The Effect of Titanium Implant on Oral Mucosal Epithelium

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Abstract

Titanium dental implant is widely spread as replacement of missing teeth now a day. Their increased use may develop a new problem that may have risk on the patients including oral cancer. This study represents the first study in Iraq that deal with the subject of genotoxic effect of titanium dental implant from cytological point of view. Aims: the aim of this study was assess the effect of titanium dental implant on oral mucosa by using cytomorphometry. Material and methods: 15 female Iraqi patients were included in this study at the implant clinic of college of dentistry –Al-Mustansiriyah university. We compared the presence of micronucleus, cluster sheets, CD, ND and the ratio of nuclear diameter to cellular diameter (N\C) of gingival mucosa of implant site(A) with adjacent normal tooth(B). The two groups were examined in two stages; one at abutment exposure visit and the second in recall visit for impression. Giemsa stain was used to stain slides for cytological investigation of exfoliated squamous epithelial cells. Results: The results for epithelial cluster shedding and micronucleus was 7(46.7%), 3(20%) for group A1 and 7(46.7%), 2(13%) for group B1. While was 12(80%) 9(60%) for group A2, 12(80%), 7(46.7%) for group B2 respectively. There was significant correlation (P<0.05) between micronucleus in group A1 and cluster shedding in groupB1 while highly significant correlation (P<0.001) with micronucleus in group B1. The mean CD and ND values were: Group A1: 3807.57 (± 710.4) and 1251.4281 (± 621.213); Group B1: 4202.9932 (± 912.8) and 1261.8046 (± 1176.1); Group A2: 4272.343 (± 650.457) and 1323.9878 (± 496.55); Group B2: 3852.2070 (± 943.8) and 1290.373 (± 559.77) urn, respectively. Correlation between the ND and CD among the groups in the two stages were highly significant (P<0.001) for nucleus diameter in group A1 with that of A2 and B2; and nucleus diameter in group A2 with that of B2, while the nucleus diameter in group B1 reversed significant (P<0.05) with cellular diameter in group B2. The ratio of N\C diameter was nearly not changed between the groups. Univariate analysis of variance (ANOVA) showed a significant group effect for cellular diameter, nuclear diameter and. Multiple comparison test by Tukey-HSD procedure revealed a significant decrease in the mean cellular diameter, increase in the nuclear diameter. The results indicate that titanium dental implant could have dysplastic effects that could be changed to oral malignancy especially squamous cell carcinoma. Conclusion: Cytomorphometric changes could be the earliest indicators of cellular alterations. There is progressive decrease in cellular diameter, increase in nuclear diameter in addition to increase in the formation of micronucleus and cluster sheet of epithelium in smears from all implant sites, as compared to normal adjacent teeth. This indicates that titanium implant could have a genotoxic effect on oral epithelium that causes cellular alteration.

Introduction

Dental implants use is a widespread treatment now a day to restore missing teeth and edentulous cases but, a successful implant treatment must be safe by the lack of inflammation or dysplastic changes in peri-implant tissues [1-4]. The increased usages of dental implant due to reduction in their costs, increasing the number of professions and patients' demand in addition to changes in the treatment plans and methods of application that means gradual increase in the number of people who carrying dental implant in their mouth that may induce a recent or unacceptable derived conditions including oral cancer.

Titanium implants represents the most common type of dental implant that consisted from titanium screw with smooth or roughened surface that is made of commercially pure titanium which is of 4 types depending on the quantity of carbon, nitrogen, oxygen and iron contained [5] that are biocompatible that covered with titanium dioxide as surface or veneer layer that encloses the other metals and preventing them from contacting bone [6].

Titanium dioxide (TiO_2) is considered as safe material because it is inert, so that it had been used from a long period of time for different purposes. However, with the development of nanotechnologies; TiO_2 nanoparticles (NP) with numerous unique and useful properties, made the demand on it and is gradually increased. Therefore there would be an expected increase in the daily exposure to TiO_2 by human, which has put its nanoparticles under toxicological category. Mechanistic toxicological studies show that TiO_2 nanoparticles principally cause unfavorable effects via induction of oxidative stress resulting in cell damage, genotoxicity, inflammation, immune response [7].

Like all metals; titanium releases TiO_2 NP particles through corrosions which become ions in the body and bind to body's proteins. Cellular uptake, subcellular localization, and capability to cause toxic effects depend on the NP properties. The two main pathways of NP uptake in the cell are active uptake by endocytosis, and passive uptake by free diffusion [8].

Since cancers arises in epithelial tissues especially squamous epithelium; so exfoliated epithelial cells may be useful for the observation of the patients who are exposed to risk factors (prevalence) like genetic and various environmental factors which may be contribute in the neoplastic process. Carcinogens affect cells by altering genetic material and thus causing instability. Cytology of the exfoliated cells was intended firstly for early detection of cervical cancer and it has been mainly applied in oral medicine practice to detect early changes in oral mucosa related to malignancy [9]. The exfoliative oral epithelial cells

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are mainly surface layer cells that rarely showed mitotic figures [10-12].

The dysplastic changes in epithelium including oral epithelium could have more than one feature. This includes abnormal nuclear cytoplasmic ratio, abnormal mitosis, presence of micronuclei (MN) and many other features. MN is representing a small nucleus that forms during cell division whenever a chromosome or a fragment of a chromosome is not integrated into one of the daughter nuclei [13,14]. The biological importance of detection of micronuclei in the buccal cells of the oral mucosa is that the micronuclei are a sign of a readily certain clastogenic event [15].

Toxins that have been associated with micronucleus are miscellaneous and include airborne pollutants, food and compounds present in various occupational hazard. Thus, the micronuclei assay is used widely test for chemicals for the ability to induce particular chromosomal damage.

Several studies show that nano-TiO_2 induces genotoxic effects, including DNA damage, and micronuclei formation which represents an indicator of chromosomal aberrations in different cell [16-18].

Although the relation of cluster shedding of epithelium was not clearly well studied by previous researches but it represents one of the criteria of epithelial tumors [19].

The increase in nuclear cytoplasmic (N:C) ratio is one of the features of cellular dysplastic changes which is used in the histopathological assessment of premalignant lesions of the oral mucosa. Many studies suggested that increased nuclear size and decreased cytoplasm size are helpful early indicators of malignant transformation by exfoliative cytology which is of value for examining clinically suspect lesions for malignancy [20].

The aim of this study was to compare the epithelial cells changes of the gingival mucosa at implant site to the adjacent normal tissue at two time points: after exposing of implant and at time of impression. These cells were examined for morphometric alterations in the area of the nucleus and cytoplasm, alterations in the nuclear/cytoplasmic ratio, and alterations in the cytological criteria for malignancy by detection of micronucleus and shedding in cluster rather than single form.

Materials and Methods

Patients

15 female patients aged 20-55 years old who were referred to the dental clinic for dental treatment were invited to participate in this study. When they agreed to participate, individuals or their legal guardians signed an informed consent form. The study was approved by the Research Ethics Committee of CDUM.

Selected individuals had no related history of smoking, alcoholism, diabetes, anemia, or debilitating diseases and

were not being treated with antibiotics or steroids during the study period.

The patients were exposed to two stages implant surgery; thus the sample were collected from them before the exposure of the implant abutment that considered group A1 and in recall visit before taking impression for crown fabrication that considered group A2.

The adjacent tooth was considered as control group to compare the results and named B1,B2 according to the study stage.

Exfoliated cell collection and preparation

Exfoliated gingival cells were collected from two sites; at implant site and around the adjacent tooth that is used as control site for comparison.

Scrapings were obtained by using a interproximal brush moistened with normal saline. Using a gentle scraping motion, exerting little pressure, cells were scraped from the clinically normal appearing gingival mucosa of the adjacent teeth, and the mucosa covered the implant site before exposure in stage one of the study and around the exposed implant in stage 2.

The scrapings were smeared on to the center of glass slide, over an area of approximately 2.5 × 2.5 cm.

The cells were fixed for 20 minutes using 95% ethyl alcohol spray to ensure proper fixation. Samples were stained using 4% Giemsa stain for 12 minutes then wash thoroughly by distal water for few minutes. Allow to dry and covered by cover slip ready to be examined under the microscope.

Cytophotometric analysis procedure

The clue cells were squamous cells epithelium of gingival mucosa that exfoliated by interproximal brush scraping.

The slides were analyzed using a digital optical microscope (Micros, Austria) at a magnification of 1000x (oil immersed objective lens = 100x with eyepiece = 10x), and 5 epithelial cells were chosen per microscope field. Within the samples, only cells that were separate, without overlapping or folds, were analyzed. The cells were selected by moving the microscope stage in "Z" shape or the zigzag method to avoid recounting of the same cell.

The observed criteria

Micronuclei: were counted if the structures had a regular border and were located inside the cytoplasm. The evaluation of micronuclei criteria was obtained just by the looking for their presence or absence in the whole sample slide due the difficulty of obtaining large number of exfoliated epithelial cells. The present case was given score (1) and the absent was given score (0) *(Figure 1)*.
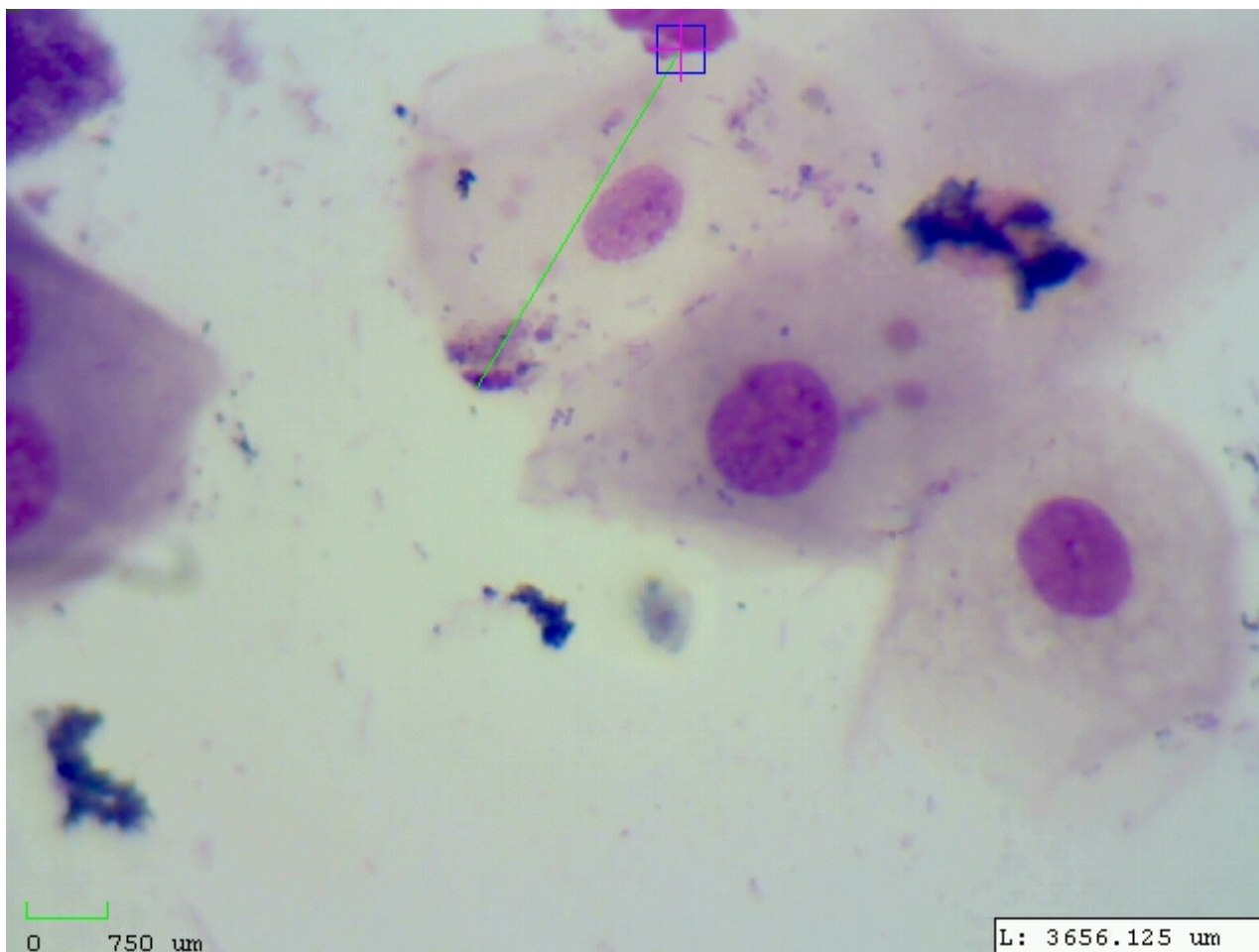
Figure 1. Micronuclei counting.

Epithelial cluster shedding: the shedding of exfoliated epithelial cells could be single or in clusters. The cases which showed marked cluster shedding had been given score (1); while few or absent of clusters was given score (0) *(Figure 2)*.
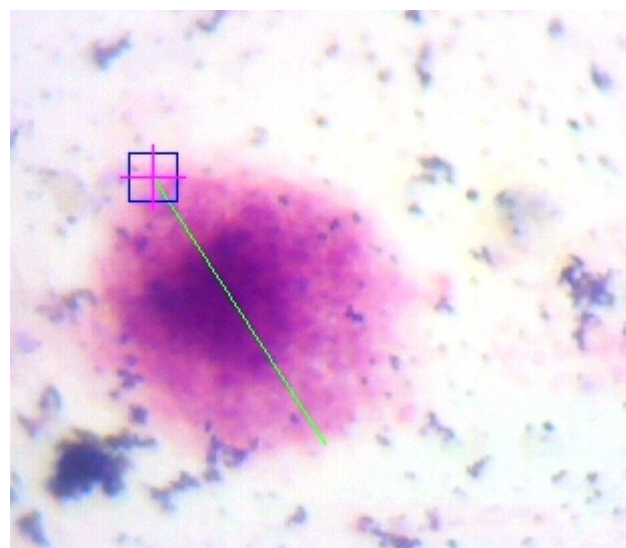

Figure 2. Epithelial cluster shedding.

Figure 3. Cellular diameter measurement 1.

Cellular diameter, nuclear diameter and nuclear/ cytoplasmic ratio criteria

Exfoliative gingival smears were evaluated for cellular, nuclear diameters, and nuclear/cellular ratios (N/C) using

computerized analysis of the digital microscope with it's special photometry system. The cellular diameter was measured by using the calibration in the microscope system by drawing a line from one side of the cell passing through the nucleus to other side *(Figures 3 and 4)*.

Statistical analysis

Variables were compared between two groups using the Pearson correlation coefficient test and the comparison between two means was done using the t-test in SPSS software version 13.0. P values <0.05 were considered significant. One way ANOVA test for multiple comparison with Tukey HSD.
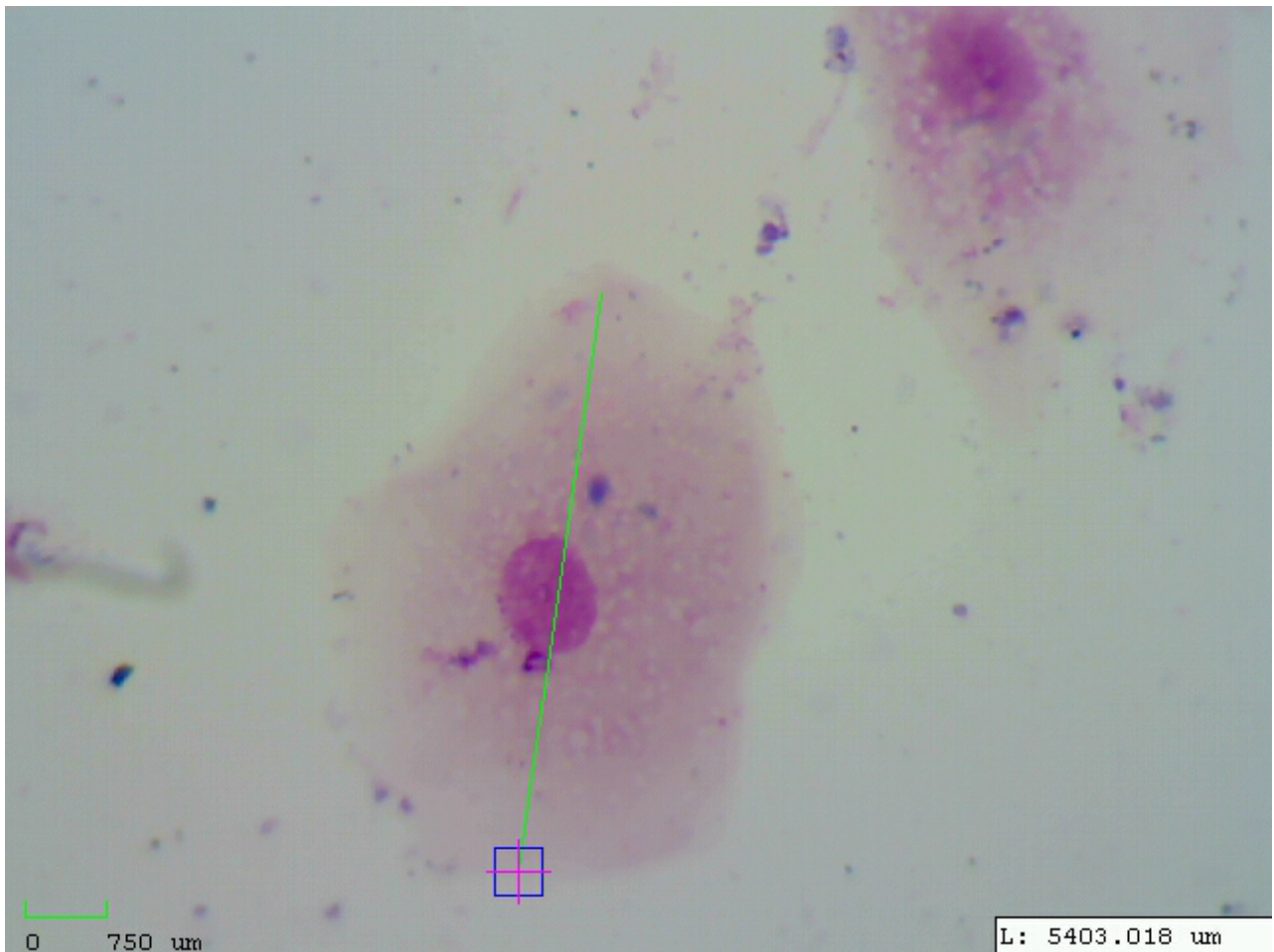

Figure 4. *Cellular diameter measurement 2.*

Results

With clinical observation of all cases; there was normal healing of the wound area with no obvious infection or complications.

Micronuclei

The presence of micronucleus was observed in 3(20%) for group A1 and 2(13%) for group B1. While 9(60%) for group A2 and 7(46.7%) for group B2 as shown in *Table 1*.

Epithelial cluster shedding

The pattern of exfoliation was also observed in each group. The exfoliation in a form of cluster sheets of parabasal cell layer more than superficial cell layer which tend to be exfoliated as single scattered cells.

The results for epithelial cluster shedding was 7(46.7%), for group A1 and 2(13%) for group B1. While was 9(60%) for group A2, 7(46.7%) for group B2 *(Table 1)*.

Table 1. *Frequencies and percentage of cluster sheets and micronucleus parameters and mean and standard deviation (SD) of cellular, nuclear diameter and nuclear to cytoplasm ratio.*

Group	Sample size	Parameter		Cellular diameter Mean ± SD	Nuclear Mean ± SD	Nuclear to cellular ratio
		Cluster sheets	Micronucleus			

A1	15	7 (46.7%)	3 (20%)	3807.57 ± 710.4	1251.4281 ± 621.213	0.33
B1	15	7 (46.7%)	2 (13%)	4202.9932 ± 912.8	1261.8046 ± 1176.1	0.3
A2	15	12 (80%)	9 (60%)	4272.343 ± 650.457	1323.9878 ± 496.55	0.31
B2	15	12 (80%)	7 (46.7%)	3852.207 ± 943.8	1290.373 ± 559.77	0.33

The correlation showed significant difference between micronucleus in group A1 and cluster shedding in group B1 ($P<0.05$) while highly significant difference with micronucleus in group B1 ($P<0.01$) as shown in *Table 2*.

Cellular and nuclear diameter

The nucleus diameter could be measured only in cells from parabasal layer and some superficial cells because the superficial cells lack of prominent nucleus.

The mean CD and ND values were: Group A1: 3807.57 (± 710.4) and 1251.4281 (± 621.213); Group B1: 4202.9932 (± 912.8) and 1261.8046 (± 1176.1); Group A2: 4272.343 (± 650.457) and 1323.9878 (± 496.55); Group B2: 3852.2070 (± 943.8) and 1290.373 (± 559.77) urn, respectively *(Table 1)*.

Table 2. Pearson correlations between groups in 2 stages for all parameters investigated.

Group	P value	significant
Micronucleus (A1) vs. cluster (B1)	0.04	S
Micronucleus (A1) vs. micronucleus (B1)	0.001	HS
Nucleus diameter(A1) vs. nucleus diameter (A2)	0.001	HS
Nucleus diameter(A1) vs. nucleus diameter(B2)	0.008	HS
Nucleus diameter(A2) vs. nucleus diameter (B2)	0	HS
Nucleus diameter(B1) vs. cellular diameter(B2)	0.04	S
*A1:implant site in stage 2, B1: control adjacent site in stage 2, A2:implant site in stage 2, B2: control adjacent site in stage2		

Correlation between the ND and CD among the groups in the two stages were highly significant difference ($P<0.01$) for nucleus diameter in group A1 with that of A2 and B2; and nucleus diameter in group A2 with that of B2. While the nucleus diameter in group B1 reversed significant difference ($P<0.05$) with cellular diameter in group B2 *(Table 2)*.

Table 3. ANOVA TEST for multiple comparison with Tukey-HSD.

Group	P value<0.01	Significant
CD(A1) XND(A1)	0.001	HS
CD(A1) XND(B1)	0.001	HS
ND(A1) XCD(B1)	0.001	HS
CD(B1) X ND(B1)	0.001	HS
CD(A2) XND(A2)	0.001	HS
CD(A2) XND(B2)	0.001	HS
ND(A2) XCD(B2)	0.001	HS
ND(A2)XND(B2)	0.001	HS
CD(B2) XND(B2)	0.001	HS

Table 3 showed the ANOVA test that showed highly significant difference (P<0.001) for CD at implant site in the 2 stages with ND of both implant and control sites in the 2 stages.

Discussion

Dysplastic changes in oral epithelium could be investigated by many biomarkers including MN rate, changes in ND and CD that could be associated with different risk factors including smocking, systemic diseases and dental materials.

In this study; the number of MN was significantly higher in implant groups than those of control group *(Table 1)*.

These observations could indicate genetic damage which correlates with future cancer of oral mucosa because most of cytological studies that deal with exfoliated cells of buccal mucosa deal with frequencies of MN in these cells as indicator of necular damage due to different risk factors [21].

There was also increasing in the number of cells that exfoliated in the form of cluster or sheets in the stage 2 of the study in comparing to stage 1 in both implanted and adjacent teeth.

These sheets of cell clusters are mostly nucleated which indicated that they are prickle or granulosium layer rather than corneum layer that lack of definite nucleus [22] in the number of cells of stratum corneum of keratinized epithelium that show abundant cytoplasm and smaller nuclei than cells from deeper layers. This hyperkeratinosis can be confirmed by an increase in the number of surface cells in examined tissue.

There was significant difference between micronucleus in implant site and cluster sheets in adjacent teeth in stage 1 (P<0.04) which indicated that epithelial changes of mucosa covering the implant site in stage 1 is higher than in stage 2 because the surface area over the implant is larger than surface area of epithelium around the dental implant in stage 2.

There was also highly significant difference (P<0.001) in stage 1 with micronucleus frequencies in adjacent teeth which indicated that titanium implant have certain genotoxic effect on oral epithelium as in the study of [23,24] that showed that increased MN incidence due to exposure to endogenous and exogenous genotoxic materials *(Table 2)*.

The higher frequency of MN in our patient's sample may be due to their female sex that usually have greater tendency to develop MN than male due to tendency of X-chromosome to be lost [24].

The genotoxic effects of titanium could be accumulative or starts after long period of time (may be years) due to corrosion

of the outer oxide layer [7] similar to other dental materials that are indirect or direct contact with oral mucosa [25,26].

Regarding CD, ND and nuclear: cytoplasmic ratio; our results showed significant difference correlation between ND in implant site in stage 1 and ND at stage 2, ND of adjacent teeth in which alteration in the ND is seen *(Table 2)*. This may be due to the effect of TiO2 nanoparticles that may affect cell divisions and cell size [7,8].

ANOVA test showed significant differences (P<0.001) for CD between the groups as well as for ND. This may indicated that CD, ND could be properly sensitive parameter in the diagnosis of cellular atypia.

These results are similar to the findings of other study that compared cell size of traumatic keratosis lesions with normal buccal cells. It also reported a statistically significant increase in the size of cells [22].

An increase in nuclear diameter and a decrease in cell diameter were observed in samples of patients with tumors in the floor of the mouth in tobacco-chewing habit and in those with smoking and tobacco-chewing habits combined [27].

Our results indicate that the increased MN and cell cluster sheets frequencies in exfoliated cells of the gingival mucosa of implant's patients may reflect genomic instability or deficiency of DNA repair capacity.

Analysis of cytophotometric values of exfoliated mucosal cells in malignant and premalignant lesions also has shown variations from the normal values.

To summarize, we believe that micronuclei assay is an effective technique aid in the diagnosis of different diseases. At this time it remains unclear whether elevated frequencies of MN and changes in CD, ND in certain tissue, such as oral epithelia, would be predictive of increased risk of future cancer.

Our study represents one of the fewest studies that correlate dental implant with cytological changes in gingival epithelium and most of the oral cytological studies that could be used as reference data were dealing with buccal exfoliated cells not with gingival epithelium.

There is a great difference between the two types of mucosa regarding location and function which reflected by the type of epithelium.

In addition; the present study used linear morphometric measurements of cell and nucleus that could be need for more measurements of surface area of both nucleus and cytoplasm to get more accurate results.

Researches like our study required more patients in longer period of time to get more accurate and beneficial results.

Conclusion

Cytomorphometric changes could be the earliest indicators of cellular alterations. There is progressive decrease in cellular diameter, increase in nuclear diameter in addition to increase in the formation of micronucleus and cluster sheet of epithelium in smears from all implant sites, as compared to normal adjacent teeth. This indicates that titanium implant

could have a genotoxic effect on oral epithelium that causes cellular alteration.

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