

Synthesis of TiO₂ Nanoparticles Biocompatibility on Human Gingival Fibroblast Cells

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Cite this paper as: Hamidreza Eghbalikhosro, Fatemeh Salehi Barzegar, Motahare Sharifyrad, Mehdi Ranjbaran, Asieh Mozaffari (2025), Synthesis of TiO₂ Nanoparticles Biocompatibility on Human Gingival Fibroblast Cells. *Frontiers in Health Informatics*, 14(2) 2751-2761

ABSTRACT

Introduction: Titanium dioxide (TiO₂) nanoparticles, known for their antimicrobial properties, low cost, and high chemical stability, are widely used in dentistry. However, their effects on human health remain insufficiently studied.

Aims: This study aimed to evaluate the cytotoxicity of varying concentrations of TiO₂ nanoparticles on human gum fibroblasts (HGFs).

Methods: In this in vitro study, TiO₂ nanoparticles at concentrations of 10, 25, 50, 75, and 100 µg/ml were applied to 10,000 HGFs (C10459) cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). TiO₂ nanoparticles were synthesized and characterized using FTIR spectroscopy, FE-SEM, and DLS. HGF viability was assessed at 24, 48, and 72 hours using the MTT method, with optical density (OD) measured via an ELISA reader. Data were analyzed using ANOVA with a significance level of 0.05.

Results: Cytotoxicity increased with higher TiO₂ concentrations in a dose-dependent manner ($P < 0.001$). Time changes were independent of concentration ($P < 0.001$). The highest OD was observed at 10 µg/ml, and the lowest at 100 µg/ml across all time points.

Conclusion: The highest viability was observed at 10 µg/ml after 24 hours, and the lowest at 100 µg/ml after 48 hours.

Key words: Cytotoxicity, Human gingival fibroblasts, MTT assay, Titanium dioxide nanoparticles.

INTRODUCTION

Nanomaterials usually refer to tiny solid particles with a diameter of 1–100 nm. Nanomaterials are (A nanomaterial is) usually defined as tiny solid particles with a diameter of 1–100 nm (a particle of matter that is between 1 and 100 nm in diameter).¹ The unique properties and application of nanoparticles are due to a variety of properties, including the similar size of nanoparticles and biomolecules such as proteins and polynucleic acids (multi-nuclei acids).² The smaller diameter of nanoparticles not only improves their mechanical properties, but also their antimicrobial effect as their surface-to-volume ratio is high.³ Titanium dioxide, also known as titania, is the naturally occurring oxide of titanium, belongs to the family of transition metal oxides.⁴ Titanium dioxide nanoparticles have photocatalytic properties when exposed to visible light or ultraviolet radiation and have different types of use in medicine and dentistry.³ In dentistry, TiO₂ nanoparticles are commonly used due to their high biocompatibility and pleasing color (lack of color).⁵

In addition, the addition of TiO₂ nanoparticles to dental composites increases their mechanical properties including modulus elasticity, micro-hardness and flexural strength.⁶ Titanium dioxide nanoparticles are often used as a matter for orthopedic implants.⁷ It has also been reported that the combination of TiO₂ with H₂O₂ significantly decreases the time needed for teeth whitening during direct contact of bleaching gel and teeth.⁸ Despite the common use of nanomaterials, there is very little information about the effect of engineered nanomaterials on human health and the environment. Because of their extremely small size, nanoparticles do not have much difficulty in passing physiological barriers in the body and therefore are effectively distributed via the vascular system in the body tissues.⁹ Cell culture techniques are very useful for the assessment of the biocompatibility of dental materials. These techniques are cheap, repeatable and reliable.¹⁰ One of the cells receiving much attention in cytotoxicity studies is fibroblast, which is the main cell in the synthesis and formation of collagen fibers and mesenchymal cells that can be divided into cementoblasts and osteoblasts.¹¹ Due to the contradictions in the studies, the present study attempts to evaluate the cytotoxic effects of TiO₂ nanoparticles on human gingival fibroblasts (C10459) at concentrations of 10, 25, 50, 75, 100 after 24, 48 and 72 hours.

Methods

Titanium (IV) isopropoxide, Isopropyl Alcohol and HCL) were purchased from Sigma-Aldrich, USA .DMEM/F- (Dulbecco's Modified Eagle Medium), Fetal bovine serum (FBS), Trypsin, MTT (GIBCO USA), etanol and chloroform (Merk, USA).

Preparation of nanoparticles

For the synthesis of titanium dioxide nanoparticles, the sol-gel method was used.¹² First, we mixed some titanium isopropoxide with isopropanol in a ratio of 1 to 4 in the Beaker and placed it on a heater and put a magnet inside it to stir and homogenize the solution. In another Beaker, we mixed an equal amount of deionized water with isopropanol and homogenized it with a magnetic stirrer for 5 minutes. We added the content of the second Beaker to the first Beaker. At this stage, the color of the solution turned into pale white. In order to reach the appropriate pH, which is about 4, we used hydrochloric acid and it was constantly assessed by pH meter (hydrochloric acid was added drop by drop to the above solution). After this step, the obtained solution was placed on a heater for a few hours and then we used ultrasonic for 30 minutes to complete the reaction. Then, we let the sol to be aged for two days. At this time, the sol turned into gel and its viscosity was increased. The obtained gel was placed in the oven for 2-3 hours at 120 degrees. Then, the dried gel was sintered at 600°C for 5 hours.¹³

Characterization of Tio₂ nanoparticle

FTIR analysis

FTIR spectrum was investigated by spectrometer (Bruker IFS 66 v/s, Tensor 27, and Biotage, Germany) at

room temperature. The spectra were determined over the range 400–4000 cm^{-1} . The Prepared samples were mixed with potassium bromide (KBr), then pressed in pellet form.

Determination of size with DLS

The average size, zeta potential and polydispersity index were characterized by dynamic light scattering (DLS) using a Zeta Sizer Nano series Nano-ZS (Malvern Instruments Ltd., Malvern, UK). Measurements were made at 633 nm with a constant angle of 90° at 25°C. This dissolution were diluted 10 times for measurements. Each dissolution was measured three times in parallel.

Particle morphology

In order to analyze the morphology and diameter of the synthesized nanoparticles were used FE-SEM (ZEISS Sigma 300). For this purpose, 10 mg of the nanoparticle powder was weighted, then sample surface coated with gold. The samples concentrated electron beam irradiation was scanned. Finally, images of topographic recorded

Cell Culture

Human gingival fibroblasts are the major cells in periodontal tissues. It cell line was purchased from the Pasteur Institute of Tehran.

MTT assay

1-Culture of human gingival fibroblasts (C10459): The cells were cultured in cell culture flasks after deforestation. For culture of these cells, DMEM medium with 10% FBS, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin were used and the cells were incubated at 37 ° C, 1 atmospheric pressure, humidity 90 Percentage and concentration of 5% carbon dioxide. Inside each of the 96-well block of plate wells, 10,000 cells were placed as a cellular layer.¹⁴ For each of the tested concentrations, 5 wells were considered and 5 wells were assigned to the control group.¹⁵

In order to evaluate the toxicity of the samples and their effect on cell growth and proliferation, direct contact method was used. First, samples were prepared at concentrations of 10 $\mu\text{g} / \text{ml}$, 25 $\mu\text{g} / \text{ml}$, 50 $\mu\text{g} / \text{ml}$, 75 $\mu\text{g} / \text{ml}$ and 100 $\mu\text{g} / \text{ml}$. DMEM culture medium was used to obtain the required concentration of nanoparticles. In order to prepare a homogeneous suspension, the samples were placed in a Sonicator Euronda at a frequency of 20 kHz for 1 minute.¹⁶

2- Placement of nanoparticles in the proximity of fibroblast cells (C10459): The cells were incubated for 24 hours at a pressure of 5% CO₂, humidity 98% and the temperature of 37 °. Then the cell culture medium was removed. And TiO₂ nanoparticles with concentrations of 10 $\mu\text{g} / \text{ml}$, 25 $\mu\text{g} / \text{ml}$, 50 $\mu\text{g} / \text{ml}$, 75 and 100 $\mu\text{g} / \text{ml}$ in the culture medium were applied to the cells in 96-well plate wells and in the wells of the control group as complete culture medium as a non-toxic substance.

3. Placement of nanoparticles in the proximity of fibroblast cells (C10459): The cells were incubated for 24 hours at a pressure of 5% CO₂, humidity 98% and the temperature of 37 °. Then the cell culture medium was removed. And TiO₂ nanoparticles with concentrations of 10 $\mu\text{g} / \text{ml}$, 25 $\mu\text{g} / \text{ml}$, 50 $\mu\text{g} / \text{ml}$, 75 and 100 $\mu\text{g} / \text{ml}$ in the culture medium were applied to the cells in 96-well plate wells and in the wells of the control group as complete culture medium as a non-toxic substance.

4. Viability assessment of fibroblast cell: Cell viability was measured at 24, 48 and 72 hours after exposure to nanoparticles using MTT assay. After the desired time, the cell culture medium was removed and 100 μl of MTT solution at a concentration of 0.5 mg / ml was poured into each well and was incubated for 4 hours. After 4 hours, the solution was taken from the cells and isopropanol was added to dissolve the purple crystals. For better dissolution of the MTT precipitate, the plate was placed on a shaker for 15 minutes. Then the

concentration of solute in isopropanol was calculated using ELISA reader (BioTek ELx808, USA) at the wavelength of 570 nm and the amount of optical absorption of TiO₂ nanoparticle wells was statistically evaluated using ANOVA test.

Statistical analysis

Data analysis was done using SPSS Statistics, version 25 (IBM Corp, Armonk, NY, USA) under the terms of the software's licensing agreement. Frequency and percentage were used to describe qualitative variables, and quantitative variables of mean and standard deviation were used. The normality of the data was checked and confirmed by the Kolmogorov-Smirnov test and Shapiro-Wilk test ($P > 0.05$). To compare the viability of fibroblasts at different concentrations and times, two-way analysis of variance (Two-way ANOVA) was used. Pair-wise comparisons were performed with Tukey post hoc test. Significance level of tests was considered $P < 0.05$.

Results

Figure 1 shows FTIR spectra TiO₂. A peak at $3300\text{--}3500\text{ cm}^{-1}$, which corresponds to OH stretching mode of hydroxyl group, indicating the presence of moisture in the sample peak at 1627.28 cm^{-1} is associated with the OH bending vibrations of the absorbed water molecules. Peaks at a range between $1000\text{ to }400\text{ cm}^{-1}$ region is ascribed to the Ti-o stretching and Ti-o-Ti bridging stretching modes.^{17,18}

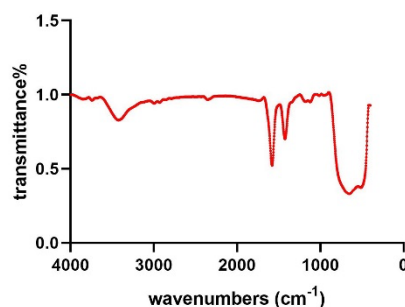
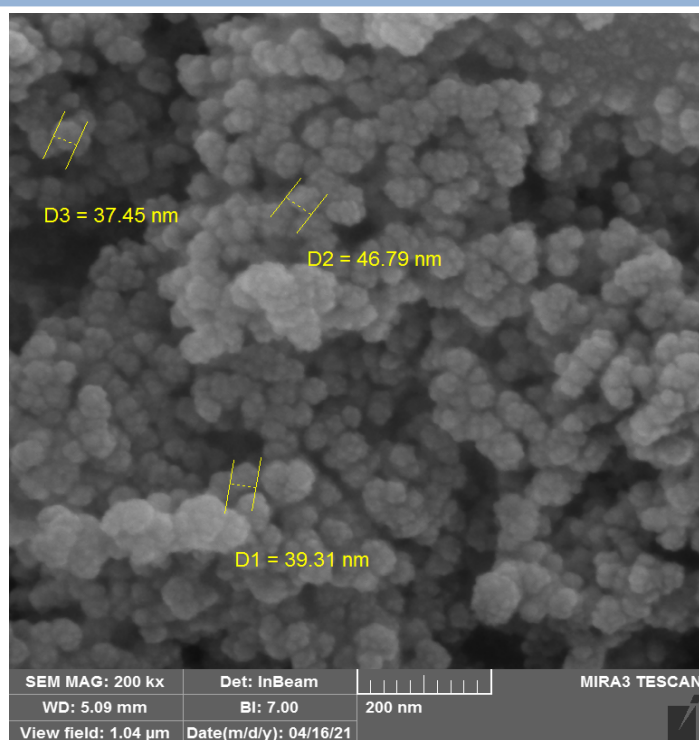


Figure 1. The spectra of FTIR TiO₂

Figure 2 (panel a) shows FE-SEM nanoparticles. In this method, spherical TiO₂ nanoparticles with an average particle size of about 10-11 nm were obtained and Figure 1 (panel b) shows DLS method, the size measured by it was reported to be $18.9 \pm 3\text{ nm}$ and it had a polydispersity index (PDI) value of 0.22. This study was performed on 5 experimental groups and a control group (6 groups totally) and on 5 samples in each period. Concentrations of $10\text{ }\mu\text{g/ml}$, $25\text{ }\mu\text{g/ml}$, $50\text{ }\mu\text{g/ml}$, $75\text{ }\mu\text{g/ml}$ and $100\text{ }\mu\text{g/ml}$ TiO₂ nanoparticles were exposed to gingival fibroblast cells (C10459) for 24, 48 and 72 hours. The amount and standard deviation of light absorption of cells, which shows their viability and the percentage of cytotoxicity compared to the control group at different times by different concentrations of TiO₂ nanoparticles are depicted in Table 1.

a)



Results

	Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 18.93	Peak 1: 19.86	94.5	6.937
Pdl: 0.212	Peak 2: 3753	5.5	1157
Intercept: 0.922	Peak 3: 0.000	0.0	0.000
Result quality : Good			

b)

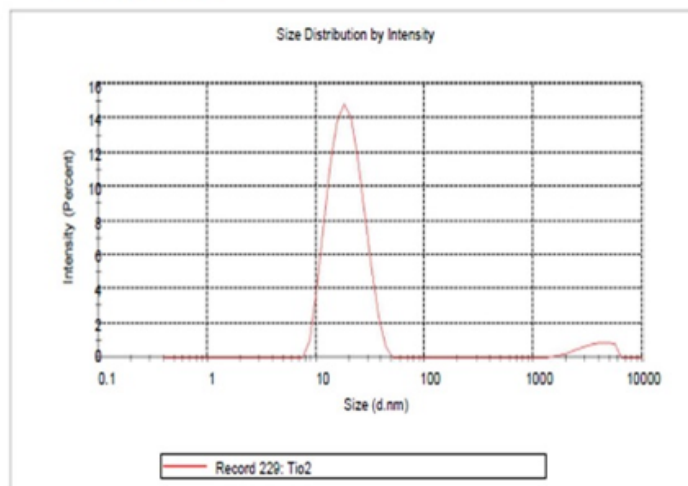


Figure 2. (panel a): Scanning electron microscope (FE SEM) image of synthesized TiO₂ nanoparticles. (panel b): Particle size distribution results using DLS test

Table 1. The amount and standard deviation of cell viability and percentages of cytotoxicity compared to the control group in terms of follow-up periods by different concentrations of TiO₂ nanoparticles

Time	24 hours	24 hours	48 hours	48 hours	72 hours	72 hours	P-value and Effect size
Nanoparticles Concentration	OD amount	Toxicity percentage	OD amount	Toxicity percentage	OD amount	Toxicity percentage	
Control (N=5)	0.24±0.01 1	-	0.29±0.01	-	0.36±0.01 6	-	Time: p< 0.001
10 µg/ml (N=5)	0.24±0.01 9	1.48	0.285±0.01 2	2.73	0.35±0.00 6	2.53	Partial Eta Squared = 0.911
25 (N=5)	0.239±0.0 24	1.56	0.280±0.01 7	4.44	0.33±0.01 5	6.92	
50 (N=5)	0.230±0.0 11	5.34	0.264±0.01 7	9.75	0.32±0.01 2	10.70	Group: P<0.001
75 (N=5)	0.22±0.01 3	6.08	0.261±0.02	11.05	0.316±0.0 1	13.28	Partial Eta Squared= 0.560
100 (N=5)	0.21±0.00 9	12.89	0.24±0.015	15.89	0.312±0.0 08	14.43	

Tukey Post Hoc Tests for Pairwise comparison of Nanoparticles Concentration

Group (I)	Group (J)	P-value	Mean Difference (I-J) (95% CI)	Group (I)	Group (J)	P-value	Mean Difference (I-J) (95% CI)
Control	10 µg/ml	0.796	0.007 (-0.009 , 0.023)	10 µg/ml	25	0.783	0.007 (-0.009 , 0.023)
	25	0.116	0.014 (-0.002 , 0.029)		50	0.006	0.020 (0.004 , 0.036)
	50	< 0.001	0.027 (0.011 , 0.043)		75	< 0.001	0.025 (0.009 , 0.041)
	75	< 0.001	0.032 (0.016 , 0.038)		100	< 0.001	0.037 (0.021 , 0.052)
	100	< 0.001	0.044 (0.028 , 0.059)		75	0.940	0.005 (-0.011 , 0.021)
25	50	0.180	0.013 (-0.003 , 0.029)	50	100	0.034	0.017 (0.001 , 0.033)
	75	0.019	0.018 (0.002 , 0.034)		75	0.274	0.012 (-0.004 , 0.028)
	100	< 0.001	0.030 (0.014 , 0.045)				

Tukey Post Hoc Tests for Pairwise comparison of Time

24 hours	48 hours	< 0.001	-0.039 (-0.039 , - 0.030)	48 hours	72 hours	< 0.001	-0.064 (-0.073 , - 0.054)
	72 hours	< 0.001	-0.103 (-0.112 , - 0.093)		-		

The percentage of cell viability was obtained by dividing the average amount of light absorption of the desired concentration by the average absorption of the control group at the same time multiplied by 100, and by deducing this from 100, the percentage of toxicity was obtained. In all time period groups, the viability of cells exposed to TiO₂ nanoparticles at a concentration of 10 µg / ml was maximum and was minimum at a concentration of 100 µg / ml (Table1, Figure 3). According to the results of Two-way ANOVA, by controlling the effect of time, a significant difference was observed in terms of fibroblast cell viability between different concentrations ($P < 0.001$). Also, time changes were statistically significant by controlling the effect of concentration ($P < 0.001$).

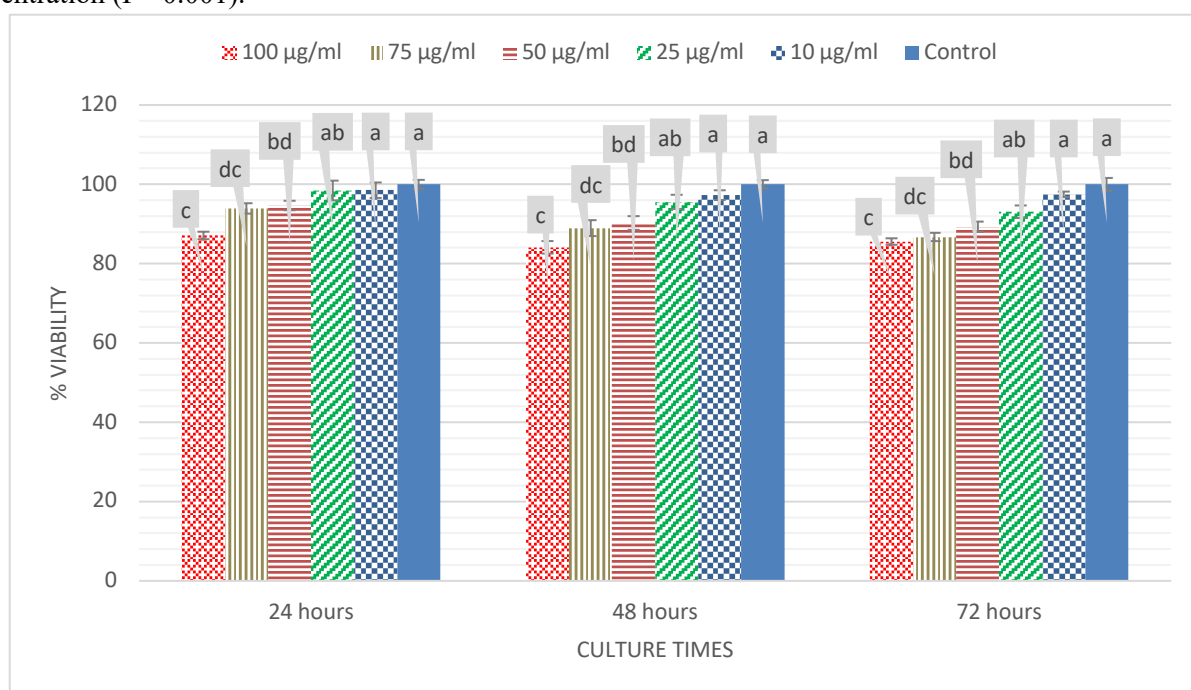


Figure 3. Average survival percentage of fibroblasts at concentrations of 10 µg / ml, 25 µg / ml, 50 µg / ml, 75 µg / ml and 100 µg / ml at 24, 48 and 72 hours. The same lowercase letters indicate no statistically significant difference between concentrations in each time. The difference between three times was statistically significant ($P < 0.001$).

Based on pairwise comparison between different concentrations, regardless of the definite time, the results showed differences in fibroblast cell viability, between control groups with concentrations of 100 µg / ml, 75 µg / ml and 50 µg / ml, between concentrations of 10 with concentrations of 50 µg / ml, 75 µg / ml and 100 µg / ml, between concentration 25 µg / ml with concentrations of 75 µg / ml and 100 µg / ml and between

concentration 50 $\mu\text{g} / \text{ml}$ with concentration of 100 $\mu\text{g} / \text{ml}$ were statistically significant ($P < 0.001$). The comparison of different times also showed a significant difference as pair-wise between all times ($P < 0.001$).

Discussion

The FTIR survive of TiO_2 nanoparticles shows the features of the formation of high purity product.

Such a discrepancy may be due to the fact that DLS measures the hydrodynamic diameter, and the size obtained by the DLS method can be larger than the value obtained by FE-SEM. PDI between 0.1 and 0.5, the particles have a good homogenous distribution.¹⁹

The results of the present study showed that the highest viability rate of fibroblast cells in 24 hours of their exposure to titanium dioxide nanoparticles with a concentration of 10 $\mu\text{g} / \text{ml}$, 98.5% was obtained and this value decreased with increasing concentration. Also, according to the results of one-way analysis of variance, there was no significant difference between the mean Optical Density (OD) at different concentrations of titanium dioxide nanoparticles at 24 hours. In other words, the mean viability of cells was not different from each other in different concentrations. Only the difference between control and concentration 100 was statistically significant by using Tukey test in pair-wise comparison between the different concentrations. The highest amount of viability of fibroblasts was in 48 hours with the exposure to titanium dioxide nanoparticles with a concentration of 10, in which 97.27% of the cells was survived, which decreased with increasing concentration. There was also a significant difference between the mean Optical Density (OD) at different concentrations of titanium dioxide nanoparticles. This difference was between the control group and the concentrations 75 and 100 and also between concentrations of 10 and 25 with concentration 100. With the evaluation of mean OD, its value was increased at the concentrations of 100, 75, 50, 25 and 10, respectively. Simply, the average cell viability decreased during 48 hours of exposure with increasing concentration. The highest degree of viability of fibroblast cells is observed in 72 hours of their exposure to titanium dioxide nanoparticles with a concentration of 10, in which 97.47% of cells survived and their values were decreased with increasing concentration. There was also a significant difference between the mean Optical Density (OD) at different concentrations of titanium dioxide nanoparticles. This was the difference between the control group with all concentrations except 10 and between concentration 10 with concentrations of 50, 75 and 100 and between the concentration of 25 with 100. The evaluation of the average OD values is based on the reduction of this value at higher concentrations. In other words, during 72 hours of exposure, the average cell viability decreased with increasing concentration. In the evaluation of the average viability of cells regardless of the studied periods, the results showed that there is a significant difference between the mean Optical Density (OD) at different concentrations. The control group with concentrations of 50, 75 and 100 showed significant difference with concentration 10 with concentrations of 50, 75 and 100, concentration 25 with concentrations of 75 and 100, concentration 50 with concentration 100. In other words, by increasing concentration, the average cell viability is decreased. Also, in all three times of 24, 48, and 72 hours, the highest average OD, which shows the amount of vital fibroblast cells, was related to the concentration of 10 and the lowest value was related to the concentration 100. The overall conclusion showed that the highest survival rate or the lowest toxicity is assigned to the concentration of 10 and in 24 hours.

The results of the research done by Tabari et al. on four types of metal oxide nanoparticles (TiO_2 , SiO_2 , ZnO and Al_2O_3) showed that cell viability and morphological changes occurred in the concentration range of 25 $\mu\text{g} / \text{ml}$ to 100 $\mu\text{g} / \text{ml}$ and in all nanoparticles. The higher concentrations and longer contact of cells with nanoparticles increases cell death, which is in line with the results of the present study.¹⁶

The study of Yu-Jin et al. showed that in cytotoxicity of titanium dioxide nanoparticles in mouse fibroblast cells, by increasing the concentration of TiO_2 nanoparticles (ranging from 3 to 600 $\mu\text{g} / \text{ml}$) in culture medium, the levels of reactive oxygen species and lactate dehydrogenase increased and the cells viability

was reduced based on the results of test, which is consistent with the results of the present study. It can be said that fibroblast cell death is partly dependent on the dose of titanium dioxide.²⁰

The study of Heravi et al. showed that the cytotoxicity of Transbond XT adhesive containing 1wt% TiO₂ nanoparticles showed lower toxicity than the pure adhesive, which indicates that this type of adhesive is suitable in orthodontic applications. The present study also mentioned the importance of using TiO₂ nanoparticles. Also, increasing the time had a significant impact on cell viability, as the cytotoxicity of both adhesives decreased with increasing time, this is not consistent with the present study. The reason is the method of sample preparation and the weight percentage of nanoparticles in the samples and different time periods from the present study. Also, the cytotoxicity of nanoparticles also depends on their physic-chemical properties including size, chemical composition, concentration and crystalline structure, and the difference in results may be related to the chemical composition of the nanocomposite.⁶

The results of Samiei et al. study examined the biocompatibility of WMTA and WMTA containing TiO₂ nanoparticles on human gingival fibroblasts and showed that by increasing the incubation time from 24 hours to 48 hours, the survival rate of human gingival fibroblasts in exposure to both substances, decreased significantly and this is consistent with the results of the present study.¹¹

In a study done by Shahoon et al. the Cytotoxicity of the rod-like hydroxyapatite nanoparticles on fibroblast cells at different concentrations at times 2, 24, 48 and 72 was investigated. The results showed that by increasing time and concentration, the average viability of cells decreased but this decrease was not significant at different times and concentrations. In the present study, the cell viability decreased with increasing concentration, and this result is consistent with the results of our study.²¹

Conclusion

Overall conclusion showed that the highest rate of viability (lowest toxicity) is assigned to the concentration of 10 µg / ml and in 24 hours. Thus, it can be said that this concentration of TiO₂ nanoparticles has acceptable biocompatibility and the lowest viability rate (The highest toxicity) is related to the concentration of 100 µg / ml in 48 hours. Given that there are different layers of connective, epithelial, and immune cells in the oral environment, and as the present study is an experimental study, the test is performed on a cultured cell layer. Therefore, generalization of the results of this study to clinical conditions should be performed with caution.

Recommendations

As the first step in a comprehensive investigation to use nanoparticles in dentistry, it is concluded that due to the dose-dependent and time-dependent toxicity of these nanoparticles, special attention is required to their safe use. These cases may be solved by combining low concentrations with biocompatible dental materials, and further research is needed to find better alternatives based on the different toxicity of these materials.

Due to the existing contradictions in biocompatibility of nanoparticles, which can be due to their specific properties and cell types used in studies and differences in the methods, and considering the importance of the subject, it is recommended to investigate the toxicity of TiO₂ nanoparticles at high concentration and during high and different intervals. Also, further studies should be conducted to evaluate the immunological and molecular reactions and the evaluation of the cytotoxicity of this matter is performed in in vivo models.

Data availability: Datasets related to this article will be available upon request to the corresponding author.

Conflict of interest: None.

Author Contribution: The participation of each author is described below, according to the authorship and co-authorship criteria: Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work – Eghbali Khosro, Sharifyrad, Ranjbaran, and Mozaffari; Drafting the work or revising it critically for important intellectual content – Eghbali Khosro,

Sharifyrad, Ranjbaran, and Mozaffari; Final approval of the version to be published – Eghbali Khosro and Mozaffari; Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved – Eghbali Khosro, Sharifyrad, Ranjbaran, and Mozaffari.

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Figure captions

Figure 1. The spectra of FTIR TiO₂

Figure 2. (panel a): Scanning electron microscope (FE SEM) image of synthesized TiO₂ nanoparticles. (panel b): Particle size distribution results using DLS test

Figure 3. Average survival percentage of fibroblasts at concentrations of 10 µg / ml, 25 µg / ml, 50 µg / ml, 75 µg / ml and 100 µg / ml at 24, 48 and 72 hours. The same lowercase letters indicate no statistically significant difference between concentrations in each time. The difference between three times was statistically significant (P<0.001).