

Vitamin C Mitigates Radiation-Induced Parotid Glands Inflammation by Downregulating TNF- α and IL-1 β Expression in Guinea Pigs

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Abstract

Background: Radiation therapy damages surrounding tissues, with parotid glands being highly vulnerable in the head and neck region. Antioxidant and anti-inflammatory properties of vitamin C may reduce radiation-induced injury. This study therefore investigated the impact of vitamin C on morphological and histological changes in guinea pig parotid glands and its impact on tumor necrosis factor-alpha (TNF- α) and interleukin-1 beta (IL-1 β) expression.

Method: 48 Dunkin Hartley guinea pigs (6-8 months, both sexes) were divided into four groups: control, vitamin C (3 mg/kg), radiation 8 Grays (Gys), and radiation + vitamin C. Animals were treated with radiation and/or vitamin C for 15 days on alternate days. Weight and saliva volume were measured on alternate days too. Parotid glands were extracted for histological and immunohistochemical analysis of TNF- α and IL-1 β . Data were analyzed using SPSS (version 21) and presented as mean \pm standard error of mean. One-way ANOVA with Bonferroni's post hoc test was applied, with $P < 0.05$ considered significant.

Results: Over 15 days, no significant differences in animal weight were observed ($P > 0.05$). However, a significant reduction in saliva production occurred in the radiation group, alleviated by vitamin C ($P < 0.05$). Histological analysis showed radiation-induced inflammation in the parotid glands, which was diminished by vitamin C. Immunohistochemistry revealed a significant increase in TNF- α and IL-1 β expression in the radiation group ($P < 0.001$), with vitamin C significantly downregulating both markers in the radiation + vitamin C group ($P < 0.001$).

Conclusion: Vitamin C may protect against radiation-induced parotid gland inflammation by downregulating TNF- α and IL-1 β , suggesting its potential as a therapy for radiation-induced tissue injury.

Keywords: Parotid glands, Radiation, Vitamin C, TNF α , IL-1 β

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Introduction

Head and neck cancer is the seventh most prevalent malignancy worldwide, with more than 660,000 new cases and 325,000 fatalities per year.^{1,2} All epithelial malignancies arising in paranasal sinuses, oral cavities, nasal cavities, larynx and pharynx, can be collectively categorized as a broader term of head and neck cancer. With very few exceptions, these are mostly squamous cell carcinomas.¹ The prevalence of this condition has been on the rise, with reports indicating changes in its etiology due to reduced smoking, especially in more industrialized countries.³

Surgery and radiotherapy have long been the major treatment approaches for head and neck cancers at all stages.⁴ However, because of the proximity of nerves, brain stem, spinal cord, and brachial plexus, salivary glands, mucous membranes, and swallowing muscles in the head and neck regions, radiotherapy inevitably causes damage to these normal tissues.⁵ Mucosal toxicities leading to oropharyngeal mucositis are common among patients receiving radiotherapy. A significant increase in the intensity and incidence of mucositis in oropharyngeal region occurs if radiotherapy is combined with chemotherapy. This is particularly pronounced with multidrug chemotherapy or accelerated or hyper-fractionated radiation.⁶

Moreover, salivary glands are highly sensitive to radiation therapy, and damage is generally irreversible at higher doses. More than half of the patients who receive radiotherapy involving major salivary glands experience the perception of hyposalivation, named radiation-induced xerostomia.^{7,8} Xerostomia is one of the most damaging long-term adverse effects of multimodal therapy in patients with locally progressed head and neck cancer.⁹ The clinical signs of xerostomia include dysphagia, painful swallowing, trouble sleeping, and difficulty in speaking. These complications compromise the quality of life of the patient and may worsen organ function over time resulting in interruption in radiotherapy use and ultimate cure.^{5,6}

Further, radiotherapy also causes significant

damage to normal cells around the tumor cells,^{10,11} and adverse effects are reported to be associated with the development of inflammation in the head and neck region. Several inflammatory markers such as tumor necrosis factor (TNF), interleukin (IL)-3, and IL-6 have been implicated in radiation associated damage.^{12,13} It has also been shown that vitamins can act as protective agents against radiation induced damage. Vitamin C (ascorbic acid), a commonly used vitamin, exhibits strong antioxidant effects due to its ability to combat free radicals.¹⁴ A study has shown that intraperitoneal administration of 3g/kg vitamin C to mice that were exposed to 7 to 8 Gray (Gy) of whole-body radiation had a higher survival rate as compared with the mice receiving only radiation. In addition, vitamin C has also been shown to reduce radiation-triggered apoptosis in bone marrow cells, and thus restores hematopoietic functions.¹⁵ Oral administration of vitamin C within 24 hours of radiation has also been reported to prevent the damaging gastrointestinal syndrome in mice. Accordingly, it has been suggested that vitamin C has the potential to be used as a protective agent against radiation induced damage.¹⁶ Vitamin C has also been found to reduce reactive oxygen species production and radiation-induced elevation of inflammatory cytokines in bone marrow cells.¹⁵ Further, Jafari et al. have also provided compelling evidence of the radio-protective efficacy of vitamin C against oxidative stress, irrespective of its administration timing relative to radioactive iodine (RAI) therapy. Notably, the study indicates enhanced benefits in the pre-RAI treatment group, suggesting a predominantly protective role rather than a mere attenuating influence. However, this investigation primarily focused on antioxidant properties of vitamin C and like other studies did not investigate its direct impact on salivary gland function.¹⁷ Notably, the improvement in salivary gland function was not consistently replicated in a separate study.¹⁸ The divergence in outcomes between these studies stems from methodological distinctions; while the former measured serum oxidative stress markers, the latter assessed salivary function through scintigraphy. As a result,

a direct comparison of these findings is not feasible. Thus, to comprehensively elucidate the potential protective effects of vitamin C, additional research efforts specifically focused on salivary gland are warranted.¹⁹ To the best of our knowledge, no study has been done yet to explore the effect of vitamin C on the expression of inflammatory markers after radiation (head and neck region) induced damage in parotid gland specifically. Therefore, this study investigated the effect of vitamin C against radiation induced damage of parotid glands using histology and further align these changes with the effect of vitamin C on the expression of inflammatory markers in parotid gland by using guinea pigs as model organism.

Methodology

Ethical approval

Before starting the study, ethical approval was taken from the Institutional review board (IRB) and Animal Ethical Committee. Approval ID is 1536/DUHS/Approval/2020 and AR.IRB-016/DUHS/Approval/2020/026, respectively. All the procedures were carried out in accordance with the Institutional Review Board's ethical guidelines and the Committee on Animal Research and Ethical Committee international guidelines for animal use and care.

Study animals, drugs and radiation dose

Total 48 (12 each group) guinea pigs weighing 500gms to 900gms were used in this study. The animals were housed under standard laboratory conditions, that is, 12: 12-hour light: dark cycle, temperature ($21 \pm 2^\circ\text{C}$), and air humidity-controlled environment. Food and water were provided ad libitum. Guinea pigs were divided into four equal groups: Group A or control group (receiving only saline), group B or vitamin C group (receiving only vitamin C; 3mg/kg), group C or radiation group (receiving only radiation; 8 Gy group D or radiation + vitamin C group (receiving both radiation and vitamin C). Vitamin C (3mg/kg) [Tablet Abbott Laboratories Pak Ltd., Karachi, Pakistan-Batch #02-132-R13] and saline were administered via intraperitoneal route to all

the animals in the respective groups on alternate days for 15 days (total 8 days). While 8 Gy of radiation was given as fractionated doses of Gamma rays given as 1 Gy/minute on alternate days for 15 days on Cobalt 60. Animals were weighed on alternate days for 15 days, while saliva was collected and measured on 3rd, 7th, and 15th day of the first exposure to radiation. All the animals were sacrificed on the 16th day and parotid glands were isolated for further histological and immune-histological studies.

Weighing of animals

Digital weighing machine (Crown Professional) was used to measure the weight of animals. Briefly the machine was set at zero gram and then guinea pigs were taken out from the cages one by one and were put on the weighing machine. When the reading was stabilized, the weight was noted. Animals were weighed on alternate days for 15 days parallel to the treatment days.

Collection of saliva

Saliva samples were collected on the 3rd, 7th and 15th day of the study protocol by putting the insulin syringe (without needle) into the animal's mouth, inside the cheeks and tapped approximately for one minute for saliva collection. Saliva was then transferred to the Eppendorf tube, centrifuged and then re-sucked in to the syringe to measure the volume.²⁰

Dissection

At the end of the experimental protocol or treatment duration i.e., on the 16th day, all animals were sacrificed under deep anesthetized by an intramuscular injection of ketamine hydrochloride (60mg/kg) and xylazine (5mg/kg). The thoracic cavity was opened through perpendicular incision and the cardiac perfusion was performed with chilled phosphate buffer saline (PBS) having heparin.

Isolation of parotid gland

The parotid gland was removed with the incision on outer canthus of eye than vertical incision till base of mandible with the help of surgical instruments as the parotid gland is located on lateral surface of ramus of mandible. After collecting both right and left parotid glands, their

gross morphological study was done, and tissue samples were saved at -80 degree centigrade for further histological and immunohistochemical investigations.

Histopathology

The standardized tissue processing steps were adopted to make tissue paraffin blocks. Three to five micrometer thick serial histological sections were obtained of each tissue block by using the microtome. In the next step, the tissue slides were stained with hematoxylin and eosin (H & E) stain by adopting the serial method.²¹ The stained tissue slides were studied under the light microscope to evaluate the histological alteration. Three fields in each section were evaluated, the microscopic anatomy of the tissue was observed in detail at 40× to evaluate the inflammatory changes.

Immunohistochemistry of TNF α and IL-1 β

After the completion of the experiment parotid gland were removed and postfixed in 10% neutral buffered formalin and then cryo-protected overnight in 30% sucrose in PBS at 4°C. Tissue molds were prepared using optimal cutting temperature (OCT) media for cryo-sectioning and then placed at -20°C to freeze the OCT media blocks. Then, 20 μ m sections were cut and laid on slides. Slides were stored at -20°C for the next day's experiment. For immunostaining, slides were placed in a humid chamber at an ambient temperature for half an hour and then were rehydrated by rinsing with PBS (three times for 5 min). Immunoblock 1% (Carl Roth, Germany) was prepared in deionized water and used as a blocking agent. After rehydration with PBS, slides were incubated with immunoblock for 20 min at a 42° C humid chamber. After incubation, slides were washed with PBS (three times for 5 min) and incubated with the primary antibody either Rabbit anti-guinea pig TNF α or Rabbit anti-guinea pig IL-1 β for 45 min at 42° C in a dark chamber and then washed with PBS for 5 min (3×). After washing, slides were incubated with secondary antibodies (Anti-rabbit FITC IgG) and again incubated for 45 min at 42° C humid chamber. After incubation slides were taken out and washed with PBS (3×) for 5 min and then 1 drop/ml of 4, 6-diamidino-2-phenylindole stain

(DAPI) (nenBlue) was added and slides were allowed to stand at room temperature for 5 min to stain cell nuclei. Slides were finally washed with PBS 3× for 5 min and cover slips were mounted with the help of immune-mount (Thermo fisher). Slides were then laid at an ambient temperature for overnight drying in a dark chamber and then stored at -20° C. The slides were examined by fluorescence microscope (Nikon Eclipse Ni-E) and images were taken through a CCD camera using NIS Element AR 3.2 software at 20× magnification. Images were further processed through NIH Image J software.

Statistical analysis

The results were analyzed by statistical package for the social sciences (SPSS) version 21 software and Mean \pm standard error (SE) of mean values were used to describe the data. Further the data were analyzed using the one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test and all the values below 0.05 were considered significant.

Results

Effect of treatment on body weight (grams)

All guinea pigs were weighed every other day throughout the 15-days course of radiation and vitamin C treatment. Figure 1 illustrates the variations observed in the weight of animals across both the control and various treatment groups. In the control group, the mean weight was initially recorded at 583.75 \pm 13.57 grams on the first day and subsequently fluctuated to 571.16 \pm 12.41 grams on the 15th day. Conversely, in the vitamin C group, the weight ranged from 574.11 \pm 13.57 grams to 558.92 \pm 12.41 grams. In the radiation-only group, it transitioned from 622.75 \pm 16.52 to 611.25 \pm 16.32 grams. In the radiation + vitamin C group, weight was observed to be within the range of 629.58 \pm 18 to 624.66 \pm 18.88. In summary, a slight decrease in weight was observed in all experimental groups by the end of the 15-day study period. However, it is important to note that these changes were not statistically significant ($P > 0.05$) (Figure 1).

Effects of vitamin C on saliva production

The impact of radiation and vitamin C

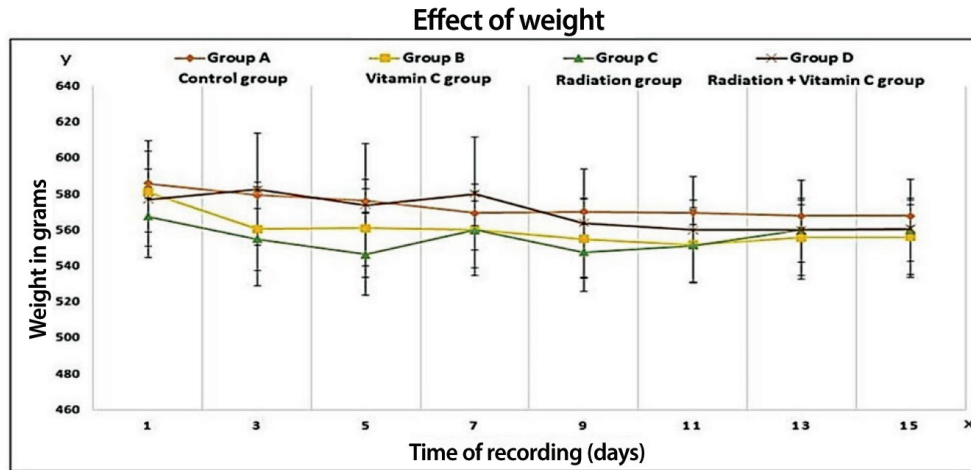


Figure 1. Effect of radiation and vitamin C treatment on the weight of guinea pigs. The graph shows variation in weight of animals in control and different treatment groups from day 1st to day 15th. No significant difference was found in weight of any of the group during 15 days of treatment ($P > 0.05$).

treatment on saliva production was evaluated by quantifying the saliva volume on the 3rd, 7th, and 15th days of the radiation protocol, as depicted in figure 2. When compared with the control group, a statistically significant increase in saliva production was observed only in the radiation + vitamin C group on the 1st day ($P < 0.05$). Furthermore, at the end of experiment duration or on 15th day the mean saliva production significantly reduced in the radiation only group as compared with the control. While there was

no significant difference in saliva production in vitamin C, radiation + vitamin C as compared with the control (Figure 2).

Gross morphology of parotid glands

Upon examination of gross morphology, all the extracted parotid glands appeared pyramidal shaped and exhibited a subtle pinkish to yellowish hue in appearance.²² However, notable differences were observed in the parotid glands of the radiated guinea pigs when compared with those of the control group, as well as the guinea pigs in the

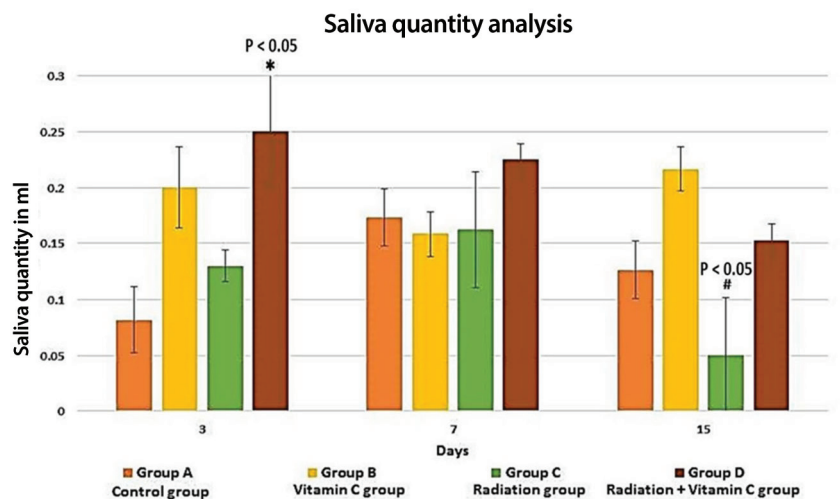


Figure 2. Effect of radiation and vitamin C treatment on saliva production. Bar graph showing volume of saliva produced by the animals in vitamin C, radiation + vitamin C and radiation groups compared from the control group on day 3, day 7 and day 15. Significant reduction in saliva production was observed in radiation groups on the 15th day ($P < 0.05$), while there was no significant difference in saliva production vitamin C only and radiation + vitamin C groups ($P < 0.05$) on day 15.

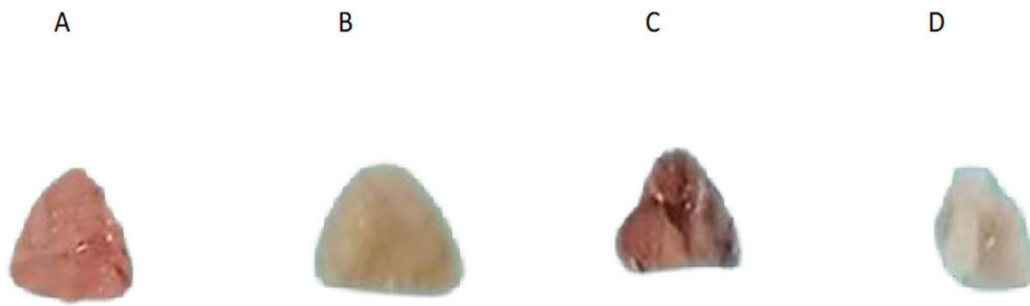


Figure 3. Photographs showing gross morphology (size and color) of parotid glands of guinea pigs in different groups. Where, A represents control group, B represents vitamin C only group, C and D represents radiation only and radiation + vitamin C group, respectively. The parotid gland appears reddish in the radiation only group (group C) compared with the other groups. No notable change in size was observed.

vitamin C only and radiation + vitamin C groups. Precisely, the parotid glands in the radiated guinea pigs appeared visibly dark reddish in color (indicating inflammation) in contrast to those in the other experimental groups (Figure 3).

Histopathological examination

To investigate the protective effect of vitamin C on radiation-induced inflammation, the histology of parotid glands was done. Briefly, the parotid glands were fixed in a 10% buffered formalin solution and subsequently subjected to

the standard paraffin-embedding protocol. Following this, sections were stained using H&E staining. All histological images were captured at a magnification of 40 \times . Microscopic examination of the images revealed the presence of cuboidal epithelium with centrally located round nuclei, acinar cells, acini, and both secretory and excretory ducts. The overall tissue architecture appeared normal, with no signs of inflammation in the parotid gland tissue in both the control and vitamin C groups. On the contrary, in the radiation

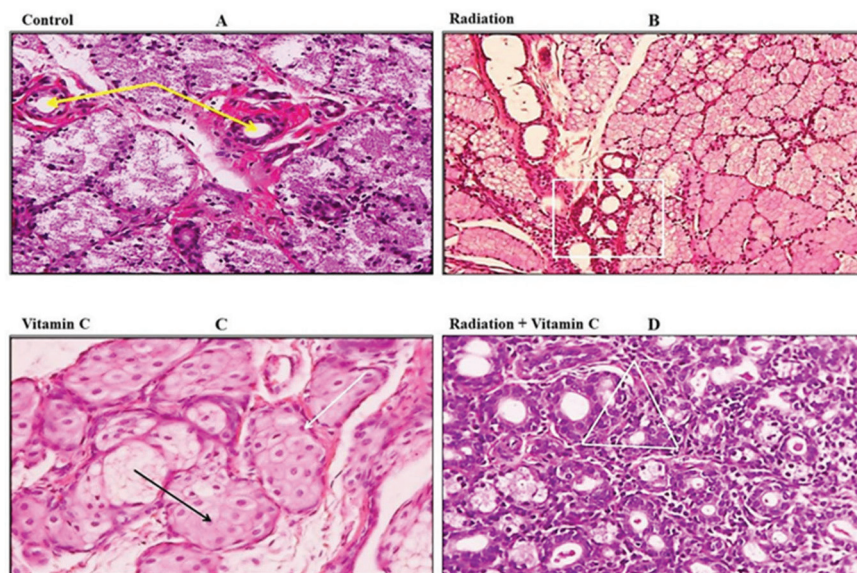


Figure 4. Histological evaluation of parotid gland in irradiated, treatment and control groups using hematoxylin and Eosin (H & E) staining. Histological images showing the cuboidal epithelium with round nucleus along with acinar cells (black arrowhead), and secretory and excretory ducts (yellow arrowhead) of parotid gland of guinea pigs. Normal histological architecture of parotid gland is visible in the control and vitamin C group images. While moderate to severe and mild to moderate inflammation is prominent in radiation (square) only and radiation + vitamin C group (triangle), respectively. All the images were taken at 40 \times magnification.

group, there was evidence of moderate to severe inflammation characterized by the infiltration of lymphocytes in the areas surrounding the acini.

Whereas the radiation + vitamin C group exhibited only mild inflammation in the parotid glands of the guinea pigs (Figure 4, A-D).

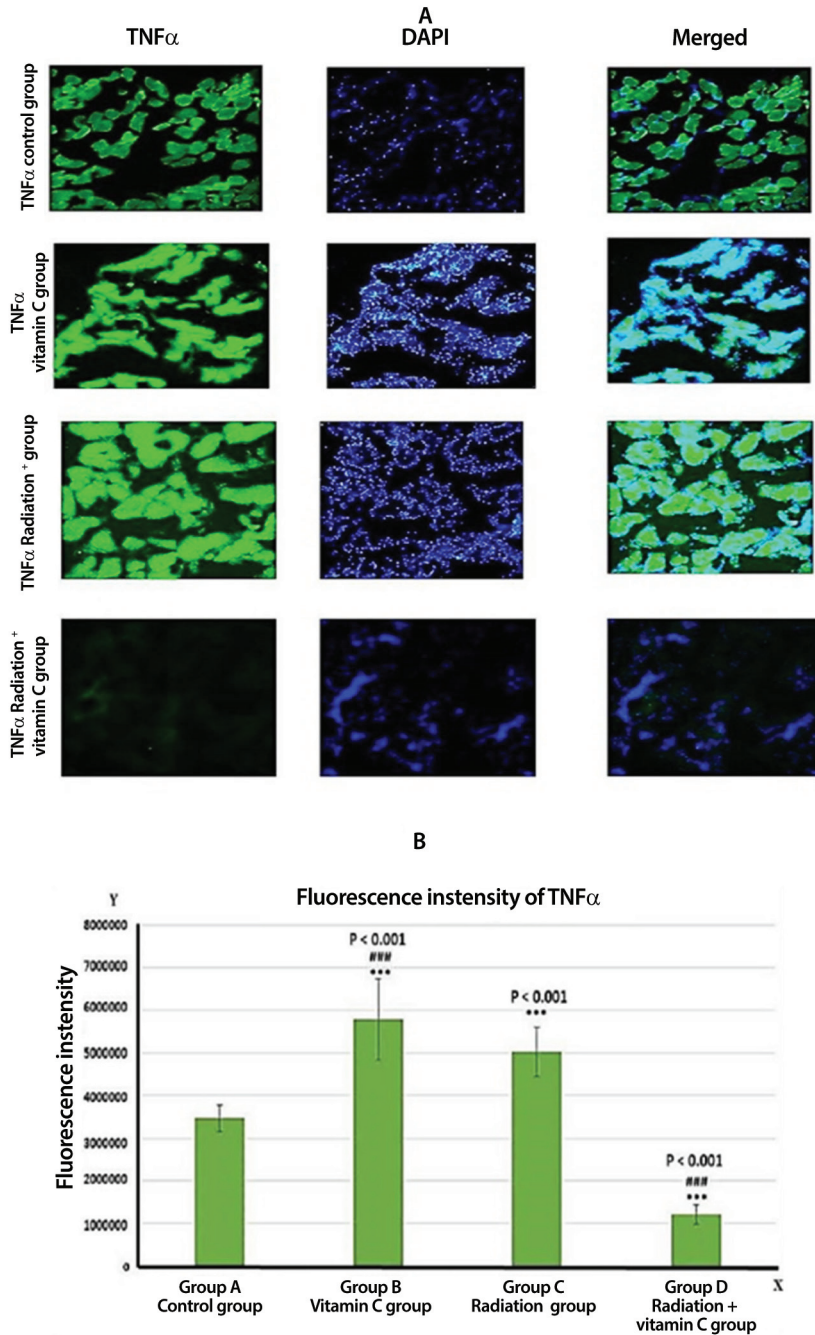


Figure 5. Representative immuno-histochemical staining for TNF α and bar graph showing α expression in parotid gland of irradiated, treatment and control groups. (A) Immuno-histological images showing increased expression of TNF α in radiation and vitamin C treatment groups as compared with controls, while prominent downregulation can be observed in combination treatment group (radiation + vitamin C) in comparison with all other groups. (B) Quantification and statistical analysis also showing significant increase in expression of TNF α in vitamin C treatment groups as compared with controls. While a significant decrease was observed in radiation + vitamin C group. Whereas *** represents a significant difference ($P < 0.001$) between control and treatment groups and ### represent significant difference ($P < 0.001$) between radiation and other treatment groups. All the images were taken at 40 \times magnification.

Expressional analysis of TNF α in the parotid glands of guinea pigs

Following the treatment duration, the animals were euthanized, and the parotid glands were subsequently extracted for analysis. The impact of radiation and vitamin C on TNF α expression was assessed through immunohistochemistry. Expression quantification was performed using Image J software. Statistical analysis, specifically one-way ANOVA followed by the Bonferroni post hoc test, revealed notable findings regarding TNF α expression. In comparison with the control group, there was a significant increase in TNF α expression in the vitamin C group ($P < 0.001$) as well as in the radiation group. Conversely, a significant downregulation of TNF α expression was observed in the radiation + vitamin C group ($P < 0.001$) when compared with both the control group and the radiation-only groups ($P < 0.001$) (Figure 5, A & B).

Expressional analysis of IL-1 β in the parotid glands of guinea pigs

Upon completion of the 15-day irradiation and vitamin C treatment period, the guinea pigs were euthanized, and their parotid glands were extracted to assess the impact of radiation, vitamin C, and their combination on IL1- β expression, which was determined through immunohistochemistry. Quantification of IL1- β expression was carried out using Image J software. The statistical analysis revealed significant downregulation of IL1- β expression in the vitamin C only group and in radiation + vitamin C group as compared with the control and radiation only group. (Figure 6, A & B).

Discussion

This study was designed to investigate the influence of vitamin C on the expression of inflammatory markers in response to radiation-induced inflammatory changes in the parotid glands. Following parameters were evaluated for the study, weight, saliva production, gross morphometry, histopathological features and immunohistochemistry of inflammatory markers. It was observed that neither radiation nor vitamin C had a significant impact on the weight of the

guinea pigs in our study. These findings align with previous research conducted by Xuexian et al. where no significant effect of radiation or vitamin C was observed on the weight of mice across various study groups.²³ On the contrary, Chen et al. reported a decrease in body weight of mice after radiation therapy. These disparate outcomes may be attributed to several potential factors, including variations in the species of animals employed, the dosage or duration of radiation exposure.²³

Furthermore, at the end of experiment duration or on 15th day the mean saliva production significantly reduced in the radiation only group as compared with the control. This effect of radiation was alleviated by vitamin C treatment as no significant difference in saliva production was observed in vitamin C and radiation + vitamin C as compared with the control. Our findings align with a previous study that demonstrated the protective effect of vitamin C against radiation-induced reductions in saliva production.²⁴ Furthermore, several previously published studies have reported reduction in saliva production on the 15th day which is consistent with our findings.^{25,26}

Moreover, there was a notable increase in saliva production on the 3rd day in the radiation + vitamin C group when compared with the control group. This observed increase in saliva production on the 3rd day may be attributed to the immediate compensatory response triggered by vitamin C against the effects of radiation.²³

We also noted that all extracted parotid glands exhibited a subtle pinkish to yellowish appearance except the parotid gland extracted from the guinea pigs exposed to radiation, they appeared reddish in color, indicating the inflamed tissue. Our findings are consistent with a study by Zhu et al.,²⁷ in which changes in the size and color of the parotid gland were observed after irradiation. However, it is worth noting that Zhu et al. were able to reduce the radiation-induced salivary hypofunction in parotid glands of mini pigs using rapamycin.²⁷ Furthermore, when examined microscopically at the histological level, the parotid glands of the control group and vitamin C group

showed normal architectural features. These features included pure serous acini, intercalated ducts and thin connective tissue septa with interlobular duct, all without any evidence of

inflammatory changes. The tissue architecture observed in our present study closely resembled that described by Ghoneim and Arafat²⁸ and Vincent et al.²⁹ In the radiation group of guinea

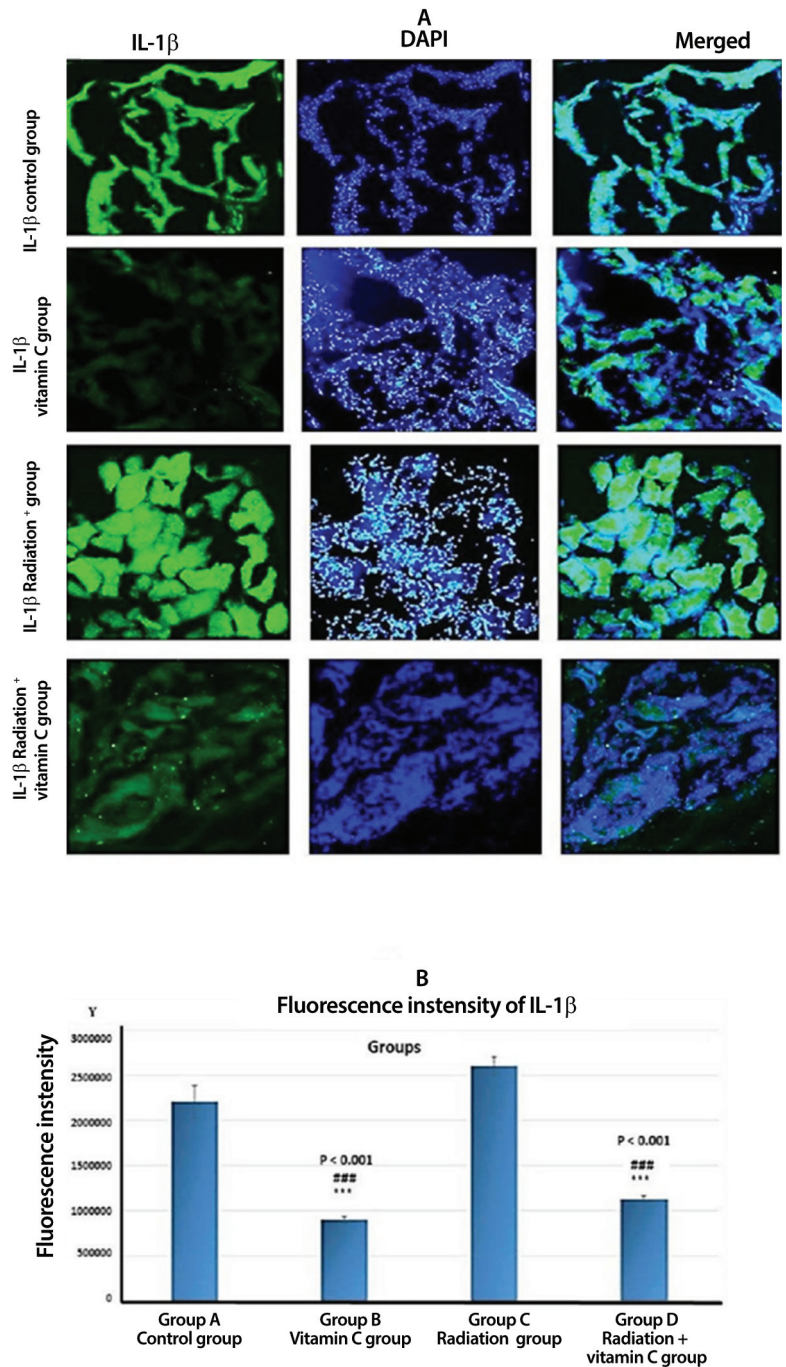


Figure 6. Representative Immuno-histochemical staining and bar graph of IL1-β expression in parotid gland of irradiated, treatment and control groups. Immuno-histological images showing mild increase in the expression of IL1-β in irradiated groups as compared with controls, while prominent downregulation can be observed in vitamin C and combination treatment group (radiation + vitamin C) in comparison with control and only radiation group. Quantification and statistical analysis also showed a nonsignificant increase in expression of IL1-β in irradiated groups as compared with control. While significant down regulation was observed in vitamin C and radiation + vitamin C group. Whereas*** represents significant difference ($P < 0.001$) between control and treatment groups and### represent significant difference ($P < 0.001$) between radiation and other treatment groups. All the images were taken at 40× magnification.

pigs, we observed moderate to severe inflammation within the parotid gland, which was effectively decreased by vitamin C treatment, leading to the observation of only mild inflammatory changes. It has been reported that the exposure of rat parotid gland to radiation lead to structural alterations which are relevant to oxidative stress effect.^{28, 30}

Further, we assessed the protective effect of vitamin C against radiation-induced inflammation at the molecular level by analyzing the expression of TNF α and IL-1 β in the parotid glands of guinea pigs. In the case of TNF α , both radiation and vitamin C increased its expression. Notably, numerous studies have investigated the impact of radiation on TNF α , yielding contrasting results. For instance, Astradsson et al. reported a significant increase in TNF α expression after radiation,³¹ on the other hand Sun et al. demonstrated no influence or a non-significant increase in TNF α after radiation treatment, which aligns with our finding.³² However, it is important to note that these prior studies assessed the effect of radiation on TNF α at the serum level. To the best of our knowledge, the present study represents the first demonstration of the specific impact of radiation on TNF α within the parotid glands themselves. Furthermore, treatment with vitamin C alone led to an increase in TNF α expression, while down regulation of TNF α expression was observed when vitamin C was administered in combination with radiation. The findings of increased TNF α expression in vitamin C-only group contrasts with studies that have shown decrease in TNF α expression following vitamin C treatment.^{33, 34} However, when vitamin C was combined with radiation, it effectively reduced TNF α expression, highlighting its protective role against radiation-induced inflammation. Regarding the expression of IL-1 β , radiation significantly increased its expression, whereas vitamin C alone successfully abrogates IL-1 β expression, both independently and in combination with radiation. This indicates that radiation-induced inflammation primarily involves IL-1 β , while the protective effect of vitamin C in the combination treatment involves both TNF α and IL-1 β . Notably, this

study is the first to demonstrate the specific impact of vitamin C and radiation on IL-1 β expression within the parotid gland. However, the study has certain limitations as only two inflammatory markers, TNF α and IL-1 β , were evaluated, whereas other inflammatory pathways and molecular mechanisms may also contribute to radiation-induced damage. Secondly, the study did not investigate different doses of vitamin C, which could help determine optimal therapeutic concentration for mitigating radiation-induced injury. Further studies are needed to explore these aspects in greater depth.

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Authors' Contributions

FH: Conception, design of research, interpreted results of the experiments, drafted, edited and revised the manuscript; AAA: Performed experiments, analyzed data, interpreted results of the experiments, prepared the figures, drafted, edited and revised the manuscript; ZAR: Performed experiments and prepared the figures; MFB: Performed experiments, analyzed data, edited and revised the manuscript; LK: Performed experiments and analyzed data; QA: Performed experiments, edited and revised the manuscript; AA: Performed experiments; AA: Performed experiments; ZH: Edited and revised the manuscript; MQ: Edited and revised the manuscript. All Authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Conflict of interests

None declared.

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