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Study on potential toxic of titanium oxide nanoparticles on osteoblasts

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ABSTRACT

Purpose: By studying the cytotoxicity and mechanism of titanium oxide nanoparticles (TiO_2-NPs) towards growing of osteoblasts, this paper provides the scientific basis for development of implantation volume for new type of Ti-based bone; Method: Establish the co-culture system for TiO_2-NPs and osteoblasts; study the cytotoxicity effect and oxidation stress response. Result TiO_2-NPs may cause the declining of osteoblasts vitality, increase of LDH content released by osteoblasts and oxidation of stress reaction. Conclusions: TiO_2-NPs may give rise to the toxic reaction of osteoblasts, and the mechanism mainly causes the oxidation stress reaction of osteoblasts. This study is provided with important theoretical significance to realize the toxicity and mechanism of osteoblasts for TiO_2-NPs and provides the theoretical basis for discussion on safe application of TiO_2-NPs in the implant field.

KEYWORDS

Titanium oxide nanoparticles; Osteoblasts; Oxidative stress; Apoptosis.

INTRODUCTION

Nanoparticles refer to the particle that the particle size is of namometer quantity grade and also called as ultrafine particle, including the engineering namometer particle and ultrafine particles in the air. Since the namometer particle is provided with unique physical and chemical property--- such as, small size effect, surface effect, macroscopic quantum tunneling effect and quantum size effect, therefore, it is widely applied in such fields[1-4] as industry, military, agriculture, living, medicine and scientific research. Titanium oxide nanoparticles（TiO_2-NPs）are deemed as a kind of namometer material with stable biological performance and relatively mature preparation technology to be widely applied in such products and biological medicine fields closely contacted with human life as drug carrier, biomarker, DNA chip, antibacterial agent, cosmetics and food additives and also brings people's doubt for its safety and potential hazard simultaneously. It is compared with traditional fabrication method of titanium dioxide that the titanium dioxide coating with namometer on the surface of oral implant may regulate the protein absorption, stimulate the cell adhesion, increase the vitality of alkaline phosphatase and improve the bone mineralization and bone mineralization function of bone and biology material interface. In addition, TiO2-NPs are provided with osteoconduction and may form the new bone within short time and shorten the healing period of implant.

However, in the long-term clinical application, due to corrosion, aseptic loosening and prosthesis friction, the bone implant, orthopaedic body or dental implant may inevitably produce the micron or nanometer wear particles in the implant/ bone interface to be released into the microenvironment. The existing study has affirmed that the abrasive dust in the titanium and titanium alloy bone implant may be released into the tissues in the periphery to cause the implant loosening, give rise to complication after operation and lead to osteolysis in the periphery[5]. Abu-Amer etc. discovers study through study that the chippings for wear particles in the tissues of bone implanting material may induce the macrophage and fibroblast to release the inflammatory cytokines, especially induce the osteoclast to secrete the dissolved bone cell factors and trigger the chronic inflammation of implant- bone interface and bone dissolution around the implant. Then, whether TiO_2-NPs is provided with adverse reaction and toxicity effect mechanism for cells in the periphery of implant especially for osteocyte is still not shown in the related literature report. The osteoblast is served as the experimental cell in this study. Through establishing the co-culture model of TiO_2-NPs and osteoblast, observe the toxicity influence on osteoblast by TiO_2-NPs with different concentration and relevant mechanism.

MATERIAL AND METHOD

Reagent and main instrument

TiO_2-NPs (anatase, <25 nm) and mycillin Penicillin-Streptomycin are purchased from sigma Company (USA). DMEM cell culture medium, fetal calf serum (FBS) and trypsin are purchased from Gibco-Invitrogen Company (UK). MTS reagent kit for cytoactive detection（CellTiter 96）is purchased from Promega Company (USA). LDH reagent kit for cytotoxicity detection is purchased from Nanjing Jiancheng Bioengineering Institute. ROS detection reagent kit and RIPA cell lysis buffer are purchased from BIYUNTIAN Biotechnology Institute. Transmission electron microscopy（TEM）: JEM-2010, Japanese Electronics Corporation. Zetasizer particle size analyzer: 3000HS, UK Malvern Instrument Co., Ltd. Zetasizer Potentiostat: UK Malvern Instrument Co., Ltd. Ultrasonic oscillation instrument : HK2200, Shanghai Kudos Ultrasonic Instrument Co., Ltd. Bechtop: Suzhou Aetna Air Technology Co., Ltd. CO2 cell incubator: Thermo Fisher Scientific (Germany). Inverted phase contrast microscope: Olympus Corporation (Japan). ELIASA: Labsystems Dragon Wellscan MK3 (Finland).

Property characteristic of TiO_2-NPs

TEM is employed to detect grain size and shape of TiO_2-NPs. TiO_2-NPs is prepared as 1 mg/l nano-particle suspended fluid by employing DMEM cell culture fluid containing 10%FBS. Zetasizer particle size analyzer and potentiometric analyzer with 600 W, 20 kHz and 30 min power ultrasound are employed to measure the grain size of particle in the solution and zeta potential.

Extraction and appraisal for osteoblast primary cell

The osteoblast of cranium parietal bone for rat that was born for 24hs is adopted. The secondary enzyme digestion is utilized to cut off the kull and place into PBS fluid as well as eliminate such connective tissues as periosteum and blood vessel. After washing twice by employing PBS fluid, shear into 1 mm×1 mm osteocomma and shift into 5 ml 0.25% pancreatin solution. Under 37 ℃, perform the predigestion for 20 mins so as to eliminate the fibrocytes. Later on, place the sclerite obtained by filtering into the petri dish, add 5 ml 0.1% Type II collagenase fluid, vibrate and digest for 60 mins under 37℃, centrifuge for 10mins under 1000 r/min, absorb off the supernatant. The sedimented cell aggregates are fabricated as cell suspension by employing DMEM nutrient solution (including 10% fetal calf serum and 100U/ml penicillin and 100 mg/ml streptomycin). Repeat above steps, place the cell obtained by two-time digestion into 37 ℃ incubator, replace the fluid once after 24hs, observe the form after cell adherence. And then, prepare the cell suspension, with 5×10^4 cells per aperture, place into 24-hole plate, after culturing for 14ds, detect the activity of alkaline phosphatase inside cell by employing the

dyeing reagent kit of alkaline phosphatase（ALP）(Shanghai Hongqiao Company). The portion dyed as purple in a view is judged as the positive area of alkaline phosphatase dyeing. The third generation of osteoblast is selected for experiment.

Co-culture of osteoblast and nanoparticle

After TiO_2-NPs is sterilized under 120 ℃ high temperature and high pressure for 2hs, DMEM culture fluid is employed to prepare the nanoparticle mixed suspension （25 μg/ml，50 μg/ml，100 μg/ml and 200 μg/ml），the ultrasound 30 min （600 W，20 kHz）dispersion and blending is exposed before cell. The negative control group is the cell free of nanoparticle treatment.

MTS colorimetry analysis method

The experiment principle means that the metabolic activity cells may be reacted with methyl thiazolyl tetrazolium to generate absorbable formyl in soluble 490 nm and indirectly reflect the quantity of viable cell through colorimetry quantification. The osteoblast is inoculated into 96-pore plate according to the density of 1×10^4/ pore. After 24h cultivation, resorb off the culture solution, add 0, 25, 50, 100 and 200 μg/mlTiO_2-NPs. There are 6 parallel samples for each concentration, after cultivation for 24hs and incubation with MTS reagent for 4hs under 37 ℃, the microplate reader 490 nm is employed to detect the optical absorption value. The survival rate per concentration is calculated by 490 nm absorption value (OD value) and expressed by the percentage of control group. Specific calculation formula: reproducibility of cell= (OD value of experiment group/ OD value of control group) *100%.

LDH release method

LDH is positioned into cell cytoplasm. Generally, LDH can't penetrate the cytomembrane. When the cytomembrane is damaged or dies, it may be released out of cell, right now, LDH activity in the cell culture fluid is proportional to cell mortality. Therefore, through detecting LDH activity in the supernatant of cell culture solution, the damage degree of cell may be judged. The osteoblast is prepared as the single cell suspension and inoculated onto 6-pore culture plate to culture for 24hs and absorb off the supernatant, add 25, 50, 100 and 200 μg/ml 2 ml cell culture solution of TiO_2-NPs. The negative control group is osteoblast free of particle exposure. After 24h culture, collect the cell culture fluid and collect the supernatant for 5 mins after 1500 r/min centrifuging, test LDH content according to the specific operation steps in reagent kit manual, adopt the spectrophotometer to conduct the color comparison at the location of 440 nm wavelength and calculate LDH content. Specific calculation formula: LDH leakage rate (colorimetric value of experiment group/ colorimetric value of control group) *100%.

Variation of cell ROS detected by FCM

Take the osteoblast, digest by 0.25% trypsin, count by cell and inoculate onto 6-pore plate according to 5.0×10^5 cell per pore. After 24h culture, resorb off the culture fluid, add 25, 50, 100 and 200 μg/ml cell culture solution containing 25, 50, 100 and 200 μg/ml TiO_2-NPs, the negative control group is the osteoblast free of particle exposure. After culturing for 6hs, resorb off the culture fluid and add 2 ml DCFH-DA application fluid diluted into cell culture fluid (adopt the cell culture fluid to dilute DCFH-DA according to proportion, with 10 μmol/L for final concentration), dyeing for 30mins by keeping out of the sun under 37 ℃. The trypsin digests and absorbs the cell into radial-flow tube, centrifuge for 5mins by 1500 r/min, resuspend into PBS fluid, immediately test it by employing the flow cytometry and calculate the mean fluorescence intensity. Specific calculation formula: ROS fluorescence intensity= (experiment intensity of experiment group/ experiment intensity of control group) *100%.

Data analysis

The data is expressed by mean ± standard difference. The statistical analysis is completed by SPSS12.0 software, and the statistical comparison is completed by variance analysis of single factor. When $p<0.05$, it is deemed that there is statistical significance.

RESULTS

Analysis on form and grain size characterization of TiO_2-NPs and Zeta potential

TEM results may refer to Diagram 1, TiO_2-NPs form presents sphere, with uniform distribution for grain size. The mean grain size TiO_2-NPs is about 20 nm, with good dispersibility, the obvious gathering isn't generated (Figure 1). In DMEM cell culture fluid containing 10%FBS, TiO_2–NPs agglomeration is more obvious, the grain size reaches 363nm. TiO_2–NPs is attached with negative electricity (TABLE 1) in DMEM cell culture fluid containing 10%FBS.

Appraisal for osteoblast

Culture 14d primary osteoblast; After dyeing by ALP reagent, it is discovered through observation under microscope that the osteoblast that is dyed as the purple within a view reaches over 95% to show that the purity of obtained primary osteoblast is higher (Figure 2).

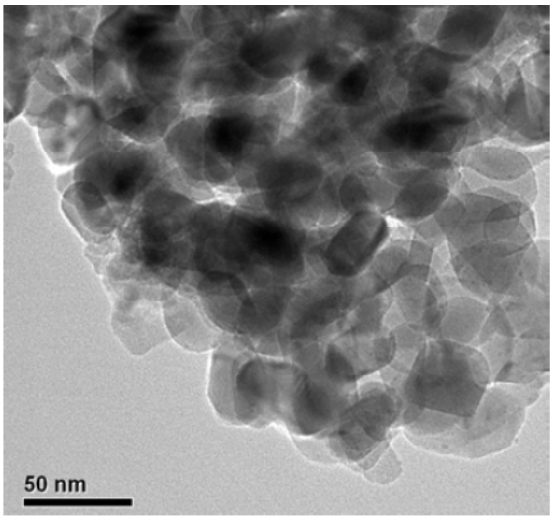

Figure 1: TEM photo of TiO_2-NPs

TABLE 1: Grain Size Distribution of TiO_2 –NPs in the normal saline and in the DMEM culture fluid containing 10%FBS and zeta potential

Particle	DLS Mean Grain Size (nm)	Zeta Potential (mv)
TiO_2 -NPs	DMEM containing 10% FBS 363	-10.9

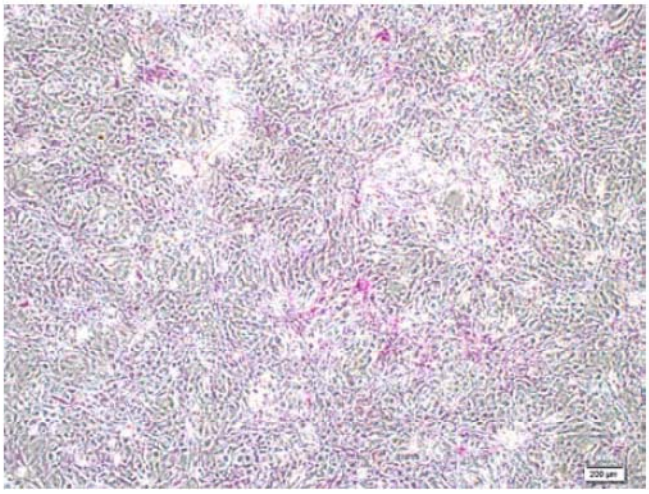

Figure 2: ALP dyeing result of primary osteoblast for 14d culture (×400)

Influence on osteoblast proliferation by TiO_2-NPs

As shown in Figure 3, within 24hs, with increase of functional dosage of TiO_2-NPs, the survival rate of osteoblast declines gradually, the obvious cytotoxicity appears while 50, 100 and 200 μg/ml, showing that the comparison difference between cell reproducibility and control group is provided with significance (p<0.05).

Osteoblast toxicity of TiO_2-NPs—LDH release method

After 24h function, it is discovered by the result of LDH leaking experiment, except for 25 μg/ml group, TiO_2-NPs for other three concentrations may cause the obvious increase of LDH release quantity for osteoblast (Figure 4, p<0.05).

Influence on oxidation stress of osteoblast by TiO_2-NPs

As shown in Figure 5, after the osteoblast is exposed to particle for 24hs, with increase of TiO_2-NPs exposure dosage, the cell shows obvious oxidation stress reaction and increases obviously through comparison between ROS and control（50, 100 and 200 μg/ml, p<0.05）

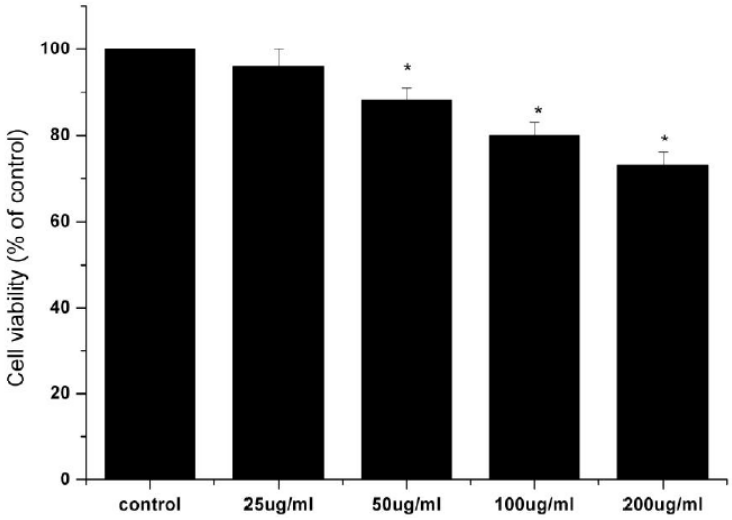

Figure 3: Inhibition function on osteoblast by TiO_2-NPs with different concentrations via detection by MTS Method (n=6, * comparison with control group $p<0.05$)

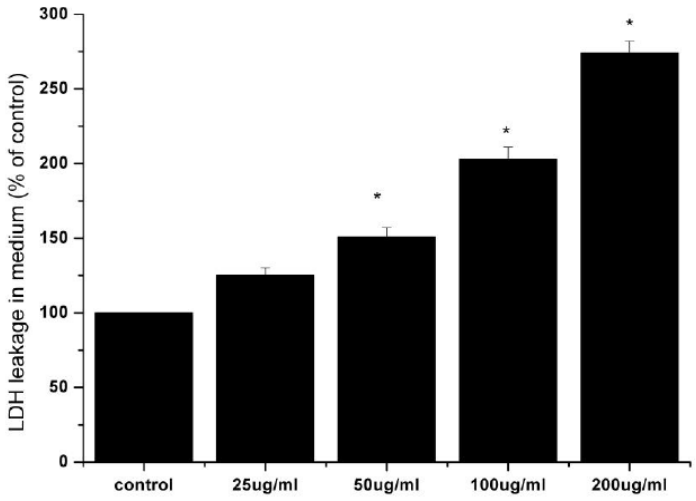

Figure 4: Influence on LDH enzymatic activity of osteoblast by TiO_2-NPs with different concentrations (n=6, * comparison with control group $p<0.05$)

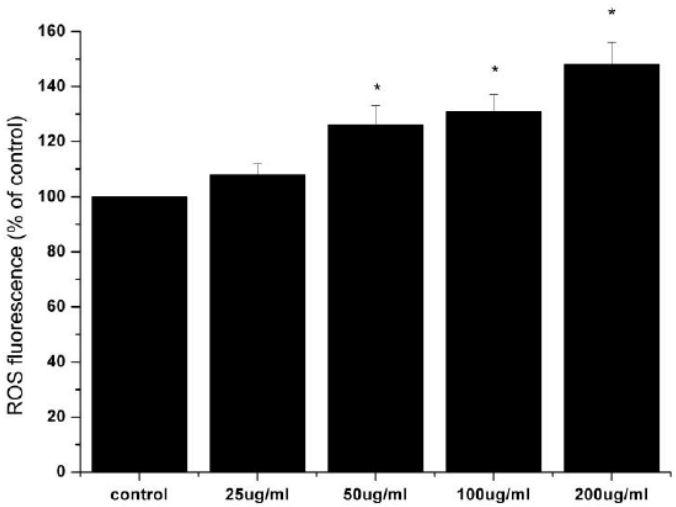

Figure 5: Oxidation damage for osteoblast by TiO_2-NPs (n=6, comparison with control group $p<0.05$)

DISCUSSIONS

With the development of nanotechnology, TiO_2-NPs is widely used in the bone implantation materials, orthotic materials and tooth implant materials, however, the toxic effect from TiO_2-NPs to the osteoblast is not clear yet at present. This study researches the damage effect to the osteoblast induced by TiO_2-NPs and explains the effect of the TiO_2-NPs on damage of the osteoblast and the mechanism through building the co-culture model of the TiO_2-NPs and the osteoblast, from the angle of the cell and molecular biology.

The TiO_2-NPs is found to be nanoscaled in the state of powder through TEM detection, with homogeneous distribution of the grain size. There is degree of agglomeration in normal saline and cell culture fluid, with agglomeration size more than 100nm (TABLE 1). The nano-particles are provided with very high activity because of small size effect, surface effect and larger specific surface area, easy to be combined with other atom to tend towards stability, resulting in agglomeration of the nano-particles each other. The testing result for surface electric potential of the nano-particles shows that the TiO_2-NPs is provided with negative electricity in normal saline and cell culture fluid. Some research report suggests that the surface charge of the nano-particles may affect the interaction between the nano-particles and the living body. Because the surface of the cell membrane is provided with negative electricity, the nano-particles with positive charge on the surface electrostatic incorporation can exist between the nano-particles with positive charge on the surface and cell membrane, which will result in the adhesion efficiency and phagocytic ability greater than that of the particles[9-10] with negative charge.

After the particles are in nanoscale, the surface effect is very prominent because the proportion of the number of the atomic on the surface is increasing in the system, but the surface of the nano-particles is the place where militating with the cell membrane and subcellular structures, is the place where the biological and toxicological reaction are in[11]. The testing result of TiO_2-NPs and MTS in this study shows that: the TiO_2-NPs with different concentration is acting on the osteoblast for 24 hours, the survival rate of the cell is decreasing with the increasing of the action concentration of the nano-particles (Figure 3). It shows that the TiO_2-NPs has inhibiting effect to the activity of the osteoblast, and has obvious dependency relationship with the action concentration. LDH is a key enzyme participating in the glycolysis in the cytoplasm, and its chemical and biological characteristics are very stable, with few leakage of LDH of the cell under normal circumstances. When the integrality of the cell membrane is damaged, and permeability is increasing, the LDH may leak from the cytoplasm into the culture fluid. The degree of damage of the cell can be assessed by measuring the leakage quantity of the LDH in the culture fluid. The measuring result of the leakage quantity of the LDH verifies the inhibiting effect of TiO_2-NPs to the activity of the osteoblast, after being exposed for 24 hours, the leakage quantity of the LDH in the cell culture fluid is rising with the increasing of the exposure dose (Figure 4), suggesting the TiO_2-NPs can cause damage to dependency of osteoblast membrane to the dosage. The tendency of the testing result of MTS in this study is consistent with the tendency of testing result of Lin equivalent[12] and Hussain equivalent[13] cytotoxicity, but the specific survival rate value of the cell is different, which may be related to the size of the nano-particles and the acted cells types.

Nel equivalent[14] has published an article in the magazine to put forward that generation and oxidative stress reaction of ROS is main way that the nano-particles cause the toxic effect for various creatures. In the view of the characteristics of the nano-particles themselves, the smaller of the particle size, the bigger of the superficial area is, the more of the points for electron acceptor and donor activity on the surface of the particles are, easier to form superoxide anion, and generate excess ROS by disproportionate reaction. The test research shows that TiO_2-NP can cause the oxidative stress reaction of the osteoblast, which is expressed specifically as after acting on the osteoblast for 6 hours, the level of the ROS of the TiO_2-NPs in the cell is increasing gradually with the increasing of the action concentration (Figure 5). The result of the test shows that the TiO_2-NPs can induce the increasing of the ROS level of the osteoblast, break the dynamic equilibrium of ROS with the activity of the antioxidant enzyme in the cell.

In conclusion, TiO2-NPs can cause the decrease of the survival rate of the osteoblast and increase of the content of the LDH released by the cell, and with dosage dependency effect, suggesting that TiO2-NPs can inhibit the activity of the cell PC12 and cause damage to cell membrane at the same time, with a degree of cytotoxicity. Meanwhile, TiO2-NPs can induce oxidative stress reaction of the cell PC12, suggesting that active oxygen may be the mechanism of action for cytotoxicity of the nano-particles. This study is of important theoretical significance for understanding the toxicity of the osteoblast of the TiO2-NPs and the mechanism, and provides theoretical foundation for discussion of security application of TiO2-NPs in implant field.

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