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Genotoxicity effects of nano bioactive glass and Novabone bioglass on gingival fibroblasts using single cell gel electrophoresis (comet assay): An *in vitro* study

Mohammad Tavakoli[1], Ensiyeh Bateni[1], Mansour Rismanchian[1], Mohammadhossein Fathi[2], Ali Doostmohammadi[3], Azim Rabiei[4], Hojat Sadeghi[5], Mahmood Etebari[6], Mina Mirian[7]

[1]Torabinejad Dental Research Center and Department of Periodontology, School of Dentistry, [5]Department of Pharmabiological Chemistry, [6]Department of Pharmacology, Isfahan University of Medical Sciences, [7]Isfahan Pharmaceutical Sciences Research Center, [2]Department of Materials Engineering, Biomaterials Research Group, Isfahan University of Technology, 8415683111, Isfahan, [3]Department of Materials Engineering, Shahrekord University, Shahrekord, [4]Dentist, Private Practice, Rafsanjan, Iran

ABSTRACT

Background: The greater surface of bioactive glass nanoparticles presents an incomparable and promising feature similar to the biological apatite. Nanoparticles improve cellular adhesion, enhance osteoblast proliferation and differentiation, and increase biomineralization for periodontal regeneration and dental implants. Considering the fact that interaction between periodontal cells and bone graft materials are important for periodontal lesion regeneration, the present study was undertaken to investigate the genotoxicity of a novel synthesized nanoscale bioactive glass and compared it with Novabone bioglass in periodontal fibroblasts cells, in order to approve the biocompatibility of nano bioactive glass.

Materials and Methods: In this *in vitro* experimental study, periodontal C165 fibroblasts cells were cultured in their logarithmic phase and the genotoxicity of novel synthesized bioactive glass nanoparticles and Novabone bioglass was studied in different concentrations and a control group using Comet assay test. By using Autocomet software, three parameters (Tail length, %DNA in tail, Tail moment) were analyzed; the genotoxicity of mentioned biomaterials and control group. Obtained data were analyzed by SPSS 11.5 software, Kruskal Wallis H and Mann Whitney tests ($P = 0.05$).

Results: No statistically significant difference was observed between the concentrations of Novabone bioglass (P value = 0.085) with control group and novel nano bioactive glass (P value = 0.437) with control group in the evaluation of %DNA in tail parameter. There was significant difference between genotoxicity of novel nano bioactive glass and control, and between Novabone bioglass and control group in concentrations of 4 and 5 mg/ml. According to significance of the mean difference, novel nano bioactive glass showed higher genotoxicity compared to Novabone bioglass in the concentration of 5 mg/ml ($P \leq 0.05$).

Conclusion: The findings of this study have demonstrated that novel nano bioactive glass had no genotoxicity in concentrations lower than 4 mg/ml. Nanoparticles have a higher surface area in comparison to microparticles and thus, the amount and rate of ion release for nanoparticles are extremely higher. This difference is the main reason for the different genotoxicity of nano bioactive glass and micro Novabone bioglass in the concentrations higher than 4 mg/ml.

Key Words: Biocompatibility, comet assay, fibroblast cell, genotoxicity, nano bioactive glass

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Address for correspondence: Dr. Ensiyeh Bateni, Torabinejad Dental Research Center and Department of Periodontology, School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran.
E-mail: nc_bateni@yahoo.com

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INTRODUCTION

Regenerative periodontal therapy comprises of procedures which are specially designed to restore those parts of the tooth-supporting apparatus which have been lost due to periodontitis. The regeneration of osseous defects caused by inflammatory

periodontal disease continues to provide an ongoing challenge in periodontal therapy. Current alternative lines of treatment include autogenous bone grafts and allografts, guided tissue regeneration, alloplastics, or a combination of techniques.[1]

Alloplastic materials are synthetic, inorganic, biocompatible, and/or bioactive bone graft substitutes which are claimed to promote bone healing through osteoconduction. Alloplastic materials such as hydroxyapatite and Tricalcium phosphate have been used in the treatment of intrabony defects. However, it has been shown that healing often occurs with encapsulation of the graft material by connective tissue and minimal or no bone formation. The bioactive glasses have been shown to bond to both bone and soft tissue. These materials differ from other bioactive ceramics in that the rate of bonding to bone can be controlled by varying the chemical composition.[2]

Bioactive glasses are biocompatible and exhibit strong interfacial bond to bone. Their bioactivity is attributed to the formation of a hydroxyl carbonated apatite (HCA) layer on their surface similar to the mineral part of bone. The rate of tissue bonding appears to depend on the rate of HCA formation, which follows a sequence of reactions between the implanted material and the surrounding tissues and physiologic fluids.[3]

The greater surface of nanostructure ceramics present an incomparable and promising character for orthopedic and dental implant formulations with better osseointegrative properties. By controlling the structural and particle size in the range of nanoscale, some properties of bioactive bioceramic such as osseoconductivity, solubility, and mechanical reliability can be improved.[4] The grain size, large surface area to volume ratio, and the ultra fine structure of nanoscale engineering bioceramics similar to the biological apatite provide surprising functional properties for these materials.[5] Recently, nanoceramics have attracted interest because surface nanostructuring allows for improved cellular adhesion, enhances osteoblast proliferation and differentiation, and increases biomineralization.[6,7]

Recently, researchers in Isfahan University of Technology have been succeeded to produce bioactive glass in nanoscale size that can be a desired substance in dentistry. Considering the fact that interaction between periodontal cells and bone graft materials are important for periodontal regeneration and according to the possibility of cellular and genetic damage of implanted material for patient and clinician, it should be concerned about the safety of mentioned biomaterials. Specifications and standards have been developed to aid producers, users, and consumers in the evaluation of the safety and effectiveness of dental products.[8] According to ISO specifications, implant devices are required to evaluate several tests such as cytotoxicity, subchronic systemic toxicity, skin irritation, intracutaneous reactivity, sensitization systemic toxicity, genotoxicity, chronic toxicity, systemic toxicity, and local effects after implantation.[9]

Wilson *et al*.[10] studied the test of solid bioglass implant in the soft tissues of rats and rabbits for time periods up to eight weeks. The surface activity in contact with bioglass which is so critical in bone adhesion was not toxic in rat tissues. Haung *et al*.[11] studied the cytotoxicity of new bioglass composite after 24 hours in cell culture, using one of the biocompatibility tests. No cytotoxic effect was observed for the composite. In addition, a significant increase in cellular activity suggested that bioglass composite was able to stimulate cellular activity by creating a favorable micromovement for cell proliferation and growth. Other studies by Vargas *et al*.[12] and Hong *et al*.[13] have proved the biocompatibility of bioglass by absence of inflammatory and toxic response.

The single cell gel electrophoresis (SCGE) assay, better known as comet assay, is a sensitive technique for detecting single and double strand break at the single cell level in DNA of any kind of cells. This test is based on the ability of DNA fragments to migrate in a weak electric field in direction of anode, given the nucleolus the appearance of comet tail when visualized by fluorescence microscopy.[14-16]

Pelaez *et al*.[15] estimated the genotoxicity of coatings containing bioactive particles on stainless steel by SCGE assay and significantly lower DNA migration in the cells of the cultures was observed. Jontava *et al*.[16] evaluated the genotoxicity of hydroxyapatite bioceramics using comet assay and showed that DNA break increased with increasing of biomaterial concentrations.

The aim of this study was to evaluate the genotoxicity of a novel nano bioactive glass and Novabone bioglass with control group in gingival fibroblasts cells using SCGE assay.

MATERIALS AND METHODS

In this *in vitro* experimental study, periodontal fibroblasts cells were cultured in their logarithmic phase and the genotoxicity of nano bioactive glass and Novabone bioglass was studied in selected cultured cells at biotechnology laboratory of pharmacology of Isfahan University of Medical Sciences. Novel nano bioactive glass was made by sol-gel method in Biomaterials Laboratory of Biomaterials Research Group at Isfahan University of Technology. Prepared nano bioactive glass was bioactive and less than 100 nanometer in size based on previous researches.[5,17,18] Novabone bioglass (NovaBone Products LLC, Alachua, FL, USA), a silica-based biocompatible material with particle size ranged between 90 to710 μm, has been examined in many studies.[19]

Comet test or SCGE assay was used in order to evaluate the genotoxicity of mentioned biomaterials on C165 fibroblasts cells which was prepared from Iran Pastor Institute in their logarithmic phase.

Cell culture

Cells were cultured in RPMI 1640 (PAA, Vienna, Austria) and 10% fetal bovine serum, FBS (GIBCO, NY, USA), and antibiotic (Penicillin, Streptomycin), then incubated in atmosphere containing 5% CO_2 at the temperature of 37°C. Before forming a confluent mono layer, the cells were harvested from the culture surface by incubation with 2 cc solution of trypsin (GIBCO, NY, USA). The cells were checked every day and passaged after 4 to 5 days. The suspension was used for the experiment when a suitable cell concentration was gained. The cells were counted and they were about 10^6 under microscope to be added experimental materials in the plates.[16]

Preparation of biomaterials

Nano bioactive glass and Novabone bioglass powders were used for preparation of 10 mg/ml concentration. Culture medium supplemented with penicillin and streptomycin was used to prepare the suspension stock of biomaterials. The suspension was shaken for 72 hours in shaker incubator, then concentrated samples were centrifuged (10 minutes, 1 800 rpm). The culture medium was aspirated with a syringe and filtered and sterilized for 30 minutes under Ultra Violet (UV) light. These procedures led to preparation of 10 mg/ml extraction of biomaterials.[16] Each test concentration of biomaterials (1, 2, 4, and 5 mg/ml) was placed in a culture plate with total 3 ml cell and culture medium of RPMI1640. As negative control (C-), the cells were cultured with medium without adding biomaterials. After storing the samples at 37°C in a 5% CO_2 atmosphere during 24 hours, they were centrifuged at 1800 rpm (10 minutes) and supernatant was removed. Finally, the cells were resuspended in culture medium and were used to measure DNA damage in an individual cell using the alkaline protocol.[15]

Comet assay

Detection of DNA damage by alkaline comet assay was used for measurement of the genotoxic activity of the biomaterials using protocol of Singh.[14] Cell suspension was mixed with Low Melting Point agarose, LMP (Sigma, NY, USA) in equal volume (250 μl), and extended it on a slide previously coated with 100 μl of 1% Normal Melting Point agarose, NMP (Sigma, NY, USA). After gelling at 4°C, the cells were lysed for 60 minutes in a solution of 5 M NaCl, 20 cc EDTA, 1 M Tris, 10 ml Dimethyl Sulfoxide, DMSO and 1 cc Triton X-100, at pH 10 and 4°C. The slides were washed three times in distilled water for 5 minutes and placed them on a horizontal electrophoresis unit, containing fresh electrophoresis buffer (300 mM NaOH, 1 Mm Ethylene Diamine Tetraacetic Acid, EDTA with pH 13). After 45 minutes of unwinding at 4°C, electrophoresis was performed for 45 minutes (20 V, 300 mA). The slides were washed 3 times in 400 mM Tris buffer (pH 7.5) and stained with ethidium bromide (2 μg/ml, 10 minutes). One hundred cells from each sample were selected randomly and analyzed by free Comet Score Software. Observations were made at magnification of X400 using an epifluorescent microscope (Ceti) coupled with CCD camera (Sony DSC H9). All steps were conducted in the dark place to prevent additional DNA damage.[14,15] Cells with damaged DNA displayed high migration of DNA fragments from the nucleus, forming a tail in comet form [Figure 1]. By using Autocomet software 3 parameters (tail length, %DNA in tail, tail moment), the genotoxicity of biomaterials was analyzed.

Data were analyzed with statistical software SPSS 11.5 and statistical test, Kruskal-Wallis H and a complimentary test, Mann-Whitney test ($P = 0.05$).

RESULTS

In this study, maximum damaged %DNA and longer tail length were observed in highest concentration

of both materials. Both higher number of comet and higher length of migrated damaged DNA was found with increasing of concentrations [Figure 2].

No statistically significant difference was observed between the concentrations of Novabone bioglass (P value = 0.085) with control group and novel nano bioactive glass (P value = 0.437) with control group in the evaluation of %DNA in tail parameter.

The comparison between cells corresponding to negative control and the concentrations of nano bioglass revealed statistically significant difference ($P \leq 0.05$) in concentrations of 4 mg/ml and 5 mg/ml in tail length and tail moment parameters [Table 1].

Nano bioactive glass generated statistically significant amount of DNA damaged at concentration of 5 mg/ml in tail length and tail moment parameters compared with negative control ($P \leq 0.05$). The concentration 4 mg/ml revealed statistically significant difference only in tail moment parameter compared with negative control ($P \leq 0.05$) [Table 1].

The comparison between cells corresponding to different concentrations of nano bioactive glass revealed higher mean compared to Novabone bioglass in all concentrations by evaluation of tail length parameter and difference was statistically significant ($P \leq 0.05$) [Table 2].

In comparison, two other parameters (%DNA in tail, tail moment) for nano bioactive glass revealed higher mean compared to Novabone bioglass in all concentrations but difference showed statistical significance ($P \leq 0.05$) only in 5 mg/ml concentration [Table 2].

DISCUSSION

In present experiment research, the comet assay was used to detect induction of DNA damage in fibroblast cells after 24 hours treatment with novel nano bioactive glass and Novabone bioglass. Results showed that DNA damage was induced in all tested biomaterials concentrations and the genotoxic effect increased with increasing the concentration

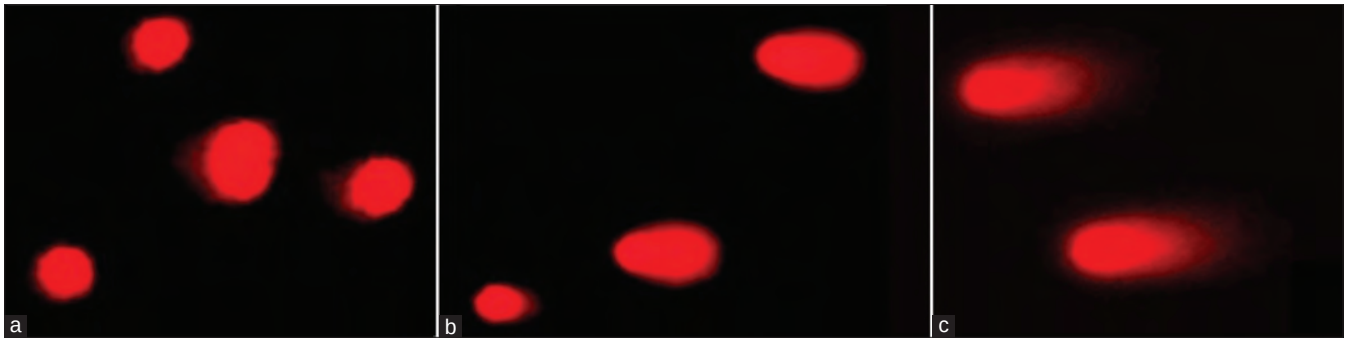

Figure 1: Microscopic view of concentration of (a) control sample, (b) 2 mg/ml nano bioactive glass, (c) 5 mg/ml nano bioactive glass (magnification of × 400)

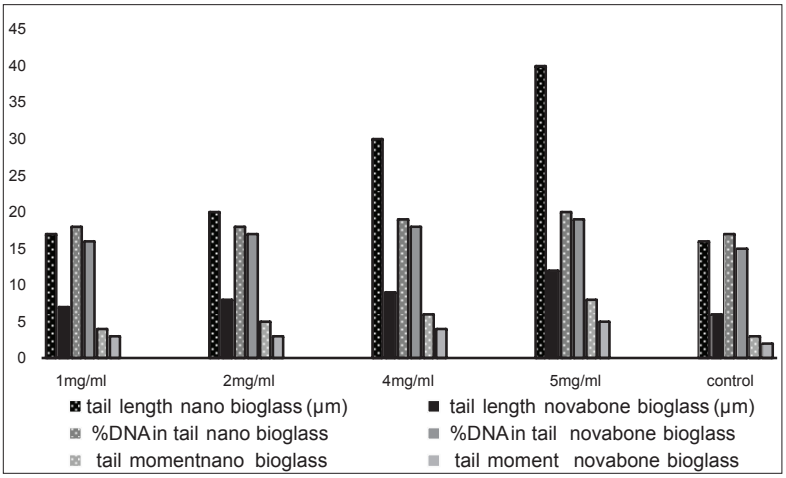

Figure 2: Mean value of comet parameter in different concentrations of nano bioactive glass and Novabone bioglass

[Figure 2]. This result confirmed the Jontava *et al.*'s[16] study. Jontava *et al.* evaluated the genotoxicity of hydroxyapatite bioceramics using comet assay and showed that biomaterials induced DNA break, which increased with increasing the bioceramic concentration. According to this fact, it could be expected that the genotoxicity of biomaterials depends on the amount of concentration and it is so critical to detect the threshold.

The comparison between cells corresponding to negative control and the concentrations of nano bioactive glass revealed statistically significant difference ($P \leq 0.05$) in concentrations of 4 mg/ml and 5 mg/ml in tail length and tail moment parameters [Table 1]. Also, no statistically significant difference was observed between the concentrations of Novabone bioglass (P value = 0.085) and nano bioactive glass (P value = 0.437) in the evaluation of %DNA in tail parameter. According to the Moller *et al.*'s study,[20] the end point measured by the traditional comet assay is a mixture of the length of the comet tail and total DNA in tail or by the tail moment (the length of the comet tail multiplied by the intensity of fluorescence in the tail); therefore, this parameter was highlighted in present study.

Table 1: Comparison of comet parameters in different concentrations of nano bioactive glass and Novabone bioglass with control sample ($P \leq 0.05$)

Parameter	Concentration	*P* value	
		Nano bioactive glass	Novabone bioglass
Tail length	1 mg/ml	0.752	0.689
	2 mg/ml	0.247	0.397
	4 mg/ml	0.004	0.080
	5 mg/ml	0.000	0.003
Tail moment	1 mg/ml	0.502	0.936
	2 mg/ml	0.412	0.082
	4 mg/ml	0.048	0.001
	5 mg/ml	0.002	0.000

According to the results, concentrations less than 4 mg/ml of novel nano bioactive glass have no genotoxicity effect, whereas biomaterials may have low, medium, or high potential risk to human safety, depending on the type and extent of patient contact.[16] Thus, for investigating the systemic toxic effect of nano bioactive glass and using it in dentistry, higher concentrations of the material are needed to be tested. Pelaez *et al.*,[15] by SCGE Assay, estimated the genotoxicity of coatings containing bioactive particles on stainless steel and significantly lower DNA migration was observed in the cells of the cultures. The present study has an advantage over Pelaez's study in evaluation of genotoxicity, because of using several concentrations of biomaterials and determining safety genotoxic threshold for tested materials. In our study, like Pelaez,[7] different materials showed different DNA damages. Pelaez use Olive tail moment parameter for detecting genotoxicity of materials and showed that the coating containing hydroxyapatite particles has lower values of Olive tail moment, which indicates less DNA damage without statistical difference with other materials.

The comparison between cells corresponding to different concentrations of nano bioactive glass showed a higher mean value compared to Novabone bioglass in all concentrations by evaluation of tail length parameter. The difference was statistically significant ($P \leq 0.05$) [Table 2]. In comparison, two

Table 2: Comparison of comet parameters in different concentrations of nano bioactive glass and Novabone bioglass ($P \leq 0.05$)

Parameter	Concentration	Mean		SD		*P* value
		Nano bioglass	Novabonebioglass	Nano bioglass	Novabonebioglass	
Tail length (μm)	1 mg/ml	16.780	7.250	19.225	11.535	0.00
	2 mg/ml	20.040	8.282	20.336	10.200	0.00
	4 mg/ml	30.410	9.090	32.250	10.686	0.00
	5 mg/ml	40.650	12.090	41.499	13.886	0.00
%DNA in tail	1 mg/ml	18.203	16.721	13.440	9.338	0.780
	2 mg/ml	18.446	17.485	11.784	7.925	0.526
	4 mg/ml	18.502	17.737	8.955	9.068	0.734
	5 mg/ml	19.606	18.354	9.572	10.465	0.007
Tail moment	1 mg/ml	4.242	3.119	6.721	6.431	0.261
	2 mg/ml	4.655	3.287	6.896	8.896	0.271
	4 mg/ml	5.984	4.363	8.467	7.677	0.192
	5 mg/ml	8.272	5.165	10.133	7.183	0.009

other parameters (%DNA in tail, tail moment), nano bioactive glass showed a higher mean value compared to Novabone bioglass in all concentrations but difference showed statistical significance ($P \leq 0.05$) only in 5 mg/ml concentration [Table 2]. These results show pieces of damaged DNA, with lower DNA percent, have migrated higher distance, so there is no statistical difference between nano bioactive glass and Novabone bioglass in concentration less than 5 mg/ml [Table 2], which can indicate safety of novel nano bioactive glass in genotoxicity according to the results of this study. These obtained results are almost similar to Wilson *et al*.[10] Haung *et al*.'s[11] and Arun *et al*.'s[21] studies proved the biocompatibility of novel composite bioglasses by other biocompatibility evaluation tests. Wilson *et al*. and Haugh *et al*.[10,11] examined the biocompatibility by implantation test in animals. Arun *et al*.[21] investigated the effect of HA-BG on chromosomal aberrations in human lymphocytes which are the other approved biocompatibility tests. However, we applied comet assay, a sensitive technique,[14] to detect induction of DNA damage in fibroblast cells. More precise results are gained by choosing fibroblast cells for detecting genotoxicity which are the most periodontal connective tissue cells, and have more contact with our materials, whereas comet assay is a technique for detecting single and double strand break at the single cell level in DNA of any kind of cells.[14-16]

The important point that must be considered in comparison of nano bioactive glass and Novabone bioglass is the difference in particle size. Nanoparticles have a higher surface area in comparison to microparticles and thus the amount and rate of ion release for nanoparticles are extremely higher. In other words, the bioactivity of nanoparticles is higher in comparison with the same mass of microparticles. This difference is the main reason for the different genotoxicity of nano bioactive glass and micro bioglass (Novabone bioglass) in the concentrations higher than 4 mg/ml.

CONCLUSION

According to the present research, concentrations less than 4 mg/ml of nano bioactive glass produced in Iran have no genotoxic effect and there is no statistical difference between nano bioactive glass and Novabone bioglass in concentrations less than 5 mg/ml. Therefore, other biocompatibility tests are needed to approve the application of new nano bioactive glass for regenerative therapy in periodontics.

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