ORIGINAL ARTICLE

Gastric toxicity of Alendronate and Methotrexate in Walker 256 carcinosarcoma jaw model

Toxicidade gástrica de Alendronato e Metotrexato em modelo de carcinossarcoma de Walker 256 em mandíbula

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Abstract

Introduction: Experimental animal models represent a key tool used to elucidate the mechanisms of action and toxicity of anticancer drugs. **Objective**: The purpose was to establish a correlation of neoplastic growth with the combinatorial therapeutic application of sodium alendronate (ALD) and methotrexate (MTX), and to evaluate the gastrointestinal toxicity of these drugs, in the rat Walker 256 carcinosarcoma inoculation model. **Methods**: Female rats were selected and randomly distributed into 5 groups (n=10): negative control (NC), positive control (PC), MTX-treated group, ALD-treated group, and MTX-ALD-treated group (MTX/ALD). Tumor cells were inoculated as a suspension of 1x106cells/mL into the alveolar cavities produced by exodontia procedures. The following parameters were evaluated: body weight, tumor volume and percentage of tumor inhibition, and gastrointestinal toxicity. **Results:** The body weight variation was statistically significant between NC animals and PC animals, and between NC animals and ALD-treated group ($p<0.01$). Tumor volume variation was statistically significant between PC animals, MTX-treated group and MTX/ALD-co-treated group ($p<0.05$). Analysis of gastric toxicity of MTX-treated group reveled slight reduction of chief (Ch) and parietal (Pr) cellular populations; ALD-treated group exhibited gastric mucosa without histological alterations of Ch cells but intense reduction of Pr cellular population; and MTX/ALD-co-treated group presented reduction of Ch and Pr cellular populations. **Conclusions**: ALD does not elicit significant antitumor effects on Walker 256 carcinosarcoma cells and decreases antitumor effects of MTX due to toxicity on the gastric epithelium, which is intensified with MTX association.

Key words: Gastric Mucosa. Carcinoma 256, Walker. Alendronate. Methotrexate.

Resumo

Introdução: Modelos experimentais em animais representam um instrumento fundamental para elucidar os mecanismos de ação e toxicidade de drogas anticâncer. **Objetivo**: estabelecer uma correlação do crescimento neoplásico com a aplicação terapêutica combinatória de alendronato de sódio (ALD) e metotrexato (MTX), e avaliar a toxicidade gastrointestinal dessas drogas, no modelo de inoculação de carcinossarcoma de Walker 256 em ratos. **Métodos:** Ratas fêmeas foram selecionadas e distribuídas aleatoriamente em 5 grupos (n = 10): controle negativo (NC), controle positivo (PC), grupo tratado com MTX, grupo tratado com ALD e grupo tratado com MTX-ALD (MTX/ALD). As células tumorais foram inoculadas como uma suspensão de 1x106 células/mL nas cavidades alveolares produzidas por procedimentos de exodontia. Os seguintes parâmetros foram avaliados: peso corporal, volume tumoral e porcentagem de inibição tumoral e toxicidade gastrointestinal. **Resultados**: A variação do peso corporal foi estatisticamente significante entre animais NC e animais PC, e entre animais NC e grupo tratado com ALD ($p <0,01$). A variação do volume tumoral foi estatisticamente significativa entre animais PC, grupo tratado com MTX e grupo tratado com MTX / ALD ($p <0,05$). A análise da toxicidade gástrica do grupo tratado com MTX revelou uma ligeira redução das populações celulares principais (Ch) e parietais (Pr); o grupo tratado com ALD exibiu mucosa gástrica sem alterações histológicas de células Ch mas intensa redução da população celular Pr; e o grupo tratado com MTX / ALD apresentou redução das populações celulares Ch e Pr. **Conclusões:** O ALD não provoca efeitos antitumorais significativos nas células do carcinossarcoma Walker 256 e diminui os efeitos antitumorais do MTX devido à toxicidade no epitélio gástrico, que é intensificada com a associação MTX.

Palavras-chave : Mucosa Gástrica. Carcinoma 256 de Walker. Alendronato. Metotrexato.

INTRODUCTION

In 2008, approximately 7.6 million deaths worldwide were attributed to cancer, and cancer-related deaths are projected to continue increasing up to 70% over the next 20 years, with an estimated 13.1 million deaths in 2030[1]. Due to the fast progression of cancer, research into potential anticancer drugs with reduced side effects has intensified.

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Conflict of interest: There are no conflicts of interest in this research.
Received 16 Set 2019; Revised: 5 Mar 2019; Accepted: 27 Mar 2019

Among the variety of antimetastatic drugs available, bisphosphonates have received considerable attention. Bisphosphonates are analogues to endogenous pyrophosphates and induce apoptosis in osteoclasts, inhibiting the initial osseous resorption promoted by neoplastic cells, thereby preventing their adhesion to the demineralized organic matrix, and significantly diminishing the incidence of osseous metastases.[2,3] Aminobisphosphonates, a subclass of bisphosphonates, were generated by the addition of nitrogen atoms to the original bisphosphonate structure in effort to increase efficacy. However, despite being approved for human use a little more than a decade ago, even the most potent aminobisphosphonates, such as sodium alendronate (ALD, 4-amino-1-hydroxybutylidene), exhibit inadequate direct antitumor activity. Therefore, it is necessary and urgent to identify chemotherapeutic agents with enhanced specificity for malignant cells[4].

Numerous drugs fit the description of tumor-specific chemotherapeutic agents. The folate inhibitor methotrexate (MTX, L-Glutamic acid,N-[4[[-(2,4-diamino-6-pteridinyl) methyl]-methylamino]benzoyl]) is still the drug of choice for the treatment of several malignancies, such as the acute lymphocytic leukemia, osteosarcoma, and breast cancer. Its action mechanism involves the inhibition of dihydrofolate reductase activity, which is necessary for the biosynthesis of the DNA base thymine. It consequently suppresses DNA replication and induces apoptosis in highly mitotic cells, such as neoplastic cells.[5] However, because it does not exhibit selectivity, MTX can non-specifically affect non-pathogenic groups of highly proliferative, resulting in clinically mild to moderate deleterious effects in the digestive tract.[6]

When applied as a single-agent therapy, MTX is well-tolerated in the majority of patients. However, its combinatorial application with ALD presents a therapeutic challenge because adding ALD results in significant side effects in the digestive tract.[7] These effects are largely attributable to the fact that it can only be orally administered.[4] The combined administration of ALD bisphosphonate and MTX has been shown to produce serious gastrointestinal complications. These side effects, combined with the onset of cancer-associated cachexia, can diminish the quality of life and life expectancy of the patients treated with these drugs.[8]

Experimental animal models represent a key tool used to elucidate the mechanisms of action and toxicity of anticancer drugs. Among these models is the rat Walker 256 carcinosarcoma inoculation model, which uses a mammary gland tumor that exhibits aggressive behavior and high invasive capacity.[9] As a breast cancer, Walker 256 carcinosarcoma is susceptible to MTX and, when inoculated into rat mandibles, represents an ideal model system for the analysis of antimetastatic and antitumor activities of ALD and MTX. Consequently, it is also an ideal model to analyze the non-specific and adverse effects of these drugs.[5,9]

The purpose of this study was to establish a correlation of neoplastic growth with the combinatorial therapeutic application of ALD and MTX, as well as to evaluate the gastrointestinal toxicity of these drugs, in the rat Walker 256 carcinosarcoma inoculation model.

METHODS

Ethical Aspects

This study was conducted in accordance with the ethical principles and regulations for animal experimentation recommended by the Brazilian College of Animal Experimentation (COBAE), an institution affiliated to the International Council for Laboratory Animal Science, and the Brazilian Legislation on Experimental Animals (Federal law nº. 6.638 -1979).

This study was submitted to and approved by the Ethics Committee of Animal Research of Federal University of Ceará (UFC).

Sample and animals identification

A total of 50 adult female Wistar rats (Rattus novergicus albinus), weighing between 140 and 220g, were studied. The rats were acquired from the Central Animal Colony of UFC and were randomly distributed into 5 groups of 10 animals each.

The animals were housed in the Sectorial Biotery of the Department of Pharmacology and Physiology of UFC in plastic cages with pine-sawdust covered floors that were maintained at a temperature of 24ºC and a dark-light cycle of 12 hours with water and commercial food (Bio-base) provided ad libitum.

Tumor inoculation

This study performed tumor implantation according to the protocol described previously.9 Briefly, the animals (n = 50) were anesthetized by intra-peritoneal (i.p.) injection of 10% chloral hydrate for the inoculation of tumor cells. These cells were inoculated as a suspension of 1×106cells/100µL into the alveolar cavities produced by exodontia procedures that were performed on the first and second inferior right molars without suturing. Next, the animals were returned to their cages and remained under observation until the dissipation of the effects of the anesthesia and the complete recovery of reflexes.

Groups and treatment protocol

After the tumor inoculation procedure, the animals were randomly distributed into 5 groups, as described in Table 1.

Body weight analysis

The body weight of the animals was measured every 2 days, starting on the day of the surgical procedure and tumor inoculation (Day 0). The animals were followed up until spontaneous death or until the established day of sacrifice (Day 35), when the survival rate was assessed. Pilot studies revealed

that the survival rate of the animals treated with MTX was approximately 22.75 days. The administration of four doses of each therapeutic regimen tested was standardized, as well as the date of sacrifice, which was defined as 7 days after the last dose of treatment, which corresponded to Day 35.

Assessment of antitumor activity

The tumor volume and the percentage of tumor inhibition were determined using the following formulas: Tumor volume (mm^3) = $(D \times d^2) \times 0.5$, where "D" represents the largest diameter (mm) and "d" represents the smallest diameter. Tumor inhibition (%) = $(1 - T \div C) \times 100\%$, where "T" and "C" represent the mean tumor volumes of the treated and control groups, respectively.10

Analysis of gastric toxicity

Three stomach sections of each animal of the experimental groups were harvested from the cardia and fundus. The fragments were identified and fixed by immersion in 10% formaldehyde for 24 hours. After fixation, the specimens were evaluated macroscopically for their morphology, presence of stomach content and the color of the stomach wall. Subsequently, the specimens were dehydrated in a series of increasing grades of alcohol, diaphanized in xylol, dipped in paraffin and melted at 60ºC. Next, the fragments were embedded into paraffin-forming blocks at room temperature. The fragments were cut into 5 µm thick sections using a semiautomatic microtome, followed by routine histological staining using hematoxylin-eosin. Using optical microscopy, we assessed the sections for histological indications of tissue damage and inflammatory reactions that were previously described to indicate any toxicity of the treatments.[11]

Statistical analysis

Body weight comparison and the analysis of antitumor activity among the five groups (Sham, control, MTX, ALD, and MTX/ALD) were performed using ANOVA, followed by Tukey's post-test at a 1% significance level to comparison of body weight and 5% significance level to analysis of antitumor activity (GraphPad Prism 5.0®).

For comparison of survival curves, the Kaplan-Meier test was used, in addition to the chi-squared test and a value of $p<0.01$ was considered statistically significant (GraphPad Prism 5.0®).

Table 1. Group distribution according to procedure, treatment, routes and days of drug administration

Group	Procedure	Treatment/routes	Days
Sham	Exodontia procedure without inoculation of tumor cells	Saline solution 0,9%; 4 doses; (p.o.)[a]	7th, 14th, 21st, 28th
Control	Exodontia procedure and inoculation of tumor cells	Saline solution 0,9%; 4 doses; (p.o.)	7th, 14th, 21st, 28th
MTX[b]	Exodontia procedure and inoculation of tumor cells	MTX 25 mg/kg; 4 doses; (i.p.)	7th, 14th, 21st, 28th
ALD[c]	Exodontia procedure and inoculation of tumor cells	ALD 3 mg/kg; 4 doses; (p.o.)	7th, 14th, 21st, 28th
MTX/ ALD[d]	Exodontia procedure and inoculation of tumor cells	MTX 25 mg/kg (i.p.) + ALD 3 mg/kg (p.o.); 4 doses	MTX: 7th, 14th, 21st, 28th ALD: 5th, 12th, 19th, 26th

aTexon®; bMTX, Methotrexate (L-Glutamic acid,N-[4[[-(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl])), Novartis®; cALD, Sodium alendronate (4-amino-1-hydroxybutylidene), Pharmacia Ind Com Ltda®; dThe treatments with methotrexate and sodium alendronate were administered on different days due to their combined toxicities and to the lack of knowledge regarding the actual side effects of these drugs when combined with bisphosphonates. p.o., orally; i.p. intra-peritoneal.

RESULTS

Due to the mortality induced by the Walker 256 carcinosarcoma, the animals of the positive control group and the ALD group did not receive treatment according to the therapeutic regimen proposed in Table 1. The control group survived only until the first two doses of saline and ALD-treated group survived only a single dose of ALD.

Analysis of body weight variation

The animals of the sham group exhibited increase in weight during the 12 days of treatment. The animals of the control group lost weight during the first 2 days, but gained weight between Day 4 and Day 6, and exhibited high weight loss at 8, 10 and 12 days, when compared to the Sham group, this loss of weight was statistically significant (Table 2).

Significantly, the animals from MTX (day 8) and MTX/ALD (day 8 and day 10) groups showed a lower weight loss compared to the control group. However, the animals of the ALD-treated group displayed similar behavior as the control group (Table 2).

Table 2. Variation of Body Weights of Animals.

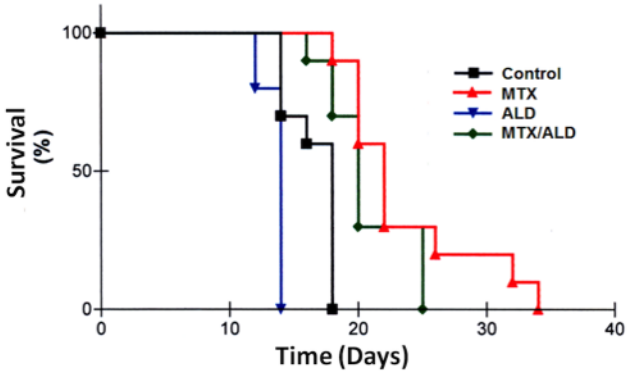
	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Sham	0 ± 0.0	-2.8 ± 0.7	6.1 ± 1.0	1.5 ± 0.6	2.0 ± 1.8	2.2 ± 1.56	1.9 ± 0.43
Control	0 ± 0.0	-5.9 ± 1.8	0.4 ± 1.6	6.7 ± 1.4	-10.6 ± 2.1[a]	-19.6 ± 2.0[a]	-12.8 ± 1.8[a]
MTX	0 ± 0.0	-1.8 ± 2.4	1.7 ± 1.3	4.2 ± 2.0	3.0 ± 1.5[b]	-3.5 ± 2.8[b]	-5.9 ± 2.7
ALD	0 ± 0.0	-4.3 ± 1.4	1.3 ± 1.0	3.0 ± 1.1	-11.0 ± 2.6	-12.0 ± 1.6	-11.6 ± 1.5
MTX/ALD.	0 ± 0.0	-8.9 ± 1.3	2.6 ± 1.8	0.3 ± 3.4	-6.2 ± 1.8	-2.8 ± 1.2[b]	-8.0 ± 2.9

a Body weights with statistically significant compared to Sham group on the same day ($p<0.01$). b Body weights with statistically significant compared to control group on same day ($p<0.01$). The variation in weight of animals was presented as the mean values ± SE. ANOVA/Tukey. MTX, methotrexate; ALD, sodium alendronate.

Survival analysis and Determination of antitumor activity

In terms of overall survival, we observed a significant difference between control group, which exhibited a mean survival time of 16.6 days, and the MTX-treated group, which exhibited a mean survival time of 23.6 days ($p=0.0001$) (Fig. 1).

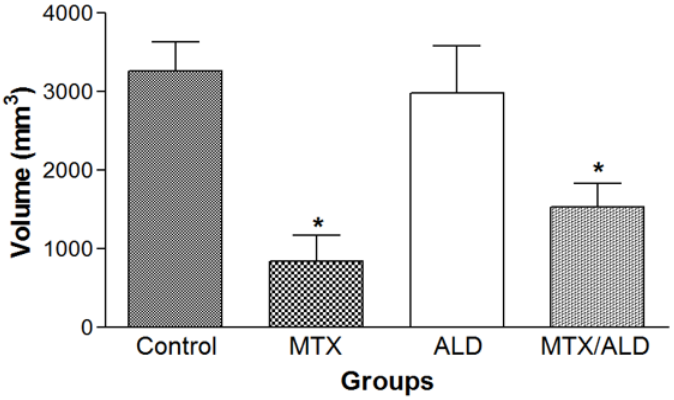
Figure 1 Survival curve of control and MTX, ALD and MTX/ALD treated groups.



The ALD-treated group exhibited a mean survival time of 13.6 days, which was significantly less than the control group ($p=0.0011$). Conversely, the experimental MTX/ALD-co-treated group exhibited a mean survival time of 20.7 days, which was significantly more than the control group ($p=0.0019$) (Fig. 1).

For the assessment of the antitumor activity, the volume of the tumors and the percentage of tumor inhibition were measured. The MTX- treated and MTX/ALD-co-treated animals exhibited reduced tumor volumes compared to the control animals (Fig. 2).

Figure 2. Variation of tumor volume after treatment with saline solution (control), MTX, ALD, and MTX/ALD (*$p<0.05$ relative to positive control).



The MTX-treated and MTX/ALD-co-treated animals exhibited 69.4% and 49.7% tumor inhibition, respectively. Compared to the control animals, the ALD-treated animals did not exhibit tumor growth inhibition.

Analysis of gastric toxicity

All of the Sham animals presented normal stomach morphologies, the presence of stomach content and gastric walls with whitish color and preserved pits. Histological analysis revealed that the gastric mucosae were lined by simple cylindrical epithelium exhibiting foveolae. Deeper within the mucosal layers were parietal cells and a predominance of chief cells (Fig. 3 - A and B). The examination of the mucosal smooth muscle layer (muscularis mucosa) revealed flanking loose connective tissue with few congested vessels, followed by the muscularis propria layer and a thin serosa layer.

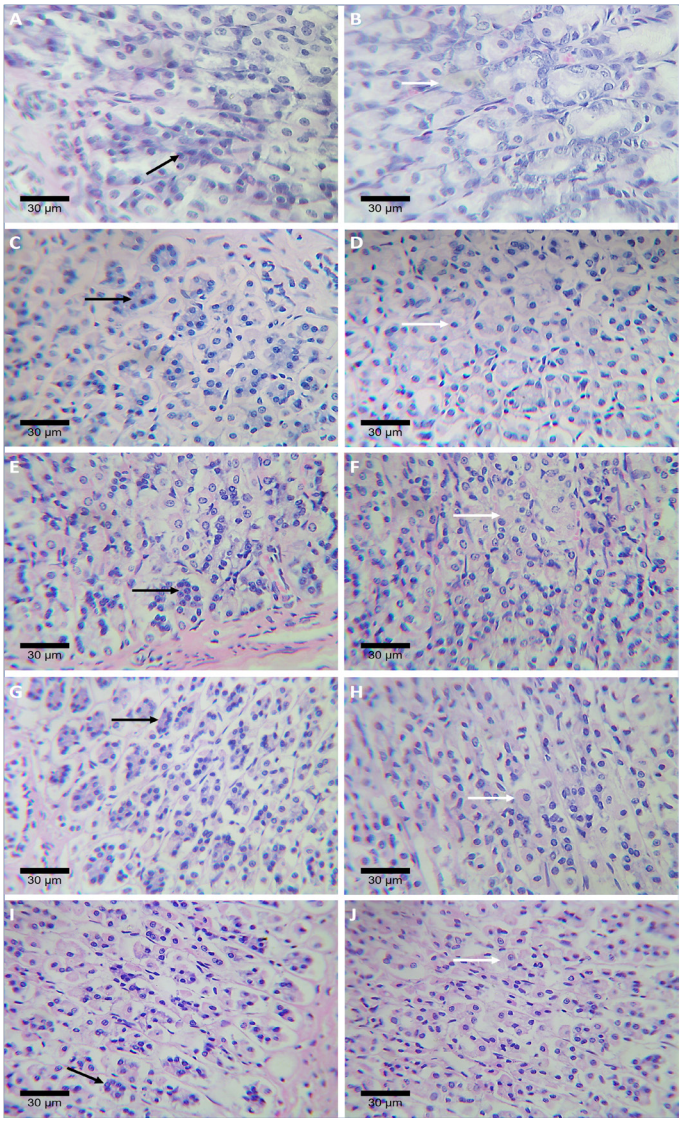
The stomachs that were removed from the control animals did not contain stomach content and presented with a whitish color, normal morphology and preserved pits. Microscopic analysis revealed that the stomachs showed the same layers described for the sham stomachs, with a few additional small sections exhibiting apparent reduced cellularity (Fig. 3 - C and D).

The MTX-treated (i.p.) animals' stomachs contained variable stomach contents, but they exhibited similar normal whitish color and preserved pits, similar to the negative control group stomachs. All of the gastric layers were found to be consistent with those of the negative control group but with a slight reduction of chief, parietal and mucous-secreting cellular populations and gradual reduction of mucosal thickness (Fig. 3 - E and F).

The ALD-treated (p.o.) animals presented stomachs that contained white-yellowish color, viscous and mucoid orange stomach contents with reddish particles in suspension and hemorrhagic striations in the stomach walls. Sections of discontinuity of the pits were observed and none of the animals had food content in their stomachs. Microscopic analysis revealed that the gastric mucosa exhibited a few sections of reduced cellularity, particularly of the parietal cells, although the submucosal microvasculature was not significantly altered (Fig. 3 - G and H).

The MTX/ALD-treated (i.p/p.o.) animals presented stomachs that contained liquid-mucous content with hemorrhagic streaks in suspension. The gastric walls exhibited a reduction of the pits and the presence of a few hemorrhagic streaks. Microscopic analysis revealed reduced cellularity, particularly of the chief, parietal and mucous-secreting cells of the gastric mucosa (Fig. 3 - I and J).

Figure 3. Photomicrographs of the gastric mucosae of the Sham, control and MTX, ALD and MTX/ALD treated groups (Black arrows: chief cells. White arrow: parietal cells). A - B) The Sham group exhibiting gastric mucosa with chief cells (A) and parietal cells (B) without histological perturbations. C - D) The control group treated with saline solution 0.9% (i.p.), exhibiting gastric mucosa with chief cells (C) and parietal cells (D) without abnormalities. E - F) The MTX-treated (i.p.) group showing a slight reduction of chief (E) and parietal cells (F). G - H) ALD-treated (p.o.) group exhibiting gastric mucosa with chief cells without histological alterations (G) and reduction of parietal cells (H). I - J) The MTX/ALD-co-treated group (i.p./p.o.) with a clear reduction of chief cells (I) and parietal cells (J). HE 400X.



DISCUSSION

MTX is a chemotherapy drug that is predominantly used to treat oncological pathologies and malignancies due to its inhibitory effects on the DNA synthesis of tumor cells. In certain malignances, such as breast cancer, it is also used simultaneously with aminobisphosphonates to diminish the possibility of bone metastasis, enhancing patient life expectancy.[4,5].

Although it is not yet approved for clinical and therapeutic application in human cancer treatment[12], ALD induces apoptosis in osteoclasts by inhibiting ATP and cholesterol synthesis, which makes it a potent inhibitor of tumor-induced osteolysis that, theoretically, can inhibit metastasis and difficult tumor implantation in bone tissue[2]. In spite of its advantages, ALD, similar to all of the other bisphosphonates, elicits many side effects, including osteonecrosis of the jaw, hypocalcemia, asthenia, headache and disorders of the gastrointestinal tract[4].

Another limiting factor regarding its use is that ALD is only commercially available as an oral formulation, a form of delivery that can directly cause accentuated damage to the surface of the gastric mucosa[7].

When the toxic effects of these drugs on the digestive tract cells are combined with the cachexia-induced by Walker 256 carcinosarcoma, the general health of the animals can be seriously compromised during oncological therapy, causing weight loss with decreased survival rate impact.

Body weight variation analysis revealed that weight loss was significant in the control group animals, confirming that malignances can consume the body's energy reserves, resulting in malnutrition and cachexia in individuals with cancer.8 Moreover, the MTX-treated animals did not exhibit similar weight loss, corroborating the antitumor effects of folate inhibitors that were previously shown to suppress uncontrolled tumor proliferation and the exacerbated consumption of body nutrients[13].

The ALD-treated animals exhibited significant weight loss, despite the antitumor and anti-angiogenic effects that ALD was previously demonstrated to elicit, particularly on malignances of bone origin[14,15]. However, when used without being combined with MTX according to the proposed protocol, ALD did not elicit significant antitumor effects and did not alter nutrient consumption. Furthermore, the use of ALD was associated with acute gastrointestinal alterations, which might have compromised the digestive action and the absorption of nutrients, thereby preventing adequate nutrition.

MTX enhanced the survival rate of the animals when used alone, a finding that can be corroborated by the reduction of tumor volume and attributed to its suppressive effects on the DNA synthesis of highly mitotic tumor cells.[5] There was a slight decrease of prevention of weight loss, of the survival curve and antitumor activity when the folate inhibitor was combined with ALD. These data suggest that the co-administration of ALD and MTX can change the antitumor activity and survival afforded by MTX. This difference might be associated with the gastrointestinal toxicity of the bisphosphonate alone[7].

Compared to the control animals, the ALD-treated animals presented a low survival rate, which is likely a consequence of several factors such as the cachexia induced by the Walker 256 carcinosarcoma,8,9 the low antitumor activity of the bisphosphonate on particular cell types, and the toxic effects of oral ALD on the gastrointestinal mucosa even with only one ALD dose[7].

Macroscopic evaluation of the stomachs presented morphological alterations in the ALD-treated and MTX-ALD-treated animals. It was demonstrated that the loss of gastric mucosal integrity due to drug toxicity could compromise nutritional absorption due to acute pain symptomatology[7].

Microscopic analysis of the MTX-treated animals revealed slight and regular decreased cellularity compared to the control animals. The toxicity of MTX can be associated with its antimetabolic effects in highly proliferative cells[5].

The ALD-treated group exhibited a reduction of gastric mucosa cells, particularly of parietal cells, highlighting the specificity of ALD on this type of cells. The selectivity of ALD toward parietal cells can be associated with the presence of an amine group and the electronegativity of its phosphate groups. Consequently, this drug exhibits increased affinity for electropositive polar environments, such as gastric acid, which is saturated with protonated hydrogen ions.16 This hypothesis is consistent with the malabsorption of ALD in the gastrointestinal tract when ingested because its protonation prevents its passage into the paracellular spaces of the small intestine[17,18].

Because the pH is extremely low in parietal cells, with an average daily range of 1.4,19 there might be a high accumulation of ALD in these cells or its proximities, making ALD largely responsible for the initial alterations of the gastric mucosa[7].

Histological analysis of the stomachs revealed that when ALD was co-administered with MTX, there was a more significant but gradual loss of gastric mucosal cells when compared to the ALD-treated group, an effect that is associated with the antimetabolic effects of MTX in highly proliferative cells,5 such as the chief and mucous-secreting cells, and with possible cytotoxic effects on the acid-producing parietal cells[7].

Taken together, our findings indicate that bisphosphonates do not appear to elicit antitumor activity on the cell lineages evaluated in our study and decrease the antitumor effect of MTX when combined whit this. ALD probably caused early toxicity on the gastric epithelium over a short treatment time and when combined with MTX, elicited a significant inhibitory effect on the turnover of gastric mucosal epithelial cells, leading to cellular rarefaction and early toxicity.

CONCLUSIONS

Therefore, ALD does not elicit significant antitumor effects on Walker 256 carcinosarcoma cells but rather elicits toxicity on the gastric epithelium, despite the short treatment time, resulting in a negative impact on the survival outcome of the treated animals decreasing antitumor effect of MTX. The combination of ALD with MTX demonstrated a significant inhibitory effect on the turnover of the gastric mucosal lining, resulting in cellular rarefaction and early toxicity.

ACKNOWLEDGEMENTS

The authors are grateful to Victor Teixeira Noronha for his assistance with biochemical approach of the protonation process of ALD. The authors wish to thank the National Agencies CNPq (National Center for Research and Development) and CAPES (Coordination of Improvement of Higher Education Personnel) for financial support. In addition, the authors also thank Cristiane Roriz for carefully proofreading the article.

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How to cite this article/Como citar este artigo:

Alves APNN, Verde MEQL, Silva PGB, Sousa FB, Mota MRL, Pessoa CO, et al. Gastric toxicity of Alendronate and Methotrexate in Walker 256 carcinosarcoma jaw model. J Health Biol Sci. 2019 Abr-Jun; 7(2):126-132.