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Article

5-Fluorouracil and Cyclophosphamide modify functional activity in submandibular gland of rats.

El 5-fluorouracilo y la ciclofosfamida modifican la actividad funcional en la glándula submandibular de las ratas.

Evelin Bachmeier.[1]
María Marcela López.[1]
Jorge Alberto Linares.[1]
Mabel Noemí Brunotto.[2]
Marcelo Adrián Mazzeo.[1]

Affiliations: [1]Chair of Physiology. School of Dentistry. National University of Cordoba, Argentina. [2]Chair of Cellular Biology "A". School of Dentistry. National University of Cordoba, Argentina

Corresponding author: Marcelo Adrián Mazzeo. Chair of Physiology of the Faculty of Dentistry of the National University of Córdoba. Haya de la Torre s/n, Ciudad Universitaria. Córdoba. Argentina. E-mail: marceloadrianmazzeo@yahoo.com.ar

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Abstract: Objective: Chemotherapy treatment against cancer produce systemic toxicities, among which are those related to important structures of the stomatognathic system and its functional activity. 5 Fluorouracil (5-FU) and cyclophosphamide (Cf) are drugs widely used in solid tumors and in bone marrow transplantation, respectively. The objective of this work was to evaluate the toxicity of these drugs regarding functional activity of the submandibular glands, by measuring the percentage of glycogen consumption in two experimental models. Material and Methods: 84 male Wistar rats aged three months were used, housed in individual cages, with controlled temperature and lighting and ad libitum diet. They were divided into four experimental groups: 1) Control (C); 2) Treated with 5-FU+leucovorin (LV) at 20 and 10mg/Kg of body weight respectively for five consecutive days; 3) treated with Cf i.p. at 50mg/Kg of body weight for two consecutive days; and 4) rats with paired feeding (PF): for five and two days respectively, the amount administered resulted from the average of the ingested food of groups 2 and 3. Both submandibular glands were excised. The submandibular glycogen concentration was analyzed at initial time (t0) and after 60 minutes of mechanical stimulation (t60). Results: the average variation changed significantly between time 0 and 60 in the groups C and PF. (p-value=0.0001), the 5-FU + LV treatment group had an average concentration higher at t0 than groups C and PF, without significant consumption at T60. While group Cf showed a lower average concentration at time 0 with respect to groups C and PF, without significant consumption at T60. Conclusion: 5-FU+LV and Cf affect the metabolism of carbohydrates, decreasing the use of glycogen as a metabolic substrate. In the present experimental model, the toxicity of these drugs affected the functional activity of the submandibular gland.

Keywords: Antineoplastic agents; 5-Fluorouracil; cyclophosphamide; glycogen; submandibular gland; rats.

Resumen: Objetivo: el tratamiento de quimioterapia contra el cáncer produce toxicidades sistémicas, entre las que se encuentran las relacionadas con estructuras importantes del sistema estomatognático y su actividad funcional. El 5-fluorouracilo (5-FU) y la ciclofosfamida (Cf) son fármacos ampliamente utilizados en tumores sólidos y en trasplantes de médula ósea, respectivamente. El objetivo de este trabajo fue evaluar la toxicidad de estos fármacos con respecto a la actividad funcional de las glándulas submandibulares, midiendo el porcentaje de consumo de glucógeno en dos modelos experimentales. Material y Métodos: se utilizaron 84 ratas Wistar machos de tres meses de edad, alojadas en jaulas individuales, con temperatura e iluminación controladas y dieta ad libitum. Se dividieron en cuatro grupos experimentales: 1) Control (C); 2) Tratados con 5-FU+leucovorina (LV) a 20 y 10mg/Kg de peso corporal, respectivamente, durante cinco días consecutivos; 3) tratados con Cf i.p.

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a 50mg/Kg de peso corporal durante dos días consecutivos; y 4) ratas con alimentación por parejas (PF): durante cinco y dos días respectivamente, la cantidad administrada resultó del promedio de los alimentos ingeridos de los grupos 2 y 3. Ambas glándulas submandibulares fueron extirpadas. La concentración de glucógeno submandibular se analizó en el momento inicial (t0) y después de 60 minutos de estimulación mecánica (t60). Resultados: la variación promedio cambió significativamente entre el tiempo 0 y 60 en los grupos C y PF. (p=0,0001), el grupo de tratamiento 5-FU+LV tuvo una concentración promedio más alta en t0 que los grupos C y PF, sin un consumo significativo en T60. Mientras que el grupo Cf mostró una concentración promedio más baja en el tiempo 0 con respecto a los grupos C y PF, sin un consumo significativo en T60. Conclusión: 5-FU + LV y Cf afectan el metabolismo de los carbohidratos, disminuyendo el uso de glucógeno como sustrato metabólico. En el presente modelo experimental, la toxicidad de estos medicamentos afectó la actividad funcional de la glándula submandibular.

Palabras Clave: *Antineoplásicos; fluorouracilo; ciclofosfamida; glucógeno; glándula submandibular; ratas.*

INTRODUCTION.

5-FU is classified as an analog of pyrimidine bases that incorporates a fluorine atom into position 5 instead of hydrogen, injuring the malignant cells by two mechanisms: inhibiting thymidylate synthetase and incorporating into RNA.[1] Within the general classification of cytostatics, (5-FU) is considered an antimetabolite like methotrexate because they inhibit the synthesis of nitrogenous bases and DNA by Enzymatic blocking. It is used in polychemotherapy treatment of solid breast, gastrointestinal, ovarian and head and neck tumors.

In recent decades it was administrated as the only standard cytostatic agent in the treatment of colorectal carcinoma, together with calcium leucovorin (LV) that modulates its action; increasing its therapeutic effectiveness. LV is a derivative of the tetrahydrofolic acid metabolite, which is an essential cofactor in the synthesis of nucleic acids.[2,3]

Although not being a cytostatic drug, it acts as a biomodulator of the drug 5-FU and its toxicity.[4] Cyclophosphamide is a cytostatic agent of the class of alkylating agents that is alkaline due to the presence of an amide group in its chemical structure. It is considered a prodrug that needs to be activated by the system of liver microsomal enzymes in order to become cytotoxic. It is activated by the cytochrome P450 system, liver metabolic pathway; it is metabolized into 4-hydroxycyclophosphamide and aldophosphamide, and in the presence of tumor cells the latter unfolds, spontaneously generating phosphoramide mustard, which has anti-tumor effects. By reacting with DNA of the tumor cells this alkylating agent forms bridges that prevent DNA replication thus causing cell death.[5]

Most of the oncological therapeutic schemes cause secondary alterations. Since the administration of these drugs is carried out systemically, adverse effects on several organ systems occur, mainly in those with a high rate of cell turn-over and functional activity, such as the stomatognathic system. The analysis of these effects is not always clear since they are supported by reports by the patients during the course of chemotherapy.[6]

Given that in many cases complications in the oral cavity can limit the continuity of oncological treatment, these should be minimized. Complications in the mouth include: xerostomia, glossitis, dysgeusia, mucositis and exacerbation of periodontal disorders.[7,8] The main dose-limiting toxicities include diarrhea, myelosuppression, and stomatitis. Other toxicities include: hand-foot syndrome, alopecia, nausea, vomiting, dermatitis, cardiac toxicity, and decreased salivary gland secretion.[9,10]

So far, the specific effect of cytostatic agents on the salivary glands has not been fully elucidated. For this reason, during the last thirty years, different hypotheses have been put forward regarding the effects of antineoplastic drugs on oral health. Several authors have described the alteration of the salivary flow and its organic and inorganic components.[11,12]

The submandibular glands contribute approximately two-thirds of the unstimulated saliva, and considering the important role of saliva, it is evident that variations in quantity and quality produce serious consequences on the homeostasis of the stomatognathic system. The oral complications related to chemotherapy are the result of complex interactions between multiple factors. The submandibular gland of the rat is a good *in vivo* model of the pathophysiology of salivary secretion, since it has rich adrenergic innervations and has the capacity of using carbohydrates as a source of energy.[13] Currently there are no protocols or drugs with universal effectiveness without

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side effects the oral cavity.[14] As depression of glandular function and its consequences in the oral cavity are a frequent problem during the course of chemotherapy, it is important that dentists exercise a relevant role in the investigation and prevention of such complications

On the other hand, studies in rats have been widely used to evaluate the effects of oncological drug on the secretion and composition of saliva, with the results extrapolated to humans, since both species share similar functional organ systems. In an attempt to address the possible alterations in the functional activity of saliva by the action of cytostatic agents, an experimental model in rats subjected to the action of 5-FU and Cf was employed.

The objective of this work was to evaluate the functional activity of the submandibular glands of Wistar rats subjected to treatment with 5-FU+LV and Cf and two experimental models through the measurement of glandular glycogen levels.

MATERIALS AND METHODS.

The experimental activity was carried out at the Faculty of Dentistry of the National University of Córdoba, Argentina. Eighty four male Wistar rats aged three months were used, housed in individual cages, under controlled temperature and lighting, and with an ad libitum diet. They were divided in four experimental groups:

1) Control (C); 2) Treated with 5-FU+leucovorin (LV) at 20 and 10mg/Kg of body weight respectively for five consecutive days; 3) treated with Cf i.p. at 50mg/Kg of body weight for two consecutive days; and 4) rats with pair feeding (PF) divided in two subgroups for 5-FU+LV and Cf during five and two days respectively. (Table 1)

The amount of food administered resulted from the average ingest of food in groups 2 and 3. The purpose of the PF group was to discard the effect of reduced ingestion by action of treatment with cytostatic agents on the functional activity of the salivary glands. (Table 2 and Table 3)

The animals fasted during 24 hrs before sacrifice and were anesthetized with an injection of ketamine and xylazine at 80 and 12.8mg/Kg of body weight. Subsequently both submandibular glands were excised. The euthanasia of the animals was practiced by cervical dislocation. The experimental activities were carried out following the protocol for care and treatment of animals approved by the Animal Bioethics Center of the Faculty of Medical Sciences of the National University of Cordoba (CICUAL). In the present work we considered the objectives and applications of the STROBE Declaration.

The concentration of submandibular glycogen at initial time (t0) was analyzed in some glandular fractions in all the experimental groups. Other fractions were analyzed 60 minutes after incubation in a solution of Krebs ringer bicarbonate solution free of glucose with mechanical stimulation and analyzed by the method of Johann & Lentini (t60).

Statistical Analysis

The statistical description of the data was made using mean±standard deviation. The comparison of the temporal variations within each treatment was evaluated with the Student's t-test for paired samples. In all cases a p-value<0.05 was established for statistical significance. In order to analyze differences between treatments at time 60, the Kruskal Wallis test and post hoc orthogonal contrasts was used, at a p-value <0.05 for statistical significance. The data was analyzed with the Infostat professional version 2018 software program.

Table 1. Experimental and control groups included in this study.

Groups	Experimental Condition
CONTROL (5-FU+ LV)	Food and drink ad libitum, without drug
CONTROL (cyclophosphamide)	Food and food ad libitum, without drug
5-FU + LV	20 and 10 mg / kg body weight i.p. of 5-FU + LV for five consecutive days
CYCLOPHOSPHAMIDE	50 mg / Kg body weight cyclophosphamide i.p. for two consecutive days.
PAIRED FEEDING (5-FU+ LV)	Average intake of animals treated with 5-FU + LV during five days without drugs.
PAIRED FEEDING (cyclophosphamide)	Average intake of animals treated with cyclophosphamide during two days without drugs.

i.p: Intraperitoneal.

Table 2. Average of food intake in experimental groups treated with 5-FU+LV.

Treatment day	Ingested food control of 5-FU+ LV (g)	Ingested food treated with 5-FU+ LV (g)	Ingested food group pair feeding without drug (g)
1	40	22	22
2	41	19	19
3	40	7	7
4	39	4	4
5	40	3	3

Table 3. Average food intake in experimental groups treated with Cf.

Treatment day	Ingested food control of cyclophosphamide (g)	Ingested food treated with cyclophosphamide (g)	Ingested food group pair feeding, without drug (g)
1	40	21	21
2	41	18	18

Table 4. 5-FU+LV. Percentage of glycogen consumption in the different experimental groups.

Group	Time 0 (µmol/g-1ps)	Time 60 (µmol/g-1ps)	*p*-value (*)	% glycogen comsumption (**)
Control	20.14 ± 0.7	10.14 ± 0.74	0.0001	49.66 %
PF	25.14 ± 0.8	12.5 ± 0.92	0.0001	50.28 %
5-Fu+LV	42.34 ± 0.73	41.5 ± 0.79	0.0204	1.98 %

ps: dry weight. **(*):** *p*-value between time 0 and time 60 minutes. Student's t-test for paired samples. **(**):** *p*-value= 0.1797C *versus* PF. *p*-value= 0.00365-FU+LV *versus* C y PF. Kruskal Wallis test and post-hoc orthogonal contrasts, for differences between treatments at time 60. (*p*-value<0.05=statistical significance).

Table 5. FCyclophosphamide. Percentage of glycogen consumption in the different experimental groups.

Group	Time 0 (µmol/g-1ps)	Time 60 (µmol/g-1ps)	*p*-value (*)	% glycogen comsumption (**)
Control	47.46±0.9	9.18±0.78	0.0001	80.66 %
PF	50.3±0.85	11.5±0.96	0.0001	77.14 %
Cf	23.97±0.75	20.68±0.68	0.0001	13.73 %

ps: dry weight. **(*):** *p*-value between time 0 and time 60 minutes. Student's t-test for paired samples. **(**):** *p*-value= 0.1797C *versus* PF. *p*-value= 0.00365-FU+LV *versus* C y PF. Kruskal Wallis test and post-hoc orthogonal contrasts, for differences between treatments at time 60. (*p*-value<0.05=statistical significance).

RESULTS.

Rats treated with 5-fluorouracil and calcium leucovorin:

In rats at time zero there was an initial concentration of 20.14±0.7µmol/g^{-1}p.s. which decreased at time 60 minutes to 10.16±0.74µmol/g^{-1}p.s., a consumption of 49.66% of the total glycogen stores.

Rats treated with paired feeding showed an initial concentration of 25.24±0.80µmol/g^{-1}p.s which decreased at time 60 minutes to 12.5±0.92µmol/g^{-1}p.s., a consumption of 50.28% of the total glycogen stores. (Figure 1)

The group 5-FU+LV showed at time zero an initial concentration of 42.34±0.73µmol/g^{-1}p.s which was reduced at time 60 to 41.5±0.79µmol/g^{-1}p.s, implying a consumption of the 1.98% of stores. (Table 4)

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Figure 1. Glycogen concentration in different experimental groups at t0 and t60.

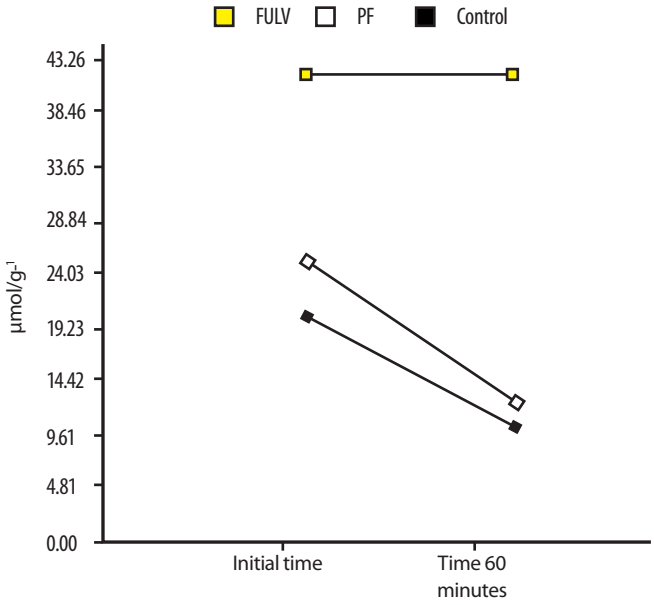
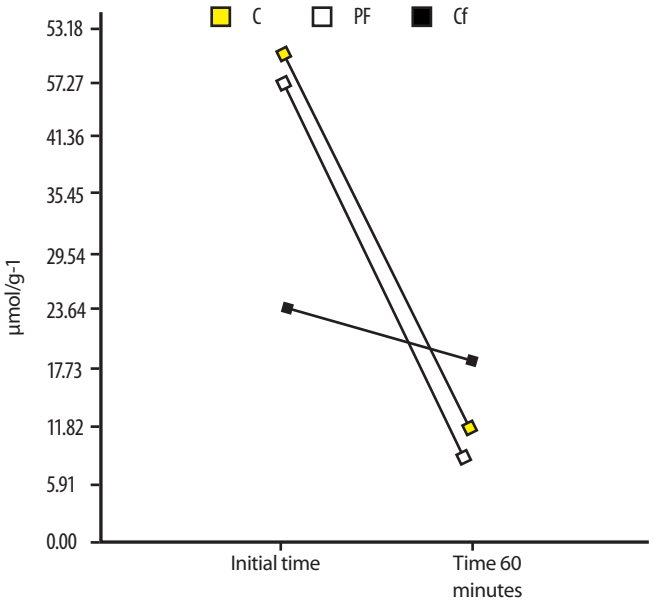


Figure 2. Glycogen concentration in different experimental groups at t0 and t60.



Rats treated with cyclophosphamide

In control rats at time zero there was an initial concentration of 47.46±0.9µmol/g^{-1}p.s. which decreased to 9.18±0.78µmol/g^{-1}p.s. in time 60, a consumption of 80.66% of the total glycogen stores. (Figure 2)

Rats treated with paired feeding showed an initial concentration of 50.3±0.85µmol/g^{-1}p.s that decreased to 11.5±0.96µmol/g^{-1}p.s. at time 60, a consumption of 77.14% of the total glycogen stores. The Cf group had a initial concentration of 23.97±0.75µmol/g^{-1}p.s, which was reduced to 20.68±0.68µmol/g^{-1}p.s, at time 60, a consumption of the 13.73% of stores. (Table 5)

DISCUSSION.

Under physiological conditions, the processes of salivary secretion require the contribution of carbohydrates as a metabolic substrate.[15] Several investigators have established the dependence of the submandibular glands on the mechanisms of glycogenolysis and glycolysis to carry out salivary secretion.[16] Thus, the variations in glandular glycogen metabolism are used *in vitro* as an indicator of their functional activity.[17]

In the present work, the basal concentration of glycogen were different in the rats treated with these cytostatic agents. There was a higher concentration of glycogen in the glands of the animals that were administered 5-FU+LV, compared to the group treated with cyclophosphamide. In both cases, this concentration was not modified by the end of the experimental period.

On the other hand, the control and feeding groups also show a significant drop in the concentration of this substrate after sixty minutes of mechanical stimulation. We have not found reports in the literature on experimental activities related to the toxic effects of these drugs on the metabolism of carbohydrates in the salivary glands.[18-19]

5-FU administration would affect glandular activity by blocking the metabolism of carbohydrates alone, while synergic action of both drugs (5-Fu+LV) would depress metabolic and nervous activity resulting in hyposalivation.[13]

However, it is important to point out that our laboratory and other authors refer to clinical alterations in the stomatognathic system and in particular in salivary glands and the quality of their secretion during the chemotherapy phase.[20-22]

Ewens *et al.*,[23] reported, among other systemic effects, the enlargement of the submandibular salivary gland as a result of the use of these drugs at higher doses. These authors also demonstrated a close correlation between drug toxicity, edema, immunosuppression, reduced salivary flow and infection.

This is attributed to the immunosuppressive effect of 5-FU, which may induce epithelial damage in the salivary gland and decreased secretion, allowing opportunistic

bacteria to settle within its stroma. From these results we could infer the toxic effect of both drugs on the metabolism of carbohydrates of the submandibular glands in the animal model used.

CONCLUSION.

5-FU+LV and Cf affect the metabolism of carbohydrates, decreasing the use of glycogen as a metabolic substrate. In the present experimental model the toxicity of these drugs affected the functional activity of the submandibular gland in rats.

Conflict of interests: The authors declare no conflicts of interest.

Ethics approval: This study approved the CICUAL Facultad de Ciencias Médicas de la Universidad Nacional de Córdoba.

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