Original Article

Morphology and Differentiation of MG63 Osteoblast Cells on Saliva Contaminated Implant Surfaces

Neda Shams[1], Mahmood Ghasemi[2]✉, Saeed Sadatmansouri[2], Shahin Bonakdar[3]

[1]Postgraduate Student, Department of Periodontics, Tehran Dental Branch, Islamic Azad University, Tehran, Iran
[2]Associate Professor, Department of Periodontics and Implant Research Center, Tehran Dental Branch, Islamic Azad University, Tehran, Iran
[3]Assistant Professor, Department of National Cell, Pasteur Institute of Iran, Tehran, Iran

✉ Corresponding author:
M. Ghasemi, Department of Periodontics, Tehran Dental Branch, Islamic Azad University, Tehran, Iran

m_ghasemi@dentaliau.ac.ir

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Abstract
Objectives: Osteoblasts are the most important cells in the osseointegration process. Despite years of study on dental Implants, limited studies have discussed the effect of saliva on the adhesion process of osteoblasts to implant surfaces. The aim of this *in vitro* study was to evaluate the effect of saliva on morphology and differentiation of osteoblasts attached to implant surfaces.
Materials and Methods: Twelve Axiom dental implants were divided into two groups. Implants of the case group were placed in containers, containing saliva, for 40 minutes. Then, all the implants were separately stored in a medium containing MG63 human osteoblasts for a week. Cell morphology and differentiation were assessed using a scanning electron microscope and their alkaline phosphatase (ALP) activity was determined. The t-test was used to compare the two groups.
Results: Scanning electron microscopic observation of osteoblasts revealed round or square cells with fewer and shorter cellular processes in saliva contaminated samples, whereas elongated, fusiform and well-defined cell processes were seen in the control group. ALP level was significantly lower in case compared to control group ($P<0.05$).
Conclusion: Saliva contamination alters osteoblast morphology and differentiation and may subsequently interfere with successful osseointegration. Thus, saliva contamination of bone and implant must be prevented or minimized.
Keywords: Osseointegration; Saliva; Dental implants; Osteoblasts; Alkaline phosphatase
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INTRODUCTION

Implant treatment success depends on achieving adequate osseointegration, which is defined as a direct and structural relationship between the surrounding living bone and the implant surface. The process of osseointegration may be compromised by errors in the drilling technique or blood and saliva contamination of the site causing subsequent histological changes [1]. Considering the continuous salivary flow into the oral cavity, risk of saliva contamination in dental procedures like periodontal surgeries, implant placement or tooth extraction is high [2]. The saliva contains antimicrobial enzymes, cytokines, growth factors, growth hormones and proteins involved in immune responses, inflammatory reactions and cell proliferation [3-6]. On the other hand, osteoblasts are the most important cells in the process of osseointegration. Electron microscopic observations have shown that, following the first day of implant placement, osteoblasts form direct and firm attachments to the implant surfaces [6].

However, to date, only one study has assessed the effect of saliva on osteoblast cells [2]. The results of the afore-mentioned study showed that the presence of saliva reduced the number and differentiation of osteoblasts compared to the non-saliva-contaminated control group; also osteoblast differentiation markers such as ALP were produced in smaller amounts in the saliva-contaminated group. Despite years of study on dental implants and the effect of various factors on their success rate, yet no experimental study has evaluated the effect of saliva-contaminated implant surface on the behavior of osteoblasts. Therefore, the aim of this in vitro study was to evaluate the effect of saliva-contaminated implant surface on the morphology and differentiation of human osteoblasts.

MATERIALS AND METHODS

Cell culture:

The cell culture medium was prepared by combining Dulbecco's Modified Eagle's Medium (DMEM) with Ham's F12 nutrient mixture and 10% fetal bovine serum (FBS). To prepare the DMEM culture medium, 10 g of powdered DMEM containing L-glutamine with 7.3 g of sodium bicarbonate powder, was dissolved in 900 mL of deionized water. Using 1 normal HCL solution, pH of the solution was adjusted to 4.7 and the medium volume was reached to 1000 mL. Finally, the medium was sterilized using 0.22 micron filter. Then, 100 µg/mL of streptomycin and 100U/mL of penicillin were added to the above-mentioned solution. The above-mentioned steps were followed for preparation of Ham's F12 medium as well. The only difference was that, 9.8 g of F-12 nutrient mixture powder, containing L-glutamine, with 1.8 g of sodium bicarbonate powder were dissolved in 900 mL of deionized water [7]. After the flask bottom was fully covered with osteoblast cells, flask medium was evacuated and the cells were washed with phosphate buffered saline (PBS). After removing the PBS, Trypsin enzyme was added to the flask and incubated at 37 ° C for 3-5 minutes. The flask was removed from the incubator and the cells detached from the bottom of the flask were transferred to a 15 mL tube. After adding the culture media to the trypsin immersed cells, the cells were centrifuged and the cell suspension was diluted to 5×10^5 cells/mL.

Saliva collection:

Saliva sample was collected immediately prior to starting the tests from a 33-year old female volunteer, who had not taken antibiotics during the past 3 months, was systemically healthy and had normal periodontium and no caries. Then, filtration with 0.22 micrometer filters (Millex-GV Filter) was done for the purpose of sterilization [2,8].

Preparation of samples:

Twelve Axiom (Anthogyr-France) dental implants (4x10mm) were divided into two groups of six each. Implants in the case group were placed in saliva containers for 40 minutes. Six other implants remained uncontaminated as the control group. Then, all implants were separately placed in a medium containing human MG63 osteoblasts in cell culture dishes for a week.

Alkaline phosphatase (ALP) activity:

ALP is an indicator of osteoblast activity [9]. One week after [10] cell culture, 6 µL of each implant medium was prepared and added to the mixture of 294 mL of diethanolamin (1 M, pH 9.8, 10 minutes), with 60 mM of 4-nitrophenylphosphate-Na and its absorbance was measured at 405 nm with a UV spectrophotometer for 5 minutes [2].

Scanning electron microscope (SEM) examination:

After one week, implants were washed twice with PBS, cells were fixed with glutaraldehyde, and washed with dehydrated alcohol. Then, samples were sputter-coated with 15 nm thickness of gold. The cells were examined using VEGA SEM (Tescan-Czech Republic).

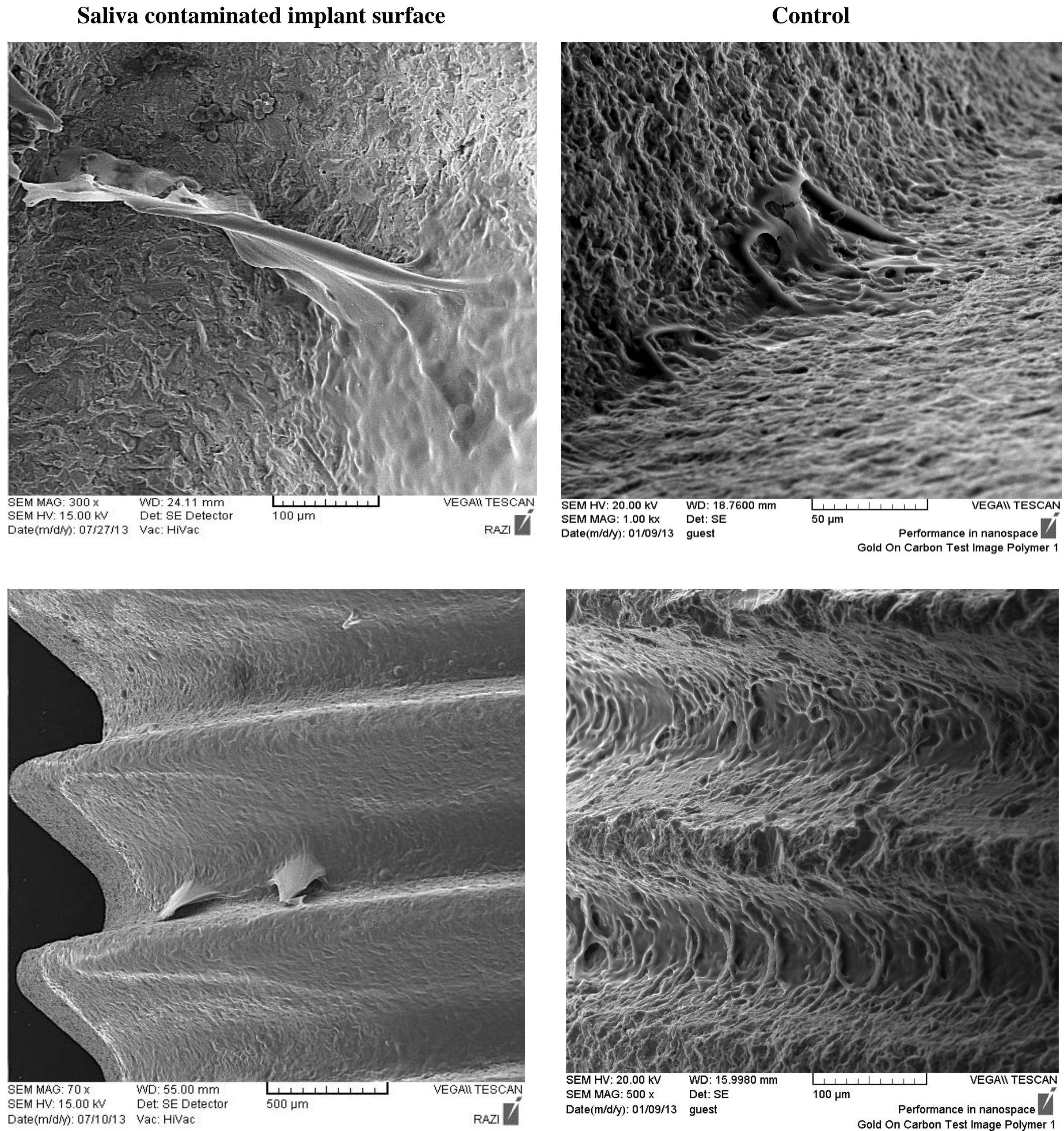

Fig. 1. SEM micrographs of saliva-contaminated implant surface (left) and control (right). Saliva contamination hampers cell proliferation; whereas the control group showed well-defined cell morphology and differentiation.

After generating vacuum, an image of the topography, morphology and cell adhesion to the implant surfaces was captured.

Statistical analysis:

In order to compare the optical density (OD) in the two groups, Kolmogorov-Smirnov test (KST) was first used to evaluate the normal distribution of data and then independent t-test was applied to compare the two groups. All calculations and statistical analyses were done using SPSS 16.0.

RESULTS

Cell morphology:

Fibroblasts attached to the saliva-contaminated implant surfaces were round or rectangular with fewer and shorter cell processes. While in the non-saliva-contaminated samples, the majority of cells were elongated and fusiform and had more and longer cell processes (Fig. 1). Due to the overlapping of cells on the implant surface, cell counting was impossible. This was observed in all cell sites [Fig. 1].

Alkaline phosphatase:

The mean and standard deviation of ALP level in the case and control groups are presented in Table 1. The level in the case group was less than that in the control group and this difference was statistically significant (P<0.05).

DISCUSSION

The interaction of cells with the surface of materials is a major topic in tissue engineering in both the clinical and experimental settings. In this respect, surfaces can directly influence tissue reaction (response), and consequently, affect the quantity and quality of newly formed tissue. Currently, the basic idea in implant treatment planning is to provide a biocompatible surface [11].

Surface of implant plays a major role in modulating bone response and implant anchorage [12-14]. Saliva contains a wide range of cytokines, growth factors and hormones playing roles in the immune and inflammatory responses and also cell growth in oral tissue [4-5]. Also, the saliva significantly accelerates oral mucosal wound healing [3,4]. However, little information is available on the response of osteoblasts to saliva contamination. The current study sought to illuminate this issue. Previous studies on cell adhesion to saliva-contaminated implant surfaces were mainly done on fibroblasts [8,15].

Zoller and Zentner observed a significant decrease in fibroblast adhesion to surfaces immersed in serum or saliva [8]. Thus, it was stated that saliva contamination of implant surfaces during placement or treatment of peri-implantitis may compromise cell adhesion. Gabriel et al, in their study on osteoblasts found the same results as ours regarding saliva or salivary mucin contaminated surfaces [15]. To the best of our knowledge, the current study is the first to assess osteoblast reactions to saliva-contaminated implant surfaces. The only study that evaluated the effect of saliva on osteoblasts has been done in a medium without implants [2]. The results of the current study showed that on non-saliva-contaminated surfaces, cells were elongated and fusiform with more and longer cell processes. In contrast, presence of saliva led to morphological changes in MG63 human osteoblasts from the elongated form with long processes to circular cells with short processes, indicative of decreased cell differentiation. Following the reduction in cell differentiation, mineralization is also decreased impairing the normal process of osseointegration and compromising the success of dental implants. These observations are consistent with in vitro findings of Proksch et al [2].

Table 1. Alkaline phosphatase level of MG63 osteoblast cells in case and control groups

Group	Minimum (OD)	Maximum (OD)	Mean ± SD	Coefficient of Variation
Case	52	75	65.5 ±7.55	0.115
Control	99	76	85.8 3 ± 9.23	0.107
P value			P<0.05	

They studied the effect of saliva on morphology and differentiation of MC3T3 mouse osteoblasts and stated that, in addition to the reduction in number of osteoblasts, the ability of cells for growth and differentiation strongly decreased and they attributed these findings to the observed reduction of osteocalcin and ALP activity, as they are the key markers of osteoblast cell differentiation. In the current study, decrease in ALP activity was also observed along with the above-mentioned morphological changes, but in samples without saliva contamination, this enzyme had normal activity. Although we did not evaluate salivary markers of inflammation or the extracellular matrix(ECM) mineral content in this study (which are important in cell behavior), others have confirmed that saliva harms osteoblast-like cells mainly by its enzymatic content rather than by triggering inflammation [2]. Regarding the ECM, it should be mentioned that more frequent presence of globular and round cells around the saliva contaminated surfaces may indicate reduced ECM consistency, although it may contain the same calcium content [2]. The higher calcium content was shown to be related to osteopontin, a sialoprotein, which does not act as a nucleator of calcium salt precipitation even though other members of the family of phosphorylated sialoproteins do so [16]. The strength point of the current study compared to previous ones is the use of human osteoblast cells which has not been done before, and this makes the present study unique. ALP is a glycoprotein and a common biochemical marker used to assess osteoblast differentiation and is considered to be involved in skeletal mineralization. ALP is abundantly found in matrix vesicles and plays a role in formation of ECM and calcification of bone. The level of ALP is increased just before the mineralization is initiated [17-18].

The observations by Proksch et al, and the present study do not confirm the published data regarding the effects of saliva on oral soft tissue cells such as fibroblasts or keratinocytes [2,4-6]. Although we did not evaluate the salivary enzyme activity, studies have indicated that mucin and salivary enzymes would best serve this purpose [17-18]. This phenomenon along with the collagenase and other enzymes capable of remodeling ECM may further confirm the existing observations [19]. Saliva preparation method in the current study was a standard method, which indicates that in spite of filtration and sterilization, saliva contains significant amounts of the afore-mentioned enzymes [2]. Thus, the reported observations and experimental evidence are not merely an in vitro artifact. From the clinical point of view, it is assumed that this mechanism causes a delay in wound healing around saliva-contaminated roots, bone and implant surfaces, as a consequence of decreased cellular adhesion [17]; however, it is important to note that the mentioned observations, may appear different to some extent in the clinical settings. In vivo, saliva contains significant amounts of microorganisms and also oral tissue cells. Therefore, it should be noted that the behavior of attached cells may even be more affected in the clinical setting. The findings of the present study indicate the need for further studies on different surface micro-designs and also in different clinical settings like in smokers to shed light on the role of saliva in implant success.

CONCLUSION

Dental implants contaminated with saliva cause a reduction in osteoblast cell differentiation, and may subsequently interfere with the osseointegration process and compromise implant success. In the clinical setting, saliva contamination of implant must be prevented during implant placement.

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REFERENCES

1- James RA. Peri-implant considerations. Dent Clin North Am. 1980 Jul;24(3):415-20.

2- Proksch S, Steinberg T, Keller C, Wolkewitz M, Wiedmann-Al-Ahmad M, Finkenzeller G, et al. Human saliva exposure modulates bone cell performance in vitro. Clin Oral Investig. 2012 Feb;16(1):69-77.

3- Dodds MW, Johnson DA, Yeh CK. Health benefits of saliva: a review. J Dent. 2005 Mar; 33(3):223-33.

4- Groschl M. The physiological role of hormones in saliva. Bioessays. 2009 Aug;31(8): 843-52.

5- Hakkinen L, Uitto VJ, Larjava H. Cell biology of gingivalwound healing. Periodontol 2000. 2000 Oct;24:127-52.

6- Oudhoff MJ, Bolscher JG, Nazmi K, Kalay H, van 't Hof W, Amerongen AV, et al. Histatins are the major wound-closure stimulating factors in human saliva as identified in a cell culture assay. FASEB J. 2008 Nov;22(11): 3805-12.

7- Borsari V, Giavaresi G, Fini M, Torricelli P, Salito A, Chiesa R, et al. Physical characterization of different-roughness titanium surfaces, with and without hydroxyapatite coating, and their effect on human osteoblast-like cells. J Biomed Mater Res B Appl Biomater. 2005 Nov; 75(2):359-68.

8- Zöller GO, Zentner A. Initial attachment of human gingival fibroblast-like cells in vitro to titanium surfaces pretreated with saliva and serum. Clin Oral Implants Res. 1996 Dec;7(4): 311-5.

9- Pivodova V, Frankova J, Ulrichova J. Osteoblast and gingival fibroblast markers in dental implant studies. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2011 Jun; 155(2):109-16.

10- Piattelli A, Scarano A, Di Alberti L, Piattelli M. Histological and histochemical analyses of acid and alkaline phosphatases around hydroxyapatite-coated implants: a time course study in rabbit. Biomaterials. 1997 Sep;18 (17):1191-4.

11- Alves SF, Wassall T. In vitro evaluation of osteoblastic cell adhesion on machined osseointegrated implants. Braz Oral Res. 2009 Apr-Jun;23(2): 131-6.

12- Buser D, Schenk RK, Steinemann S, Fiorellini JP, Fox CH, Stich H. Influence of surface characteristics on bone integration of titanium implants: A histomorphometric study in miniature pigs. J Biomed Mater Res. 1991 Jul;25 (7):889-902.

13- Szmukler-Moncler S, Reingewirtz Y, Weber HP. Bone response to early loading. The effect of surface state. In; Davidovitch, Z., Norton, L. A. Biological Mechanisms of Tooth Movement and Craniofacial Adaptation, Harvard Soc. Adv. Orthodontics, Boston: 1996: 611-16.

14- Klokkevold PR, Johnson P, Dadgostari S, Caputo A, Davies JE, Nishimura RD. Early endosseous integration enhanced by dual acid etching of titanium: A removal torque study in the rabbit. Clin Oral Implants Res. 2001 Aug; 12(4):350-7.

15- Gabriel MO, Grünheid T, Zentner A. Glycosylation pattern and cell attachment inhibiting property of human salivary mucins. J Periodontol. 2005 Jul;76(7):1175-81.

16- Boskey AL. Osteopontin and related phosphorylated sialoproteins: effects on mineralization. Ann N Y Acad Sci. 1995 Apr 21; 760: 249-56.

17- Oudhoff MJ, van den Keijbus PA, Kroeze KL, Nazmi K, Gibbs S, Bolscher JG, et al. Histatins enhance wound closure with oral and non-oral cells. J Dent Res. 2009 Sep;88(9): 846-50.

18- Heaney TG. Inhibition of fibroblast attachment. J Clin Periodontol. 1986 Nov;13 (10): 987-94.

19- Hannig C, Hannig M, Attin T. Enzymes in the acquired enamel pellicle. Eur J Oral Sci. 2005 Feb;113(1):2-13.