ORIGINAL RESEARCH

Concomitant Chemoradiotherapy Induced Nuclear Abnormalities in Normal Buccal Mucosa Cells: A Serial Cytological Evaluation

Sadia Minhas[1], Muhammad Kashif[1], A.H. Nagi[2]

ABSTRACT

Introduction: Oral squamous cell carcinoma is a growing malignancy in Pakistan. Concurrent chemo-radiotherapy (CCRT) has a fundamental role in the management of loco regionally advanced head and neck tumours and a survival advantage for this approach in comparison to radiation alone is now generally acknowledged. The objective of this study was to evaluate the relationship among duration of concomitant chemo-radiotherapy and nuclear abnormalities in normal buccal mucosa cells collected by serial scrape smears from oral squamous cell carcinoma patients.

Materials and Methods: The study included 70 patients of OSCC treated by concomitant chemo-radiotherapy. Serial scrape smears were taken from normal buccal mucosa on specific days of therapy i.e. before and at the end of therapy with the help of wooden spatula. The smears were then stained with H&E, Pap and Giemsa stains and evaluated by light microscopy. The frequencies of karyolysis, karyorrhexis, micronucleation, binucleation, nuclear budding, prominent nucleoli and multinucleation were recorded.

Result: Among 140 smears from normal buccal mucosa, karyolysis in n=71 (50.7%), karyorrhexis in n=71 (50.7%), binucleation in n=77 (55%), nuclear budding in n=68 (48.5%), micronucleation in n=73 (52.1%), prominent nucleoli in n=85 (60.7%) and multinucleation in n=54 (38.5%) smears were seen. The CCRT induced nuclear abnormalities i.e. karyolysis, karyorrhexis, binucleation, micronucleation, nuclear budding and multinucleation in normal buccal mucosa cells have statistically significant relationship with duration of CCRT and this increase in nuclear abnormalities was more evident at the end of therapy.

Conclusions: Nuclear abnormalities can occur in normal buccal mucosa cells due to CCRT which may lead to atypical and even dysplastic changes in radiation field.

Keywords: OSCC; CCRT; Nuclear abnormalities; Buccal mucosa; Cytology

How to cite this article: Sadia Minhas, Muhammad Kashif, A.H. Nagi. Concomitant chemoradiotherapy induced nuclear abnormalities in normal buccal mucosa cells: a serial cytological evaluation. International Journal of Contemporary Medical Research 2015;2(4):960-965

[1]PhD Research Fellow, [2]Professor of Pathology & HOD, Department of Morbid Anatomy and Histopathology, University of Health Sciences Lahore, Pakistan

Corresponding author: Sadia Minhas, BDS; M.Phil, Department of Morbid Anatomy and Histopathology, University of Health Sciences Lahore, Pakistan

Source of Support: Nil

Conflict of Interest: None

INTRODUCTION

Head and neck cancers are characterized according to the area of the head and neck from where they arise, and these consist of pharynx, nasal cavity, larynx, oral cavity, salivary glands and paranasal sinuses.[1] More than 90% of oral cancers are oral squamous cell carcinomas (OSCC) arising in the mucous membranes of the oral cavity and oropharynx.[2,3]

Globally, oral cancer is the eighth most common cause of cancer-related deaths, although many people are unaware of its presence.[4] Developing countries have the world's maximum reported occurrence of oral cancer with squamous cell carcinoma for being the most common histological type.[5] The current treatment modalities available for OSCC patients are surgery or radiotherapy alone, surgery in combination with radiotherapy and combine chemo-radiotherapy with or without surgery.[6] A good loco-regional and systemic control for advance cases of OSCC is an important goal of concurrent chemo-radiotherapy.[7]

Attempts to elaborate the tests which could calculate the reaction of normal buccal mucosa to radiotherapy, have been made in earlier period and are still being follow. Early researchers used biopsies from the buccal mucosa or lesional site in order to predict response to radiotherapy. Afterwards, Graham opened up the use of exfoliative cytology to calculate radiation associated changes in cervical cancer and established them helpful in predicting the reaction to the treatment.[8] Nowadays, more emphasis has been given towards the role of ex-

foliative cytology as an assay to predict the radiation induced nuclear changes in normal buccal mucosa to consider field cancerization and development of secondary tumours. Cowpe and his associates suggested that it is an investigative test for precancerous and cancerous lesions presenting in the oral cavity.[9] Numerous nuclear changes, as a result of radiotherapy, including karyolysis, karyorrhexis, micronucleation nuclear budding and multinucleation were described in a number of studies.[10,11]

The current exfoliative cytological smears study was carried out to evaluate the relationship among various nuclear changes in contralateral normal buccal mucosa smears with duration of concomitant chemo-radiotherapy and also to investigate the possibility of utilizing exfoliative cytology as an assay to predict normal buccal mucosa response to concomitant chemo-radiotherapy.

MATERIAL AND METHODS

The study was conducted in the Department of Morbid Anatomy and Histopathology, University of Health Sciences Lahore, Pakistan. The study group was comprised of 70 patients with histologically confirmed squamous cell carcinoma of oral cavity treated by concomitant chemo- radiotherapy at the Department of Radiotherapy, Institute of Nuclear Medicine and Oncology Lahore (INMOL), Pakistan.

Each patient received a total of 70 to 90 Gy of external beam radiation in 33 fractions of 2Gy each given daily, five times a week, over a period of six weeks in combination with chemotherapy i.e. cisplatin and 5-FU with varying doses, adjusted by chemotherapist. The patients undergoing for CCRT treatment for the first time were selected for the study. While the patient treated with other treatment modalities, like radiotherapy or chemotherapy alone, patients suffering from co- morbid conditions and patients who were already undergone through CCRT treatment were excluded from the study.

After getting informed consent, a detailed oral examination, comprehensive clinical findings and case history of each patient were recorded in a proforma. Three scrape smears were taken (One for each Haematoxylin and Eosin, Papanicolaou and for staining with May-Grunwald Giemsa stains) from the normal appearing contralateral buccal mucosa (opposite to tumoural area). Subsequently smears were taken on similar pattern from the same sites at the end of CCRT. Scrapings were taken with the help of a sterile wooden spatula wet in distilled water. The collected material was smeared without delay on a grease free super frosted glass slide. Smears to be stained with haematoxylin and eosin and Papanicolaou stain were immediately fixed in equal volume of ether and alcohol, while those to be stained with Giemsa stain were air dried and then fixed in methyl alcohol.

A total of five hundred random epithelial cells were counted in each smear from the contralateral normal buccal mucosa of each patient and the results were made as number of cells presenting nuclear abnormality per 1000 cells. Cells clumps, cells with disintegrated or vague nuclear membrane and poorly preserved cells were not counted. Nuclear changes were assessed under light microscope i.e. karyolysis, karyorrhexis, micronucleation, nuclear budding, binucleation and multinucleation.

STATISTICAL ANALYSIS

The data was entered and analyzed using SPSS 22.0. Comparisons between clinical and microscopic parameters were performed with the sample t-test. A difference of $p < 0.05$ was considered to be significant.

RESULTS

All the nuclear abnormalities that were studied i.e. karyolysis, karyorrhexis, micronucleation, prominent nucleoli, nuclear budding, binucleation and multinucleation, proved a dose dependent increase in response to concomitant chemo-radiotherapy (Table 1).

Karyolysis

It was observed in n = 71 (50.7%) smears on both days and it was raised significantly from baseline n=1 (1.4%) to n = 70 (100%) smears taken at the end of CCRT. Significant statistical association was noticed among days of CCRT and karyolysis (p = 0.000).

Parameters	Before CCRT (Baseline)	At the end of CCRT
Karyolysis	n = 1 (1.4%)	n = 70 (100%)
Karyorrhexis	n = 1 (1.4%)	n = 70 (100%)
Binucleation	n = 7 (10%)	n = 70 (100%)
Prominent nucleoli	n = 15 (21.4%)	n = 70 (100%)
Multinucleation	n = 0 (0%)	n = 54 (77.1%)
Nuclear Budding	n = 0 (0%)	n = 68 (97.1%)
Micronucleation	n = 3(4.3%)	n = 70 (100%)

Table-1: Frequency of nuclear abnormalities before and at the end of CCRT.

Karyorrhexis

It was observed in n = 71 (50.7%) smears on both days and raised significantly from baseline n=1 (1.4%) to n = 70 (100%) smears taken at the end of CCRT [Figure 1]. Significant association was observed among days of CCRT and karyolysis (p = 0.000).

Micronucleation

A raise in the counts of cells, showing micronucleation, was observed with increasing radiation dose. Micronucleation was observed in n=73 (52.1%) smears on both days. The frequency of cells with micronuclei was increased from a baseline value n=3 (4.3%) and was more predominantly observed at the end of CCRT in n= 70 (100%) smears [Figure 2]. This increase was also statistically significant (p = 0.000).

Nuclear budding

Nuclear budding was observed in n=68 (48.5%) smears. Cells showing nuclear budding increased significantly (p=0.000) from their baseline value where it was absent in smears however the smears taken at the end of CCRT showed pronounce increase in nuclear budding and was observed in n=68 (97.1%) end therapy smears.

Binucleation

Binucleation was observed in n=77 (55%) on both days. These number of binucleated cells increased from the baseline values in n=7 (10%) to n=70 (100%) smears taken at before and end of CCRT respectively. Strong association was observed between days of treatment (CCRT) and binucleation (p = 0.000).

Multinucleation

Multinucleated cells increased significantly from n=0 (0%) in before CCRT smears to n=54 (77.1%) end therapy smears. A significant association was seen between days of CCRT and multinucleation (p = 0.000).

Prominent Nucleoli

Prominent nucleoli were observed in n = 85 (60.7%) smears. They were seen in n = 15 (21.4%) and in n = 70 (100%) smears taken before and at the end of CCRT respectively [Figure 3]. Statistically significant association was noticed among days of CCRT and prominent nucleoli (p = 0.000).

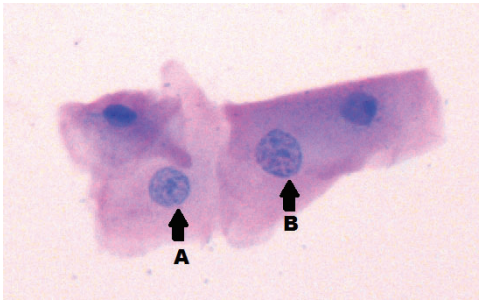

Figure-1: Photomicrograph showing feature of nuclear atypia, prominent nucleoli (A arrow) and karyorrhexis (B arrow) in normal oral buccal mucosa exfoliated epithelial cells (H&E stain, 40X).

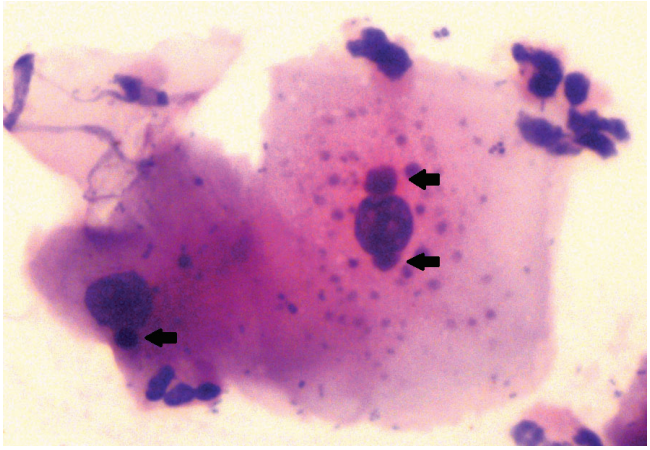

Figure-2: Photomicrograph showing micronuclei (arrow) in normal oral buccal mucosa exfoliated epithelial cells (H&E stain, 40X).

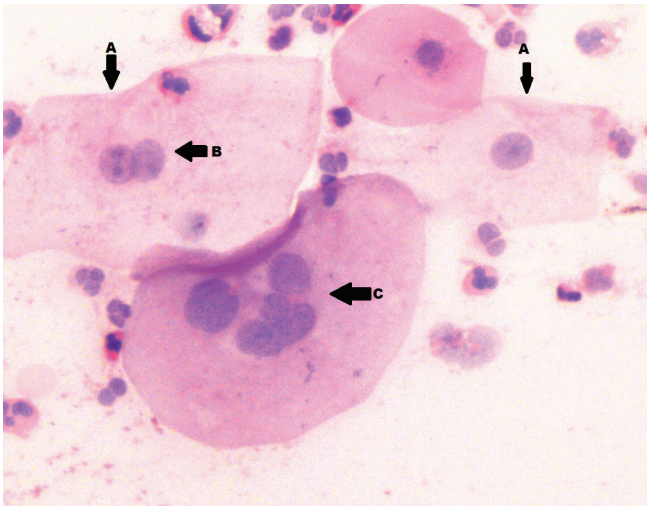

Figure-3: Photomicrograph showing features of nuclear atypia, prominent nucleoli (A arrow), binucleation (B arrow) and nuclear budding (C arrow) in normal oral buccal mucosa exfoliated epithelial cells (H&E stain, 40X).

DISCUSSION

Concomitant chemo-radiotherapy exerts its effects on normal buccal mucosa which may affect the field of irradiation and causing chromosomal injury, the end results of which can be distinguished by the occurrence of micronuclei in dividing cells. Micronuclei are abnormal fragments of a chromosome that fall behind during

cell division and are not included in the main nucleus. These are formed due to genotoxic damage to the cell e.g. radiation brings about chromosomal injury and characterized by a fatal genomic injury. The micronucleus test in epithelial cells and peripheral blood lymphocytes has been broadly used for observing genotoxic damage, caused by chemicals,[12] ionizing radiations,[13] and as well as the efficiency of chemo-preventive drugs used for cancer treatments.[14] Similarly another study is in accordance with the present study which stated that micronucleation was increased due to chemotherapy genotoxicity.[15] Thus as the CCRT dosages increased the frequency of micronucleation was also raised on contralateral normal buccal mucosa in current study also.

Micronuclei may result from DNA repair method taking place at low radiation doses leading to rejoining of DNA strand breaks during the S phase of cell cycle prior to the cell go into the mitotic phase.[16] With raise in the radiation dose, the repair capacity of the cell reduces while the quantity of chromosomal damage increases leading to accumulation of un-repaired DNA fragments which may lead to formation of further increased number of nuclear buddings and micronuclei. A dose-response association among the micronucleated cells and radiation has earlier been stated both in vivo and in vitro[16,17] as well as in OSCC patients receiving radiation therapy.[10,11,18-20] Hence the results of this study stated the view that micronucleus is a responsive marker of chromosomal damage caused by ionizing radiation.

A rise in number of cells showing nuclear budding was also seen with increasing radiation dosage but this increase was more significant at the end of CCRT.

Nuclear budding is considered to stand for a micronucleus which has been partly cover by the main nucleus or partly throw out during karryokinesis and thus remains continuous with the main nucleus. It may also be a symbol of straight consequence of radiation on the nuclear membrane leading to nuclear blebing.[18]

Radiation brings about per-oxidation of membrane lipids. It can also stimulate damage to cell membrane which may end in failure of cytokinesis (cytoplasmic division) following nuclear division and may lead to development of a binucleated cell.[18] The number of binucleated cells also increased at the end of CCRT, which is in agreement with previous studies. Binucleation is not considered to be caused by a direct effect of radiation on the nucleus but infact it may represent damage to cell membrane due to radiation which causes per-oxidation of membrane lipids which in turn lead to inability of the cell to undergo cytokinesis.[11] A binucleated cell may also be produced by definite non radiation-induced methods and it might correspond to a mitotic cell which has not been gone through cytokinesis as up till now.[11,18]

A considerably higher count of binucleated cells in the pre-treatment (CCRT) smears as noted in the present study supports the assumption that a subset of these cells may be produced by some other non radiation influenced mechanisms. This is also maintained by the fact that binucleated cells have also been reported in smears from normal oral mucosa of healthy controls as well as OSCC patients without any genotoxic exposure.[21] A part of these binucleated cells can be a symbol of lethal concomitant chemo-radiotherapy injury, whereas a few may stand for undamaged dividing cells as tissues are identified to repopulate after experience to fractionated radiation. However in smears it is not possible to distinguish between binucleated cells produced by non radiation-induced mechanisms or those produced due to radiation injury, the importance of binucleation as a marker of concomitant chemo radiation damage in oral smears remains unanswered.

Two mechanism of radiation induced multinucleation have been proposed. Radiation induced damage to peri-centriolar matrix leading to multipolar mitosis and another mechanism is that cell membrane damage can lead to binucleation and a further division in one or both of the nuclei in a binucleated cell may lead to multinucleation.[11] At higher doses of radiation there are unrepaired damage to the cell membrane and may result in an increase in the binucleated and multinucleated cell too. In present study though a net raise in the count of multinucleated cells is seen, it is significant only at end of therapy [Table 1]. In addition the counts of multinucleated cells were lower than the counts of other nuclear abnormalities at end of treatment.

Karyolysis is defined as dissolution of the nucleus of the cell/complete dissolution of the chromatin of the dying cells due to enzymatic degradation. There is no such study conducted on the presence of karyolysis in the smears from normal buccal mucosa area in CCRT receiving patients. However the study conducted in India on serial cytological smears from OSCC patients receiving radiotherapy reported that karyolysis was observed in OSCC smears and more obvious at the end of radiotherapy.[10] Similarly another study is in accordance with the present study which stated that karyolysis was increased due to chemotherapy genotoxicity.[15]

Karyorrhexis is the destructive fragmentation of the nucleus of a dying cell whereby its chromatin is distributed irregularly throughout the cytoplasm. It signi-

fies nuclear breakup into smaller fragments. However there is no such study conducted on CCRT induced karyorrhexis on normal buccal mucosa. Whereas the study conducted in India by Bindu and his associates on serial cytological smears of OSCC noted that the karyorrhexis was more pronounced at the end of therapy.[10] Another study carried out in India on serial cytological smears from OSCC reported the mean percentages of karyorrhexis raised with increased radiotherapy dosage.[22] Similarly, another study is in accordance with the present study which stated that karyolysis was increased due to chemotherapy genotoxicity.[15]

Prominent nucleoli are small, typically round granular bodies, composed of protein and RNA in the nucleus of a cell. It is usually associated with a specific chromosomal site and involved in ribosomal RNA synthesis and the formation of ribosomes. There is no such study conducted on the prominent nucleoli and its incidence in normal buccal mucosa in CCRT receiving patients however the study conducted in USA reported that the incidence of prominent nucleoli was raised in smears obtained from benign prostate glands after radiotherapy.[23] A significant association was observed between days of CCRT and prominent nucleoli ($p = 0.000$) in present study.

The results of current study show a dose related increase in all the nuclear parameters that were studied i.e. binucleation, micronucleation, multinucleation, karyolysis, karyorrhexis, prominent nucleoli and nuclear budding. However there is no such study conducted on combined chemo-radiotherapy effects on normal buccal mucosa and nuclear abnormalities. The following graph [Figure-4] shows that the frequencies of a variety of nuclear abnormalities increase with the increased CCRT dosages.

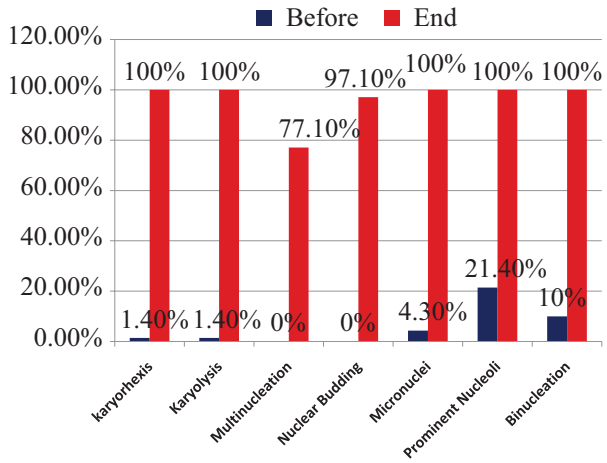

Figure-4: Bar chart shows an increase in the frequencies of a variety of nuclear abnormalities before the start and at the end of therapy smears.

It is also noted that reading of scoring criteria is eventually related with the subjective evaluation of each laboratory and scorer and that is the reason that a number of laboratories might be extra careful in accepting a cell presenting with micronucleation than others. Another practical restriction may be the features of the optics of microscope used. It has been recommended that the scorer's knowledge and understanding plays a significant role in scoring the cells.[24] Similar inter-laboratory differences may also be seen in case of other radiation induced nuclear changes. In light of this large inter-laboratory inconsistencies, assessment of data from different studies may not be very precise.

CONCLUSION

The findings of the present study show a direct dose-response relationship among the concomitant chemo-radiotherapy and duration of various nuclear abnormalities in contralateral normal buccal mucosa by studying chronological smears from patients undergoing fractionated radiotherapy and chemotherapy. Nuclear budding and micronucleation come out to be as a consistent markers for concomitant chemo-radiation damage. There is a requirement for additional studies with larger sample size and longer follow-up for better understanding of the role of these changes in calculating the normal buccal mucosa response to concomitant chemo-radiotherapy.

ACKNOWLEDGEMENTS

The authors acknowledge the encouragement extended by Vice Chancellor of UHS, also laboratory staff of Histopathology Department specially Mr. Sameer Anjum (Lab Technician), Usman Ali (Lab Manager) and Waqas Latif (Biostatistician), Sadia Maqbool (Research assistant) and all the staff of INMOL Hospital Lahore for their technical and logistic support.

REFERENCES

1. NCI, 2013. Head and Neck Cancers. National Cancer Institute Fact sheet. National Cancer Institute at the National institutes of health. [Online] Available at: <http://www.cancer. gov/cancertopics/factsheet/Sites-Types/head-and-neck> [Accessed 10th April 2015].
2. Scully C, Bagan J. Oral Squamous Cell Carcinoma Overview. Oral Oncology 2009; 45: 301-8.
3. Scully C, Epstein JB. Oral health care for the cancer patient. Oral Oncol Eur J Cancer 1996; 32:

281–92.
4. Papadimitrakopoulou VA, Lee JJ, William WN, et al. Randomized trial of 13-cisretinoic acid compared with retinylpalmitate with or without betacarotene in oral premalignancy. J Clin Oncol 2009; 27: 599–604.
5. Zheng JW, Qiu WL, Zhang ZY. Combined and sequential treatment 1. of oral and maxillofacial malignancies: an evolving concept and clinical protocol. Chin Med J (Engl) 2008; 121:1945-52.
6. NCI, 2010. Radiation Therapy for Cancer. National Cancer Institute Fact Sheet.National Cancer Institute at the National institutes of health. [Online] Available at: >http://www. cancer.gov/cancertopics/factsheet/Therapy/radiation< [Accessed 17th- March 2015].
7. Seiwert TY, Salama JK, Vokes EE. The chemo-radiation paradigm in head and neck cancer. Nat Clin Pract Oncol 2007;4: 156-71.
8. Graham RM. The effect of radiation on vaginal cells in cervical carcinoma; description of cellular changes. Surg Gynecol Obstet 1947;84:153-65.
9. Cowpe JG, Longmore RB, Green NW. Quantitative exfoliative cytology of abnormal oral mucosal smears. J Roy Soc Med 1988;81:509- 13.
10. Bindu L, Balaram P, Mathew A, et al. Radiation-induced changes in oral carcinoma cells - a multiparametric evaluation. Cytopathology 2003;14:287-93.
11. Mehrotra R, Madhu, Singh M. Serial scrape smear cytology of radiation response in normal and malignant cells of oral cavity. Indian J Pathol Microbiol 2004; 47:497-502.
12. Sarto F, Tomanin R, Giacomelli L, et al. The micronucleus assay in human exfoliated cells of nose and mouth: application to occupational exposure to chromic acid and ethylene oxide. Mutat Res 1990;244:345-51.
13. Kormos C, Koteles GJ. Micronuclei in X-irradiated human lymphocytes. Mutat Res 1988;199:31-5.
14. Benner SE, Lippman SM, Wargovich MJ,et al. Micronuclei, a biomarker for chemopreventive trials: results of a randomized study in oral pre-malignancy. Int J Cancer. 1994;59:457-9.
15. Torres-Bugarín O, Ventura-Aguilar A, Zamora-Perez A, et al. Evaluation of cisplatin + 5-FU, carboplatin + 5-FU, and ifosfamide + epirubicine regimens using the micronuclei test and nuclear abnormalities in the buccal mucosa. Mutat Res 2003: 539:177-86.
16. Mitchell JC, NormanA. The induction of micronuclei in human lymphocytes by low doses of radiation. Int J Radiat Biol Relat Stud Phys Chem Med 1987; 52: 527-35.
17. Jagetia GC, Ganapathi NG. Radiation-induced micronucleus formation in mouse bone marrow after low dose exposure. Mutat Res 1994; 304:235-42.
18. Bhattathiri NV, Bindu L, Remani P, et al. Radiation-induced acute immediate nuclear abnormalities in oral cancer cells: serial cytologic evaluation. Acta Cytol 1998; 42:1084- 90.
19. Mehrotra R, Goel N, Singh M,et al. Radiation-related cytological changes in oral malignant cells. Indian J Pathol Microbiol 2004; 47:343-7.
20. Kumari R, Chaugule A, Goyal PK. Karyoanomalic frequency during radiotherapy. J Cancer Res Ther 2005; 1:187-90.
21. Ramirez A, Saldanha PH. Micronucleus investigation of alcoholic patients with oral carcinomas. Genet Mol Res 2002; 1:246-60
22. Agarwal D, Khan N, Siddhiqui SA, et al. Assessment of Various Cytological Changes for Predicting Radiosensitivity of Oral Cavity Cancer by Serial Cytology. JK Science 2011;13: 4.
23. Cheng L, Cheville JC, Bostwick DG. Diagnosis of prostate cancer in needle biopsies after radiation therapy. Am J Surg Pathol 1999; 23: 1173-83.
24. Fenech M, Bonassi S, Turner J, et al. Human MicroNucleus project. Intra- and inter-laboratory variation in the scoring of micronuclei and nucleoplasmic bridges in binucleated human lymphocytes. Results of an international slide- scoring exercise by the HUMN project. Mutat Res 2003; 538:185-6.