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Comparative investigation of the biocompatibility of various silicon nitride ceramic qualities *in vitro*

A. NEUMANN*, T. RESKE, M. HELD, K. JAHNKE
Department of Otorhinolaryngology, University of Essen, Hufelandstr. 55, 45122 Essen, Germany
E-mail: andreas.neumann@uni-essen.de

C. RAGOß, H. R. MAIER
Institute for Ceramic Components in Engineering, Nizzaallee 32, 52072 Aachen, Germany

There is a controversy about the biocompatibility of silicon nitride ceramics contained in the literature, which appears to be related to a factor of the individual chemical composition of different qualities of silicon nitride ceramics and of the different surface properties. This study attempts to investigate the cytotoxicity of different qualities of industrial silicon nitride ceramics applying an L929-cell culture model in a direct contact assay combined with a cell viability assessment. Five different qualities of industrial standard silicon nitride ceramics were chosen for *in vitro* testing. The chemical composition was determined by EDS analysis. Different biomedically approved aluminium oxide qualities, a titanium alloy, glass and polyvinylchloride (PVC) served as control materials. L929 mice fibroblasts were incubated directly on the materials for 24 h, stained with bisbenzimide and propidium iodine for double fluorochromasia viability testing, and evaluated by inversion-fluorescence microscopy to control cell morphology, viability and cell counts compared to empty well values. Scanning electron microscopy was applied to additionally investigate cell morphology. There was no observation of cytotoxic effects on the silicon nitride ceramic samples; moreover cell morphology remained the same as on aluminium oxide and titanium. Viability testing revealed the presence of avital cells exclusively on PVC, which served as a negative control. Cell counts on all polished surfaces showed significantly higher numbers, whereas some rough surface samples showed significantly lower numbers. We conclude that silicon nitride ceramics show no cytotoxic effects and should be considered for biomedical application owing to its favourable physiochemical properties, especially its superior resistance to mechanical stress, which would be useful for compression loaded conditions. Polished surfaces would appear to promote advanced biocompatibility.

1. Introduction

Numerous investigations show the biocompatibility of various ceramics [1, 2]. Most applications concern orthopaedic indications, which are sterile implantation sites. Possible indications in the authors' field of otorhinolaryngology include ossicular chain reconstruction prostheses, disks for reconstruction of anterior or lateral skull base defects, obliteration of paranasal sinuses and middle ear cavities and applications in traumatology such as reconstruction of the floor of the orbit or osteosyntheses in other parts of the face and skull. In contrast to most other surgical and orthopædic indications, these postulated implantation sites in otorhinolaryngology, are referred to as ''semi-open'' and thereby potentially at risk of bacterial colonisation. Direct attachment between implant and the mucosa of the respiratory tract is a common feature of implants in this field. Therefore, ceramics as well as other biomaterials must withstand high demands on biocompatibility, including these adverse conditions [3]. Taking the example of the middle ear, it was shown that aluminium oxide (Al_2O_3) ceramic is coated by proteins immediately after implantation. This is believed to lead to an immunocoating, thus preventing foreign body reactions [4]. Al_2O_3 is subsequently sealed with mucosa within a few weeks [5].

Based on our good experiences with Al_2O_3, it was our aim to search for a ceramic which is biocompatible and has a high strength so that it may remain *in situ* even under conditions of mucosal attachment and possible infections, and could at the same time serve as a stable material for osteosynthesis. This may be of special advantage in treating fractures of mucosa-attached bone such as the paranasal sinuses. Silicon nitride (Si_3N_4) is a

*Author to whom all correspondence should be addressed.

ceramic known for its high performance characterised by fracture toughness, high wear resistance and low coefficient of friction. Therefore, it has already been considered as biomaterial, in particular for orthopædic and dental indications.

Controversy exists concerning the biocompatibility of Si_3N_4 among the limited number of authors dealing with this material [2, 6]. Depending on the industrial process of sintering, as well as the surface properties, Si_3N_4 ceramics may have different physicochemical features, which may influence the biocompatibility and experimental assessment of the material [6]. This study aims to reinvestigate the biocompatibility of several qualities of Si_3N_4 ceramics by cytotoxicity testing using the L929-cell culture model in a modified direct contact assay [7, 8] and also cell viability assessment by double fluorochromasia. Another interest of investigation is the influence on cell culture parameters of the chemical composition and surface properties of Si_3N_4.

2. Materials and methods

2.1. Testing materials

Five qualities of industrial standards of silicon nitride ceramics (SiN) were chosen for *in vitro* testing. These were cut into discs measuring 10 mm in diameter with a thickness of 1 mm.

SiN I: Ceram Tec GS 120, sintering process disclosed (CeramTec AG, Plochingen, Germany).

SiN II: N3208, gas pressure sintered (Cfi GmbH (now called HCStarck Ceramics, Selb, Germany) and Co. KG, Rödental, Germany).

SiN III: N5301, sintered reaction bonded (Cfi GmbH (now called HCStarck Ceramics, Selb, Germany) and Co. KG, Rödental, Germany).

SiN IV: N7202, atmospheric pressure sintered (Cfi GmbH (now called HCStarck Ceramics, Selb, Germany) and Co. KG, Rödental, Germany).

SiN V: SHM 400 Z sintering process disclosed (SHM GmbH, Aachen, Germany).

In accordance with EN/ISO 10993-12 (''Biological evaluation of medical devices: sample preparation and reference materials'', 1996) and recommendations of other authors [9–13], the following materials were chosen as reference:

1. Aluminium oxide ceramics (AO):

AO I: HPA (Condea Chemicals, Ceralox Division, USA) , processed according to ISO 6474.

AO II: Rubalit 710 (Ceram Tec AG, Plochingen, Germany).

AO III: Biolox forte (CeramTec AG, Plochlingen, Germany), processed according to ISO 6474.

2. Titanium alloy: TiAl6V4 (Zapp Werkstofftechnik, Düsseldorf, Germany).

3. Cover glass slides.

4. Polyvinylchloride (PVC) with softening agent (IKV, Aachen, Germany) served as negative verification.

The materials were analysed with electron-dispersive X-ray analysis for quantity measurements of their element dispersion (Table I).

Discs were kept unpolished, or polished using a diamond free lapping technique and the surface roughness was analysed by profilometry. The polished surface roughness measured $Ra < 0.03\ \mu m$, the unpolished surface 0.32–0.82 μm.

The preparation of the test articles for modified direct contact assay, comprised cleansing the discs using chromosulphuric acid for 14 days, which was followed by repeated rinsing with Aqua bidest and insertion into HCL–isopropanol 96% for 48 h. Cleaning and sterilisation was completed by insertion into ultrasonic 96% grade HCL–isopropanol bath, rinsing with ethanol and steam autoclaving at 134 °C for 20 min except PVC (121 °C). All test materials were handled with PVC forceps to avoid friction and/or scratching by metallic instruments.

2.2. Cells and culture conditions

The purified L929 Cell Line (aneuploid fibroblasts isolated from subcutaneous tissue of C3H/An Mice) was provided by the Institute of Cell Biology, University of Essen Medical School. The cells were maintained in sterile 50 ml tissue culture grade polstyrene culture flasks (Beckton Dickinson Labware, Franklin Lakes, NJ, USA) using Dulbecco's Modification of Eagle's Medium (DMEM, Sigma Aldrich Co. Ltd., Irvine KA12 8NB, UK) with the addition of 10% fetal calf serum (Biochrom KG, Berlin, Germany), antibiotics/antimycotics (penicillin 100 E/ml, streptomycin 100 μg/ml, amphotericin B 2.5 μg/ml, Gibco Chemicals, Paisley PA4 9RF, UK) and L-glutamin 290 mg/ml. During the 57-week testing period, the cell line was subcultivated 66 times. The culture flasks were kept at 37 °C in a humidified atmosphere containing 5% CO_2. Bacterial and viral contamination of the cell line was regularly excluded by microbiological assessment. The cell kinetics over a

TABLE I Element composition (At-%) of testing materials (EDS). TiAl6V4, titanium alloy; AO, aluminium oxide; SIN I–V, different industrial qualities of silicon nitride

TiAl6V4		Al_2O_3	AO I	AO II	AO III	Si_3N_4	SiN I	SiN II	SiN III	SiN IV	SiN V
Al	10.3	Na	0.1	0.2	0.1	N	55.6	50.8	53.8	48.9	55.1
Ti	87.2	Mg	0.2	0.4	0.2	Mg	0.1	0.0	0.1	0.5	0.1
V	2.5	Al	39.8	39.5	39.8	Al	1.7	2.4	1.1	2.4	1.7
		Si	0.0	0.1	0.0	Si	37.3	40.7	39.8	41.5	38.3
		Ca	0.0	0.0	0.0	Y	1.0	1.0	1.5	1.1	0.9
		O*	59.9	59.9	59.9	O*	4.3	5.1	3.8	5.6	4.0

*Stochiometric difference

period of 120 h verified that the cells were in their exponential growing phase during the first 24 h.

2.3. Cytotoxicity testing

Experiments were carried out on sterile Falcon Multiwells (Becton Dickinson Labware, Franklin Lakes, NJ, USA) 3.8 cm^2 diameter per well, maximal volume 7 ml. Each well was fitted with material discs and filled with 2 ml DMEM supplemented as described. Cells were seeded at a density of 6×10^5 cells/well. Each passage of investigation was carried out with two discs of each material. Altogether 10 passages were processed. After a period of 24 h incubation, the wells were rinsed with a phosphate buffered saline solution (HBSS, Gibco Chemicals, Paisley PA4 9RF, UK), the cells were stained with the DNA intercalating fluorescent dyes bisbenzimide (H33342) and the viability stain propidiume iodine (both: Sigma Aldrich Co. Ltd., Irvine KA12 8NB, UK) for 10 min followed by rinsing with phosphate buffered saline (PBS). All manipulations concerning the fluorescence staining and evaluation were carried out in darkness. For inversion-fluorescence microscopy (Axiotech Zeiss, Germany, wave length: 320 nm, filter: 310–400 nm, magnification: 200×) the discs were transferred into Petri dishes filled with PBS in order to allow the objective to immerse into the fluid. Three random sectors of each disc were examined for cell count per microscopic section, morphology and viability. The cell counts per section on each material were referred to the cell population in empty culture flasks.

Cell morphology testing was done by scanning electron microscopy (SEM). The incubated cells on the disks were rinsed twice with Hanks medium. The attached cells were fixed on the disks with 2.5% glutaraldehyde in 0.1 m sodium cacodylate buffer solution for 7 h at 4 °C, pH = 7.4. The discs were then washed with cacodylate buffer and postfixed with 1% osmium tetroxide for 1 h followed by dehydration through a graded series of acetone. The attached cells were then incubated in hexamethydisilazane and sputter coated with gold palladium. The cell bearing surface of each disk was examined using a Zeiss Leo electron microscope using a magnification of 100× and 2500× . Thirty random cells per material were investigated.

2.4. Statistical analysis

The cell counts of each test material were compared for statistical significance using the nonparametric Mann, Whitney, Wilcoxon U-test the SPSS 10.0 software. All tests were conducted at the $p < 0.05$ significance level.

3. Results

3.1. Cytotoxicity testing

The toxic response to each of the five silicon nitride ceramics qualities, assessed by viability testing showed no cytotoxic features. About 100% of the cells retrieved from the control polystyrene wells, aluminium oxide ceramics, glass, titanium alloy TiAl6V4 and silicon nitride discs were viable indicated by propidium iodine testing and green bisbenzimide fluorescence of the nuclei (Fig. 1). On fluorescence microscopy spindle-shaped cells with round-oval nuclei were seen on all samples. There was no observation of cell clusters with polymorph

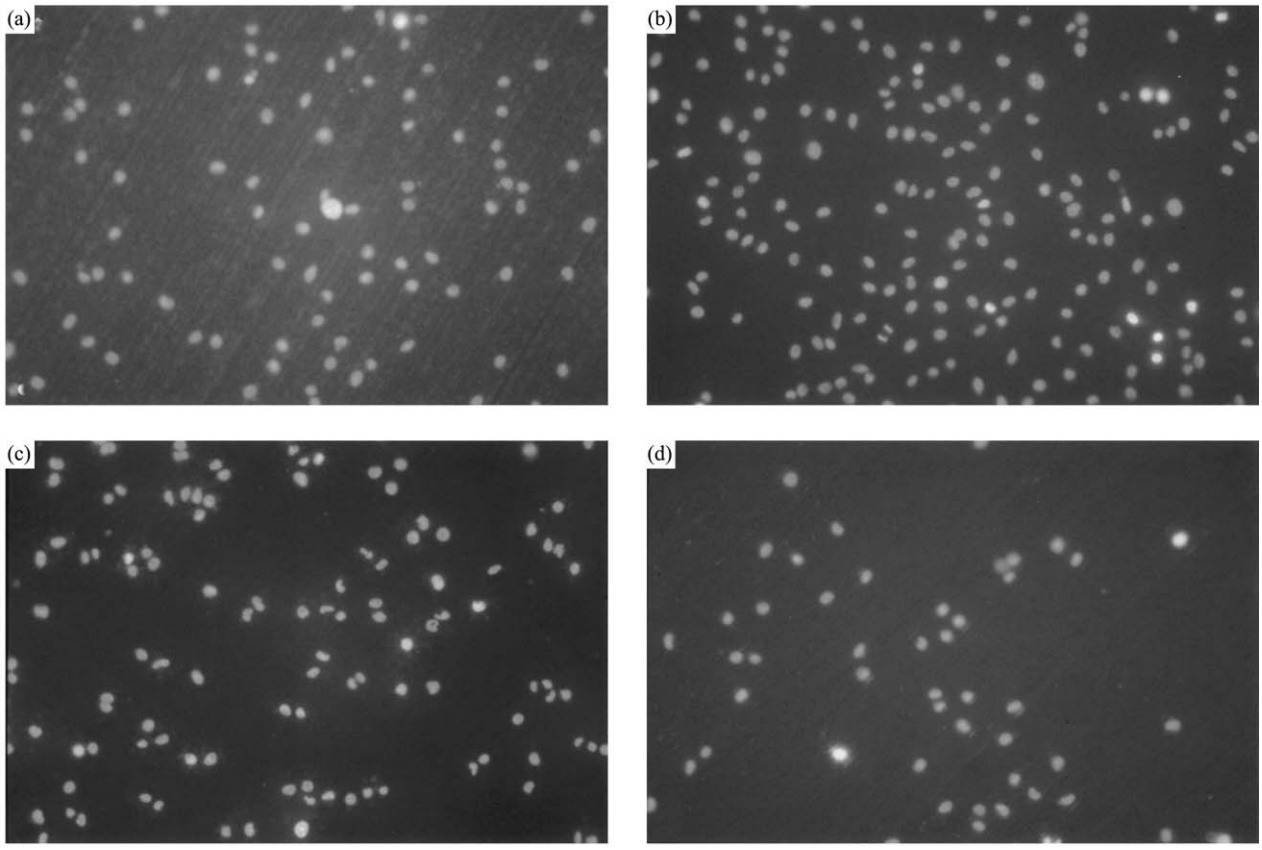
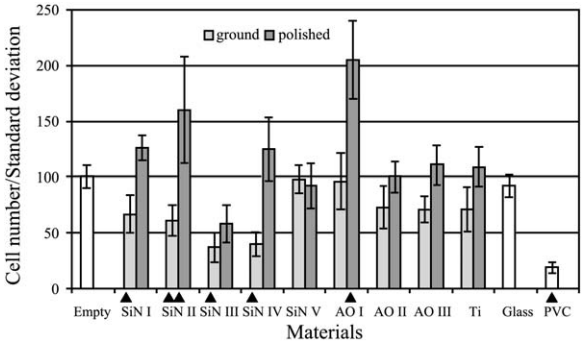


Figure 1 Fluorescence microscopy of viable, bisbenzimide-stained L929 cell nuclei on (a) ground, (b) polished silicon nitride (SiN II), (c) aluminium oxide (AO II), (d) titanium.

TABLE II Cell counts per microscopic section on testing materials, normalised to empty well value = 100. Total of 60 counts per material. SiN I–V: Different qualities of silicon nitride. AO I–III: Different qualities of aluminium oxide. Arrows indicate values significantly deviating from empty well value



fibroblasts, no multinuclear cells or nucleus–plasma relation shifts. The cells were distributed evenly on the discs. Staining with propidium iodine indicated dead cells only on the PVC negative control, in addition, cells on PVC showed multinuclear cells, cell clusters and cells with less cytoplasmic extensions.

3.2. Cell proliferation

The cell proliferation indices of the different samples are presented in Table II. Surface structures seem to have an impact on cell proliferation. The effect of surface polishing of the different materials shows significantly higher cell yields in comparison to cell growth on corresponding unpolished discs, with the exception of SiN V. Furthermore, cell yields of polished materials were higher than empty well values with the exception of SiN III and V, significance levels of this issue were gained only for SiN II and AO I. In contrast, all cell yields of ground surfaces were lower than the empty well value, significance gained for SiN I–IV. Considered together, the cell counts show a large variance. However, the low cell counts of both ground and polished SiN III are striking.

3.3. Cell morphology

There was no visible morphologic difference between cells retrieved from the polystyrene control, silicon nitride or positive controls by light microscopy. The SEM analysis showed cells tightly attached to the surface of silicon nitride discs. The same typical fibroblastic morphology with widespread cells showed up on the positive controls aluminium oxide and titanium alloy (Fig. 2). Filiform cell extensions are shown in higher magnification in Fig. 2(b), indicating biocompatible attachment to the material surface. There was no presence of membrane defects.

4. Discussion

Ceramics are increasingly evaluated for biomedical application. Al_2O_3 is the prototype of a bioinert ceramic and is frequently used in hip joint replacement. In otorhinolaryngology it is a material in favour for ossicular chain reconstruction prostheses [14]. Si_3N_4 has not been introduced for implantation in humans, although the physicochemical properties suggest that it is appropriate for medical application. Its superior resistance to wear, thermal shock and mechanical stress promoted industrial use for cutting tool inserts, bearings, highly stressed wear parts and applications for heat engines, among many others [15]. The mechanical stability exceeds that of Al_2O_3 which would be of use for applications in stress bearing implantation sites.

So far, only a few authors have investigated the biocompatibility features of Si_3N_4 or its composites [1, 2, 6, 16–18]. Howlett *et al*. [6] investigated the biocompatibility of Si_3N_4 ceramic *in vitro* by showing attachment and differentiation of marrow stromal cells. *In vivo*, porous intramedullary Si_3N_4 rods implanted in rabbit femurs supported bony ingrowth. Further *in vivo* studies showed successful osseous reconstruction using femoral segmental Si_3N_4 endoprostheses implanted in adult rabbits [6].

The biocompatibility features described by the above mentioned authors are in accordance with our results applying the L929 cell line model. L929 cell line is in frequent use for cytotoxicity testing [19, 20] and one of the recommended cell lines with optimum sensitivity and ease of culture for biocompatibility testing [20]. It is a reliable and reproducible test compared to *in vivo* implantation studies in rats [21]. The direct contact test method for cytotoxicity screening applied in this study is based on the techniques described by Rosenbluth *et al*. in 1965 [8] and was confirmed by other authors [20]. In accordance with ISO 10993 [22], the method was modified in such a way, that cells were incubated directly on the materials as was proposed by Johnsson and Hegyeli [23]. This method combined with inversion-fluorescence microscopy has the advantage of assessing the cells without further manipulation or dislocation. The cell membranes of viable cells exclude propidium iodine which specifically binds to DNA and RNA [24]. Dead cells therefore appear with red fluorescence emission. In contrast, bisbenzimide is a low cytotoxic, membrane permeable DNA staining agent suitable for viabilty testing [25, 26]. The nuclei of live cells show a green fluorescence signal.

Our investigations revealed no indications of cytotoxic effects on the test materials except PVC which served as a negative control. Cell morphology and viability on Si_3N_4 did not differ from the observations made on Al_2O_3 which is already established in human medical applications. The comparison of cell counts with the distribution of elements in the different silicon nitride samples shows no correlations. The possible influence of chemical composition on different biocompatibility performance of a material has been noted by Howlett *et al*. [6] in reply to Griss *et al*. [2], who found Si_3N_4 the least compatible material in rat femur implantation model compared to Al_2O_3, stainless steel and bioglass.

Cell counts showed higher numbers on all but one polished material and significantly lower numbers for most ground surface samples. This is in accordance with the findings of Kue *et al* [17], showing that polished surfaces of Si_3N_4 have the ability to propagate human

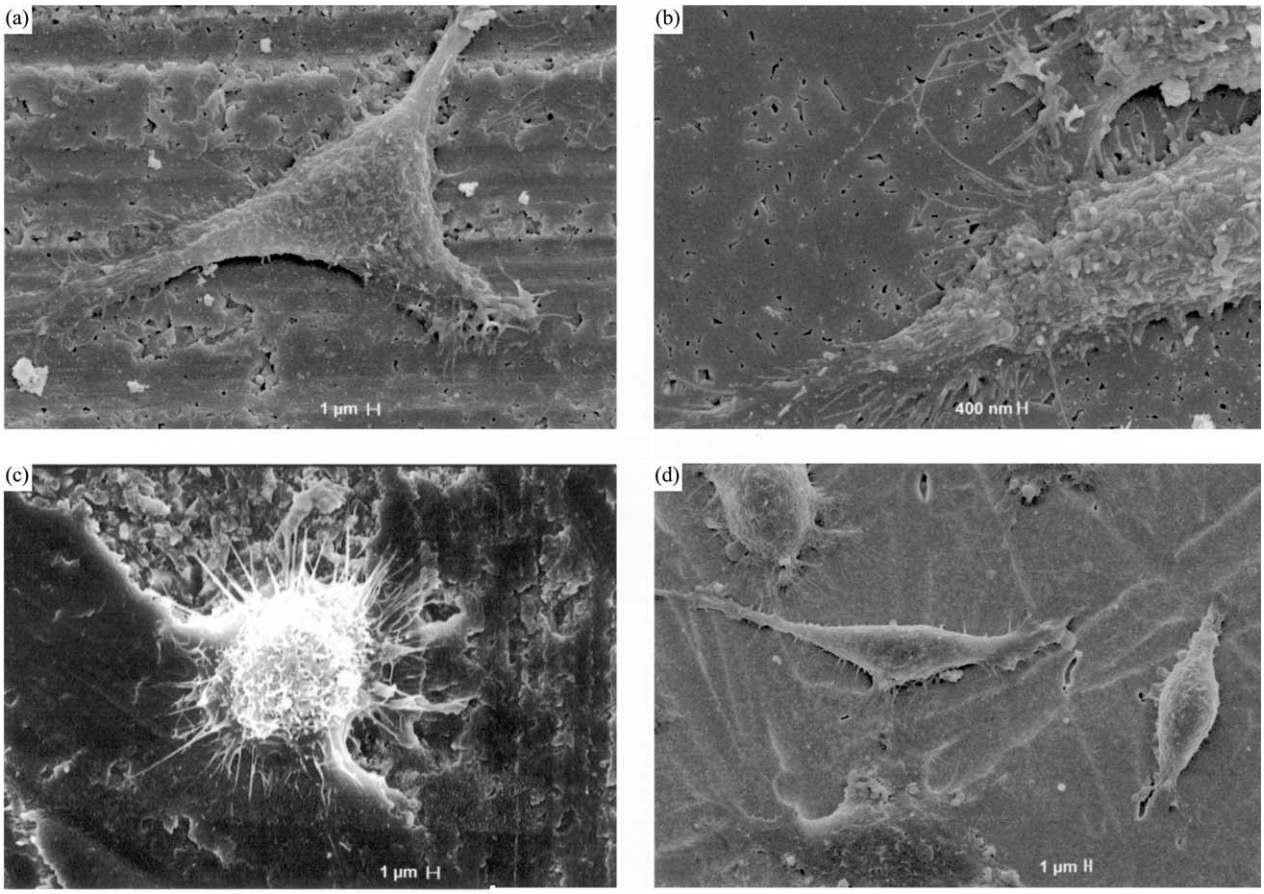


Figure 2 Scanning electron microscopic appearance of L929 cell on (a) ground, (b) polished silicon nitride (SiN II), (c) aluminium oxide (AO II), (d) titanium.

osteoblast cell growth *in vitro*. Cells proliferation capacity was similar to that shown on a polystyrene surface and a higher level of osteocalcin was produced [17]. Analogous *in vitro* studies also demonstrated the biocompatibility of this material in both block and particulate forms [17, 27]. However, the value of cell counts should not be overestimated, especially if one considers that there are no signs of cytotoxicity. If the processes of sintering of the Si_3N_4–ceramics substantially influence cell culture parameters, this has not been investigated systematically in this study.

Although cell culture models have shown good correlation to *in vivo* assays and even larger sensitivity than the animal implantation method within the limits of acute toxicity [7, 8, 28], they can only serve as a first step screening method of biocompatibility and cannot substitute animal experiments [29]. The latter are subject of present investigation.

5. Conclusions

We conclude that silicon nitride ceramic shows no cytotoxic effects and should be considered for biomedical application due to its favourable physicochemical properties especially its superior resistance to mechanical stress, which would be of use for compression loaded conditions. Polished surfaces seem to promote advanced biocompatibility.

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