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ORIGINAL ARTICLE

CHANGES ON FOOD INTAKE, BODY WEIGHT AND SALIVARY AMYLASE SYNTHESIS IN THE SUBMANDIBULAR GLAND OF WISTAR RATS TREATED WITH BEVACIZUMAB AND CYTOSTATICS

Cambios en la ingesta de alimentos, peso corporal y síntesis de amilasa salival en la glándula submandibular de ratas Wistar tratadas con Bevacizumab y Citostáticos

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ABSTRACT

Background: Bevacizumab together with 5-fluorouracil and oxaliplatin inhibit microvascular growth of tumor blood vessels and tumor proliferation. Few reports state the effect of these therapeutic schemes on salivary glands.

Materials and Methods: Food consumption, body weight and salivary amylase activity were assessed in the submandibular gland of rats. Adult male Wistar rats, of three months old with 350/400 grams body weight, under 12-hour light/dark cycles respectively, were divided into the following experimental groups: G1) Control group, G2) 5-Fluorouracil and leucovorin calcium treated group, G3) Bevacizumab treated group, G4) Oxaliplatin treated group, G5) Bevacizumab, oxaliplatin, 5-fluorouracil and leucovorin calcium treated group and G6) Drug-free paired feeding treated group. Assessment of treatment effect was performed by one-way ANOVA. A value of $p<0.05$ was set for statistical significance.

Results: Salivary amylase activity in gland homogenate was G1: 137.9 ± 4.64, G2: 60.95±4.64, G3: 120.93 ± 4.96, G4: 26.17 ±4.64, G5: 10.77 ±4.64 and G6: 82.87 ±4.64 U/mg protein (mean ± S.D.) Amylase activity in the G1 group was higher relative to the other experimental groups $p<0.0001$.

Conclusions: The drugs 5-fluorouracil and oxaliplatin altered salivary amylase activity by serous granules of the submandibular gland interpreted as a mechanism of impaired acinar function. Bevacizumab administered in isolation did not alter salivary amylase activity compared to the control group. While the lower intake of the matched feeding group affected salivary amylase activity compared to the control group, the effect was significantly greater in animals treated with the oncology drugs used in the present animal model.

Keywords: *Chemotherapy; Antibodies, monoclonal; Dietary intake; Body weight; Amylase; Submandibular gland.*

RESUMEN

Antecedentes: Bevacizumab, junto con 5-fluorouracilo y oxaliplatino, inhiben el crecimiento microvascular de los vasos sanguíneos tumorales y la proliferación tumoral. Pocos reportes establecen el efecto de estos esquemas terapéuticos sobre las glándulas salivales.

Materiales y Métodos: Se evaluaron el consumo de alimentos, el peso corporal y la actividad de amilasa salival en la glándula submandibular de ratas Wistar macho adultas, de tres meses de edad con 350/400 gramos de peso corporal, bajo ciclos de luz/oscuridad de 12 horas respectivamente, se dividieron en los siguientes grupos experimentales: G1) Grupo control, G2) Grupo tratado con 5-Fluorouracilo y Leucovorina cálcica. , G3) Grupo tratado con bevacizumab, G4) Grupo tratado con oxaliplatino, G5) Grupo tratado con bevacizumab, oxaliplatino, 5-fluorouracilo y leucovorina cálcica y G6) Grupo tratado con alimentación emparejada sin fármacos. La evaluación del efecto del tratamiento se realizó mediante ANOVA unidireccional. Se estableció un valor de $p<0,05$ para significación estadística.

Resultado: La actividad de amilasa salival en homogeneizado de glándula fue G1: 137,9 ± 4,64, G2: 60,95 ± 4,64, G3: 120,93 ± 4,96, G4: 26,17 ± 4,64, G5: 10,77 ± 4,64 y G6: 82,87 ± 4,64 U/mg de proteína (media ± S.E.). La actividad de amilasa en el grupo G1 fue mayor en relación con los otros grupos experimentales $p<0,0001$.

Conclusión: Los fármacos 5-fluorouracilo y oxaliplatino alteraron la actividad de la amilasa salival mediante gránulos serosos de la glándula submandibular interpretados como un mecanismo de deterioro de la función acinar. Bevacizumab administrado de forma aislada no alteró la actividad de la amilasa salival en comparación con el grupo de control. Mientras que la menor ingesta del grupo de alimentación combinada afectó la actividad de la amilasa salival en comparación con el grupo de control, el efecto fue significativamente mayor en los animales tratados con los medicamentos oncológicos utilizados en el grupo. modelo animal actual.

Palabras Clave: *Quimioterapia; Anticuerpos monoclonales; Ingestión de alimentos; Peso corporal; Amilasa; Glándula submandibular.*

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INTRODUCTION

Under physiological conditions, angiogenesis plays a vital role in the generation of vascular neo-proliferation during the process of embryogenesis and is depressed in living adults, with transient activation during healing and during the female sexual cycle. Although angiogenesis is tightly controlled by an intricate interplay of pro- and anti-angiogenic factors, it can be precipitated by solid tumors during their differentiation. This phenomenon, commonly referred to as the *"angiogenic switch"*, is a situation recognized as a hallmark of tumor growth.[1]

Vascular growth inhibitor (anti-VEGF) drugs have been shown to be an effective therapeutic alternative in combination with other conventional cytostatic agents for the treatment of various types of systemic cancer.[2]

Bevacizumab is a monoclonal antibody currently used for the treatment of metastatic breast cancer, non-small cell lung cancer, glioblastoma, renal cell carcinoma, ovarian and cervical cancer.[3]

Currently there are few reports of the possible adverse effects of bevacizumab administered in combination with chemotherapy for the treatment of colorectal cancer in relation to dietary intake and body weight recording.[4]

Although some alterations in the oral cavity such as oral mucositis have been described in the scientific literature, there is no clear evidence about the effect that the combination of monoclonal antibodies and chemotherapy could have on the functional activity of the salivary glands.[5]

Based on this background, the aim of the present study was to analyze the pharmacological scheme: bevacizumab, 5-fluouracil, leucovorin calcium and oxaliplatin and its effect on habitual food intake, body weight and on the functional activity of the submandibular gland in an animal model. In the present study, we have considered incorporating calcium leucovorin, a derivative of the metabolite tetrahydrofolate acid, which is an essential coen-zyme for nucleic acid synthesis. Despite not being a cytostatic drug, it acts as a biomodulator of 5-fluorouracil, enhancing its toxicity and consequently increasing its therapeutic effectiveness.[6,7]

MATERIALS AND METHODS

Forty-eight adult male Wistar rats of 350/400 g body weight, inbred animals from the biotherium of the Department of Physiology of the Faculty of Dentistry, National University of Cordoba, maintained under 12-hour light/dark cycles, were employed, distributed in the following experimental groups (n=8):

G1: Control.

G2: Administration of 5-fluorouracil and leucovorin calcium.

G3: Bevacizumab administration.

G4: Oxaliplatin administration.

G5: Administration of bevacizumab, oxaliplatin, 5-fluorouracil and leucovorin calcium.

G6: Paired drug-free feeding.

Animals were housed in individual cages with ad libitum feeding and drinking for groups one, two, three, four and five. Animals in group six received the average daily intake of animals treated in group five during treatment with antineoplastics and bevacizumab This group was intended to rule out the effect of lower dietary intake on the action of bevacizumab and cytostatics on the parameters analyzed.

Light/dark cycles were twelve hours each for all experimental groups. Animals in group two received a joint intraperitoneal injection for five consecutive days of 5-fluorouracil and leucovorin calcium at 20 mg/kg and 10 mg/kg body weight. Group three animals were injected with an intraperitoneal dose of bevacizumab 10 mg/kg

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body weight on days one and 15 of treatment. Group four animals were injected with an intraperitoneal dose of oxaliplatin 6 mg/kg body weight on days one and 15 of treatment.

Animals in group five received an intraperitoneal dose of 5- fluorouracil and Leucovorin calcium of 20 mg/kg and 10 mg/kg body weight respectively on days one, two, three, four and five and bevacizumab and oxaliplatin, 10 mg/kg and 6 mg/kg body weight respectively on days one and fifteen of treatment (Figure 1).

Daily weighing of food and body weight was performed in all treatment groups. At the end of the experimental stage, they were fasted for 24 hours prior to slaughter in order to achieve the same basal operating conditions. Subsequently, they were anesthetized with a combined dose of Ketamine and Xylazine (80 and 12.8 mg/kg body weight respectively) and both submandibular glands were removed. At the end of the surgical procedure, the animals were killed by cervical dislocation.

The protocol was approved by the CICUAL (Res: 6/2018 Commission for Care and Use of Laboratory Animals, Facultad de Ciencias Médicas y facultad de Odontología Universidad Nacional de Córdoba). The National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) was followed. All efforts were made to minimize the number of animals and their suffering. The following variables were evaluated:

Dietary intake

This was assessed throughout the experimental period by means of daily intake records. 50 g of GEPSA RAT/MAT commercial feed were weighed on a precision balance and subsequently the pellet not ingested was recovered for the quantification of the real intake. The centesimal composition of the feed was as follows: protein 24%, ether extract 6%, fiber 7%, calcium 1.2%, phosphorus 0.9%, total minerals 8%. Every 24 hours, the remaining capsules were collected to record the feed consumed.

Body weight

Body weight was monitored daily throughout the experimental period, keeping a weighing schedule and measuring instrument (ad hoc balance) in all groups.

Determination of salivary alpha amylase activity of the submandibular gland:

After anesthetic induction, both submandibular glands were excised to measure glandular salivary amylase concentration by spectrophotometric method, considered as a marker of functional activity.

Sample size calculation

To compare the means and/or medians of the treated groups, considering a two-sided alpha error of 5% and a power of 80%, and a standardized deviation (SD) as the ratio between the mean differences d and the standard deviation s, the following formula was used: n = 16/(SD)^2. Thus, the minimum number of animals for the planned evaluations was determined to be 8 individuals per treatment group.[8]

Statistical analysis

For data analysis, the first step involved testing for variance normality. Since the dataset contained fewer than 50 observations, the Shapiro-Wilk test was chosen. In this regard, when a *p*-value of ≥0.05 was obtained, the variable under analysis exhibited normality in its data. Conversely, when the *p*-value of the test was <0.05, the variable showed no normality in its data.

To assess whether there is a difference between the means or medians (depending on the distribution), the ANOVA test and Bonferroni post

hoc test were conducted for variables with normal responses. Meanwhile, for variables with abnormal responses, the Kruskal-Wallis test was performed. A *p*-value <0.05 was set for statistical significance. Data were analyzed using Infostat Professional 2020.

RESULTS

Dietary intake

Mean dietary intake at day 5 of treatment were 41.13, 4, 6.25, 19.25, 19.25, 9.88 ± 0.58 grams in groups G1, G2, G3, G4 and G5 respectively (*p*-value ≤ 0.0001). While dietary intake at day 15 of treatment showed a mean value of 42, 14,5, 11 and 4.75 ± 0.67 grams in groups G1, G3, G4 and G5 (*p*-value: < 0.0001) respectively, minus group G2 which had completed the therapeutic scheme on day 5 of the experimental activity, (Figure 2).

Body weight

Body weight showed the following mean values at initial and final stage G1:322 *versus* 338.38, G2: 341.88 *versus* 299.38, G3: 343.12 vs.284.38, G4: 348.75 *versus* 275, G5: 334.38 *versus* 241.88 and G6: 316.88 *versus* 276.88 grams ± 5.58 and 5.8 respectively (*p*-value ≤ 0.001 and ≤ 0.0001), (Figure 3).

Determination of salivary α amylase activity in submandibular gland homogenate

The means were 137.9±4.64, 60.95±4.64, 120.93 ±4.96, 26.17±4.64, 10.77±4.64 and 82.87±4.64 U/ gram gland for G1, G2, G3, G4, G5 and G6.

Group G1 showed a significantly slower salivary amylase concentration in relation to the other experimental groups *p*<0.01 *versus* G1, G2, G3 y G6, (Figure 4).

Figure 1. Scheme with the different treatments for each experimental group. The number of animals for each group was eight rats. All animals received *ad libitum* feeding except G6.

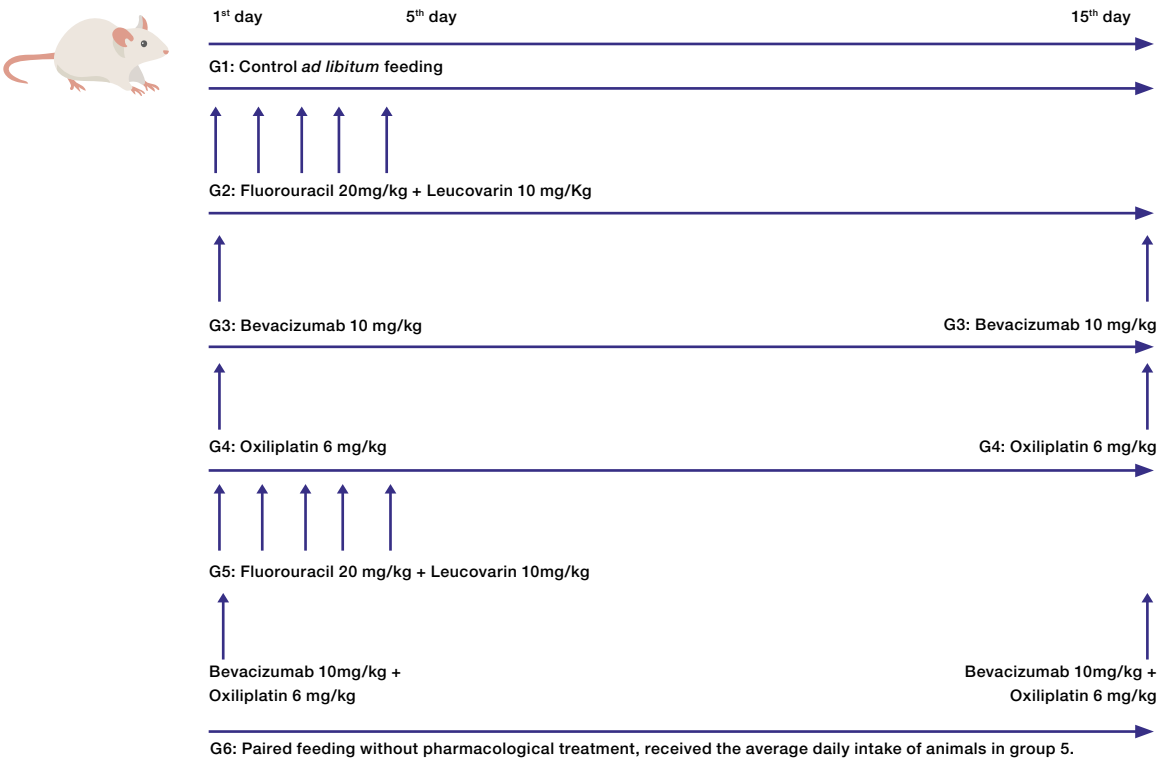

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Figure 2. Different treatments for each experimental group. The number of animals for each group was eight rats. All animals received *ad libitum* feeding except G6.

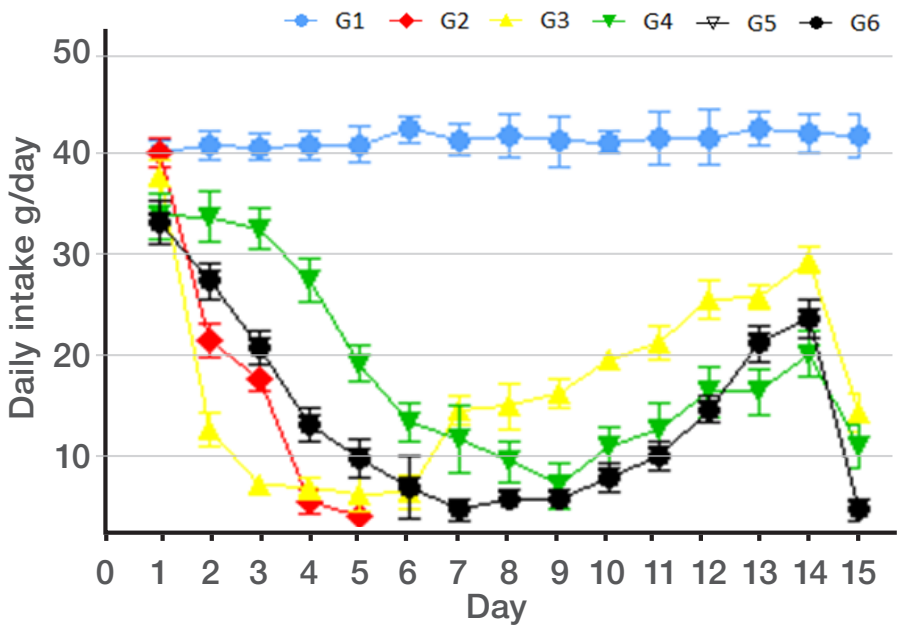
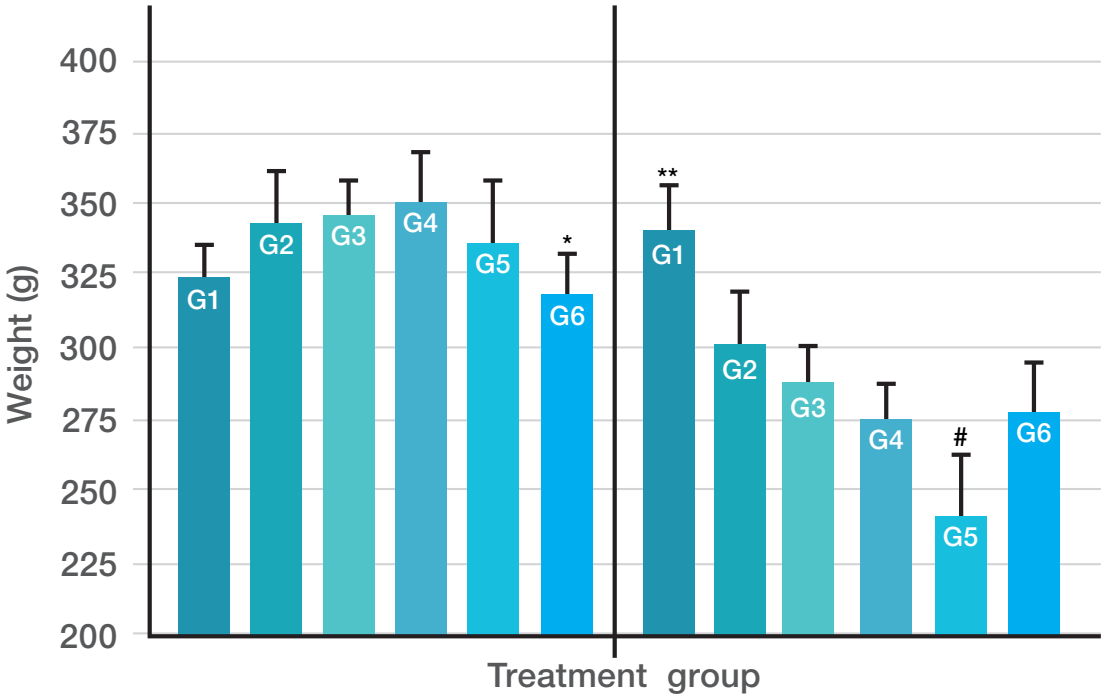


Figure 3. Weight evolution of each group.



***p*=0.00** *versus* G2, G4 and G6 (Baseline). ****p*=0.02** *versus* G2, G3, G4, G5 and G6. **#*p*=0.001** *versus* G2, G3, G4 and G6 (Final).

Figure 4. Data represents mean±S.E. n=8 per experimental group. *$p<0.01$ *versus* G, G2, G3 y G6.

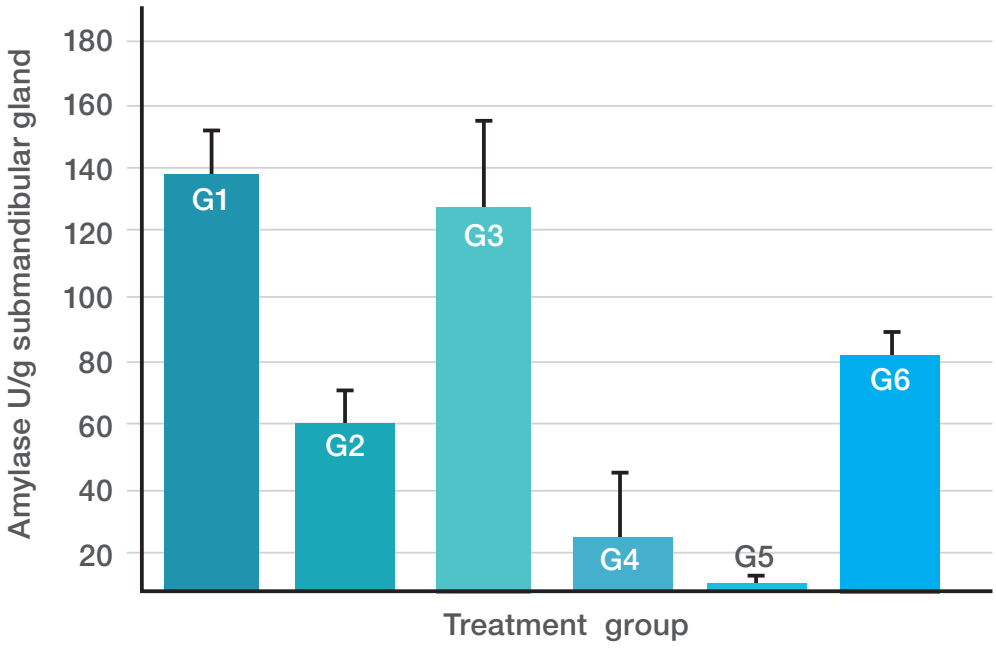


Table 1. Biochemical parameters in the different experimental groups.

Group	Red Blood (cells/ mm³)	Hemoglobin (g/dL)	Hematocrit (%)	White blood (cells/mm³)	Platelets (/mm³)	Urea (mg/dL)	Creatinine (mg/dL)	GOT (UI/L)
G1	7,047,500	13.35	37.88	7,825	412,250	0.65	0.60	78.75
G2	5,356,666	14.67	43.00	3,800	197,000	0.65	0.67	70.33
G3	7,846,666	14.77	40.00	6,400	140,666	0.72	0.73	48.67
G4	5,356,666	14.50	41.00	5,600	162,333	0.77	0.65	71.00
G5	5,883,333	11.10	31.67	1,700	115,000	0.94	0.86	142.33
G6	6,333,333	13.30	38.00	8,766	413,333	0.66	0.63	67.67

G1: control. **G2:** 5-FU+LV. **G3:** Bevacizumab. **G4:** Oxaliplatin. **G5:** 5-FU+LV/Bevacizumab/Oxaliplatin. **G6:** Matched feeding (without drugs).

DISCUSSION

Conventional cancer chemotherapy treatments remain controversial because numerous cytostatics applied, in addition to shrinking or eliminating a given tumor and/or metastasis, often affect other organs and systems. This has been described by several authors who reported adverse effects due to the non-selective action of drugs such as 5-Fluorouracil and oxaliplatin.[9]

With the advent of immunotherapy and its combination in schemes with these cytostatics, antineoplastic treatments showed a significant therapeutic advance, however, some deleterious effects on metabolism and function on other organ systems were reported.[10]

In addition to other clinical and hemodynamic variables, one of the aspects most considered in this type of therapy is the nutritional status

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of cancer patients, since protocols combining chemotherapy and immunotherapy could also alter this parameter.

For their part, several authors have warned that these treatments may be better tolerated when the nutritional status is optimal, a situation that would predispose to the maintenance of a certain state of general homeostasis of the organism, while trying to cure or halt the progression of a given tumor.[11-13]

From a clinical point of view, bevacizumab was approved for use in combination with intravenous 5-fluorouracil, Irinotecan or oxaliplatin for a first or second line treatment of metastatic carcinoma of the colon or rectum. Although this drug is generally well tolerated, it may cause some adverse reactions that may be intensified depending on the chemotherapy regimen with which it is associated. The most common reactions in this particular regimen are proteinuria, epistaxis, respiratory infections, anorexia, stomatitis, diarrhea, fatigue and exfoliative dermatitis.

Clinical manifestations such as gastrointestinal perforation, bleeding, arterial thrombosis, hypertensive crisis, neutropenia, nephrotic syndrome and congestive heart failure have occasionally been described. However, there is no clear experimental or clinical evidence demonstrating alterations on intake parameters, body weight and salivary glands.[14,15]

In the present study, significant differences were observed in the average intake in the different groups analyzed. In fact, at the end of the experiment, the combined action of bevacizumab, 5-fluorouracil and oxaliplatin showed a significantly lower food intake pattern than the other experimental groups. Compared to the untreated animals whose intake remained unchanged, the other groups significantly reduced their feeding. The second parameter analyzed was body weight, which, coinciding with intake, showed a similar behavior. From these results, we could hypothesize that the greater deleterious effect on intake and body weight would be due more to the action of 5-fluorouracil and oxaliplatin than that induced by the monoclonal antibody.

However, bevacizumab reduced both parameters compared to untreated animals. It often happens that, as a consequence of the administration of these drugs, many animals could go through a state of cachexia, caused by the decrease of nutrients, altering the general homeostasis of the organism during the treatment. In the present work, from the clinical observation on these animals, this fact was not so noticeable, although numerous hematological indices, plus those analyzed, suffered severe modifications as usually happens in these schemes used to treat these carcinomas (Figure 1). In contrast to another similar study carried out in rats, but with a significantly higher dose (40mg/kg body weight for 4 consecutive days), the applied dose of 5-fluorouracil would be a conditioning variable that could have modified the fact that the animals treated in the present model did not reach cachexia.[16]

In another sense, although we do not have evidence from other studies that can be contrasted with ours, it is inferred that the lower body weight of the paired feeding group, without the deleterious effects described by chemotherapy, would only be related to a lower intake induced over a prolonged period of time. This group of animals was intended to rule out the effect of lower intake on the toxicity of the applied drugs on various functional parameters.[17]

It has been widely demonstrated that oxaliplatin can cause diarrhea during conventional chemotherapy in both clinical and experimental schemes. The dose of oxaliplatin tested in this animal model, in agreement with other authors, has confirmed this effect both in the administration scheme of this drug alone or together with bevacizumab.[18]

Among other clinical evidence, and in agreement with another author, the animals in the groups with conventional chemotherapy and chemotherapy associated with bevacizumab showed piloerection and discontinuous alopecia in the craniofacial region, neck and trunk. Several specimens showed signs of spontaneous hemorrhage in forelimb and hindlimb phalanges.[19] From a pharmacological point of view, bevacizumab together with 5-fluorouracil and oxaliplatin have the capacity to inhibit neovascularization and tumor proliferation. In relation to the oral cavity, few reports refer to its effect. Some authors have described some events on the mucosa and bone level of the stomatognathic system due to the action of Bevacizumab due to its anti-angiogenic effect.[20-22]

In fact, in the stomatognathic system, there is no evidence reported at either clinical or experimental level regarding possible toxicities caused by this pharmacological scheme in relation to salivary gland activity. One way to evaluate these parameters is to quantify their functional activity by determining the concentration of the enzyme salivary alpha amylase or ptyalin, present in the serous granules of the parotid and submandibular or submaxillary glands.[23] In agreement with other experimental results previously obtained in our laboratory, the drugs 5-fluorouracil and oxaliplatin altered amylase synthesis by the serous granules of the submandibular gland with impaired acinar function. Bevacizumab alone did not alter glandular amylase concentration. While lower intake affected the synthesis of this enzyme, cytostatics had a significantly greater effect. From this perspective, it could be inferred that the combined administration scheme between cytostatics and monoclonal antibodies would alter the functional activity of submandibular glands only by the deleterious action of the antineoplastic drugs.[24]

In the present work it has been demonstrated that the drugs 5-fluorouracil and oxaliplatin altered the synthesis of salivary amylase by the serous granules of the submandibular glands, interpreted as a mechanism of impaired acinar function. Bevacizumab administered alone did not alter salivary alpha amylase concentration compared to the control group. Although the lower intake of the Matched Feeding group affected salivary alpha amylase concentration compared to the control group, the effect was significantly greater in animals treated with the oncology drugs used in this animal model.

Possibly the quantification of this enzyme would allow for the evaluation of changes in the functionality of the submandibular glands, in this type of oncological therapies. It would be appropriate to extend the present study on the parotid gland, as it is the main responsible for the synthesis of salivary amylase. It would also be interesting to contrast these preliminary results in cancer patients treated under this same scheme by measuring the concentration of this metabolic substrate in total saliva.

Future clinical studies on total saliva of patients undergoing these treatments could corroborate the results obtained in the present experimental study, with the aim of correlating the functionality of the processes of salivary synthesis and secretion.

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CONFLICT OF INTERESTS

All authors declare that there are no potential conflicts of interest regarding the authorship and/or publication of this article.

ETHICS APPROVAL

Approved by the Institutional Committee for the handling and care of laboratory animals, Facultad de Ciencias Médicas y de Odontología, Universidad Nacional de Córdoba.

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AUTHORS' CONTRIBUTIONS

Dubersarsky C: Investigation, supervision and writing-review and editing.
Bachmeier E: Investigation, supervision and writing-review and editing.
Francia M: Investigation.
Rivoira M: Methodology and resources.
Porta D: Investigation.
Brunotto M: Formal analysis.
Mazzeo MA: Methodology, funding acquisition, investigation, writing-original draft and review and editing.

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