Scientific Research Publishing

Open Journal of Stomatology, 2025, 15(5), 85-97
https://www.scirp.org/journal/ojst
ISSN Online: 2160-8717
ISSN Print: 2160-8709

Effects of N-Acetyl-Cysteine in the Prevention of 5-Fluorouracil Induced-Oral Mucositis Hamsters

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How to cite this paper: Sá, O.M.S., Lopes, N.N.F., Alves, M.T.S., Oliva, M.L.V., Caran, E.M.M. and Carvalhaes, J.T. (2025) Effects of N-Acetyl-Cysteine in the Prevention of 5-Fluorouracil Induced-Oral Mucositis Hamsters. *Open Journal of Stomatology*, **15**, 85-97.
https://doi.org/10.4236/ojst.2025.155008

Received: April 1, 2025
Accepted: May 23, 2025
Published: May 26, 2025

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Abstract

Oral mucositis is the most common and dose-limiting complication of cancer treatment. N-acetylcysteine (NAC) is an antioxidant that minimizes oxidative stress and the lipid peroxidation of cellular membranes. **Objective:** The present study aimed to evaluate the effects of NAC as a treatment for 5-fluorouracil-induced oral mucositis in hamsters. **Methods:** The animals were divided into two groups: therapeutic NAC (group I; n = 15) and control group (group II; n = 15). Mucositis was induced in hamsters by intraperitoneal injection of 5-fluorouracil on days 0 and 2, and the left cheek pouch was irritated by superficial scratching on days 3 and 4. In group I, oral mucositis was induced and treated with NAC intraperitoneal infusion during seven days. In group II, mucositis was induced, and no treatment was done. Oral mucosa was photographed from days 4 and 7. Photographs were randomly scored according to the severity of induced mucositis (0 to 5). Excisional biopsies of the palatal mucosa were performed, and the hamsters were sacrificed. Tissue sections were evaluated for morphological and histological analysis. Samples of cheek pouches were removed from animals per group for Malondialdehyde (MDA) measurement. **Results:** Group I showed better prevention of oral mucosa structural damage than group II. Clinical evaluation demonstrated that group I had a better outcome and faster healing. Group I presented more granulation tissue and fibroblasts. According to Malondialdehyde values, group I showed a decrease in lipid peroxidation. **Conclusions:** The results suggest that NAC treatment had a positive effect in reducing mucositis severity and a pronounced

effect in treating established mucositis.

Keywords

N-Acetylcysteine, Chemotherapy, Oral Mucositis, Neutrophils, Malondialdehyde, Inflammatory

1. Introduction

Oral mucositis (OM) is characterized by erythematous and ulcerative lesions of the oral mucosa, frequently encountered in patients undergoing chemotherapy and/or radiation therapy targeting the oral cavity. These lesions can be exceptionally painful, compromising nutrition and oral hygiene while heightening the risk of both local and systemic infections [1] [2]. The management of oral mucositis necessitates exploring various therapeutic strategies, including pharmacological, nutritional, and supportive care approaches. Current treatments range from mouth rinses (e.g., saline and sodium bicarbonate) and topical agents (e.g., anesthetics and antimicrobial agents) to systemic therapies targeting the inflammation and damage caused by cancer treatments [3]-[6]. Systemic agents such as palifermin, a keratinocyte growth factor, have also been utilized with some success in reducing the incidence and severity of OM [7] [8]. However, despite advancements, effective management of OM remains a challenge, particularly in mitigating its impact on patients' quality of life.

The pathophysiology of oral mucositis, whether due to radiation therapy, chemotherapy, or a combination of both, is a complex process outlined in a five-phase model by Sonis [9]. This model includes initiation, signaling, amplification, ulceration, and healing. Initially, tissue injury occurs as a result of treatment, leading to the death of basal epithelial cells and the subsequent formation of reactive oxygen species [10]. These reactive species provoke direct cellular death and enhance inflammatory pathways, causing further cellular damage. This cascade includes notable amplifiers such as tumor necrosis factor-alpha (TNF-α). Following this process, mucosal ulcerations develop alongside increased inflammation, ultimately culminating in the epithelial healing phase marked by cell proliferation [10].

The pathogenesis of OM encompasses direct damage to epithelial and submucosal cells, alongside the inflammatory response triggered by high-dose chemotherapy (HDC). The activation of nuclear factor kappa B (NF-κB) and elevated levels of cytokines such as IL-1β, IL-6, and TNF-α have been linked to the development of OM [11]. Additionally, chemotherapy induces oxidative stress and generates reactive oxygen species, which exacerbate mucosal cell damage [9]. Consequently, the utilization of antioxidant compounds presents a promising avenue for alleviating the severity or incidence of OM complications. Various studies have investigated the efficacy of antioxidant agents such as vitamin E, β-carotene, selenium, and zinc sulfate, yielding mixed results regarding their preventive effects

against chemotherapy- and radiation-induced OM [12]-[15].

Evaluations for oral mucositis typically rely on clinical history and physical examinations, as laboratory tests and radiography are often less informative. If ulcers are observed on the hard palate, attached gingiva, or the dorsum of the tongue, cultures should be obtained to exclude viral or fungal etiologies. Severity assessment is conducted using established scales, with several developed over time [5].

N-acetylcysteine (NAC), a thiol-containing antioxidant and an amino acid derivative of cysteine, is well established for use as a mucolytic agent and as an antidote for acetaminophen-induced hepatotoxicity. NAC promotes glutathione synthesis and scavenges free radicals [16]. It is absorbed by N-deacetylase, hydrolyzed to form cysteine, which is subsequently utilized in glutathione synthesis [17]. The glutathione peroxidase enzyme family, particularly glutathione peroxidase-1, utilizes glutathione to protect cells from oxidative damage [18]. Animal studies have indicated that NAC may reduce the generation of reactive oxygen species, lower myeloperoxidase activity, and inhibit xanthine dehydrogenase/xanthine oxidase activity, all of which contribute to oxidative stress [19]. Furthermore, NAC has been shown to prevent the activation of NF-κB, thereby attenuating the inflammatory response [20].

Given that oral mucositis is the most common and dose-limiting complication of cancer treatment, exploring effective treatment modalities is crucial. N-acetylcysteine's properties as an antioxidant may help minimize oxidative stress and lipid peroxidation of cellular membranes. The present study aims to evaluate the effects of NAC as a treatment for 5-fluorouracil-induced oral mucositis in hamsters. We will assess the effects of systemic NAC supplementation on the clinical severity of OM, the degree of inflammatory response through histological evaluation of neutrophil infiltration, and the level of oxidative stress by measuring the final product of lipid peroxidation.

2. Materials and Methods

This study was approved by the Ethics Committee of São Paulo Federal University (UNIFESP) (1916/08). Thirty female Golden Syrian hamsters (*Mesocricetus auratus*), 8 weeks old and weighing approximately 150 g each, were used. The animals were kept in groups of six per plastic container, with food and water available ad libitum.

2.1. OM Induction Protocol

A well-accepted published protocol for chemotherapy-induced oral mucositis in hamsters was utilized [21]. Briefly, all animals received an intraperitoneal injection of 80 mg/kg of the chemotherapy drug 5-Fluorouracil (5-FU) on day 0, followed by an additional 40 mg/kg of 5-FU administered intraperitoneally on day 2. The right cheek pouch of each animal was everted, and the mucosa was irritated by superficial scratching using the tip of an 18-gauge needle. The scratching was consistently performed in the same location, central, and of the same size (5 cm)

by the same operator on days 3 and 4.

2.2. NAC Supplementation

The animals were randomly divided into two groups of 15 animals each. Animals in Group 1 received a 2 mg/g of body weight intraperitoneal injection of NAC (Exir Pharmaceuticals Company, Boroujerd, Iran), diluted in saline at a concentration of 5%. Treatment with the NAC was initiated on day 0, with application once per day (in the morning), for seven days. Animals in Group 2 served as controls and did not receive any glycine supplementation, but were treated identically in all other respects.

2.3. Clinical Evaluation of OM

Two blinded evaluators performed a clinical evaluation of OM. On days 3 and 7, the right cheek pouch of all animals was turned outward for the clinical evaluation of the severity of the mucositis. Mucositis scores from 0 to 5 were assigned based on the method described by Sonis *et al.*, with higher scores indicating greater severity [22] (**Table 1**).

2.4. Histological Evaluation of OM

All animals were sacrificed on day 7, and the right cheek pouch was removed. The cheek pouch samples were labeled, immediately cooled in isopentane for 10 s, and then flash frozen in liquid nitrogen. The fragments were positioned in such a way as to provide cross-sectional slices during microtomy. Serial slices (10 μm) were obtained in a cryostat at a temperature of −20˚C, placed on salinized glass slides, submerged in acetone, and dried at room temperature for 10 min. The serial sections of each sample were stained using hematoxylin-eosin staining and examined under a light microscope by a blinded pathologist. The absence or presence of microscopically visible ulceration and the severity of neutrophil infiltrate were each separately scored, using the scale described by Lopes *et al.* [23] (**Table 1**).

2.5. Measurement of Oxidative Stress: Determination of Malondialdehyde (MDA) Levels

Determination of Malondialdehyde (MDA) is a final product of lipid peroxidation and a well-established measure of the level of free radicals in intestinal tissue [24] [25]. To determine MDA levels, the Thiobarbituric acid (TBA) reaction proposed by Kohn and Liversedge [26] was used. Tissue samples were defrosted and weighed, and a volume equivalent to five times the weight of TRIS 0.01 M/pH 7.4 buffer solution was then added. Tissue samples were homogenized in an ice bath four times, for 30 seconds each, and subsequently centrifuged for 5 minutes at 10,000 rpm, at 4˚C. The protein content of the homogenate was determined by the Coomassie brilliant blue (CBB) procedure, as described by Kohn and Liversedge [26]. Briefly, the CBB reactant interacts with protein, enabling its quantification by using a standard albumin curve with known concentration.

For MDA measurement, 400 microliters of the centrifuged homogenate supernatant were collected and added to 1 ml of 20% trichloroacetic acid and 400 ml of 1.6% TBA. The mixture was incubated for 30 minutes at 95˚C. Lipids were extracted by adding n-butanol (1.6 ml) and stirring vigorously. The sample was again centrifuged for 10 minutes at 3000 rpm. Absorbance of the organic layer was determined through reading at 510, 532, and 560 nm. The following equation, proposed to minimize the interference of both heme pigments and hemoglobin in the measurement of MDA (26, 27), was used:

$$\text{MDA } 532 = 1.22\ [(A532) - (0.56)\ (A510) + (0.44)\ (A560)]$$

The calibration curve was drawn with 1,3,3-tetramethoxypropane (also known as malondialdehyde bis. MDA levels were calculated and expressed in nmol MDA/mg of protein.

2.6. Statistical Analysis

The Kappa coefficient (k) was calculated to determine inter-examiner agreement for clinical assessments of OM. Qualitative variables (clinical and histological scoring of mucositis severity) were compared using the Pearson's Chi square test and the Fisher test. Quantitative variables (MDA levels) were compared using the analysis of variance (ANOVA). All statistical analyses were performed with a significance level of 5% ($\alpha = 0.05$).

Table 1. Scales used for clinical and histological evaluation of oral mucositis.

	Grade	Criterion
Clinical Evaluation	0	Pouch is completely healthy. No erosion or vasodilatation.
	1	Erythema, but no evidence of mucosal erosion.
	2	Severe erythema, vasodilation and superficial erosion.
	3	Formation of ulcers in one or more places, but not affecting more than 25% of the surface area of the pouch.
	4	Severe erythema and vasodilation cumulative ulcer formation about 50% of pouch surface area.
	5	Virtually complete ulceration of the pouch mucosa. Loss of pliability.
Histological Assessment: Neutrophil infiltrate	0	Absent or rare neutrophil
	1	Moderate or severe neutrophil infiltrate
Histological Assessment: Ulceration	0	Ulceration absent
	1	Ulceration present

3. Results

3.1. Clinical Evaluation of OM

There was excellent inter-examiner agreement on the clinical assessment of OM Kappa = 0.80 for Group 1 (NAC supplementation) and 0.90 for Group 2 (controls). These data demonstrate that there was adequate calibration for evaluation of the clinical characteristics of OM. The mucositis induction protocol consistently caused erythema, hemorrhage and ulceration in the right cheek pouch of all animals. Thus, all animals in both groups were scored as having Grade 3 mucositis on day 3 (Figure 1). However, by day 7, there was a marked reduction in Clinical mucositis severity in most animals in the NAC group, with the majority showing healing of ulcerations. In comparison, the clinical mucositis severity in control animals stayed the same or worsened (Table 2). This difference between groups was clinically and statistically significant ($p < 0.001$).

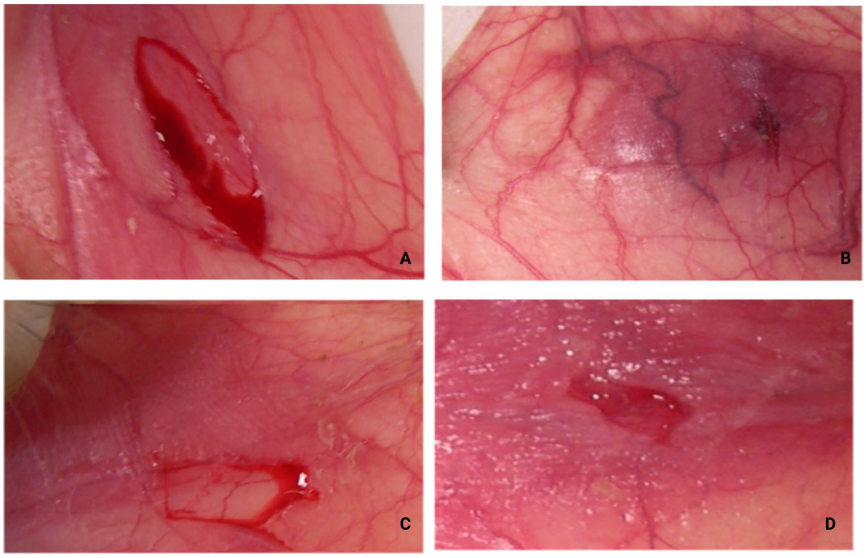

Figure 1. A representative photograph of the cheek pouch of hamsters at magnification × 400. NAC group: A) Day 3 (ulcer present) and B) Day 7 (reepithelialization of the mucosa). Control group representatives: C) Day 3 (ulcer present) and D) Day 7 (persistent ulcer).

3.2. Histological Evaluation of OM

Histopathological findings in control animals on day 7 were consistent with those previously described for this animal model and mucositis induction protocol [21]. In general, control animals demonstrated an intense cellular infiltration with prevalence of neutrophils, hemorrhagic areas, severe vascular hyperemia, edema, and ulceration. Focal points of surface bacterial colonization and abscesses were seen (Figure 2). In contrast, the NAC group generally exhibited a less intensive histopathological reaction, with discreet vascular hyperemia and slight inflammatory infiltration. On day 7, 100% of animals in the control group had a moderate-severe neutrophil infiltrate (grade 1), as compared to only 25% of animals in the NAC group (Table 2). The remaining 75% of animals in the NAC group demon-

strated minimal neutrophil infiltrate (grade 0) ($p < 0.001$). On day 7, 100% of animals in the control group demonstrated microscopic ulceration (grade 1), as compared to only 35% of the animals in the NAC group (Table 2). The remaining 65% of animals in the NAC group demonstrated re-epithelialization and healing, with absence of microscopic ulceration (grade 0) ($p < 0.001$).

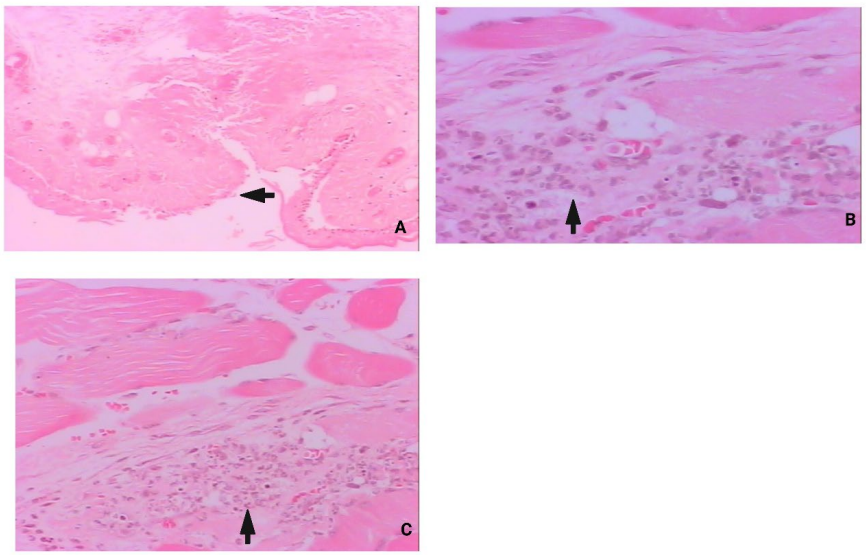

Figure 2. A representative photomicrograph of hamster oral mucosa on day 7 at magnification × 400, demonstrating ulceration and inflammatory infiltration in epithelial cells. NAC group representative: A) Absence of ulceration and inflammatory infiltration; Control group representatives; B) Moderate inflammatory infiltration; C) Intense inflammatory infiltration, ulceration and bacterial colonization.

Table 2. Clinical, histological evaluation and malondialdehyde (MDA) levels of oral mucositis.

		Clinical					
Day	**Group**	**Mucositis grade**					
		0	1	2	3	4	5
3	NAC				15		
	Control				15		
7	NAC*	1	12	1	1		
	Control				10	5	
		Histological evaluation					
		Neutrophil Infiltrate		**Ulceration**			
		Grade 0*	**Grade 1***	**Grade 0***	**Grade 1***		
7	NAC*	10	5	11	4		
	Control		15		15		

Continued

		Malondialdehyde Levels		
		MDA Nmol MDA/mg protein		
7		**Mean**	**Standard Deviation**	n
	NAC*	0.194*	0.110	15
	Control	1.072*	0.200	15

a. The table represents quantification of data, representing the number of animals with each grade of oral mucositis at each time-point, grade of neutrophil infiltrate and ulceration, at day 7 and measurement of oxidative stress: Determination of malondialdehyde (MDA) levels at day 7; *p < 0.001*.

3.3. Measurement of Oxidative Stress: Determination of MDA Levels

At day 7, the mean MDA levels in the cheek pouch of animals in the control group were more than 5-fold the MDA levels in the NAC group (Table 2). Treatment with NAC thus significantly reduced this marker of lipid peroxidation and free radical production ($p < 0.001$).

4. Discussion

In the present study, our results demonstrated that N-acetylcysteine (NAC) significantly reduces the incidence of severe oral mucositis (OM) (grades 3), decreases neutrophil infiltration and ulceration, and minimizes oxidative stress. These findings are important for the nutritional treatment of oral mucositis and highlight significant interest in effective nutrients, anti-inflammatory and antioxidant.

Oral mucositis is a complex process that involves not only direct cellular injury caused by chemotherapy or radiation, but also a cascade of biological events [27]. The process begins with clonogenic cell death and the release of reactive oxygen species (ROS), progressing through a series of steps during which multiple biological pathways are activated and amplified. This ultimately culminates in ulcer development and, eventually, healing [28]. Investigations into the pathogenesis of OM emphasize the critical role of the inflammatory response, which involves numerous inflammatory mediators, including nuclear factor kappa B (NF-κB) [2] [9] [29] [30], cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and platelet activating factor (PAF) [2] [31] [32], as well as the cyclooxygenase pathway [2] [29] [30].

The choice of the 5-fluorouracil (5-FU) protocol for this study is well documented in the literature, association between 5-FU administration and development of oral mucositis [1]-[3] [33] and model described and accepted by Sonis [21]. Establishing it as a relevant model of oral mucositis to investigate potential therapeutic interventions, such as NAC. Understanding the mechanisms underlying 5-FU-induced mucosal injury allows the development of targeted strategies to mitigate these adverse effects of chemotherapy.

NAC serves as a free radical-scavenging antioxidant [34]. Several studies have

reported its efficacy in reducing inflammation in mucous membranes, enhancing the elimination and excretion of sputum in inflammatory respiratory diseases, and inhibiting cytokine production [35]. Our current results support this anti-inflammatory role for NAC. Using a well-established animal model of chemotherapy-induced OM, we found that NAC supplementation significantly reduced the severity of clinical mucositis. The attenuated clinical severity was accompanied by a marked reduction in neutrophil infiltration, suggesting that NAC suppressed the inflammatory response associated with mucositis. Furthermore, NAC also reduced the production of damaging free radicals, as indicated by diminished malondialdehyde (MDA) levels.

The selection of a 2 mg/g dosage of NAC for this study is substantiated by prior research demonstrating its efficacy in reducing oxidative stress and inflammation associated with oral mucositis and similar conditions. NAC is recognized for its antioxidant properties and its role as a precursor to glutathione, a key antioxidant. Studies such as those by Oka *et al.* [20] have shown favorable outcomes with NAC doses ranging from 100 to 500 mg/kg, resulting in a significant reduction in oxidative stress markers in animal models. Furthermore, clinical evidence suggests that NAC dosages between 600 mg and 1,200 mg daily are effective in managing inflammatory conditions, supporting the extrapolation of similar therapeutic benefits for oral mucositis at 2 mg/g [36]. Additionally, research conducted by Jahangard-Rafsanjani *et al.* [14] demonstrated that NAC effectively alleviated the severity of oral mucositis in patients undergoing hematopoietic stem cell transplantation, reinforcing the rationale for the selected dosage. Collectively, these findings support the use of a 2 mg/g NAC dosage as a reasonable and scientifically informed choice for mitigating chemotherapy-induced oral mucositis.

The anti-inflammatory and antioxidant effects of NAC are believed to be mediated, at least in part, by its mechanism of action. NAC acts primarily as a precursor to glutathione, one of the most critical antioxidants. By increasing glutathione levels, NAC helps neutralize reactive oxygen species and reduce oxidative stress, which plays a significant role in inflammatory processes. Additionally, NAC may modulate pro-inflammatory cytokines, further contributing to its anti-inflammatory properties. These combined actions make NAC a potential therapeutic agent for various inflammatory conditions [37]. Previous animal studies indicate that NAC may decrease the production of reactive oxygen species, myeloperoxidase activity, and xanthine dehydrogenase/xanthine oxidase activity (sources of ROS) [19]. Furthermore, NAC prevents the activation of nuclear factor kappa B, which is associated with increased inflammatory responses [20].

NAC exerts its therapeutic effects on oral mucositis through several mechanisms. One pivotal action of NAC involves the suppression of NF-κB, a crucial transcription factor that regulates the expression of pro-inflammatory cytokines. By inhibiting NF-κB activation, NAC reduces the production of cytokines such as TNF-α and IL-6, which are known to mediate inflammatory responses and contribute to the pathogenesis of oral mucositis [28]. This modulation of cytokine

levels explains the observed reductions in MDA levels, a marker of lipid peroxidation, and neutrophil infiltration noted in the study. Furthermore, NAC enhances glutathione levels, which neutralize reactive oxygen species, thereby reducing oxidative stress and inflammation [38] [39]. Consequently, these findings underscore NAC's role not only as an antioxidant but also as an anti-inflammatory agent.

Nutritional supplementation with anti-inflammatory and antioxidant effects plays a crucial role in the management of oral mucositis. Sa *et al.* [40] demonstrated the positive effects of glycine supplementation in healing oral mucositis by enhancing collagen maturation and mitigating oxidative stress, thereby positioning it as a supportive therapy for managing chemotherapy-induced mucosal injuries associated with 5-fluorouracil (5-FU). Our results indicate similar effects with N-acetylcysteine (NAC), which modulates oxidative stress and inflammatory markers due to its antioxidant properties. These findings support the use of nutritional strategies, including NAC supplementation anti-inflammatory and antioxidant to effectively manage oral mucositis.

This study has limitations that should be considered. Firstly, the short follow-up period of only 7 days restricts the evaluation of long-term healing and the durability of NAC's effects on oral mucositis, potentially overlooking sustained impacts beyond the immediate outcomes observed. Secondly, the reliance on an animal model limits the generalizability of the findings to human populations, where individual variability, concurrent therapies, and nutritional differences could significantly influence treatment outcomes. Lastly, further research is needed to explore NAC's effects at different stages of mucositis development and its impact on other relevant markers of oral mucositis. Addressing these limitations emphasizes the necessity for extended follow-up periods and clinical trials in human subjects to validate and optimize the therapeutic benefits of NAC.

5. Conclusion

In conclusion, NAC supplementation significantly reduced chemotherapy-induced oral, mucosal injury, neutrophil infiltrate and free radical production in an animal model of oral mucositis.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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