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# Anticipating Oral Mucositis In Oral Cancer Patients Undergoing Fractionated Radiotherapy: A Cytological Correlation.

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**ABSTRACT**

Oral mucositis is one of the most common side effects of fractionated radiation therapy. Along with clinical changes, radiation also induces cytological changes which include both cytoplasmic as well as nuclear alterations. The present study was carried out todocument and correlate theclinical and cytological changes in the oral epithelial cells of patients undergoing fractionated radiation therapy for head and neck malignancies. 24 patients undergoing fractionated radiotherapy for head and neck malignancies were selected and observed during the course of therapy for development of clinical changes like erythema, ulceration and trismus. Simultaneously smears from oral mucosa were taken and studied for increase in cell size, cytoplasmic changes like granularity, vacuolization and fragmentation and nuclear changes like micronuclei, binucleation and multinucleation. Significant linear dose dependant correlation was seen between the radiation doses and clinical and cytological changes. The present study is critical in its method to predict the development of erythema and ulceration by assessing genotoxic cellular markers like micronuclei, binucleation and multinucleation which are direct indicators of molecular changes. As these markers correlate well with the molecular changes, namely the reactive oxygen species (ROS), nuclear factor kappa β (NF-kβ) and cytokines, the treatment regime to improve control of pain and irritation during mucositis can be planned more efficiently.

**Keywords**: head and neck malignancies, fractionated radiotherapy, oral mucositis, genotoxic damage

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## INTRODUCTION

Oral mucositis is one of the most common adverse reactions associated with radiation which is a common modality for treatment of head and neck cancer. Oral mucositis is characterized by ulceration in the oro-esophageal and gastrointestinal mucosa that results in pain, dysphagia, and dysfunction depending on the tissue affected [1]. Changes have been reported in almost all tissues exposed to therapeutic and/or palliative radiation. The cytoplasm and nuclei of oral epithelial cells have been observed to undergo change upon exposure to radiation. Prominent nuclear changes include nuclear budding, micronuclei, binucleation and multinucleation. Cytoplasmic changes reported include oedema, granularity and vacuolization, besides an overall increase in cell dimensions [2-5]. The present study was undertaken to evaluate the dose-response relationship between radiation and various clinical, cytoplasmic and nuclear changes in oral epithelial cells, collected by sequential scrape smears from patients undergoing radiotherapy for head and neck malignancies. The study also aimed to find a correlation between clinical and cytological changes seen in the oral cavity during radiotherapy.

## MATERIALS AND METHODS

The study included 24 patients undergoing fractionated radiation therapy (2 Gy per day for 30 days) for head and neck malignancies in our hospital. Ethical clearance was obtained from the Institutional Ethical Committee. Informed consent was obtained from the patients in the study sample and the forms were duly signed by the patient. The clinical changes seen in the oral cavity were noted in a proforma.Smears were obtained from the irradiated sites in the oral cavity on alternate days (corresponding to the fractionated radiation dose) to obtain the exfoliated cells. They were stained to evaluate the morphology of the cells and nuclear abnormalities like micronuclei (Countryman and Heddle criteria),[6] nuclear budding, binucleation and multinucleation and cytoplasmicchanges like granularity, oedema and vacuolization. The cytoplasmic and nuclear abnormalities were visualized and assessed using Papanicolaou (PAP) stain under light microscopy and Acridine Orange stain under fluorescent microscopy respectively. Also nuclear-cytoplasmic dimensions were evaluated using image analysis software (Image J analysis software Version 1.46, NIH USA) in 50 cells of PAP stained smears.Repeated measures Analysis of Variance(ANOVA) was applied to the statistical data and descriptive mean values of each parameter was calculated at 5 progressive doses of fractionated (0, 4, 8, 12, 18 Gy).

## RESULTS

The study included 24 patients whounderwent radiotherapy for head and neck malignancies. The malignancies involved different sites in different patients with varied histology and clinical stages (Fig 1). Repeated measures of ANOVA was applied to the statistical data and descriptive mean values of each parameter was calculated at 5 progressive doses of fractionated (0, 4, 8, 12, 18 Gy). Of the 24 patients in the study, a majority of the patients (n=20) developed oral mucositis upon completion of the fifth dose of radiotherapy and one patient developed mucositis upon the fourth dose (at 12 Gy), while three patients developed mucositis after the fifth dose of radiation. Therefore, the statistical analysis was performed maintaining the average at the fifth interval, thereby effectively reducing the sample size available for analysis to 23.

**Table 1: Correlation between clinical and cytological changes**

| Parameter | Radiation dose | | | | |
|---|---|---|---|---|---|
| | 0 Gy | 4 Gy | 8 Gy | 12 Gy | 18 Gy |
| Erythema | Absent | Absent | Absent | Present | Present |
| Ulceration | Absent | Absent | Absent | Absent | Present |
| Micronuclei | 1.48 | 2.22 | 3.35 | 5.09 | 6.00 |
| Binucleation | 2.61 | 3.00 | 4.65 | 6.22 | 8.65 |
| Multinucleation | 0.17 | 0.13 | 0.22 | 0.65 | 1.65 |
| Nuclear area (micron) | 69.75 | 83.52 | 92.01 | 102.52 | 126.14 |

In the present study, it was found that oral mucositis and radiation-induced trismus(Fig 2,5) were the most common clinical changes produced, while micronucleation, binucleation and multinucleation(Fig3,6) were observed in cytological examination of the irradiated oral epithelial cells. The severity of clinical changes

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correlated with an increase in cytological changes.(Table 1) Erythema was noted around 12 Gy of radiation dose while ulceration (Fig 5) was observed upon receipt of 18 Gy of radiation dose. Along with nuclear changes, there was a progressive increase in cytoplasmic abnormalities (granularity, vacuolization and fragmentation) with increasing dose of radiation (Fig 3,7). The nuclear dimensions were also increased significantly with progressive doses of radiation (Fig 4).

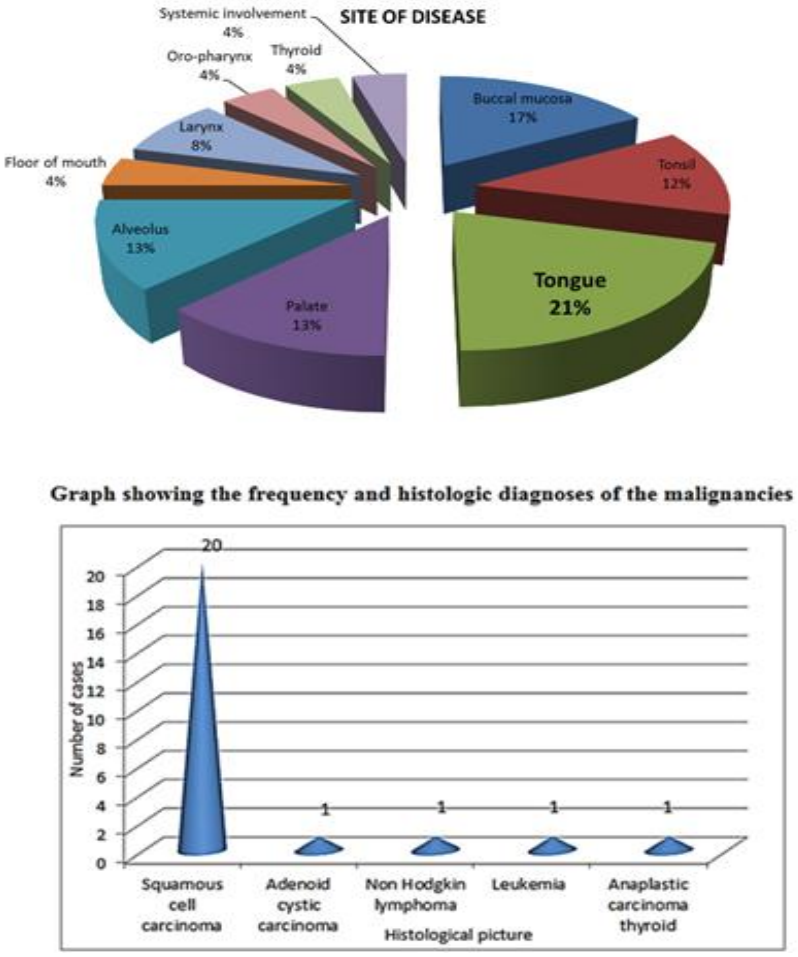


**Figure 1: Graphs showing the frequency of sites and histologic diagnosis of head and neck cancer**

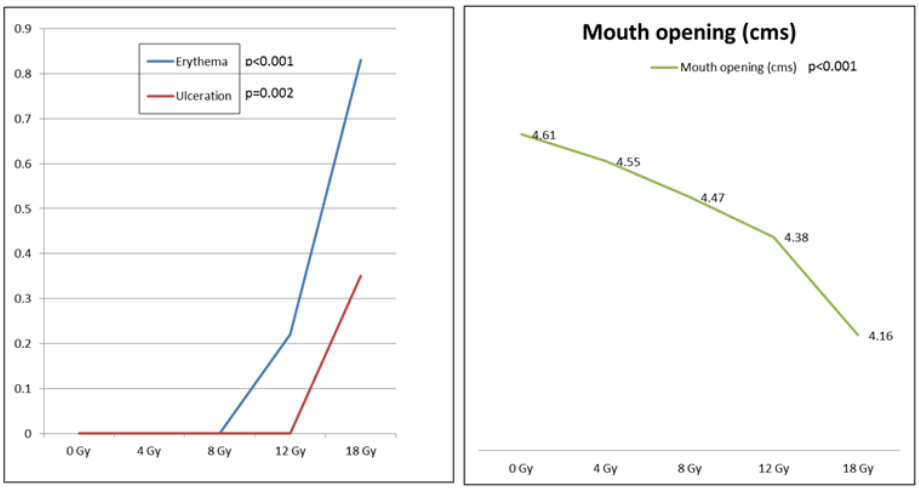


**Figure 2: Line graphsdepicting a linear association between erythema, ulceration and reduced mouth opening (features of oral mucositis) in relation to fractionated dose of radiotherapy (5 intervals – 0, 4, 8, 12, 18 Gy)**

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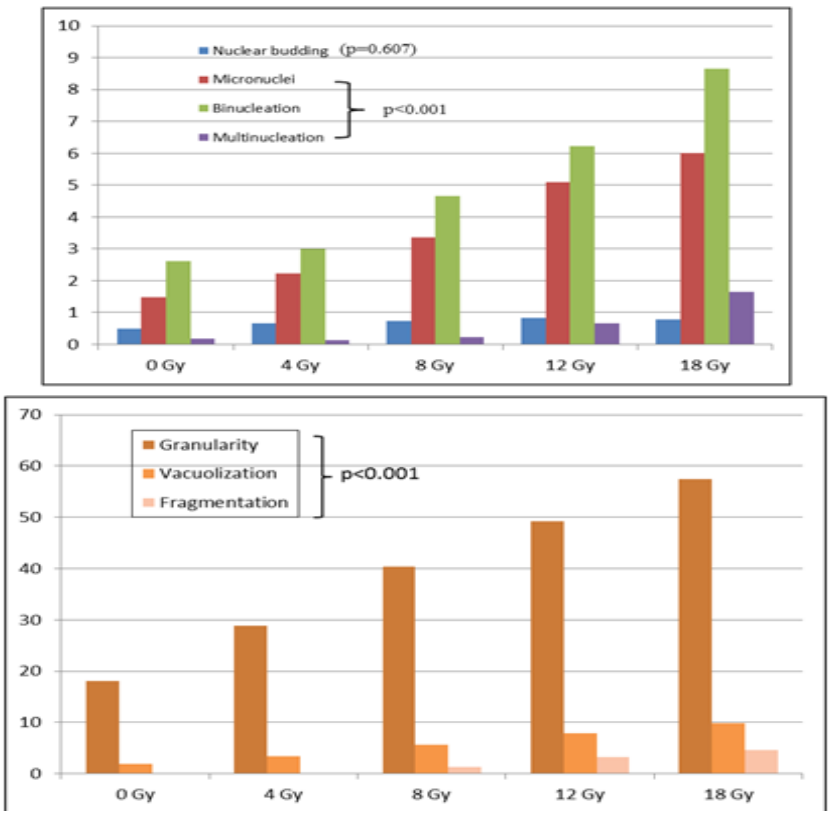


**Figure 3: Bar graphs depicting nuclear and cytoplasmic changes in relation to fractionated dose of radiotherapy (5 intervals – 0, 4, 8, 12, 18 Gy)**

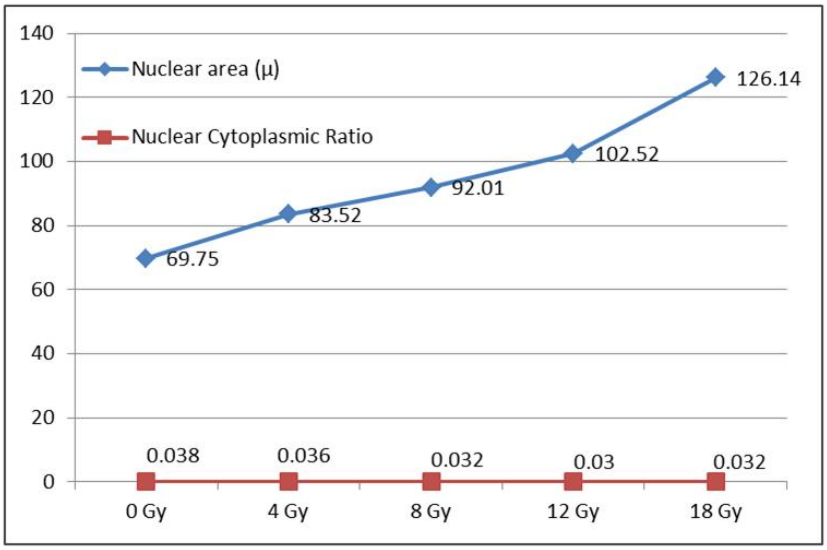


**Figure 4: Line graph depicting changes in nuclear dimension in relation to fractionated dose of radiotherapy (5 intervals – 0, 4, 8, 12, 18 Gy)**

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**Figure 5: Oral clinical changes in the form of erythema and ulceration (arrow) with radiotherapy**

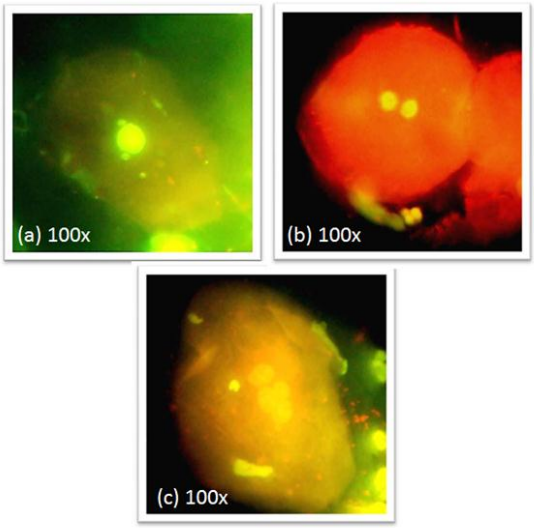


**Figure 6: Nuclear changes like (a) micronuclei, (b) binucleation and (c) multinucleation seen in exfoliated oral epithelial cells stained with Acridine Orange under fluorescent microscopy (x1000)**

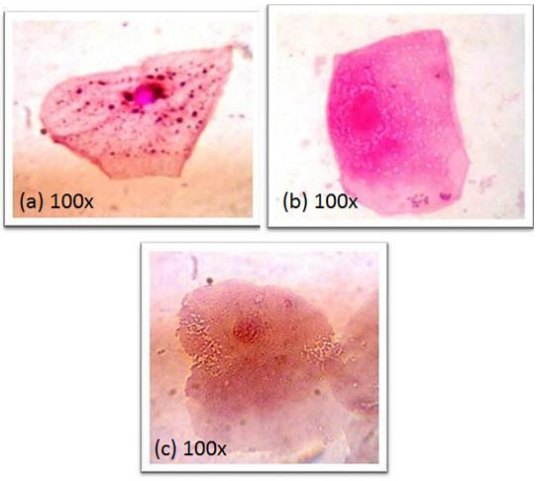


**Figure 7: Cytoplasmic changes like (a) granularity, (b) vacuolization and (c) fragmentation seen in exfoliated oral epithelial cells stained with Papanicoloau stain under light microscopy (x1000)**

## DISCUSSION

Oral mucositis is a common complication seen in association with fractionated radiotherapy used for the treatment of cancer. The first clinical signs of mucositis occur at the end of the first week of a conventional

seven-week radiation protocol (daily dose of 2Gy, five times a week). It is characterized by *erythema* which is followed by *ulceration* [1,7].

In the present study, it was observed that erythema developed after 12 Gy (sixth fraction of radiation dose) of radiation while ulceration developed around 18 Gy of radiation (Fig 5) (ninth fraction of radiation dose), which is in accordance with the findings of Triester and Sonis [8]and Sonis. [1,9-11]From the beginning of the fractionated radiation therapy, till the development of erythema and ulceration, there was a progressive increase in genotoxic changes (micronuclei, binucleation and multinucleation) as evident in our study. The observation that nuclear damage increased with the progress of mucositis indicates involvement of molecular events in its development. These genotoxic changes appear before the presentation of clinical symptoms.

The development of mucositis involves a complex interplay of radiological toxicity and the tissues which are irradiated. In the initiation phase, reactive oxygen species (ROS) that are generated within the tissues, by exposure to radiation therapy result in DNA strand breaks and damage to cells, tissues and blood vessels, which ultimately cause apoptosis. Such damage triggers activation of transcription factors such as nuclear factor kappa B (NF-κB), which in turn causes signalling and amplification through gene up-regulation, and increases production of cytokines. Increasing levels of cytokines like interleukins (IL-1β and IL-6) trigger the initiation of various pathways that damage epithelial cells and surrounding fibroblasts. Increased apoptosis of epithelial cells cause epithelial atrophy while the interleukins stimulate vascularity. This is clinically seen as erythema [9,11].

Pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), further increase the activity of NF-κB, causing a feedback loop that promotes the cycle of inflammation, pain, and functional impairment. Action of matrix metallo-proteinases and cytokines, cause further apoptosis of epithelial cells causing a breach in the continuity of the epithelium. This is clinically seen as ulceration which facilitates colonization by oral bacteria and increases the risk of sepsis [9,11].

The present study is critical in its method to predict the development of erythema and ulceration by assessing genotoxic cellular markers like micronuclei, binucleation and multinucleation which are direct indicators of molecular changes. As these markers correlate well with the molecular changes, namely the reactive oxygen species (ROS), nuclear factor kappa β (NF-kβ) and cytokines, the treatment regime to improve control of pain and irritation during mucositis can be planned more efficiently.

With the increasing levels of genotoxic damage, various treatment modalities can be instituted. ROS scavengers (e.g. amifostine) are more efficacious at early stages of erythema,which correlated with average occurence of 5 micronuclei, 6 binucleation and 1 multinucleation in 500 cells counted per case. (Table 1) Similarly, sphingomyelinase pathway inhibitors (N-Acetyl Cysteine), apoptosis inhibitors (pallifermin) and growth factors are more efficacious at the early stages of ulceration,which correlated with average occurrence of 6 micronuclei, 9 instances of binucleation and 2 counts of multinucleation in 500 cells counted. Cytokine inhibitors (e.g. N-acetyl cysteine and benzydine hydrochloride) are efficacious in preventing both erythema and ulceration. All these agents, if used before/at these threshold values of genotoxic damage, can prevent the development of clinical symptoms of mucositis, increasing patient compliance and thus helping in better management of the patient [8,10,11].

*Trismus* presents as a verycommon complication of the movement of the mandible following surgery and/or radiotherapy (RT) in head and neck cancer. It has been described as any type of restriction in opening of the mouth including radiation and conditions after trauma, surgery, or tetanus.In the present study, it was seen that mouth opening reduced progressively with increase in radiation, and severity hampered function when the number micronuclei were greater than 4 in 500 cells (Table1). In radiation therapy, there is damage to the oral mucosa.The injured tissue attempts to undergo fibrotic remodelling which serves to seal off the damaged region. However, if the extent of remodelling is exuberant, the surrounding tissue becomes involved, resulting in a loss of elasticity that produces detrimental functional consequences.Radiation-induced fibrosis is characterised by presence of infiltrating inflammatory cells, atypical fibroblasts, and large amounts of various extracellular matrix components. The result of this fibrosis limits the mouth opening, with major effects on nutrition, dental hygiene, swallowing, and phonation [12]. The severity of trismus is dependent on the configuration of the radiation field, the radiation source and the radiation dose [13].

Fibrosis occurs in the healing phase of mucositis. In trismus, the ensuing fibrotic response mediated by radiation is associated with an increase in ECM (extracellular matrix) deposition. Fibroblasts, when activated, produce various ECM components and can also differentiate into myofibroblasts, which are more contractile and more readily synthesize ECM, resulting in a greater loss of tissue elasticity [14]. Another key stromal cell type demonstrated to promote fibrosis is the epithelial cell. Macrophages produced in response to mucositisdown-regulate inflammatory responses. They produce transforming growth factor β (TGF-β) which in turn, result in a transition of resident epithelial cell phenotype towards a motile, ECM-producing mesenchymal cell. This process is referred to as epithelial to mesenchymal transition (EMT) and is understood to contribute to lung, liver and kidney fibrosis [14]. The progression of trismus is an irreversible change and the deterioration of trismuscannot be reversed after conventional therapy [13]. Anti-inflammatory drugs and fibrotic inhibitory drugs can be given when the number of micronuclei are≥4. As a result, there will be less production of macrophages leading to reduced production of TGF-β and consequently a lesser degree of fibrosis.

The efficacy of the present study revolves around the simple procedure of exfoliative cytology, whichhas been tried in the past for prediction of oral cancer radio-sensitivity. Bindu et al and Mehrotra et al have reported various radiation-induced findings in benign oral squamous epithelial cells [15,2].

In the present study, there was a gradual increase in all nuclear changes (micronuclei, binucleation, multinucleation) as well as cytoplasmic changes (granularity, vacuolization, fragmentation) (Table 1)(Fig 3,6,7) including an increase in nuclear areas. Similar changes have been found by other authors both in benign oral epithelial cells of patients undergoing radiotherapy. Hannah Peters (1958) noted an increase in cell size, cytoplasmic granules and nuclei (binucleation and multinucleation) in normal squamous epithelium which received radiation along with cancerous epithelium.[3]Memonand Jafarey(1970)observed changes like cell enlargement, cytoplasmic vacuoles, multinucleation, nuclear enlargement and other nuclear changes in the decreasing order of their frequency in the benign epithelial cells affected by radiation.[16]Silverman and Sheline (1961)also studied occurrence of cytoplasmic vacuolization, multinucleation, nucleo-cytoplasmic enlargement and nuclear changes in the benign irradiated cells [4].

The nuclear and cytoplasmic changes can be explained in terms of their repair mechanism.A wide variety of radiobiologic data indicate that the nucleus is more radiosensitive (in terms of lethality) than the cytoplasm, especially in dividing cells. This means, that the nuclear changes are first hastened and thus are reliable in detecting radiation-induced damage.

In fractionated radiation therapy, there is continuous and repeated exposure of the tissue to external radiation, but the amount of radiation dose is not very high so as to kill the benign epithelial cells. In case of fractionated therapy, even though the benign cells sustain injury and undergo repair, they are not able to revert back to their original state showing some cytoplasmic and nuclear abnormalities in the process. Since the cells are repeatedly exposed to increasing doses of radiation, the amount of these abnormalities also increases. The molecular interplay within the nucleus is observed as micronuclei, binucleation and multinucleation.

Nuclear budding is an early phase of micronucleation. It may be induced by a direct, localized effect on the nuclear membrane. As a result there is incomplete karyokinesis, giving rise to nuclear budding. Expulsion of the nuclear bud into the cytoplasm gives rise to micronuclei. On the other hand, binucleation occurs due to radiation-induced peroxidation of lipids in the cell membrane causing structural and functional alteration to the membrane. There is a block in cytokinesis (as a result of radiation-induced damage to cytoplasmic organelles), resulting in the formation of a binucleated cell and further division of one or both nuclei leads to multinucleation. Multinucleation can also occur if there is damage to the peri-centriolar matrix (PCM), which can lead to multi-polar mitosis and multinucleation [15,17]. Membrane damage coupled with accelerated proliferation of the nucleus can be expected to result in an inability of the membrane to keep up with nuclear division leading to multinucleation and clonogenic death [2,15,17].

An increase in nuclear size was also reported in the present study, a finding which has been well documented previously in literature [5,18]. The present study aimed to document and correlate the clinical and cytological changes in the oral epithelial cells of patients undergoing fractionated radiation therapy for head and neck malignancies. Radiation induced genotoxic damage to the oral epithelial cells was reflected in the form of clinical complications such as oral mucositis. These changes can allow a clinician to pinpoint the

underlying molecular processes and thus efficiently treat symptoms, that otherwise would develop complications and impair patient compliance with therapy. However, development of a good system based on clinic-cytologic correlation for grading oral mucositismore objectively necessitates a larger sample size. This could help the physician to anticipate and intervene in the progression of these symptoms, thus improving the patient's compliance and help to prevent unwanted interruptions in the therapy that directly affects the prognosis.

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