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Cytotoxicity of Zirconium Oxide Nanoparticles on Diabetic Rabbit Tooth Gum Cells

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Abstract

The cytotoxicity mechanism of zirconium oxide nanoparticles (ZrO_2-NPs) has not been clarified to date. Nevertheless, generation of reactive oxygen species (ROS) by ZrO_2-NPs has been considered as an important mechanism in some in vivo studies. The present study was conducted to identify the potential effects of ZrO_2-NPs on diabetic rabbit tooth gum cells as well as the mechanisms of their cytotoxicity. ZrO_2-NPs were characterized using scanning electron microscopy (SEM) and dynamic light scattering (DLS). Cell viability, ROS level, lipid peroxidation (MDA), mitochondrial membrane potential (MMP), glutathione count (GSH/GSSG), lysosome damage and apoptosis/ necrosis were evaluated. Biodistribution studies were conducted on tooth gum, liver, kidney, heart, and brain tissues in the diabetic rabbit using the inductively coupled plasma optical emission spectrometry (ICP/OES). ZrO_2-NPs increased ROS, MMP collapse and MDA. In contrast, GSH/GSSG and apoptosis/ necrosis were found to be changed. The ZrO_2-NPs accumulated significantly more on diabetic rabbit tooth gum tissues compared with other organs ($P < 0.05$). The study provided evidence that ZrO_2-NPs cannot be considered completely biocompatible in the gum cell tissues of the diabetic rabbit. Before these nanoparticles can be used for human dental applications, further investigations on a wide range of cell death signaling should be performed.

Keywords: Cytotoxicity; Diabetic Rabbit; Reactive oxygen species; Tooth gum; Zirconium oxide nanoparticles

Abbreviations: ROS: Reactive Oxygen Species; DLS: Dynamic Light Scattering; MMP: Mitochondrial Membrane Potential; ICP/OES: Inductively Coupled Plasma/Optical Emission Spectrometry; DM: Diabetes Mellitus; BSA: Bovine Serum Albumin; FBS: Fetal Bovine Serum; IDF: International Diabetes Federation; SEM: Scanning Electron Microscopy; PB: Peripheral Blood; RBC: Red Blood Cell; HB: Hemoglobin, HCT: Hematocrit; MCHC: Mean Corpuscular Hemoglobin Concentration; MCH: Mean Corpuscular Hemoglobin, PDW: Platelet Distribution Width; PLT: Platelet Distribution width or Thrombocyte count; MPV: Mean Platelet Volume; WBC: White Blood Cell; SSC/FSC: Side Scattering/ Forward Scattering; MMP: Mitochondrial Membrane Potential; LPO: Lipid Peroxidation; TBARS: Thio Barbituric Acid Reactive Substances; GSH: Glutathione Redox; BMV: Bone Marrow Volume; TBV: Trabecular Bone Volume Tissue; OS: osteocytes; OB: osteoblasts; MPT: Mitochondrial Permeability Transition

Introduction

The use of nanomaterials has increased notably in medicine and other biomedical fields. Although nanomaterials blend with the environment (air, water, and soil), there are serious concerns about their impact on human health as well as environmental pollution that results from the distribution of kiln dust (nanoparticles or microparticles) [1]. The concentration of pollutants increases because of the increasing use of metal nanomaterials [1]. Nanomaterials such as single- and multi-walled

carbon nanotubes, nanofibers, quantum dots and metal oxide nanoparticles have been reported to be cytotoxic to human cells, bacteria, and rodents [2]. For example, cobalt nanoparticles cause damage to the human skin, and causes diseases such as rhinitis, asthma, allergic dermatitis, and cardiomyopathy [3]. Silver nanoparticles possess the highest degree of human cytotoxicity and environmental toxicity. Zinc oxide (ZnO), titanium dioxide (TiO_2) and aluminum trioxide (Al_2O_3) nanoparticles adversely affect cell proliferation and viability in human lung epithelial cells [4]. Over the past decade, zirconium oxide nanoparticles (ZrO_2-NPs), and TiO_2-NPs have been extensively used in the semiconductor, thermoelectric, electro-optical, piezoelectric, painting, and dielectric materials industries. These nanoparticles are also incorporated into sunscreens, cosmetics, deodorants, and topical ointments. More recently, ZrO_2-NPs have been used in biomedicine as drug delivery systems in conjunction with near-infrared light irradiation for anti-cancer therapy [1], as well as for diagnostic purposes. They have also been used for environmental decontamination because of their unique physicochemical features [1-6].

Dental materials and implants are an efficient alternative to traditional prostheses or bridges to restore missing teeth [7]. For the effectiveness of osseointegration, and the long-time survival of dental implants, the direct connection and capability between the implant and bone, without the intervention of soft tissues after implantation is very important [8]. Any disturbance in this biological mechanism will have a detrimental effect on the treatment outcome [8]. Diabetes mellitus (DM) is one of the chronic diseases that damage dental implant and provided peri-implantitis and poor osseointegration [8]. The Studies show a direct correlation between diabetic patients and bacterial infection around the implant [9]. Therefore, for the effectiveness of osseointegration in diabetic patients, it is necessary to use new materials. Zirconium oxide nanoparticles are used considerably in the ceramics industry and as dental and optical coatings. Previous studies reported that the cytotoxicity of these nanoparticles is dependent on their size and shape [10-11]. Although the toxic effects of ZrO_2-NPs have not completely been elucidated, in vitro and in vivo studies have identified that ZrO_2-NPs generate free radicals that damage vital intracellular macromolecules such as proteins and lipids. The free radicals are also responsible for apoptosis of different cell types [12-13]. Recently, high-strength ZrO_2 dental implants have been introduced as an alternative for the well-established titanium implants because of their improved fracture resistance and excellent flexural strength. Although ZrO_2 implants are becoming increasingly popular in oral implantology, the effects of their application on osteogenesis induction of mesenchymal stem cells have not been thoroughly investigated. Some studies reported that ZrO_2 dental implant systems do not possess clinical longevity [14-18]. Zirconia implants that replace maxillary first premolars have been evaluated for their long-term survival [19]. The biocompatibility of ZrO_2-NPs and white Portland cement has been evaluated [20]. Investigation of the extent of extracellular matrix (ECM) mineralization around ZrO_2 dental implants after 2 and 4 weeks of surgical implantation indicated that a slightly higher degree of bone apposition was achieved compared to titanium implants [21]. Accordingly, the cytotoxicity of ZrO_2-NPs was investigated in the present study.

Material and Methods

Materials

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfide (DMSO), 2′,7′-dichlorofluorescein diacetate (DCFH-DA) probes, Rhodamine 123 (Rh 123), Malondialdehyde (MDA), Thiobarbutiric acid (TBA), n-butanol, Tetramethoxypropane (TEP), O-Phthalaldehyde (OPA) probe, N-Ethylmaleimide (NEM) probe, Acridine orange (AO), Ethylene Diaminetetraacetic acid (EDTA) were purchased from the Millipore Sigma. Annexin V FITC Apop Dtec Kit I, BD, Franklin Lake, USA were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA), fetal bovine serum (FBS) was purchased from Nichirei Bioscience, Tokyo, Japan and used without further purification. Zirconium (IV) tert-butoxide was purchased from the Merck KGaA, Darmstadt, Germany. Tetracycline hydrochloride and xylocaine/epinephrine were purchased from Showa Yakuhin Kako Co., Ltd., Tokyo, Japan. Alloxan monohydrate was provided from Sigma Aldrich BVBA, Overijse, Belgium.

Instruments

During the testing process Fourier-transform infrared spectroscopy, IRTracer-100, Shimadzu, Kyoyo, Japan; Scanning electron microscopy, ALS -2100, Pooraka, South Australia, Australia; Zetasizer Nano ZS system, Malvern PAN alytical Inc, Westborough, MA, USA; Incubator 37°C, Sensor CO_2 Sanyo, Japan MCO; Vapor bath stark eliwellewpc 800T, UKA; Refrigerated Centrifugation, Sanyo, Harrier 18/80, Japan; Floremetry, Shimadzu RF-500, Japan; digital scale Japan; Shaker ,REAX2000 Iran; ELISA reader ,In finite 200 M, Tecan, Basel Switzerland; Flowcytometry, BD Biosciences FACS Calibure TM flow cytometer, hematological auto-analyzer ,Orphee Mythic 22 hematology analyzer, Diamond Diagnostics, Holliston, MA USA were used.

Preparation of ZrO_2-NPs

Stearic acid (569 g, 2 mol) was melted in a beaker at 73°C. Zirconium (IV) tert-butoxide (383.7 g, 1 mol) was added to the melted stearic acid under magnetic stirring. The mixture was heated in an oven at 300-400°C for 60-70 min. Impurities derived from stearic acid were removed in the form of H2O, CO_2 and CO. The mixture was then calcined at 850-900°C for 5 hours. Thereafter, the mixture was cooled to produce ZrO_2-NPs [22].

Characterization of ZrO_2-NPs

Fourier-transform infrared spectroscopy was used to analyze the synthesized NPs. Scanning electron microscopy (SEM) was

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used to examine the morphology of the NPs. Size distribution of the NPs was determined using a Zetasizer Nano ZS system [22].

Antibacterial activity of ZrO_2-NPs

Antibacterial evaluation was conducted for both Gram-positive (Staphylococcus aureus; ATCC 11775; American Type Culture Collection, Manassas, VA, USA) and Gram-negative bacteria such as and Escherichia coli bacteria (ATCC; 25923). Antibacterial activities were evaluated using the modified Kirby-Bauer disk diffusion procedure. Briefly, the cells cultures were seeded in a Muller-Hinton chamber at a temperature of 35 ± 2°C in a rotary spinner. For microbial growth, 100 µg of fresh bacterial culture was diluted to 10^6 colonies forming units (CFU)/mL on the agar plates using a sterile glass-rod propagator. Afterward 10 min, 8 mm wells were connected to the anchor plates for testing the antimicrobial activity of the nanomaterials. All wells were sealed with agar to block the permeability of ZrO_2-NPs from the bottom of the wells. Using a micropipette, 100 µL (50 µg) of ZrO_2-NP suspension was delivered to each well. Inhibition zones, if any, were measured after incubation overnight at 35°C ± 2°C. Rhea pure solvent and the antibiotic tetracycline were used as negative and positive control, respectively [23].

Animals

Male rabbits (250-300 g each, 6 months old) were purchased from the Pasteur Institute of Iran. The animals were housed under controlled temperature (20-12°C) and relative humidity (50-60 %), as well as a 12-h light/dark cycle; they had free access to tap water and standard food. Five animals were used for each group (control and treatments; n = 5). The study protocol was approved by the Animal Research Ethics Committee (AREC) of Iran Medical University. Efforts were made to minimize the number of animal experiments. After decapitating the rabbits, their tooth gum tissues were rapidly dissected and completely rinsed with isotonic saline. Peripheral blood (PB) samples were collected from the animals for isolation of the cells for subsequent characterization.

Diabetic model procedure

All rabbits received a single dose of alloxan monohydrate (100 mg/kg period time,1 min) using an intravenous cannula. To reduce risk of nephrotoxicity from hyperuricemia, a 7 ml/kg body weight iv injection of 0.9% saline was given immediately after the injection of alloxan at the rate of 1 mL/min. According to previous studies, to relieve the pain and distress from alloxan injection, the injection was performed under anesthesia. (medetomidine 0.25 mg/kg IM /ketamine 25 mg/kg bolus). A subcutaneous injection of 5% glucose solution (5 ml) was performed. Based on previous studies, animals that showed blood glucose levels above 250 mg/dL were considered diabetic [24].

Surgical procedure

Operations were performed under sterile conditions. Each diabetic rabbit was anesthetized by intracardiac injection of sodium pentobarbital. Anesthesia was maintained by inhalation of halothane (1.5-2.0 vol %). The root of the nose was shaved and disinfected using 70% ethanol. Local anesthesia was implemented using 2% xylocaine/epinephrine. A mucosal flap was made to expose the tooth gum laterally. Dental holes were made at eye level and 7 mm lateral toward the midline into the tooth gum area, using a 1-mm diameter, 4-mm long slow-speed dental drill under sterile saline irrigation. The ZrO_2-NPs were inserted into the hole at a concentration of 0.003 g using a sterile spatula. Each hole was then filled with amalgam. Tetracycline hydrochloride paste was injected into the surgical site to prevent bacterial infection [25].

Stereological examination

The total volume of tissues was calculated using the Cavalieri method. A grid was superimposed over the images using stereological software. The volume was estimated using the formula [26]

$$V = \sum P \; x \; a \; / \; p \; x \; t$$

where "ΣP" is the total points hitting the thrombus section, "a/p" is the zone related to any point, and "t" is the distance between the sampled sections. The numerical density of cells was determined using the optical dissector method. The position of the microscopic fields was selected by systematic uniform random sampling, with a moving stage in equal distances in x- and y-directions. A disinterested counting plate with inclusion and exclusion borders was superimposed on the images. A mikrokator was then attached to the stage of the microscope to measure the z-axis. The numerical densities (NV) were estimated using the formula:

$$Nv = \left[\frac{\sum Q^-}{\sum P \times \frac{a}{f} \times h} \times \frac{t}{BA} \right]$$

where "ΣQ" is the number of the cells coming into focus and counted, "ΣP" is the total number of the counting frames in all fields, a/f is the area per frame, "h" is defined as the height of the dissector, "t" represents the real section thickness measured using the mikrokator when Q- was counted, and BA represents the block advance of the microtome [27].

Experimental design

The diabetic rabbits were randomly divided into two main groups. Group 1, health (0 day: before exposure) and group 2, experimental (0 day: before exposure) and six subgroups (1,15 and 30 days, after exposure ZrO_2-NPs). In a pilot study, the NPs were prepared in different weight concentrations. After optimization of the isolated tooth gum cells on ZrO_2-NPs, experiments were subsequently conducted using 0.003 g of ZrO_2-NPs in normal saline. The ZrO_2-NPs were inserted in each diabetic rabbit's incisor tooth. The cells were isolated based on the experimental design.

Nanoparticle distribution

Tooth gum tissues were analyzed by elemental analysis to determine the Zr content in the ZrO_2-NPs using a 10 mg L-1

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standard stock solution (Agilent Technologies, Les Ulis, France). The samples were digested in ultrapure nitric acid overnight. Then, 0-3 mL of H_2O_2 (30% (v/v) was added to the solution and heated at 25 ◦ C until the samples were completely digested. The remaining solutions were diluted by 3 mL nitric acid. The detection limit of ZrO_2-NPs was 0.1 µg/mL by coupled plasma – optical emission spectrometry (ICP/OES). Data were expressed as ng/g of fresh tissue [28].

Hematological analysis

Each diabetic rabbit was anesthetized through inhalation of 5% isoflurane. A blood sample (about 2 mL) was taken and placed into 2 polypropylene tubes. All blood samples were treated with EDTA to prevent coagulation. Serum was generated after the samples were centrifuged at 3,000rpm for 10 min. A hematological auto-analyzer was used to identify various hematological parameters such as red blood cell (RBC), Hemoglobin (HB), Hematocrit (HCT), corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), platelet distribution width percent (PDW%), platelet distribution width or thrombocyte count (PLTs%), mean platelet volume (MPV), white blood cell (WBC) distribution width, lymphocytes (%), neutrophils (%), monocytes (%).

Cellular toxicity assay

Fibroblast isolation

The diabetic rabbits' tissues were extracted after 1 month. Under local anesthetic, a small section of the gingiva (2×1×1mm) was removed using a scalpel. The tissues were immediately rinsed with boric acid for prevention of fungal infection. The gingiva tissues were placed in Dulbecco's Modified Eagle Medium (DMEM) that was supplemented with 10% FBS and penicillin 100 IU/mL, streptomycin 100 µg/mL and amphotericin B 100 µg/mL. The specimens were then rinsed with sterile PBS (PH ~7.4) and transferred to a petri dish containing DMEM, where it was mechanically minced. The tissue suspension and fibroblast cells were centrifuged (200 g for 5 min). The re-suspended pellet was placed in a plastic bottle containing the culture medium. The cells were incubated at 37˚C in humidified air containing 5% CO_2. The medium was renewed every 2 days [29].

Cell viability

Cells (~1×10^6 cells) isolated were subjected to flow cytometry cell sorting. The crude cells were dissolved in 0.5 mL PBS. One hundred microliter of aliquots was redistributed to the BD flow cytometry tube. Results of the side scattering/forward scattering (SSC/FSC) were analyzed for at least ~10,000 counts per sample in the flow cytometer [26]. Cell viability was evaluated by MTT staining. The cell viability (1×104 cells) test was based on the reduction of tetrazolium salt by intracellular dehydrogenases of vital cells to purple formazan. The latter was dissolved in dimethyl sulfoxide for measuring the absorbance at 570 nm using a plate reader. The experiment was conducted in triplicate [30].

Reactive Oxygen species production

The cells (1×10^6 cells) were fed with both culture medium and ZrO_2-NPs. The Reactive Oxygen species production (ROS) level was evaluated before and after the treatment. After the treatment, cells were washed with PBS as previously described. Ten microliter of DCFH-DA was used to measure intracellular ROS after interaction with fluorescent DCFH-DA. Fluorescence was measured using a spectrophotometer at $\lambda_{exitation}/\lambda_{emission}$ = 490/535 nm [31].

Mitochondrial membrane potential (MMP)

The cells (1×10^6 cells) were treated with both the culture medium and ZrO_2-NPs before and after culture. Cell aliquots were incubated with Rh 123, a lipophilic, cationic fluorescent dye, at room temperature for 20 min in the dark with diluted PBS. The fluorescence intensity of the dye is quenched when it accumulates in the mitochondria. Dye uptake was measured with a spectrophotometer. Fluorescence was measured at $\lambda_{exitation}/\lambda_{emission}$ = 490/535 nm. The uptake capacity of the cells was also measured [32].

Lipid peroxidation

the lipid peroxidation (LPO) was assayed by the determination of the amount of thiobarbituric acid reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides on isolated cells (1×10^6 cells/well) by following the absorbance at 532 nm in a spectrophotometer analyzer by determining the MDA level following the manufacturer's instructions. Each test/group was examined with three replicates for each sample [33].

Glutathione redox state

On isolated cells, (1×10^6 cells/well), glutathione redox (GSH) by OPA probe and glutathione oxidase (GSSG) by NEM probe are the most important scavengers of ROS that can be utilized as a biomarker of the redox balance. Each sample was measured in quartz cuvettes using a fluorimeter set at $\lambda_{exitation}/\lambda_{emission}$ 350 /420 nm wavelengths. Each test/group was examined with three replicates for each sample [33].

Lysosome membrane integrity

Cell suspension (1×10^6 cells/well) was stained with AO (5 µM), an indicator of lysosome membrane permeabilization. The cells were separated from the incubation plate by centrifugation at 1000 rpm for 1 min. The cell pellet was re-suspended in 2 mL of fresh culture medium and rinsed twice to remove the fluorescent dye. AO redistribution in the cell suspension was

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measured colorimetrically using a spectrophotometer at $\lambda_{exitation}$/ $\lambda_{emission}$= 490/535 nm. Lysosome membrane damage is defined as the difference in the redistribution of AO from lysosomes into the cytosol between the treated and control cells [34].

Apoptosis and Necrosis assay

After the treatment, the cells (1×10^6 cells/well) were stained with 5 ml of Annexin V and 5 ml of PI at room temperature for 20 min. The cells were diluted in the banding buffer (400 ml) and analyzed with flow cytometry. The fluorescence signals of Annexin V and PI were measured by flow cytometry on the FL1 and FL3 channels. Supplied with the softwaring 1.2.5 and each determination is based on the mean fluorescence intensity of 10,000 counts and following the manufacturer's instructions.

Statistical analysis

All data sets were normally distributed and homoscedastic. Hence, the data were analyzed using one-way and two-way analysis of variance followed by post-hoc Tukey and Bonferroni tests, respectively. Results were presented as mean ± standard deviation (n = 5), with statistical significance pre-set at α= 0.05.

Results

ZrO_2-NP characterization

The morphology of the prepared ZrO_2-NPs is shown in Figure 1A. Size distribution of the NPs, as measured by their hydrodynamic diameter (dh) is presented in Figure 1B.

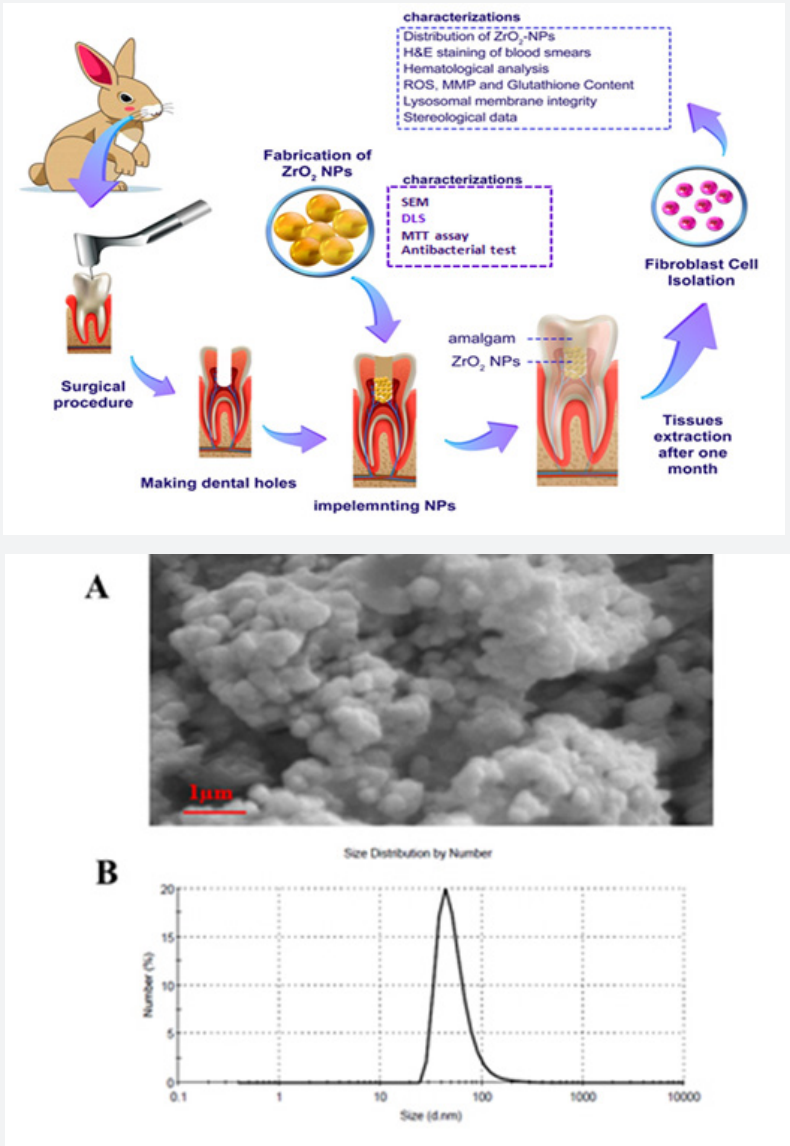

Figure 1: : Characterization of ZrO2-NPs by SEM (A), size distribution of the hydrodynamic diameter of the ZrO2-NPs (B).

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Distribution of ZrO_2-NPs

Distribution of ZrO_2-NPs in the brain, heart, lungs, kidneys, and liver at days 0 (before exposure of NPs) 1, 15 and 30 (after exposure to the NPs) is shown in Table 1. In ZrO_2-NP treatment (CD) group, the NPs were accumulated predominantly in the tooth gum after 15 and 30 days. Accumulation of ZrO_2-NPs was barely detectable in the brain, heart, kidneys, lungs, and liver. Accumulation of the ZrO_2-NPs in the tooth gum tissues was 1.1 ± 0.11 µg/g after 1 days, 2±0.1 µg/g after 15 days and 1.4±0.1 µg/g after 30 days (*P < 0.05) and 0 (before exposure of NPs) in all of tissue had not any accumulation of ZrO_2-NP.

Table 1: Distribution of ZrO_2-NPs in the brain, heart, lungs, kidneys, and liver of diabetic rabbits at 0 (before exposure time), 1, 15 and 30 days (after exposure time). Values are expressed as means ± SD (n = 5). ns: non-significant; *P<0.05; compared to control cells before exposure.

ZrO_2-NP content in different tissues				
Tissue	Concentration of ZrO_2 (µg/g)			
	Before	After		
	0 day	1 day	15 days	30 days
Tooth gum	<0.1	1.1 ± 0.1*	1.2 ± 0.1*	1.4 ± 0.1*
Brain	<0.1	<0.1 ^{ns}	<0.1 ^{ns}	<0.1 ^{ns}
Heart	<0.1	<0.1 ^{ns}	<0.1 ^{ns}	<0.1 ^{ns}
Lungs	<0.1	<0.1 ^{ns}	<0.1 ^{ns}	<0.1 ^{ns}
Kidneys	<0.1	<0.1 ^{ns}	<0.1 ^{ns}	<0.1 ^{ns}

Antimicrobial properties

The ZrO_2-NPs did not exhibit antibacterial activity against Gram-positive nor Gram-negative bacteria (Figure 2A). As shown in Figure 2B, no inhibition zones were identified when disks containing ZrO_2-NPs were in placed in contact with S. aureus or *E. coli*.

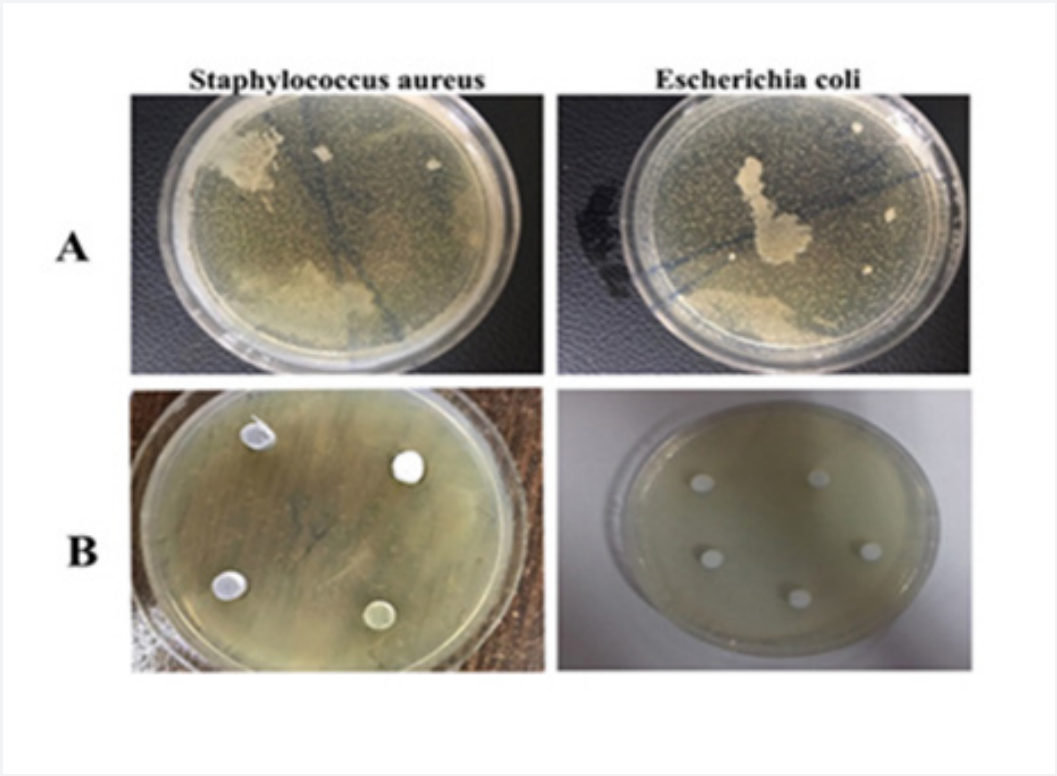

Figure 2: Agar diffusion methods: disk-diffusion of microbial extracts using Staphylococcus aureus and Escherichia coli as test microorganisms (A). Antibiogram test in agar culture of S. aureus and E. coli of ZrO2-NPs.

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Stereological examination

Images from the control health group (CH) and ZrO_2-NP treatment group (CD) (B) are shown in Figure 3A volume of epithelium (V. E mm) and connective tissue (V. CT), and number of neutrophils (N.N) in tooth gum tissue. B. Estimation of the total volume of bone (TV), Figure 3B bone marrow volume (BMV), trabecular bone volume tissue (TBV), osteocytes (OS) and osteoblasts (OB) in the tooth gum tissue showed no significant differences between the CH and CD groups (Table 5A, B).

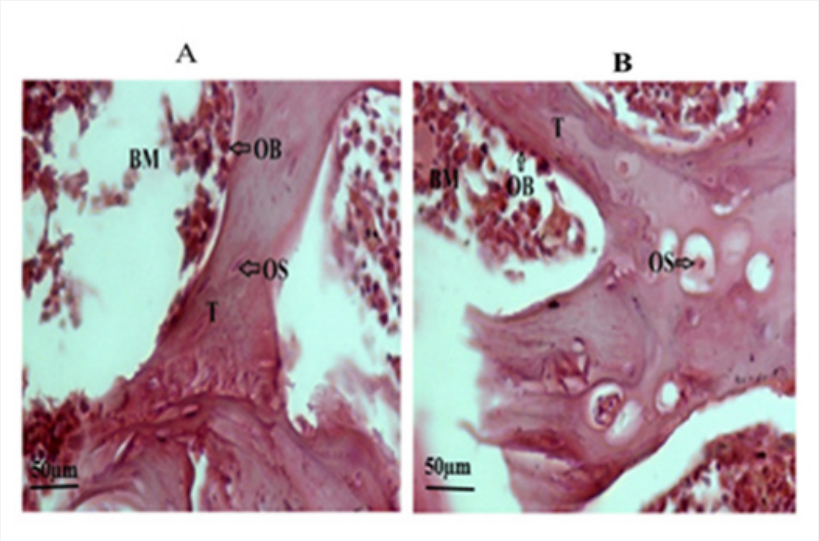

Figure 3: Stereological examination of tissue bone marrow volume (BM), trabecular bone volume tissue (T), osteocyte (OS) and osteoblast (OB). There is no significant difference between the control health(A) and ZrO2-NP treatment (CD) (B) groups. The density of tissue is equal to 50 µm.

Table 2: Difference in hematological parameters between control health (CH) and ZrO_2-NP treatment (CD) at 0 (before exposure time), 1, 15 and 30 days (after exposure time). Values are expressed as means ± SD (n = 5). ns: non-significant; *P<0.05; *P<0.01; compared to control cells before exposure.

White blood cells (count; cells 10^6)								
Parameter	Control				Experimental			
	Before	After			Before	After		
	0 day	1 day	15 days	30 days	0 day	1 day	15 days	30 days
WBC	5.44±0.05	5.62±0.08 ^{ns}	5.72±0.07^{ns}	5.75±0.04^{ns}	5.55±0.11^{ns}	6.50±0.43*	6.62±0.41*	6.69±0.36*
Lymphocytes	12.0±1.0	12.4±1.0 ^{ns}	17.3±1.15^{ns}	17.0±1.0^{ns}	12.22±3^{ns}	15.7±3.1^{ns}	19.7±2.3*	24.0±4.0**
Monocytes	3.0±0.41	3.33±0.57 ^{ns}	3.33±0.57^{ns}	3.66±1.10^{ns}	3.20±0.57^{ns}	3.33±0.57^{ns}	3.99±0.01*	4.66±0.57*
Neutrophils	46.22±1	46.7±1.5 ^{ns}	44.0±1.0 ^{ns}	45.0±1.0 ^{ns}	45.02±2.0^{ns}	46.0±1.0^{ns}	64.0±1.0**	66.0±1.0**

Table 3: Hematological evaluation of various parameters on control health (CH) and ZrO_2-NP treatment (CD) at 0 (before exposure time), 1, 15 and 30 days (after exposure time). Values are expressed as means ± SD (n = 5). ns: non-significant; *P<0.05; **P<0.01; ***P<0.001; compared to control cells before exposure.

Hematological evaluation of various parameters								
Parameter	Control				Experimental			
	Before	After			Before	After		
	0 day	1 day	15 days	30 days	0 day	1 day	15 days	30 days
RBC	4.40±0.06	4.45±0.03^{ns}	4.53±0.02 ^{ns}	4.54±0.02 ^{ns}	4.22±0.02 ^{ns}	4.103±0.005 ^{ns}	4.14±0.05 ^{ns}	4.19±0.10 ^{ns}
HB	14.12±0.10	14.26±0.15 ^{ns}	14.12±0.12 ^{ns}	14.25±0.02 ^{ns}	14.23±0.1 ^{ns}	14.44±0.11 ^{ns}	14.53±0.10 ^{ns}	14.64±0.09 ^{ns}

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MCH	25.33±0.05	25.42±0.03 ns	25.45±0.02 ns	25.54±0.01 ns	25.1±0.1*	27.00±0.51*	27.15±0.53*	27.45±0.83*
MCHC	30.11±0.07	30.12±0.02 ns	30.13±0.01 ns	30.14±0.02 ns	35.14±0.33**	37.14±0.71**	37.41±0.29***	37.53±0.21***
PLTs	150±1.0	154±1.5 ns	155±1.7 ns	156±1.5 ns	203±12**	219±16.5***	222±16.9***	227±16.1***
HCT	39.07±0.1	39.7±0.1 ns	39.53±0.6 ns	40.4±0.3 ns	39.88±0.5 ns	39.92±0.3 ns	40.88±1.0*	42.81±3.63*
MCV	88.33±0.10	88.70±0.17 ns	88.67±0.01 ns	88.73±0.02 ns	88.80±0.40 ns	88.84±0.33 ns	88.86±0.43 ns	88.97±0.38 ns
PDW %	11.32±0.10	11.66±0.15 ns	11.00±1.00 ns	11.55±0.01 ns	12.33±0.21 ns	12.44±0.19 ns	12.62±0.13 ns	12.73±0.21 ns
MPV	8.09±0.06	8.11±0.02 ns	8.24±0.03 ns	8.33±0.02 ns	8.06±0.01 ns	8.46±0.08 ns	8.54±0.10 ns	8.61±0.11 ns

Table 4: Ratio of Intracellular GSH and extracellular GSSG concentrations on control health (CH) and ZrO_2-NP treatment (CD) cells (1×10^4 cells/well) at 0 (before exposure time), 1, 15 and 30 days (after exposure time). Values are expressed as means ± SD (n = 5). ns: non-significant; *P<0.05; *P<0.01; compared to control cells before exposure.

Glutathione content (µg/mg protein)								
Parameter	**Control**				**Experimental**			
	Before	**After**			**Before**	**After**		
	0 day	**1 day**	**15 days**	**30 days**	**0 day**	**1 day**	**15 days**	**30 days**
GSH	286±0.008	286±0.008 ns	279±0.006 ns	277±0.007 ns	224±0.07*	232±0.005*	246±0.004*	213±0.004*
GSSG	360±0.020	360±0.020 ns	365±0.014 ns	363±0.021 ns	557±0.01**	561±0.001**	594±0.011**	614±0.005**
GSSG/GSH	1.3±0.020	1.3±0.020 ns	1.3±0.014 ns	1.3±0.021 ns	2.4±0.01*	2.4±0.001*	2.4±0.011*	2.9±0.005*

Table 5: Stereological study. A. Estimation of the V. E, V. CT, N.N in tooth gum tissue. B. Estimation of the total volume of TV, BMV, TBV, OS and OB in jawbone tissue on control health (CH) and ZrO_2-NP treatment (CD). Values are expressed as means ± SD (n = 5). *P<0.05; **P<0.01; ***P<0.001; compared to control health before exposure.

Stereological Examinations (µm)								
Groups	**A: Tooth gum**			**B: Jawbone**				
	N. N	**V.CT**	**V.E(mm)**	**TV (mm^3)**	**BMV (mm^3)**	**TBV (mm^3)**	**O_s**	**OB**
CH	37	35.9	52.6	429.5	25.5	115.8	32	27
CD	132***	58.1**	91.1**	417.3*	24.1*	108.6*	31*	28*

Hemotoxicity

Hematoxylin and eosin-stained blood smears of the control health group (CH) (A) and ZrO_2-NP treatment group (CD) are shown in Figure 4. At 1 day's post-treatment, hemotoxicity was absent from the peripheral blood samples obtained from diabetic rabbits treated with ZrO_2-NPs compared control health group (before time).

Hematological analysis

Increase of white blood cell (WBC) count in ZrO_2-NP treatment groups after 1, 15 and 30 day (*P<0.05), The percentage of lymphocytes population in 15 (*P<0.05) and 30 days (**P<0.01), The percentage of monocytes population in 15 (*P<0.05) and 30 day (*P<0.05), The percentage of neutrophils population in 15 (**P<0.01) and 30 days (**P<0.01). All of group were not significant compared with control health (CH, 0day) group all of time (Table 2). counts of RBC count in the experimental group after administration of the ZrO_2-NP was not significant in all of time compared with control health (CH, 0 day) (Table 3). HB did not significantly change after treatment with ZrO_2-NPs in the experimental and health group after 1, 15 and 30 days compared with control health (CH, 0 day) (Table 3). MCH increased in 0, 1, 15 and 30 days after treatment of the ZrO_2-NP in experimental group compared with control health (CH, 0 day) (Table 3). Significant induction of MCHC after exposure ZrO_2-NPs in the experimental group in 0, 1 day (**P<0.01), 15 and 30 day (***P<0.001) compared with control health (CH, 0 day) and other groups were not significant change in factor (Table 3). PLTs was increased after ZrO_2-NP administration in experiment groups after 0, 1, 15 and 30 days (**P<0.01) compared with control health (CH, 0 day) and other groups were not significant change in factor (Table 3). HCT was increased after ZrO_2-NP administration in experiment groups after 0, 1, 15 and 30 day (*P<0.05) compared with control health (CH, 0 day) and other groups were not significant change in factor (Table 3). In MCV, PDW% and MPV were not any change on experimental groups after exposure ZrO_2-NPs all of time (Table 3).

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Cell viability

The percentage of total cell viable (Q1) population were CH~ 94.3%, CH.1~ 92.88%, CH.15~ 98.56%, CH.30~ 90.64%, CD~ 89.78%, CD.1~ 78.86%, CD.15~ 72.35%, CD.30~ 59.37%. Changed garrulity (Q2) population were CH~ 1.41%, CH.1~ 0.48%, CH.15~ 0.1%, CH.30~ 4.90%, CD~ 0.99%, CD.1~ 0.97%, CD.15~ 0.23%, CD.30~ 11.06%. Changed morphology/granulity (Q3) population were CH~ 2.82%, CH.1~ 1.09%, CH.15~ 0.11%, CH.30~ 0.09%, CD~ 2.09%, CD.1~ 1.89%, CD.15~ 10.50%, CD.30~ 15.49%. Changed morphology (Q4) population were CH~ 1.62%, CH.1~ 5.64%, CH.15~ 1.23%, CH.30~ 4.37%, CD~ 7.13%, CD.1~ 18.28%, CD.15~ 16.90%, CD.30~ 13.97% (Figure 5A). cell viability as evaluated by MTT staining, was significantly reduced after 0, 1,15 days(**P<0.01), 30(***P<0.001) incubation of cells with ZrO_2-NPs in experimental group compared with control group (CH, 0 day) (Figure 5B).

Reactive oxygen species and mitochondrial membrane potential

Application of ZrO_2-NPs significantly elevated ROS generation in cells obtained from tooth gum tissue (Figure 6A); generation of ROS was concentration- and time-dependent. Administration of ZrO_2-NPs in experimental group after 1,15 (*P<0.05) and 30 days(**P<0.01) compared with control group (CH, 0 day). Significantly reduced the mitochondrial membrane potential in tooth gum cells in a concentration- and time-dependent manner, as indicated by the increase in cellular uptake of Rh123 after exposure of ZrO_2-NPs in experimental group on 15 and 30 days(***P<0.001) compared with control group (CH, 0 day). (Figure 6B).

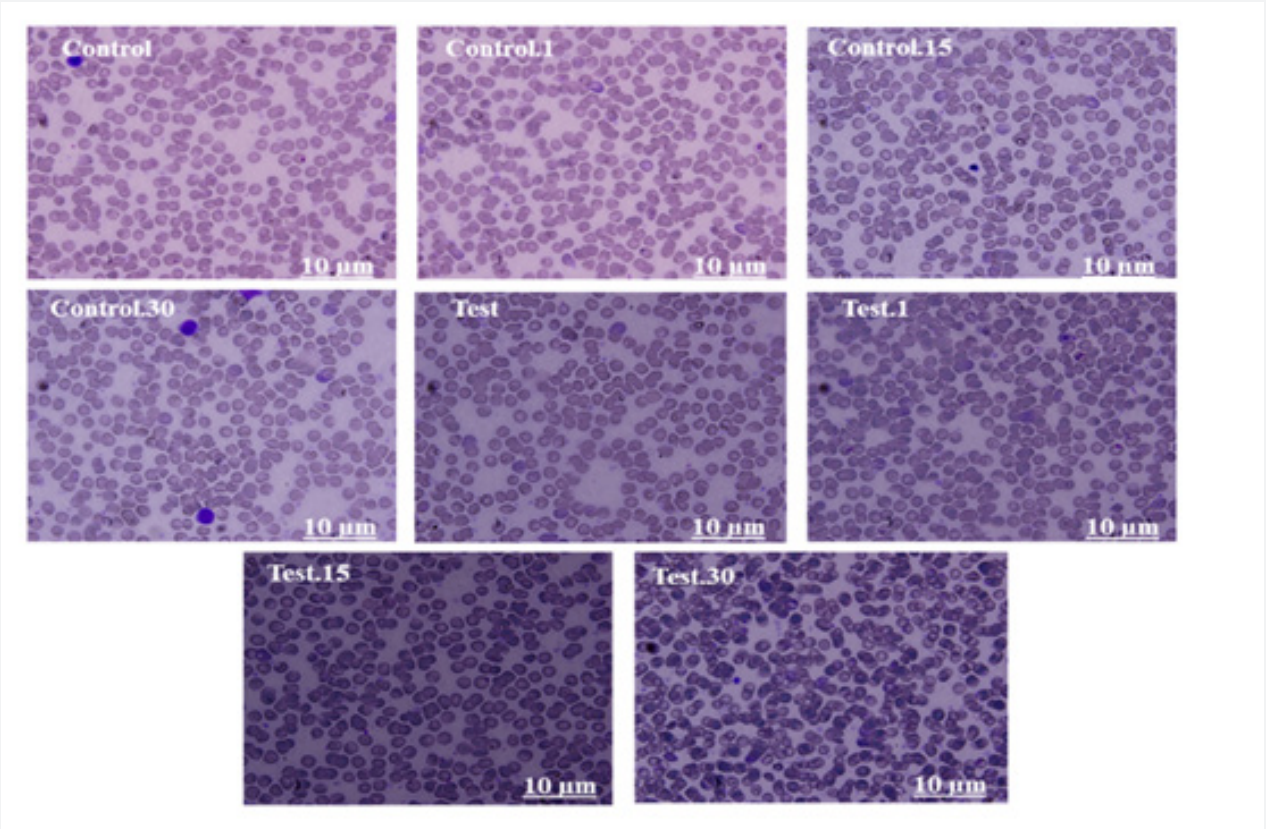

Figure 4: Hematoxylin and eosin staining. Photomicrographs of the collected blood on control health (CH) and ZrO2-NP treatment (CD) at 0 (before exposure time), 1, 15, 30 days (after exposure time). Magnification x100.

Glutathione content

Significant decrease GSH levels in CD group (0 day) and administration of ZrO_2-NPs in experimental group after 1,15 and 30 day (*P<0.05) compared with control group (CH, 0 day). Cells in CD group (0 day) and exposed to ZrO_2-NPs in experimental groups and significantly increased on GSSG contents after 1,15 and 30 days (**P<0.01) compared with control group (CH, 0 day) and finally GSSG/GSH was changing treatment groups (Table 4).

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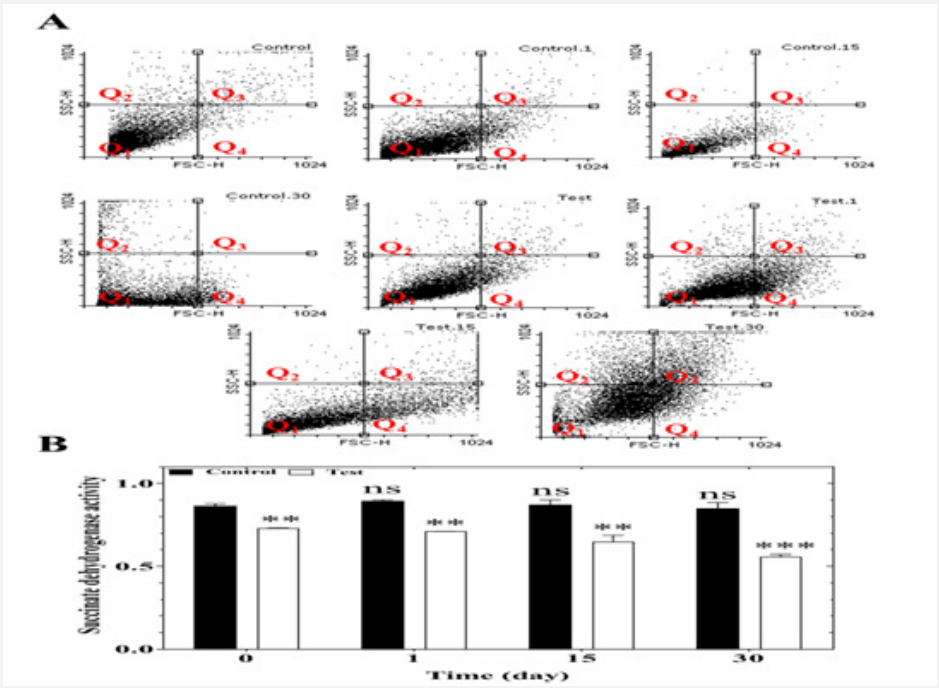

Figure 5: Flow cytometry of viable tooth gum cells (10,000 counts) (A) and cell viability assay (1×104 cells/well) (B) on control health (CH) and ZrO2-NP treatment (CD) at 0 (before exposure time), 1, 15, 30 days (after exposure time). Values are expressed as means ± standard deviations (n = 5). ns: non-significant; **P<0.01; ***P<0.001; compared to control cells.

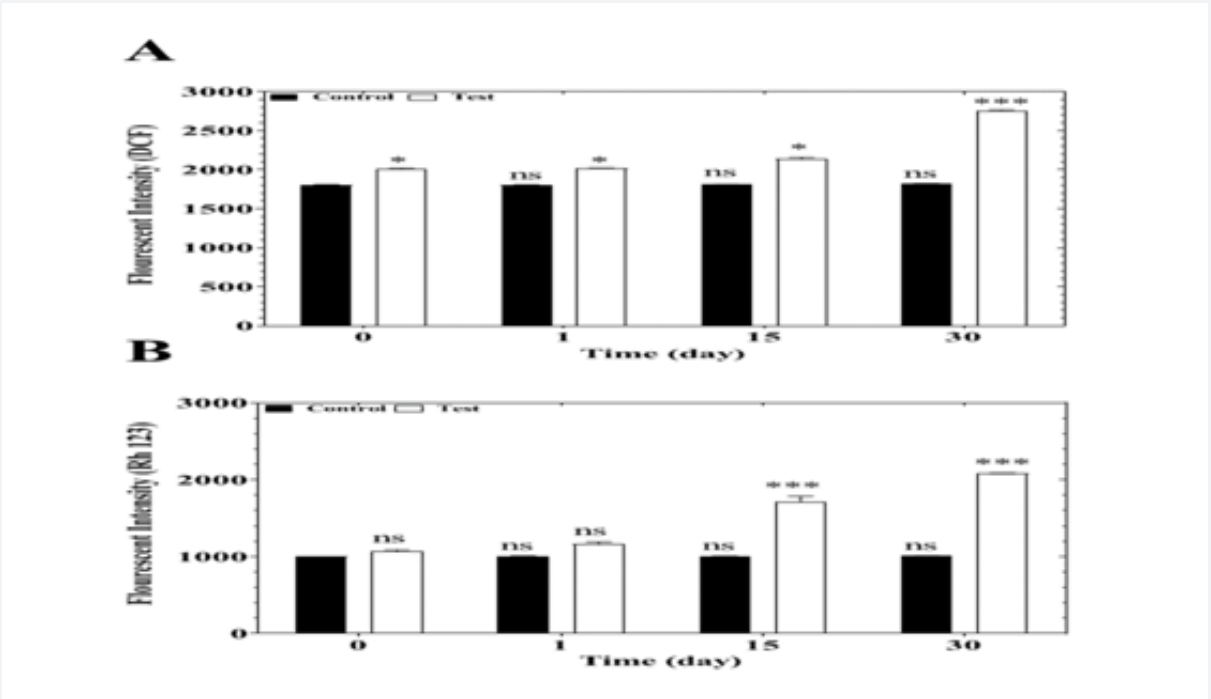

Figure 6: ROS formation was expressed as fluorescent intensity (1×104 cells/well) (A)and MMP was expressed as fluorescent intensity (1×104 cells/well) (B) on control health (CH) and ZrO2-NP treatment (CD) at 0 (before exposure time), 1, 15 and 30 days (after exposure time). Values represented as mean ± SD (n = 5). ns=no significant, *P < 0.05 and ***P < 0.001 compared with control health (CH) group.

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Lipid peroxidation and lysosomal membrane integrity

Administration ZrO_2-NPs in experimental groups caused a significant increase in the MDA levels in the tooth gum cells at day 1 (**P < 0.01), 15 and 30(***P < 0.001). Thereafter, there was no significant difference in the MDA levels between health groups before and 1,15, 30 days (Figure 7A). There were also significant increases in lysosomal membrane permeabilization in the experimental group before (*P < 0.05) and after exposure to ZrO_2-NPs for 11 (**P < 0.01), 15 and 30 days1 (***P < 0.001), as revealed by the increase in uptake of AO (Figure 7B).

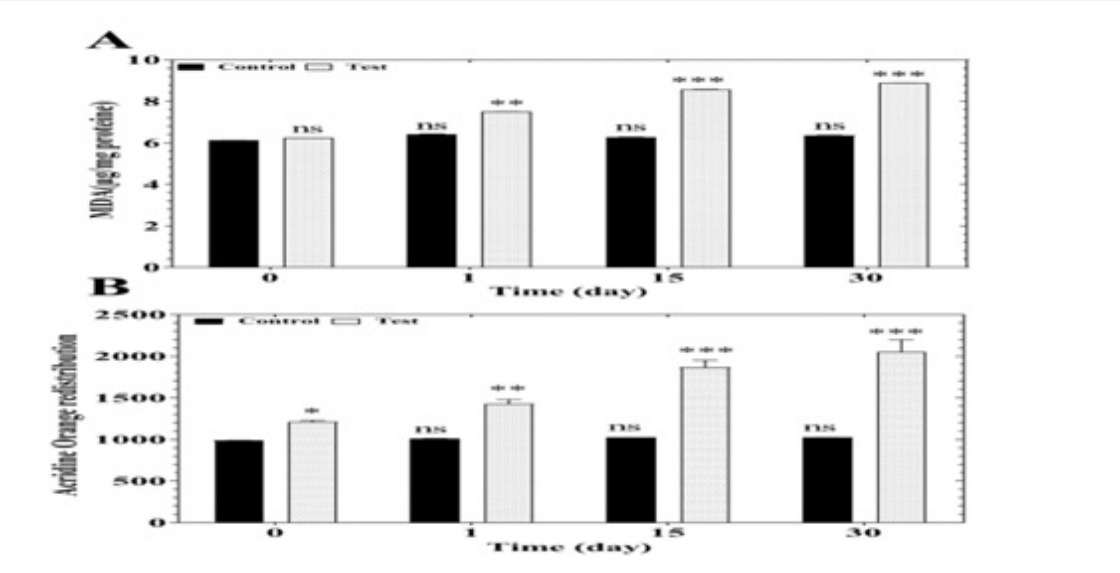

Figure 7: Thiobarbituric Acid Reactive Substances assay for quantification of MDA, the end-products of lipid peroxidation (1×104 cells/well) (A) on control health (CH) and ZrO2-NP treatment (CD) at 0 (before exposure time), 1, 15, 30 days (after exposure time). Lysosomal membrane integrity (1×104 cells/well) (B) shows that there is a significant difference between the integrity of lysosomal membranes on control health (CH) and ZrO2-NP treatment (CD) at 0 (before exposure time), 1, 15 and 30 days (after exposure time). Values represented as mean ± SD (n = 5). ns=no significant, *P < 0.05; **P < 0.01 and ***P < 0.001 compared with control health (CH)group.

Apoptosis and Necrosis

The apoptosis was quantified by the externalization of PS (phosphatidylserine). PI stains the nuclear and, thus, it was used as an indicator of membrane integrity and finally apoptosis/necrosis assessed by annexin V/PI double staining at cell viable (Q1) population were CH~ 99.27%, CH.1~ 99.20%, CH.15~ 99.17%, CH.30~ 99.17%, CD~ 93.67%, CD.1~ 87.45%, CD.15~ 75.72%, CD.30~ 60.63%. necrosis (Q2) population were CH~ 0.56%, CH.1~ 0.13%, CH.15~ 0.16%, CH.30~ 0.07%, CD~ 5.70%, CD.1~ 0.37%, CD.15~ 10.29%, CD.30~ 28.75%. Early apoptotic (Q3) population were CH~ 0.6%, CH.1~ 0.24%, CH.15~ 0.30%, CH.30~ 0.42%, CD~ 0.51%, CD.1~ 3.60%, CD.15~ 11.14%, CD.30~ 8.01%. Late apoptotic (Q4) population were CH~ 0.09%, CH.1~ 0.43%, CH.15~ 0.37%, CH.30~ 0.34%, CD~ 0.08%, CD.1~ 8.58%, CD.15~ 2.72%, CD.30~ 2.43% (Figure 8).

Discussion

Diabetes mellitus is known as an endocrine disorder and the number of sufferers has been increasing in recent years. According to the prediction of the International Diabetes Federation (IDF), the number of diabetics will increase to 578.4 million people by 2030 and to 700 million people by 2045. In most cases, due to the lack of effective control, this disease causes significant complications [7]. The various complications include neurological disorders, narrowing of blood vessels, blindness in adulthood, end-stage renal disease, ischemia, peripheral vascular disease, microbial infections, and loss of dental implants [8]. A few studies have shown that despite high bone density in female diabetic patients compared to their male counterparts, the fracture rate in womsen is higher, which indicates a qualitative difference in bone [35]. In addition, in diabetic patients, osteoblastic activity is limited, and parathyroid hormones change calcium and phosphorus metabolism. As a result, collagen fiber formation is reduced, and bone cells may undergo apoptosis and prevent bone formation [36].

Zirconium oxide nanoparticles are used considerably in the ceramics industry and as dental and optical coatings. Previous studies reported that the cytotoxicity of these nanoparticles is dependent on their size and shape [13-14]. It has been speculated that ZrO_2-NPs are potentially cytotoxic because of their potential to induce cellular oxidative stresses. The latter, in turn, results in increased ROS production, reduced GSH levels, and augmented

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lipid peroxidation. The lack of evidence supporting these hypotheses in antecedent studies prompted us to investigate the in vitro and in vivo cytotoxicity of ZrO_2-NPs on diabetic rabbit tooth gum cells.

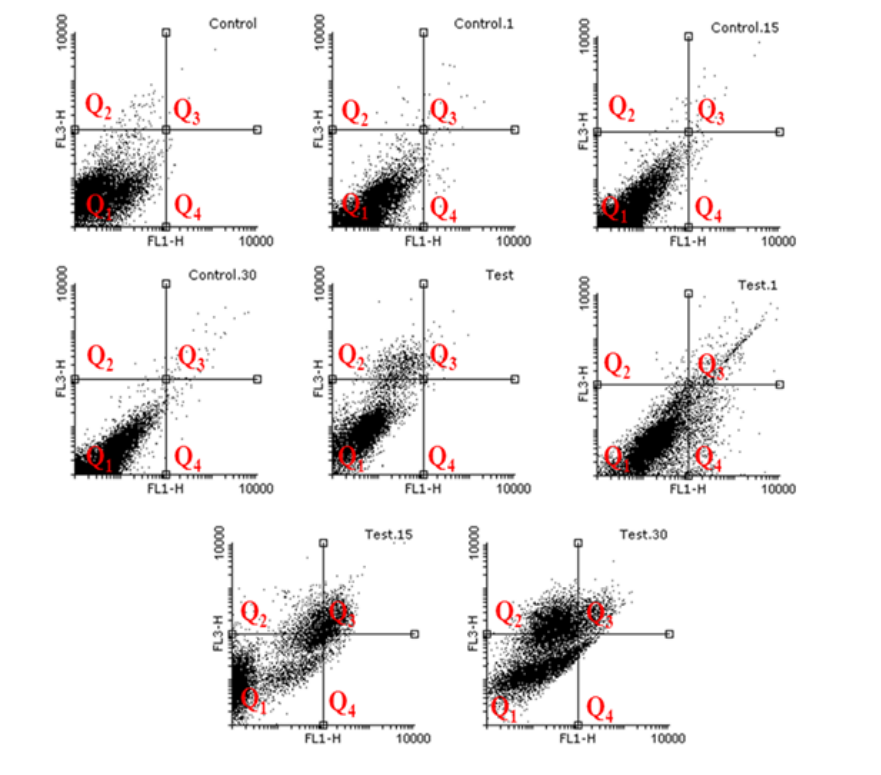

Figure 8: determination of apoptotic and necrotic (annexin V/PI) markers on cells (1×104 cells/well) by flowcytometry on control health (CH) and ZrO2-NP treatment (CD) at 0 (before exposure time), 1, 15 and 30 days (after exposure time).

Results obtained from the present study indicate that increased intracellular ROS after exposure to ZrO_2-NPs is responsible for the cytotoxicity of these NPs in vitro and in vivo. These results are in general agreement with previous reports in the literature. The generation of ROS is caused by electron ‘‘leakage’ of the mitochondrial respiratory chain, which can be evaluated by the production of dichlorofluorescein, the highly fluorescent oxidized derivative of dichloro- fluorescein diacetate. These ROS originate from superoxide production either from complex I or from ubiquinone/complex III, due to partial damage and/or inhibition of the respiratory chain complexes [33]. Several authors claimed that ROS generation stimulates pro-apoptotic cell signaling via cytochrome c release from mitochondria [32,33].

Although the effect of ROS on cellular damage has not been completely elucidated [33], some studies suggested that lipid peroxidation plays a crucial role in NP-induced cytotoxicity [36, 37]. This hypothesis was supported by the results obtained from the present study. Increased ROS generation results in oxidation of the thiol group of membrane proteins. This, in turn, induces opening of the mitochondrial permeability transition (MPT) pores, which precipitate mitochondrial dysfunction. The latter is manifested by unlimited proton movement across the inner mitochondrial membrane as well as induction of MMP disruption and uncoupling of oxidative phosphorylation [38]. It has been proposed that cell membrane damage occurs via lipid peroxidation, which initiates a chain of reactions that further results in cellular degradation [39]. Several authors have reported that ZrO_2-NPs generate ROS and oxidative stress of occurs through induction of death intracellular signaling pathways [38,39]; ROS generation adversely affect DNA and transcription of proteins that are responsible for membrane integrity, subsequently resulting in the loss of membrane integrity [40-44]. Others suggested that ZrO_2-NPs induce apoptosis in T-helper lymphocytes at concentrations higher than 5 mM, through increasing the release of tumor necrosis factor-alpha [45].

Contrary to these reports, Damestani et al. reported that ZrO_2-NPs are biocompatible and do not induce acute inflammatory responses in the host tissue [46]. Renu et al. reported that ZrO_2-NPs cause an increase in GSH and decrease in lipid peroxidation levels [43]. To validate the results of the previous study, the toxicity of ZrO_2-NPs was investigated in the present work using antioxidant enzymes (glutathione peroxidase) and non-enzymatic

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assays (lipid peroxidation and hematological analysis), apoptosis and necrosis. Our results showed that there was a significant reduction in lipid peroxidation and GSH levels in the tooth gum tissues of diabetic rabbits that had been injected with ZrO_2-NPs. These findings were consistent with the study by Chakraborty et al., who reported increased lipid peroxidation in the liver and heart in the presence of ZrO_2-NPs [48]. Several studies examined the reaction of animal cells to ZrO_2-NPs using MTT assay, wherein cytotoxicity was estimated through evaluation of cell proliferation. The authors reported cytotoxicity associated with ZrO_2-NPs [49-57]. Results from the present study showed ZrO_2-NPs decreased succinate dehydrogenize activity and decreased the viability of diabetic rabbit tooth gum cells.

Conclusion

Within the scope of the present study, it may be concluded ZrO_2-NPs are not as biocompatible as they were previously assumed. While zirconia may be a compatible biomaterial, reduction of the metal oxide to nano particulates triggers increases in oxidative stress within the mitochondria and disruptions in lysosomal membrane integrity. The cytotoxicity of ZrO_2-NPs is non-uniform toward body tissues and the most toxic of them is to diabetic rabbit dental tooth gum cells. Accumulation of the NPs in other organs of the diabetic rabbit appears to be minimal. While there is no problem associated with the use of zirconia in bulk, such as in the case of dental implants. Due to the possible concern about the cytotoxicity of zirconia as functional nanoparticles for human dental implants, many specialized investigations should be done in the future.

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Author Contributions

Conceptualization, Parvaneh Naserzadeh, Behnaz Ashtari; Methodology, Parvaneh Naserzadeh; Software, Parvaneh Naserzadeh, Seyed Arash Javad Mossavi; Formal analysis, Parvaneh Naserzadeh, Seyed Arash Javad Mossavi; Investigation, Parvaneh Naserzadeh, Fatemeh Ghanbary, Behnaz Ashtari, Masoud Akhshik; Data curation, Parvaneh Naserzadeh; Writing original draft preparation, Parvaneh Naserzadeh, Behnaz Ashtari; Writing review and editing, Parvaneh Naserzadeh, Behnaz Ashtari, Masoud Akhshik; Visualization, Parvaneh Naserzadeh, Behnaz Ashtari; supervision, Parvaneh Naserzadeh; project administration, Parvaneh Naserzadeh, Behnaz Ashtari. All authors have read and agreed to the published version of the manuscript.

Disclosure Statement

The authors declare no conflict of interest.

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