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ARTICLE

DOES SYSTEMIC ANTICANCER GEMCITABINE COMPROMISE ORAL SOFT TISSUE WOUND HEALING?

¿La gemcitabina anticancerígena sistémica compromete la cicatrización de heridas en tejidos blandos orales?

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ABSTRACT:

Background: Numerous types of cancer are of substantial medical and social concern, posing a major challenge to modern medicine. Chemotherapeutic drugs include the use of nucleosides, which are composed of nucleic acid and sugar.

Objective:This study aims to assess the impact of systemic chemotherapeutic drugs at a therapeutic dose on the wound healing process of the oral mucosa.

Material and Methods: 30 healthy rats were randomly divided into two main groups based on the study material, 15 rats in each group. Group A (control) was given a single dose of normal saline (1ml/kg, intraperitoneal), and Group B (study) a single injection of gemcitabine (50 mg /Kg, intraperitoneal). After anesthesia, a full-thickness soft tissue incision (0.5 cm length) on the right side of the buccal mucosa was made in the animals of both groups. Each group was subdivided according to the time of sacrifice into 3, 7, 14 days after surgery, at the end of the experimental periods, specimens were collected for histopathological study, and samples of blood were obtained from retro-orbital venous plexus and collected in microfuge tubes and levels of antioxidant enzymes were measured by ELISA. The data were analyzed statistically at a 0.05 level of significance.

Results: Gemcitabine delayed the onset of wound cascade (inflammation and re-epithelization) which lead to worsening healing of the oral tissue; it also resulted in a decrease of the antioxidant activity of glutathione peroxidase and catalase, as well as activated caspase 3, which induces cell apoptosis.

Conclusion: Gemcitabine showed negative feedback on oral tissue wound healing through delayed wound healing cascade and by inducing apoptosis.

KEYWORDS:

Mouth mucosa; Antineoplastic agents; Gemcitabine; Wound healing; Inflammation; Apoptosis.

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RESUMEN:

Antecedentes: numerosos tipos de cáncer son motivo de gran preocupación médica y social, lo que representa un gran desafío para la medicina moderna. Los fármacos quimioterapéuticos incluyen el uso de nucleósidos, que están compuestos de ácido nucleico y azúcar.

Objetivo: Este estudio tiene como objetivo evaluar el impacto de los fármacos quimioterapéuticos sistémicos a una dosis terapéutica en el proceso de cicatrización de heridas de la mucosa oral.

Material y Métodos: 30 ratas sanas se dividieron aleatoriamente en dos grupos principales según el material de estudio, 15 ratas en cada grupo. Al grupo A (control) se le administró una dosis única de solución salina normal (1 ml/kg, intraperitoneal) y al grupo B (estudio) una inyección única de gemcitabina (50 mg/kg, intraperitoneal). Después de la anestesia, se realizó una incisión de tejido blando de espesor total (0,5 cm de longitud) en el lado derecho de la mucosa bucal en los animales de ambos grupos. Cada grupo se subdividió de acuerdo al tiempo de sacrificio en 3, 7, 14 días después de la cirugía, al final de los períodos experimentales se colectaron especímenes para estudio histopatológico, se obtuvieron muestras de sangre del plexo venoso retroorbitario y se recolectaron en tubos de microcentrífuga y los niveles de enzimas antioxidantes se midieron por ELISA. Los datos se analizaron estadísticamente a un nivel de significación de 0,05.

Resultados: La gemcitabina retrasó el inicio de la cascada de heridas (inflamación y reepitelización) que condujo a un empeoramiento de la cicatrización del tejido oral; también resultó en una disminución de la actividad antioxidante de la glutatión peroxidasa y la catalasa, así como de la caspasa 3 activada, que induce la apoptosis celular.

Conclusión: La gemcitabina mostró retroalimentación negativa sobre la cicatrización de heridas del tejido oral a través de una cascada de cicatrización retardada y mediante la inducción de apoptosis.

PALABRAS CLAVE:

Mucosa Bucal; Antineoplásicos; Gemcitabina; Cicatrización de Heridas; Inflamación; Apoptosis.

INTRODUCTION.

Chemotherapy is one of the most important cancer treatment strategies. Gemcitabine is an example of a nucleoside analog used in the treatment of neoplasms. Gemcitabine hydrochloride (HCl) is one of the antimetabolite cytotoxic drugs available on the market in the freeze-up form of aqueous solution identified as Gemzar which is administered as an infusion.[1,2,3]

Gemcitabine, a prodrug, is intra-cellularly transformed by deoxycytidine kinase to difluorodeoxycytidine monophosphate, which is then converted sequentially to two active metabolites, gemcitabine triphosphate and gemcitabine diphosphate.[4] The metabolite of gemcitabine has the extraordinary activity of enhancing biological processes of global inhibitory activities of cell growth and inducing apoptosis.[5]

Apoptosis encompasses sequences of tightly organized proceedings, categorized by blebbing of the membrane, cell shrinkage, DNA condensation, fragmentation, and positional organelle loss.[6]

Normal and malignant cells are affected by chemotherapy drugs via modifying activity through one or more phases of the cell cycle, the normal cells show a superior capacity to heal and recover than malignant cells, this fact can be used to attain the therapeutic effect of cytotoxic chemotherapy.[7,8] Multiple methods are used to treat cancer patients, all of which affect their ability to repair wounds. The formation of reactive oxygen species (ROS) is required during the wound healing process as a defense against bacterial infections. On the other hand, excessive ROS exposure causes oxidative stress and inhibits wound healing.[9] To the best of our knowledge, there are no available studies

about the effect of gemcitabine anticancer on oral wound healing. The purpose of this research was to examine the effects of intraperitoneal injection of gemcitabine at a therapeutic dose on the healing of buccal mucosa wounds from a histological and biochemical standpoint.

MATERIALS AND METHODS.

Study design and sample size

All animal procedures involved in this study were conducted in compliance with the guidelines of the National Institutional Health Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985).

Ethical standards were considered in all steps of performing procedures and animal handling and the study was approved by the Ethical Committee [Approval letter No. UoM.Dent/A.L.66/21].

The sample size was calculated based on the single mean formula [n = (z r/D)2]. In this study, n was considered as the number of sample subjects', z (constant) = 1.96 for 95% confidence, r (standard deviation) = 0.4mm,[10] and D (precision) = 0.2 unit. The resulting number was adjusted, and the final sample size in each group = n + (n_0.2). Thirty healthy albino male rats, whose weight was about 200-250 grams were collected from the animal house of the experimental research unit at the College of Veterinary Medicine.

They had been kept in rodents' plastic cages with wire mesh covers at a temperature of (23°C) and fed standard laboratory food and water on a 12 hr light/12 hr dark cycle ad libitum.

Experimental design

A total of thirty rats were included in this study. This study is a single-blinded randomized study in which animals were randomly divided into two groups (15 rats each) based on the study material: Group A (control) was given a single dose of normal saline of 0.9% (1ml/kg, intraperitoneal), Group B single injection of gemcitabine anticancer (50 mg/g, intraperitoneal) (Gemtu®, Turkey).

Surgical procedure

Intraperitoneally, rats in groups A and B were administered ketamine hydrochloride (anesthetic) at 50mg/kg combined with 10mg/kg xylazine (muscle relaxant, sedative, and analgesic).

The rats' reflexes were examined after 5 minutes to establish that an anesthetic had been successfully administered. Full-thickness soft tissue incision (0.5cm length) in the right side buccal mucosa is incised in both groups. The operated animals were kept in separate cages until they were fully recovered from anesthesia.

Their physical activity was extensively monitored during the first twenty-four hours after surgery. Within 3 and 4 hours postoperatively, all animals resumed regular daily activities including eating. Each main group (A and B) were subdivided according to the time of sacrifice as follows: 3, 7, 14 days after surgery (of 5 rats each). At the end of the experimental periods, blood samples were obtained from the retro-orbital venous plexus under mild ether anesthesia using microhematocrit capillary tubes and stored in microfuge tube to determine levels of the antioxidant enzymes catalase and glutathione peroxidase, as well as levels of malondialdehyde (MDA) and caspase-3 by ELISA.

Tissue preparation for histological study

The animals were euthanized, and the buccal mucosa of each rat was prepared for histological examination by immersing in a 10% buffered formalin solution for 24 hours to fix it; following that, the tissues were dehydrated by gradually increasing alcohol concentrations ranging from 30% to 100% during a 5-minute interval for each concentration.

After clearing the samples in two successive xylene changes, they were embedded in paraffin wax for final sectioning. Following that, the cross-sections of the samples were marked with hematoxylin and eosin (H&E) stains to assess the histological changes using a light microscope, as described 11-13 with minor modifications. The data collected was subjected to statistical analysis.

Statistical Analysis

The statistical analysis was performed using Statistical Package Social Statistics (SPSS) version 21

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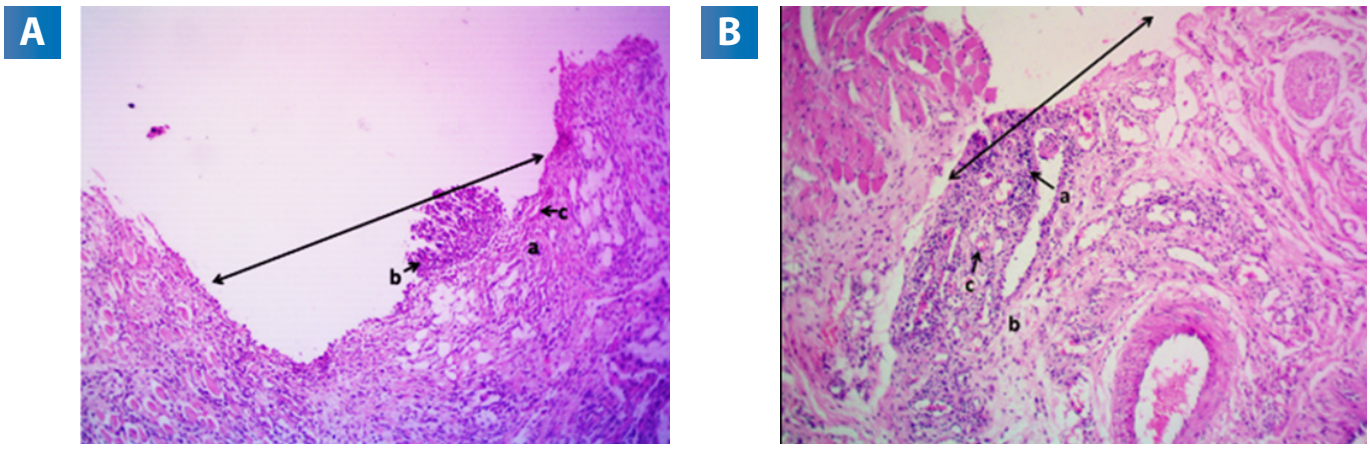
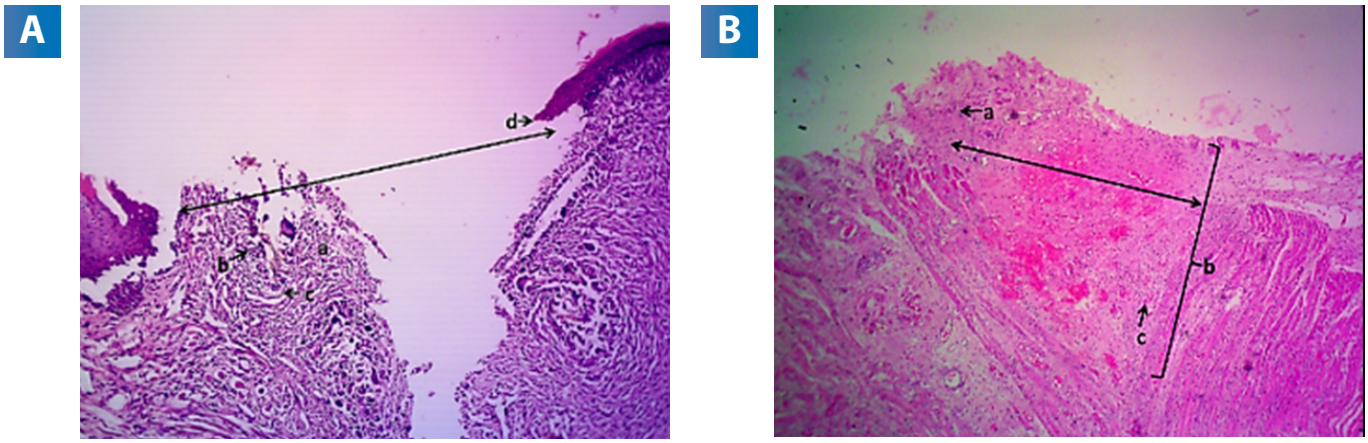
for Windows software. To assess histopathological examination differences between the control and study groups, a Mann-Whitney test was performed. An independent sample T-test was used to compare the differences in biochemical findings. A *p*-value of 0.05 was considered statistically significant.

Figure 1. Photomicrograph of oral mucosa at three days post treatment for both groups.



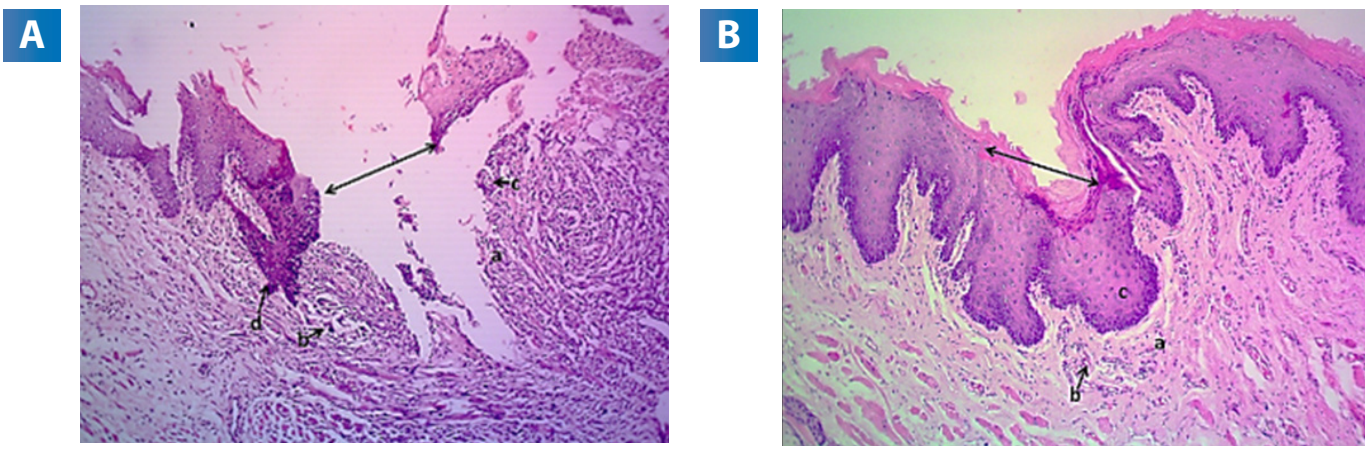
A: For the control group, a thin mucosa covered the wound, with an irregular arrangement of granulation tissue (b&c), with fewer inflammatory infiltrated cells (a).
B: For the gemcitabine treated group, there was no re-epithelialization over the wound edge, with a low infiltrate of inflammatory cells (a&b) and few blood vessels © and fibroblasts. Hematoxylin and eosin, x100.

Figure 2. Photomicrograph of oral mucosa at seven days post treatment for both groups.



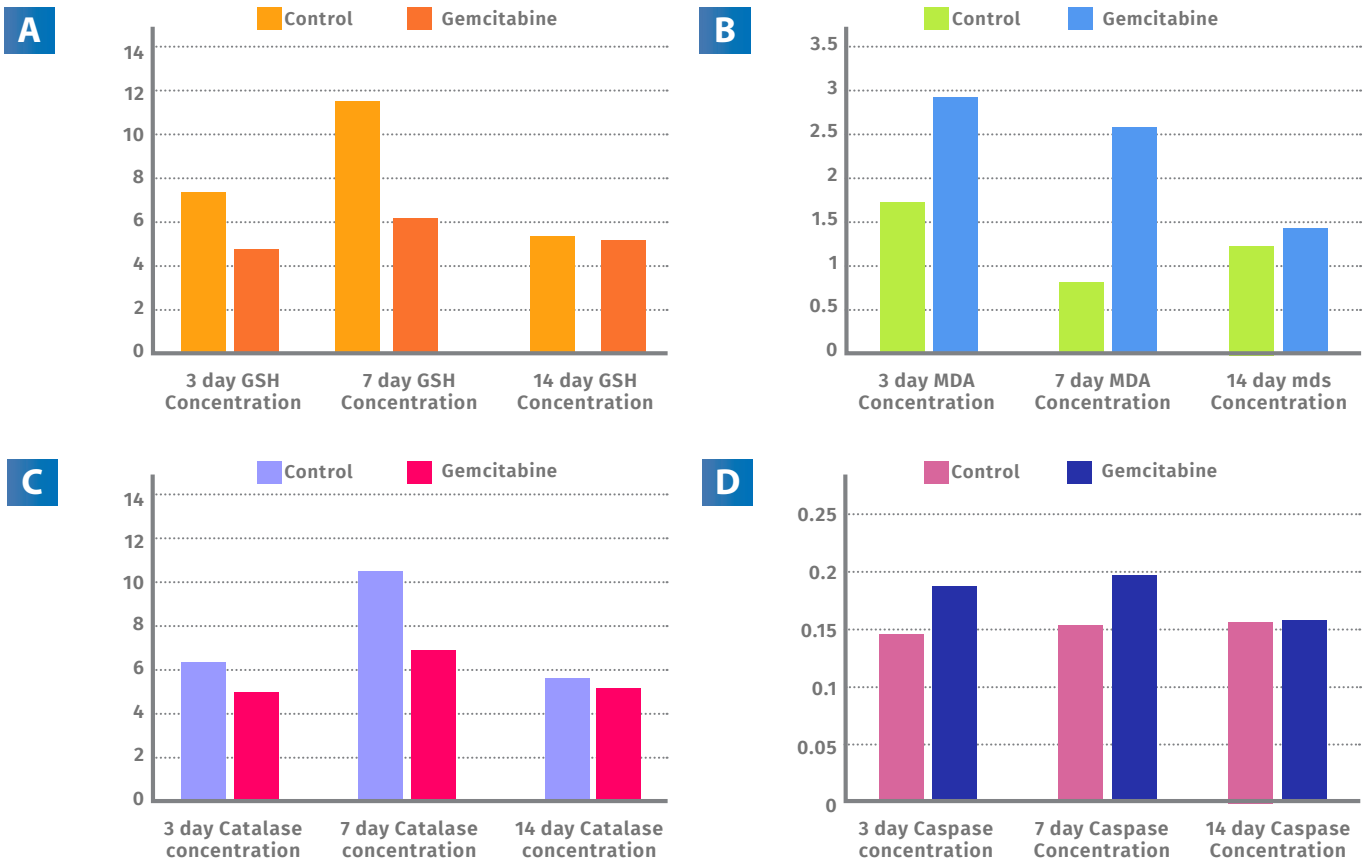
A: For the control group, the scab remained attached to the thin mucosa that covered the incision, whereas the submucosa was filled with fibrous tissue (d), well-vascularized contains numerous blood vessels were visible (b&c), as well as a minimal amount of granulation tissue (a) with absences of inflammatory cells.
B: The treated group still showed absence of re-epithelialization over the wound edge with an infiltrate with inflammatory cells (a) and few blood vessels and fibroblasts (b&c). Hematoxylin and eosin, x100.

Figure 3. Photomicrograph of oral mucosa at 14 days post treatment for both groups



A: In the control group the wound was completely reepithelialized
B: The treated group exhibited normal epithelial thickness and appearance, with difficulties identifying the wound line and complete lack of inflammation. Hematoxylin and eosin, x100.

Figure 4. Mean concentration of four biochemical parameters in blood serum in control and treated group at three different time points post intervention.



A: Mean concentration of glutathione peroxidase (GSH ng/ml) in blood serum in control and treated group at three diferent time points post intervention.
B: Mean concentration of malondialdehyde (MDA, ng/ml) in blood serum in control and treated group at three diferent time points post intervention.
C: Mean concentration of catalase (ng/ml) in blood serum in control and treated group at three diferent time points post intervention.
D: Mean concentration of caspase-3 (ng/ml) in blood serum in control and treated group at three diferent time points post intervention.

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Table 1. Histopathological assessment of gemcitabine on oral wound healing. Mann-Whitney statistical test was used to assess differences between two independent groups.

HEALING PERIODS	PATHOLOGICAL READINGS	MEAN RANK Control group to Study group	*p*-value
3 days N=(10)	Inflammation	8.0	.005*
		3.0	
	Granulation Tissue	7.5	.018*
		3.0	
	Reepithelialization	8.0	.005*
		3.0	
	Angiogenesis	8.0	.005*
		3.0	
7 days N=(10)	Inflammation	8.0	.005*
		3.0	
	Granulation Tissue	8.0	.005*
		3.0	
	Reepithelialization	8.0	.005*
		3.0	
	Angiogenesis	8.0	.005*
		3.0	
14 days N=(10)	Inflammation	5.5	1.00
		5.5	
	Granulation Tissue	6.6	.212
		4.4	
	Reepithelialization	5.4	.906
		5.6	
	Angiogenesis	4.0	.221

*: Statistical significance.

RESULTS.

Histological assessment of buccal mucosal wound repair:

After three and seven days of healing

Gemcitabine's therapeutic effect persists throughout the oral mucosal healing process reflected in (Table 1). The recruitment of inflammatory cells began on day three and lasted up to day seven (Figure 1 and Figure 2). There was a 3 to 7-day lag in the anticancer drug's effect on granulation tissue formation and reepithelialization. Consequently, this impeded the wound's ability to generate new blood vessels, resulting in a slowing of the healing process.

After fourteen days of healing

Table 1, there were no statistically significant diffe-rences between the groups for any of the histopa-thological assessments at day 14.

All cells eventually returned to normal after 14 days of wound healing. The scar from the wound had disappeared, and the area had fully healed.

On day 14, histological examination of an oral mucosal incision revealed complete re-epithelialization with minimal granulation tissue and normal epithelial thickness in both groups.

There were no signs of any inflammation in both groups.

Biochemical Finding

Blood serum glutathione peroxidase (GSH)

The level of GSH in serum significantly decrease after three and seven days in rats exposed to a single injection of gemcitabine (4.68±0.1, 6.2±0.05 respectively) in comparison to control (7.268±0.05, 11.47±0.4, at days three and seven respectively), whereas there was no significant difference in GSH between control and treated groups at day 14 post treatment (5.35±0.7, 5.1±0.10 respectively) (Figure 4A).

Blood serum malondialdehyde (MDA)

The level of MDA in serum significantly increase in rats which exposed to a single injection of gemcitabine after three and seven days (2.91±0.02, 2.56± 0.5 respectively) in comparison to control on days three and seven (1.71±0.2, 0.8±0.31 respectively), whereas there was no significant difference in MDA levels between control and treated groups at day 14 post injection of gemcitabine (1. 2±0.7, 1.42±0.10 respectively). (Figure 4B).

Blood serum catalase

The level of catalase in serum significantly decrease after three and seven days in rats exposed to a single injection of gemcitabine (5.05±0.1, 7.0±0.5 respectively) in comparison to control on days three and seven (6.4±0.05, 10.51±0.2 respectively), whereas there was no significant difference in catalase levels between control and treated groups at day 14 post treatment (5.6±0.7, 5.15±0.10 respectively). (Figure 4C)

Blood serum caspase-3

The level of caspase-3 in serum significantly increased after three and seven days in rats exposed to a single injection of gemcitabine (0.187±0.04, 0.196±0.05 respectively) in com-parison to control on days three and seven (0.147±0.02, 0.152±0.023 respectively), whereas there was no significant difference in caspase-3 b levels between control and treated groups at day 14 post injection of gemcitabine (0.157±0.07, 0.160±0.01 respectively). (Figure 4D).

DISCUSSION.

The healing intervals (three days, seven days, and fourteen days) were selected as they encompass the stages of inflammation and re-epithelization. There are studies that support the hypothesis about impediment of cell metabolism, cell division, and angiogenesis by chemotherapy drugs.

In our study we assessed whether gemcitabine, a chemotherapy drug, decreases the inflammatory reaction involved in wound healing.[14-16] When the patient's immunological status decreases due to chemotherapy, there is a change from the normal microbiota of the oral cavity to opportunistic bacteria, simultaneously there is a worsening of the wound repair capacity, which exposes patients to the danger of developing oral wound infections.[17]

Histological sections of gemcitabine-treated rats at three and seven days revealed that the neutrophil cells are present in a low amount at the site of incision of buccal mucosa in treated rats, conducive to a delayed wound healing process.

Furthermore, gemcitabine interferes with replication, transcription, and translation,[18] resulting in decreased new blood vessel formation, in addition to decreased collagen fiber production as a result of restrained fibroblast proliferation, as shown in this study.

During re-epithelialization, keratinocyte migration is guided by both intracellular and extracellular events such as basement membrane remodeling, cell-cell and cell-matrix interactions, and proteinase-mediated matrix modification.

Many of these events are settled by gemcitabine, which were illustrated in the result of this study by the absence of re-epithelization at seven days, increasing the possibility of infection. Likewise, angiogenesis is also restricted. At fourteen days of the healing process, the inflammatory reaction of both groups had completely subsided with complete re-epithelialization observed.

One of the main causes inhibiting wound healing is an increase in reactive oxygen species (ROS) in wound tissue. Although the generation of ROS is required for the defense against bacterial pathogens,

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excessive ROS exposure causes oxidative stress.[19,20] In the present study, gemcitabine single-dose injection (50 mg/kg/day, i.p.) influences biochemical parameters through a significant decrease in GSH and catalase enzyme activity and an increase in MDA levels in the blood serum of rats. This result is in agreement with a previous study that showed the administration of the anticancer drug cisplatin resulted in a decrease in GSH and catalase activity.

Antioxidants may be beneficial in attenuating gemcitabine-induced tissue damage. 21 Lipid peroxidase is a critical sign of oxidative stress, and malondialdehyde (MDA) is a marker of an increase in lipid peroxidase. Initiator caspases are triggered in response to proapoptotic stimuli such as cytotoxic stimulation or particular ligand binding to cellular receptors (caspase-2, -8, -9, and -10). These caspase initiators convert inactive or pro-caspases to active caspases (caspase-3, -6, and -7). The triggered caspase can react against the initiator caspase in a variety of diseases.[22,23]

Caspases are cysteine proteases key to programmed cell death.[24] In this study gemcitabine promoted apoptosis, by increasing caspase-3 activity, which is necessary and important for apoptosis. The increase in caspase 3 in the present study is in agreement with a previous study that suggested the administration of gemcitabine to treat multiple myeloma cells caused apoptosis due to increased activation of caspase-3; the caspase activation was shown to be a key step in the induction of apoptosis by gemcitabine in multiple myeloma cell lines.

Increased DNA breakage and activation of caspase-3 activity suggest that gemcitabine produces apoptosis in a caspase-dependent way,[25,26] illustrating that caspase-3 is an important mediator in the chemotherapy-induced apoptosis pathway.[27]

The limitations of the present study are represented first in terms of the small size of the induced lesion, the response to a larger injury needs to be assessed. The interval of follow-up is short and needs to be extended to confirm that injury in the gemcitabine group is not associated with any late complications or infections.

CONCLUSION.

The present study demonstrated that gemcitabine significantly induced damage, manifested by delayed wound healing that results from inhibiting inflammation and re-epithelization, with oxidative stress produced by the drug, by decreasing the antioxidant activity of glutathione peroxidase and catalase enzymes.

Gemcitabine activated caspase 3, key for apoptosis in tissues, which could mediate the delayed oral wound healing observed by increasing rate of cell death rates.

Conflict of interests:

No conflict of interest to declare.

Ethics approval:

All animal procedures involved in this study were conducted in compliance with guidelines of the National Institutional Health Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985). Ethical standards were considered in all steps of performing procedures and animal handling and the study was approved by the institutional ethical committee [Approval letter No. UoM.Dent/A.L.66/21].

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Authors' contributions:

Naser AI: Conception; Data curation; Analysis; Research; Methodology; Resources; Software; Supervision; Validation; Display; Writing - original draft; Drafting - revision and editing.

Hamed RS: Acquisition of funds; Research; Methodology; Project administration; Resources; Software; Supervision; Validation; Display.

Taqa GA: Conception; Data curation; Analysis; Research; Methodology; Resources; Supervision; Validation; Display; Writing - Original draft; Drafting - Revision and Editing.

The manuscript has been read and approved by all the authors.

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