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ORIGINAL ARTICLE

Effects of dose-dependent response to gamma radiation on circumvallate papilla by expression of caspase-3 in vivo

Khaled El-Haddad [a,b,*], Nabil El-Faramawy [c]

[a] Department of Oral and Maxillofacial Surgery and Diagnostic Sciences, College of Dentistry in ArRass, Qassim University, Qassim Region, Saudi Arabia
[b] Department of Oral Biology, Faculty of Dentistry, Ain Shams University, Abbassia, Cairo, Egypt
[c] Physics Department, Faculty of Science, Ain Shams University, Abbassia, Cairo 65511, Egypt

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Abstract *Background:* Radiotherapy is one of the most significant treatment modality of head and neck cancers. However, it has various hazards on the normal tissues in the radiation field. One of these affected tissues is the lingual mucosa with their papillae such as circumvallate papilla. The effects of radiation on the lingual specialized mucosa may be represented by radiation-induced mucositis and taste alteration including partial or complete loss of taste.

Objectives: The aim of the study was to evaluate the dose-dependent response of circumvallate papillae to gamma radiation by immunohistochemical expression of caspase-3.

Material and methods: Twenty-four adult male albino rats were divided into 3 equal groups irradiated at 2.0, 4.0 and 6.0 Gy whole-body gamma radiation doses. Six non-irradiated rats were used as the control group. The radiation effects on circumvallate papillae were evaluated three days after irradiation via histomorphometric investigation of the papillary size and taste buds' distortion in addition to an immunohistochemical assessment of the apoptotic activity using Caspase-3 marker.

Results: Dose-related changes were observed in the circumvallate papillae size and morphology and taste buds affection. The changes were obviously detected in rats irradiated at 4 Gy and 6 Gy doses. The detection of caspase-3 marker was evident in a dose-dependent manner in all the irradiated groups, more noticeably in the taste bud cells.

* Corresponding author at: Department of Oral and Maxillofacial Surgery and Diagnostic Sciences, College of Dentistry in ArRass, Qassim University, Qassim Region, Saudi Arabia.
E-mail addresses: k.elhaddad@qu.edu.sa (K. El-Haddad), nabil_elfaramawi1@sci.asu.edu.eg (N. El-Faramawy).
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Conclusions: It could be concluded that circumvallate papillae are adversely affected in a dose-dependent manner by gamma radiation particularly in 4 Gy and 6 Gy doses.

1. Introduction

Lingual papillae are small, nipple-shaped structures located on dorsal and lateral surfaces of the tongue giving the typical rough texture (Norton, 2007). Circumvallate papillae differ in number according to the species. Rats have only one circumvallate papilla placed posteriorly on the midline of the tongue. The papilla is surrounded by circular mucous membrane depression (furrow or trench) which contains numerous taste buds. Ducts of lingual serous salivary glands (Von Ebner's glands) drain into the base of the furrow (Hosley and Oakley, 1987; Nanci, 2013). The lingual papillae are subjected to many factors, which are claimed to predispose defects in the papillary structure and functions (Scully, 2013). Consequently, there are several reported taste disorders caused by multiple influences (Wang et al., 2009).

Radiotherapy is an integral part of head and neck cancer treatment. However, ionizing irradiation causes damage in the normal tissues located in the field of radiation (Vissink et al., 2003). Radiotherapy induces adverse effects such as trismus, delayed teeth eruption, defects in hard dental tissues and hyposalivation (El-Faramawy et al., 2011, 2013, 2015; Kawashita et al., 2020). Osteoradionecrosis of jaws is a severe and complex complication of head and neck tumors radiotherapy (Ribeiro et al., 2018). Regarding the effects of gamma radiation on the lingual tissues, gamma radiation, particularly 6 Gy dose, produced defective changes in the interior structures of the tongue. The most noticeably affected tissues are the serous acini of the lingual minor salivary glands while the least affected tissues are the intrinsic muscles (El-Haddad and Metwaly, 2018).

Oral mucositis is a severe side effect of head and neck radiation and chemotherapy, affecting patient daily routine life. This may be due to associated severe pain, and difficulty in mastication and swallowing (Kawashita et al., 2020). Accordingly, radiation-induced oral mucositis is considered a major factor in the dose determination in head and neck cancer patients (Maria et al., 2017), as the progress of oral mucositis is associated with radiation type, dose, and regime in addition to other factors related to patients (Kusiak et al., 2020).

Additionally, taste alteration is one of radiation-induced disorder; thus, patients subjected to radiotherapy develop a partial or complete loss of taste during treatment. This could be due to the high taste buds' sensitivity to radiation and that they are included in the beam of radiation for most oral cancer treatments. However, the cells of taste buds usually regenerate within 4 months after treatment (Silverman, 1999). Three models have been proposed to explain irradiation-induced taste dysfunction. The first model is the disruption of the contact between taste cells and nerves, which leads to taste cell death. The second model is the direct damage of taste cells while the third model suggests that, the affection of the taste progenitors could interrupt the new taste cells production. The combination of the second and third models was also suggested (Nguyen et al., 2012).

The present work aimed to evaluate the effect of gamma radiation on the circumvallate papilla with different doses to correlate between the applied gamma doses and the response of the lining mucosa of the papillae and the taste buds. In addition, this could shed light on the optimum dose, which could avoid mucosal injury and taste bud degeneration.

2. Materials and methods

2.1. Grouping and irradiation

Thirty adult male albino rats of average 250 g body mass were used in the present study and divided into four groups. The sample size in our work was sufficient based on an equation proposed by Ilyas et al., (2017): [E = Total number of animals – Total number of groups]. Any sample size having E value between 10 and 20 is considered enough. The application of this equation in our study is as follows: (E = 30–4 = 26), which is enough sample size with minor excess added only to the irradiation groups. The present work is a cross-sectional animal study; we utilized the rats for the study from animal occupation until scarification with the permission of animal house in Ain Shams University, Al-Demerdash region, Cairo, Egypt.

Twenty-four rats were irradiated once in a chamber to a definite dose of total body irradiation. The rats were divided equally into 3 groups (8 rats each) according to the irradiation dose (groups R2, R4, R6) received 2, 4 and 6 Gy respectively. In addition, the control group consisted of 6 rats, which received no radiation. The irradiation process was achieved in the Radiation Department in Atomic Energy Authority in Egypt (AEAE).

2.2. Histomorphometric and immunohistomorphometric analysis

All rats were sacrificed three days after irradiation. The tongues were separated and prepared for the histological and immunohistochemical examinations. Samples were placed in 10% buffered formalin for seven days at least. The fixed samples were prepared for staining by hematoxylin and eosin (H&E) stain for histological examination and histomorphometric analysis. Caspase-3 immunohistochemical marker was used to evaluate the apoptotic changes related to radiation in the circumvallate papillae. Caspase-3 is considered as the executioner caspase in apoptosis for its role in organizing the destruction of cellular structures (McIlwain et al., 2013). The positive reaction to the Caspase-3 marker is indicated by brown color in the epithelium and connective tissue of the papillae.

The image analysis of the samples was performed using "Image J" program (version 1.52a, Wayne Rasband National Institute of Health, USA). The area of circumvallate papilla and the area of the connective tissue core were measured. In

addition, the percentage of the affected taste buds in relation to the total number of taste buds in the field was calculated. Regarding the immunohistomorphometric analysis, the area fraction of the immunopositive reaction was measured. The results were tabulated and statistically analyzed. by Kruskal Wallis test to determine the differences among groups according to their means' values using SPSS program version "24". We selected The Kruskal Wallis Test as it is an appropriate alternative for the parametric test (ANOVA) in case of a relatively low sample size of each group (6–8 samples).

3. Results

3.1. Histological results

The examination of H&E stained sections of the control group revealed regular characteristic inverted cone circumvallate papillae covered by keratinized stratified squamous epithelium. The papillae were surrounded by trench lined with epithelium containing numerous taste buds (Fig. 1a). The irradiated groups exhibited variable morphological features, group R2 (irradiated with 2 Gy) showed almost similar features as the control group (Fig. 1b). On the other hand, groups R4 and R6 (irradiated with 4 Gy and 6 Gy respectively) revealed more considerable shift from the normal features. The outlines of the papillae were distorted, and the furrow was relatively wider in comparison to the control and 2 Gy groups (Fig. 1c and d).

The epithelial covering of the control group and Group R2 presented layers of keratinized stratified squamous epithelium with well-defined nuclei (Fig. 1e). Group R2 showed almost no changes regarding the epithelial layers (Fig. 1f). Group R4 revealed ill-defined nuclei in some regions of stratum spinosum (Fig. 1g). Clear spaces, indicating signs of degeneration, were observed in the connective tissue of groups R4 and R6 in addition to rarely detected loss of keratin in group R6 (Fig. 1g and h). Regarding the taste buds, group (C) showed numerous buds densely packed with cells particularly in the bases of the taste buds (Fig. 2a). The irradiated groups displayed degenerative changes in the cellular elements of taste buds (Fig. 2b and c). Some taste buds of group R6 showed almost loss of half of the cellular content (Fig. 2d).

3.2. Immunohistochemical results

Examination of control group specimens stained with caspase 3 immunohistochemical marker revealed very little positive reaction in the circumvallate papillae in epithelial and connective tissue cells except for some taste buds and basal and parabasal cells and occasionally in the connective tissue (Fig. 3a and 4a). All the irradiated groups presented more observable positive reactions in both epithelium and connective tissue (Fig. 3b to d and Fig. 4b to d).

3.3. Histomorphometric analysis

The descriptive changes in the papillary size and the taste bud's distortion generated the requisite for the histomorphometric analysis for these parameters (Fig. 5). The area of the papillae decreases in a dose dependent manner with sharp decline from 2 Gy dose to 4 Gy. While the area of the connective tissue cores showed more regular decrease in size with increasing doses. Regarding the affected taste buds, there was a gradual increase in the percentage of the defective taste buds in groups R2 and R4 with sharp upgrading in group R6. The differences in area of the papillae and connective tissue were statistically significant. While the differences in the percentage of the taste buds was nonsignificant.

3.4. Immunohistomorphometric analysis

The immunopositive cells were quantified and the percentage (fraction) of the immunopositive cells per field was calculated. The area fraction of the immunopositive cells increased in abrupt manner from the control group to the group R2. Then

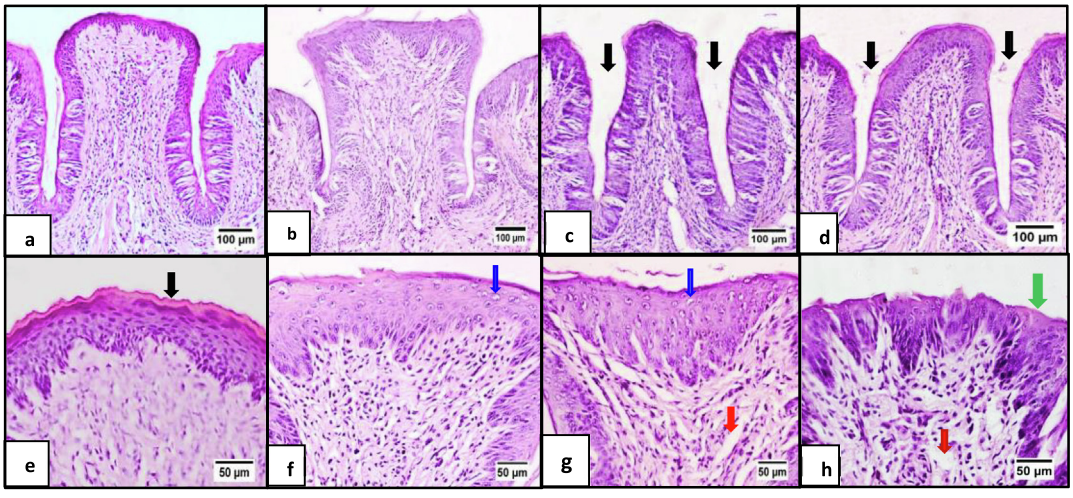

Fig. 1 (a): non-irradiated sample, (b): group R2 with regular papillae (c): group R4 and (d): group R6 with papillary distortion and wide furrow "arrow". (e): Non-irradiated sample with keratinized stratified squamous epithelium (arrow) and underlying connective tissue, (f): Group R2 showing similar results except for ill-defined nuclei in some cells "blue arrow" (g): Group R4 with ill-defined nuclei in some cells "blue arrow and minute spaces in the connective tissue "red arrows". (h): Group R6 with minute spaces in the connective tissue "red arrows" and rare loss of keratin "green arrow". [H&E, Original magnification: a-d: x200 – e-h: x400].

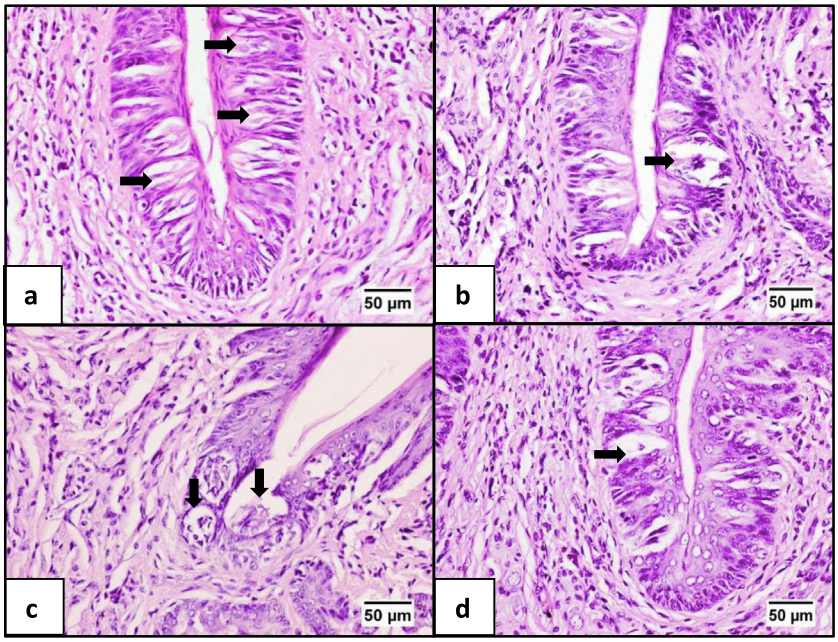

Fig. 2 (a): control group showing taste buds densely packed with cells "arrows", (b): Group R2, (c): Group R4 and (d): Group R6 revealed degenerative changes "arrows. [H&E, Original magnification: x400].

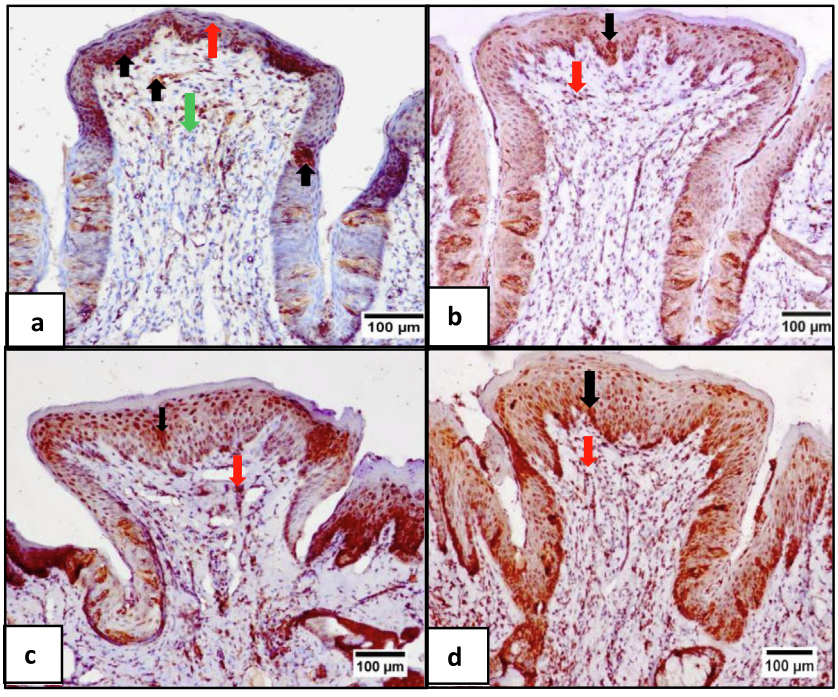

Fig. 3 (a): Non-irradiated group with negative reactions in epithelium "red arrow" and connective tissue "green arrow" and positive reactions in connective tissue, basal and parabasal cells and few taste buds "black arrow". (b, c, d): Groups R2, R4 and R6 respectively, with positive reactions in all epithelial layers "black arrows" and connective tissue "red arrows". [Caspase-3. Original magnification: x200].

the increase took a more regular style in the following doses. The change in the area fraction of the immunopositive cells between the groups was statistically insignificant. The mean and standard deviation in all parameters of all groups is demonstrated in Table 1.

4. Discussion

Total body irradiation is an appropriate treatment modality when the malignant cell population is not uniformly

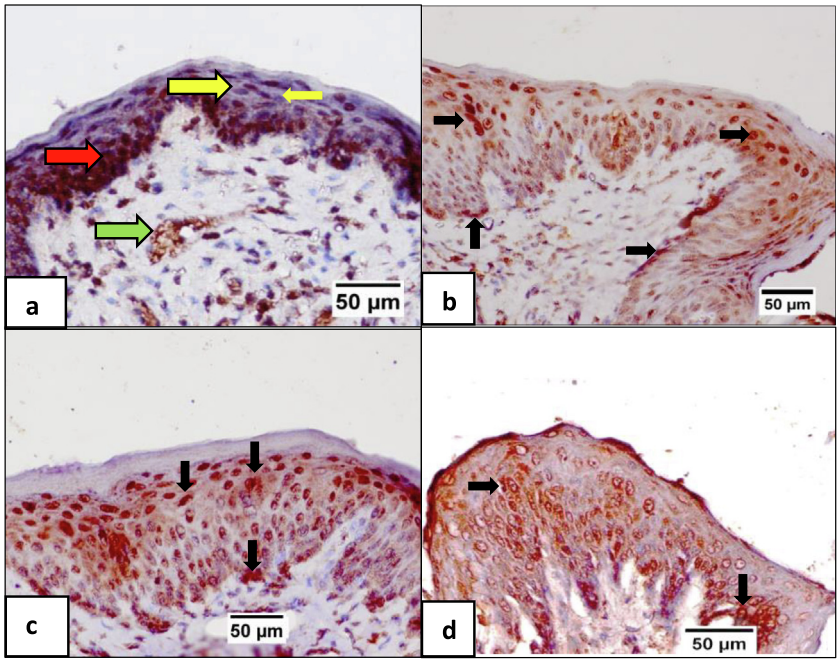

Fig. 4 Higher magnification showing: (a) Non-irradiated sample showing negative reaction in the epithelium "yellow arrow" with positive reactions in basal and parabasal cells "red arrow" and connective tissue "green arrow". (b, c, d): R2, R4 and R6 Groups respectively, with positive reaction in all epithelial layers "black arrows". [Caspase-3. Original magnification: x400].

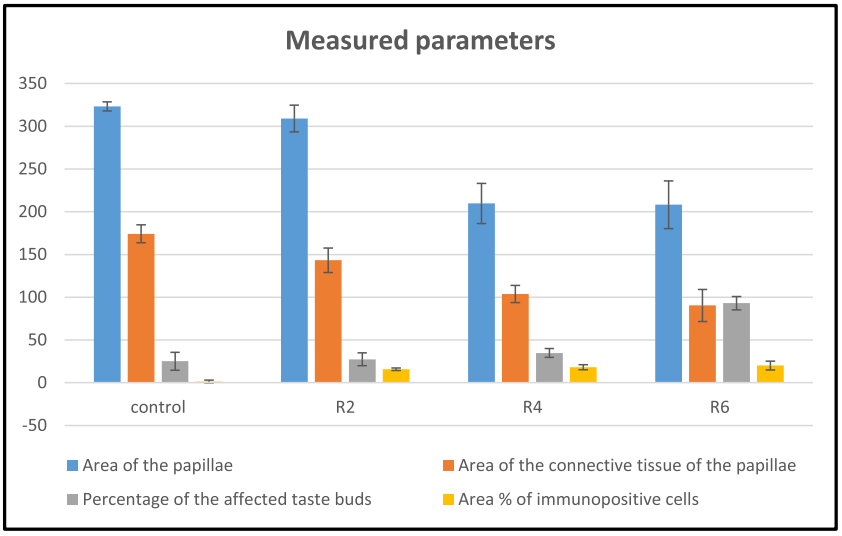

Fig. 5 Bar chart of the mean values and standard deviation of the measured parameters in all groups.

distributed throughout the body as in lymphoma (Wheldon, 1997). This required the investigation of the possible response of the different tissues to this mode of treatment. Thus, the local response of several tissues including the lingual mucosa to the total body irradiation has been investigated by many authors (Zhao et al., 2009). The current research aimed to clarify the dose related response of specific region in lingual mucosa to the total body irradiation. The doses that were given to the rats were 2, 4 and 6 Gy to assess the optimum dose causing the least undesirable effect to recommend in radiotherapy to avoid the hazards of radiotherapy on oral mucosa as well as taste sensation. We selected the duration of 3 days after irradiation as this duration has been reported by Indran et al., (1991) to be suitable for assessment of the maximum damage caused by radiation.

The present work aimed to evaluate the dose dependent effect of gamma radiation on the circumvallate papillae. We selected circumvallate papilla as an indicator of oral mucosal changes with gamma radiation. Moreover, there is an opportunity to evaluate the taste bud reaction as circumvallate papillae gathering numerous taste buds in one localized region (Mostafa et al., 2019).

The histological examination of the epithelial covering of circumvallate papillae revealed numerous cells with pyknotic

Table 1 The mean values and standard deviation and the significance of the measured parameters.

Group	Parameter	Mean	Std. Deviation	Kruskal Wallis test	p-value
control	papillae areas	323.2	5.33	9.538	0.023
R2		309.0	15.58		
R4		209.7	23.44		
R6		208.3	27.93		
control	papillae connective tissue areas	174.17	10.56	10.236	0.017
R2		143.29	14.26		
R4		103.74	10.12		
R6		90.38	18.70		
control	percentage of the affected taste buds	25.18	10.51	7.45	0.059
R2		27.42	7.5		
R4		34.8	5.3		
R6		93.02	7.79		
Control	Area % of immunopositive cells	1.41	1.57	6.945	0.074
R2		15.68	1.46		
R4		18.07	3.00		
R6		20.06	5.06		

or even lost nuclei. This finding was in accordance with a previous study on the human buccal mucosa that revealed formation of micronuclei in cell cytoplasm (Bazyka et al., 2017)

The immunohistochemical results in this study were indicated by caspase-3 immunohistochemical marker which was previously used to detect apoptosis in the literature (Bucci et al., 2006). We detected immunopositive reaction in the control group basal and parabasal cells which coincide with Veeravarmal et al., (2016) who reported the nuclear expression of caspase-3 in basal and parabasal layers of normal epithelium. In the present work, we revealed dose dependent immune-positive reaction of many cellular elements. This agreed with Michelin et al., (2004) who studied the cellular death in cell culture using caspase antibody as an immunohistochemical marker; the authors stated that the radiation induced cellular death, 24 h post-irradiation which was dose-dependent. The radiation induced apoptosis in this work coincides with enormous studies that claimed that radiation causes cell death (Veit et al., 2015).

Although the signs of cell death were observed in all the irradiation doses, nevertheless mild effects were seen in the dose 2 Gy in comparison to 4 and 6 Gy. This was consistent with Bishay et al., (2000) and Goo et al., (2013) who reported dose dependent cell death with more observable signs in doses over 2 Gy. Furthermore, this result coincides with preceding studies that recommended fractionation of radiotherapy dose into separated doses claiming that 2 Gy is a safe dose to the normal tissues in the field of radiation (Vissink et al., 2003).

The apoptotic activity in the current study was noticeable in a statistically significant manner particularly in 6 Gy irradiated rats. This agreed with Siles et al., (1998) who reported the apoptotic death induced after treatment with 6 Gy of gamma-irradiation on different cells. The histomorphometric and immunohistochemical results displayed injurious changes in their cellular elements of taste buds. This might explain taste dysfunction that was reported as a common problem after radiotherapy for head and neck cancer (Vissink et al., 2003; Kawashita et al., 2020).

Interestingly, we noticed that the damage in taste buds was the most striking effect on the papillae despite the short period of the study. This was in accordance to Nguyen et al., (2012) who reported that taste progenitor cells undergo cell cycle arrest or apoptosis within 1–3 days after irradiation. Moreover, we observed that the apoptotic effects of taste bud cells appeared from the dose of 2 Gy. This might be attributed to the extreme radio-sensitivity of taste buds because of their location in the tongue (Silverman, 1999).

There are several hypotheses explaining the mechanism of degenerative effects of gamma radiation on taste buds. Nguyen et al., (2012) reported the radio-sensitivity of the taste nerves. This coincides with the studies revealed the direct relation between injury or denervation of taste nerves and the loss of integrity of taste buds. The taste denervation was reported to be related to the apoptotic and degenerative effects on taste buds of guinea pig circumvallate papillae (Huang and Lu, 2001). Another explanation could be that gamma radiation might induce inflammation, which sequentially produce taste disorders. Wang et al., 2009 have reported that inflammation can predispose to taste disorders and increase apoptotic cell death in taste buds. This could explain the current results particularly when we relate with a study that declared that inflammatory reaction onset could be observed at 2 Gy dose (Veit et al., 2015).

5. Conclusions

From the results presented in this work, it could be concluded that the size and morphology of the circumvallate papilla are affected by gamma radiation in a dose dependent manner. Gamma radiation adversely affects the taste cells. The radiation induced degenerative changes are minimal with 2 Gy dose compared to those associated with 4 and 6 Gy doses. Further investigations are needed to assess the reversibility of the effects with different doses.

Ethical statement for solid state ionics

Hereby, I/Khaled-ElHaddad, consciously assure that for the manuscript ‘‘Effects of dose-dependent response to gamma radiation on circumvallate papilla by expression of caspase-3 in vivo” the following is fulfilled:

1) This material is the authors' own original work, which has not been previously published elsewhere.

2) The paper is not currently being considered for publication elsewhere.

3) The paper reflects the authors' own research and analysis in a truthful and complete manner.

4) The paper properly credits the meaningful contributions of co-authors and co-researchers.

5) The results are appropriately placed in the context of prior and existing research.

6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.

7) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

The violation of the Ethical Statement rules may result in severe consequences.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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