MTX-OLME 0.5 or 1 mg/kg (p>0.05, Figs. 6g, h, i, j, k, l, 7). These results were histopathologically obvious in the MTX-OLME 5 mg/kg group, which exhibited reduced tissue damage, preserved areas of the villus, and reductions of cel-
In this study, the rats that were treated with MTX experienced severe gastrointestinal toxicity, including the induction of intestinal mucositis. The latter is defined by the presence of ulcerative and inflammatory lesions in the mucosa of the gastrointestinal tract.19) Grade 3 intestinal mucositis was observed throughout the small intestine, with involvement of the duodenum, jejunum, and ileum. Previously, MTX-induced mucositis has been characterized by inflammation and increased expression and levels of MPO. 20) In the present study, high levels of MPO activity were also detected throughout the small intestines of the MTX-treated rats. Gastrointestinal toxicity due to MTX is largely due to the high rate of proliferation that enterocytes undergo, and the subsequent cell death that is induced by this pro-oxidant compound that depletes dihydrofolate pools. As a result, villous atrophy and crypt hyperplasia occurs in the small intestine, and this disrupts hydrolase activity for the intestine. The clinical manifestation of mucositis includes the presence of mucosal ulcerations,21) as well as diarrhoea and subsequent weight loss. All of the animals in the MTX group experienced diarrhoea, with two rats experiencing mild diarrhoea, mild diarrhoea with three animals, and another two rats experiencing severe diarrhoea.

Weight loss was also observed for rats in the MTX group on days 2, 3, and 4. However, the difference in the mean weights for the MTX group between the first day and the last day of the experiment were not significant.

**DISCUSSION**

In this study, the rats that were treated with MTX experienced severe gastrointestinal toxicity, including the induction of intestinal mucositis. The latter is defined by the presence of ulcerative and inflammatory lesions in the mucosa of the gastrointestinal tract.19) Grade 3 intestinal mucositis was observed throughout the small intestine, with involvement of the duodenum, jejunum, and ileum. Previously, MTX-induced mucositis has been characterized by inflammation and increased expression and levels of MPO. 20) In the present study, high levels of MPO activity were also detected throughout the small intestines of the MTX-treated rats. Gastrointestinal toxicity due to MTX is largely due to the high rate of proliferation that enterocytes undergo, and the subsequent cell death that is induced by this pro-oxidant compound that depletes dihydrofolate pools. As a result, villous atrophy and crypt hyperplasia occurs in the small intestine, and this disrupts hydrolase activity for the intestine. The clinical manifestation of mucositis includes the presence of mucosal ulcerations,21) as well as diarrhoea and subsequent weight loss. All of the animals in the MTX group experienced diarrhoea, with two rats experiencing mild diarrhoea, mild diarrhoea with three animals, and another two rats experiencing severe diarrhoea.

**Fig. 4.** Levels of MPO Activity Detected in the Treated and Control Rats; MPO Activity Detected in the Negative Control, MTX, MTX+OLME (0.5, 1 and 5mg/kg) and OLME (0.5, 1 and 5mg/kg). Significantly lower levels of MPO activity were detected for the MTX+OLME 0.5, MTX+OLME 1, and MTX+OLME 5 groups (**p<0.001). In addition, significantly lower levels of MPO activity were detected for all of the rats treated with OLME (OLME 0.5 and OLME 5, ***p<0.001; OLME 1, *p<0.05). Software GraphPad Prism 5.

**Fig. 5.** MTX Elevated Levels of IL-1β (p<0.05) and TNF-α (p<0.05) than the Negative Control. The IL-1β and TNF-α levels were decreased in the MTX-OLME 5mg/kg (p<0.01 and p<0.05). Levels of IL-1β were significantly decreased in the MTX-OLME 0.5mg/kg and MTX-OLME 1mg/kg (p<0.05) groups, compared to the MTX group. Software GraphPad Prism 5.

OLME is an angiotensin II receptor antagonist and represents a class of antihypertensive agents with a low adverse event profile. Research over the past several years has characterized the anti-inflammatory properties of OLME, which includes inhibition of TNF-α, MPO, MDA, and IL-1β.22) The MTX+OLME 0.5, MTX+OLME 1, and MTX+OLME 5 groups exhibited lower levels of MPO enzyme activity, and these levels were significantly lower than those of the MTX group after taking into consideration all of the segments of the small intestine that were assayed (p<0.001). The IL-1β and TNF-α levels were decreased in the MTX-OLME 5mg/kg (p<0.01 and p<0.05). Levels of IL-1β were significantly decreased in the MTX-OLME 0.5mg/kg, MTX-OLME 1mg/kg and MTX-OLME 5mg/kg (p<0.05) groups, compared to the MTX group. Chemotherapeutic drugs cause upregulation of stress response genes, that upregulate the production of proinflammatory cytokines such as interleukin-1β (IL-1β), Interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α).22) Olmesartan can decrease these proinflammatory cytokines, which are responsible for initiating inflammation in response to tissue injury. When the HE scores were analysed, a generally lower HE score was observed for...
Fig. 6. Histopathological Analysis of Duodenum, Jejunum, and Ilium Small Intestine Tissue Sections Obtained from the Treated and Control Rats

Images from negative control, MTX, MTX-OLME 0.5, MTX-OLME 1.0 and MTX-OLME 5.0 groups are shown in panels a–c, d–f, g–i, j–l and m–o. Note that the MTX (d–f) alone rats’ exhibited intestinal mucositis with significant loss of crypt architecture and signs of crypt remodelling, severe villous epithelial atrophy, degeneration and shortening of the villus, and polymorphonuclear leukocyte infiltration in the lamina propria. Intestinal damage was reduced in animals treated with MTX-OLME 5 mg/kg (Figs. 6j–l) compared with animals that received MTX-OLME 0.5 mg/kg or MTX-OLME 1 mg/kg (Figs. 6g–l, respectively). These results were histopathologically obvious in the MTX-OLM 5 mg/kg group, which exhibited reduced tissue damage, preserved areas of the villus, and reductions of cellular infiltration and areas of haemorrhage or ulceration. Magnification 20×, scale bar=100 μm.
the animals treated with MTX and OLME, especially for the MTX+OLME 5 group. In the ileum sections, a score of grade 1 was assigned, thereby indicating that constipation capillaries were present in the villi.

All of the rats in the MTX+OLME 0.5, MTX+OLME 1, and MTX+OLME 5 groups experienced progressive diarrhoea, with increased faeces flow observed throughout the experiment. Correspondingly, increasing weight loss was observed. However, these symptoms could not be justified by MTX-induced intestinal mucositis or by other inflammatory processes. Furthermore, animals in the OLME 0.5, OLME 1, and OLME 5 groups also had diarrhoea, though of lesser intensity in number and degree. Of the six animals with diarrhoea, the condition observed was recorded as mild to moderate. When weight loss was analysed, taking into account the arithmetic mean of the animals in each group on the first day and the fourth day of the experiment, weight loss was observed in MTX+OLME 1 group (p<0.01). Only the levels of sucrose activity were found to decrease. In particular, a significant decrease in sucrose activity was observed in the tissues obtained from the animals treated with OLME (1 mg/kg) and in the tissues from the animals treated with MTX and OLME at all dose levels (p<0.05), which may explain the diarrhoea and weight loss that was associated with these animals, and was classified as malabsorptive diarrhoea. Pink showed patients with a disaccharide deficiency have very few symptoms whilst others are quite severely troubled with diarrhoea. In this patient we tried to elucidate the cause of the diarrhoea, which might be due to osmotic effects of sucrose and low

Fig. 7. Histopathological Scoring of Duodenum, Jejunum, and Ilium Small Intestine Tissue Sections

The duodenum tissues treated with OLME alone received significantly lower histological scores than the duodenum tissues that were treated with MTX+OLME (**p<0.001), especially compared with the MTX+OLME 1 and MTX+OLME 5 tissues (*p<0.05). The jejunum samples from the rats treated with 5 mg/kg OLME did not exhibit a significant reduction in histopathological score compared with the MTX+OLME 5 sections (**p<0.001). Similarly, the ileum samples from the rats treated with OLME alone did not receive significantly lower histopathological scores compared with the ileum samples from the MTX+OLME 1 and MTX+OLME 5 rats (**p<0.001). Software GraphPad Prism 5.

In the ROADMAP study, treatment with 40 mg of OLME produced intestinal adverse effects in 2232 patients. When OLME treatment was suspended for patients with idiopathic enteropathy that mimics celiac disease (with diarrhoea and weight loss being the main symptoms), clinical remission was achieved in all of these patients. Consequently, in March 2013, the Federal Drug Administration (FDA) approved a change in the labeling of OLME medoxomil to include enteropathy celiac disease-like adverse event as the use of OLME. However, it remains unclear whether the mechanism of action of OLME is responsible for this adverse event.

Overall, anti-inflammatory activity was observed in rats with MTX-induced intestinal mucositis that were treated with OLME. However, further studies are needed to elucidate the adverse effects of OLME, which may mimic celiac disease and induce a worsening of diarrhoea symptoms and weight loss in animals with intestinal mucositis induced by MTX.

In our study, enteropath characterized by diarrhoea and weight loss of the animals in groups MTX+OLME 0.5, MTX+OLME 1, MTX+OLME 5, OLME 0.5, OLME 1, and OLME 5 can not be justified by histopathologic changes intestinal mucositis induced by methotrexate, or for inflammatory events. This then is a malabsorptive diarrheal syndrome that mimics celiac disease known as Celiac Disease-induced olmesartan like. One possible explanation as to why this adverse event is the decrease in the sucrase found in groups OLME 0.5, OLME 1, and OLME 5. Another fact that confirms this claim activity does sugar absorption in patients diagnosed with Celiac Disease is impaired.

Probably, this mechanism explains the increased severity of diarrhoea in animals treated with methotrexate and olmesartan than those treated with olmesartan. The methotrexate also disrupts the activity of sucrase, and added to the fact of olmesartan cause reductions in the activity of this enzyme has a higher concentration of simple carbohydrates not digested in the intestinal lumen and consequent increase in osmolarity, triggering desabsorviva diarrhea with higher dimensionality. Then observed a synergistic action to methotrexate in reducing the activity of hydrolases. In groups where+MTX and MTX+OLME 1, OLME 5 there were two one deaths, respectively. Other animals showed severe diarrhoea. Probably the main cause of death was hypovolemic shock and the secondary cause metabolic acidosis, both resulting from severe diarrhoea.

In conclusion, olmesartan has anti-inflammatory activity in methotrexate-induced intestinal mucositis. However we recommend further studies to elucidate the effects and adverse effects of olmesartan enteropathy that mimics celiac disease, worsen symptoms of diarrhoea and weight loss of animals with
intestinal mucositis induced by methotrexate.

Conflict of Interest  The authors declare no conflict of interest.

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