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Original Article

In vitro cytotoxicity evaluation of dental porcelain using human cell lines

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Abstract The *in vitro* cytotoxic potential of locally produced dental porcelain was evaluated in this study. The cellular response of human osteoblast and fibroblast cell lines were assessed using MTT assay by incubating with the fluid extract of dental porcelain powder and dental porcelain discs (direct test). Aging process was carried out by submerging the discs into 3% bovine serum albumin (BSA) solution. Tests on extracts showed that dental porcelain was significantly different from the control at a concentration of 250 mg/ml. Direct test showed that dental porcelain after aging was not significantly different from the control with a mean (SD) of 89.2 (13.4)%, whereas, it was significantly different from the control before conditioning of BSA with a mean (SD) of 88.5 (12.1)%. However, the dental porcelain caused mild suppression of succinate dehydrogenase activity (<25%), which is considered to be accepted clinically and hence can be ranked as being non-cytotoxic.

Keywords: Dental Porcelain, cell line, cytotoxicity, MTT assay

Introduction

Dental porcelain is increasingly an important material for the restoration of teeth. The demands of patients for tooth colored restorations and the availability of new types of this material have increased its uses in a variety of clinical restorative situations (Denry, 1996). The relative incidence of biological side effects of porcelain as compared with other restorative materials is considered low. In general, conventional dental porcelain is considered to be the most inert dental restorative material (Sjögren *et al*., 2000). However, since inert porcelain restorations are in close contact with oral tissues for extended periods under a load-bearing biological environment, the biological effects of this material should be taken into consideration (Li *et al*., 1993). The issue of biocompatibility has profound social, ethical, technical and legal effects on prosthodontic practice (Wataha, 2001). Development of an intraoral material for prosthodontic applications that meets the requirements of biocompatibility would indeed be extremely valuable in clinical practice. Today, with the increased applications of dental porcelain, efforts have continued to develop new biologically compatible materials, which in turn, help to expand the use of these materials in a variety of clinical situations. One common method of testing the biocompatibility of biomaterials is *in vitro* cytotoxicity tests (Wataha and Lockwood, 1998). This system is based on cell culture studies, which are useful tools for the investigations of dental materials. Cell culture techniques have been widely used in the initial evaluation of biocompatibility of biomaterials (Hyakuna *et al*., 1989) for

which established cell lines are preferred (ISO, 2009). Though primary cells as well as many mammalian cells are used for this purpose, rodent or human cells are found to be the most useful. Of the human cells used for basal cytotoxicity, NHK or fibroblasts are probably the cells most frequently used with good results in validation studies (Sina *et al*., 1995; Harbell *et al*., 1997). Hence, human cell lines were chosen to conduct this study. This study aimed to detect the potential ability of locally produced dental porcelain in inducing toxic effects on human cell lines and to detect the cellular responses to changes in dental porcelain tested after exposure to cells. It was hypothesized that the porcelain material tested is a satisfactory biocompatible material in terms of toxicity potential at the cellular level.

Materials and methods

Biomaterial

Locally produced dental porcelain, which was developed by a research group at the School of Materials and Minerals Resources Engineering, Universiti Sains Malaysia (USM), Malaysia, was used in this study. The composition of this dental porcelain is presented in Table 1. It was obtained in powder and solid "disc" forms. The porcelain discs were 5 mm in diameter and 2 mm thick. Before the experiments, the porcelain discs and powder were sterilized by autoclaving at 121°C (186 kPa pressure) for 20 minutes.

Cell lines and culture

Human osteoblast (HOS) and fibroblast (MRC-5) cell lines were obtained from American Type Culture Collection (ATCC, USA) and used for cytotoxicity testing. Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM), which was supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (All from GIBCO, Grand Island, USA). Cells were cultured in T-75 tissue culture flasks (NUNC, Denmark) and the incubations were carried out at 37°C and 95% relative humidity in an air atmosphere containing 5% CO_2.

Table 1 Composition of locally produced dental porcelain

Composition	Weight Percentage
Na-feldspar	27.91
K-feldspar	51.46
Kaolin	0.70
SiO_2	11.0
Al_2O_3	1.06
Na_2CO_3	1.38
K_2CO_3	2.95
$MgCO_3$	0.28
$CaCO_3$	1.29
$SrCO_3$	0.08
$BaCO_3$	0.28
TiO_2	0.115
Li_2CO_3	1.50

Cytotoxicity test on extracts

Cytotoxicity of the porcelain material was evaluated by testing on extracts of porcelain according to ISO 10993-5: 2009 (ISO, 2009). Human osteoblast (HOS) cell line was used in this test. Cells at the 6-8th passages were employed. Cells were incubated for 72 hours at a density of 1x10^4 cells / well in 200 µl fluid extract using 96-well cell culture plate (NUNC, Denmark). Untreated cell culture medium was used as a control. The extract of dental porcelain powders was prepared by placing the dental porcelain powders (50, 100, 150, 200 and 250 mg) after being sterilized in universal bottle vessels with 1 ml of culture medium, which was added into each vessel; the vessels were then sealed and placed at 37°C in the CO_2 incubator for 24 hours. Following incubation, vessels containing extracts of dental porcelain were vigorously shaken and then filtered using 0.22 µm filter (Schieicher and Schuell, Germany). The resulting undiluted extracting solutions were decanted into sterile glass containers and used directly after its preparation for cytotoxicity. The test was carried out in 8 replicates for each concentration and repeated two times to ensure that variables such as cell population and variability in solutions did not introduce any systematic error. Minimum sample size required by ISO 10993-5:2009 (ISO, 2009) is 3 replicates.

Cytotoxicity direct contact test

Cytotoxicity of porcelain tested was also evaluated by direct contact method according to ISO 10993-5:2009 (ISO, 2009). Human fibroblast (MRC-5) cell line was used in this test. Passage numbers were 4-5. Cells were plated at a density of $3x10^4$ cells/well using 24-well cell culture plate (NUNC, Denmark). The cells were incubated for 24 hours to enable attachment to the wells. All porcelain discs (n=12; 5 mm in diameter and 2 mm thick; surface area of 70.6 mm^2) were placed on top of a pre-cultured cell layer and the time of contact with cells was 72 hours. The test was carried out in 6 replicates and repeated two times.

Cytotoxicity direct contact of aging porcelain disc

The sterilized discs of porcelain were submerged into 3% Bovine Serum Albumin solution (BSA) (MACS, Miltenyi Biotec) for 96 hours. The discs were then rinsed once by dipping into sterile water and later tested for cytotoxicity as described under the cytotoxicity direct contact test.

Cellular response assessment: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cellular response was assessed using MTT assay for measuring the mitochondrial succinate dehydrogenase (SDH) activity. MTT assay is based on the ability of mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals, which is largely impermeable to cell membrane, thus resulting in its accumulation within viable cells. The number of viable cells is directly proportional to the level of the formazan product created (Freimoser *et al*., 1999). MTT (Calbiochem, Germany) solution was prepared by dissolving 5mg of MTT powder in 1ml Phosphate Buffered Saline (PBS) and then filtering using 0.22 µm syringe filter. Then, 20 µl of the resulting MTT solution was added into each of the 96-well cell culture plate followed by incubation for 4 hours at 37°C in the CO_2 incubator. The media was removed after the incubation and 200 µl Dimethyl Sulfoxide (DMSO) (Sigma, France) was added to dissolve the blue formazan crystals. The plate was shaken and put in the CO_2 incubator for 5 minutes and then transferred to ELISA (Enzyme Linked Immunosorbent Assay) reader (Tecan, DKSH). Optical densities of the resulting solutions were measured at 570 nm wavelength using absorbance measurement mode (ELISA plate reader, Tecan, Switzerland). The optical density for each well was converted to percentage based on the control values.

Statistical analysis

The SPSS (Statistical Package of Social Science) version 12.0.1 was used for data entry and statistical analysis. The differences of cellular viability means of control with the porcelain tested were compared using one-way ANOVA and Tukey Post-Hoc comparisons at a significance level of $p<0.05$.

Results

The potential ability of the extracts of the porcelain material in inducing toxic effects on HOS cell line at various concentrations was tested, the results of which are shown in Table 2. The results obtained varied according to the concentrations of the dental porcelain and was significantly different from the control at a concentration higher than 150 mg/ml. The potential ability of the discs of the porcelain material in inducing toxic effects on MRC-5 cell line was also tested, the results of which are shown in Tables 3 and 4. The result of the dental porcelain was significantly different from the control ($p=0.032$), but had no effect in cell culture as the suppression of SDH activity was mild (88.5% compared to 100%). The results obtained after the aging process showed that the porcelain material tested showed cellular viability of 89.2% compared to 100% of the control ($p=0.05$).

Table 2 Response of human osteoblast (HOS) cell line to the extracts of dental porcelain tested at various concentrations

Concentration	Percentage of cellular viability Mean(SD)		*F* Statistic[a] (*df*)	*p* value[a]
	Control	Test material		
50 mg/ml	100(16.8)	102.2(12.8)	4.84 (5,90)	0.998
100 mg/ml	100(16.8)	98.9(10.3)	4.84 (5,90)	1.000
150 mg/ml	100(16.8)	89.4(15.8)	4.84 (5,90)	0.325
200 mg/ml	100(16.8)	86.7(14.6)	4.84 (5,90)	0.113
250 mg/ml	100(16.8)	82.9(16.5)	4.89 (5,90)	0.016

n = 16
[a] One-way ANOVA

Table 3 Response of human fibroblast (MRC-5) cell line to the discs of dental porcelain tested at various concentrations

Percentage of cellular viability Mean(SD)		*F* Statistic[a] (*df*)	*p* value[a]
Control	Test material		
100(12.5)	88.5(12.1)	5.28 (1,22)	0.032

n = 12
[a] One-way ANOVA

Table 4 Response of human fibroblast (MRC-5) cell line to the discs of porcelain tested test after the exposure to BSA solution

Percentage of cellular viability Mean(SD)		*F* Statistic[a] (*df*)	*p* value[a]
Control	Test material		
100(12.3)	89.2(13.4)	4.29 (1,22)	0.050

n = 12
[a] One-way ANOVA

Discussion

In this study, human osteoblast and fibroblast cell lines were used to assess the cytotoxicity of the locally produced dental porcelain. The uses of these cell lines for cytotoxicity evaluation of biomaterials have been previously reported (Bagambisa *et al*., 1994; Messer *et al*., 2003). A long-term advantage of using human cells is that the human cell cytotoxicity data derived from this approach can be added to human toxicity databases to facilitate the development of methods that may later predict acute human lethality. In general, human NHK or fibroblasts are probably the cells most frequently used for basal cytotoxicity due to its good results in validation studies (Sina *et al*., 1995; Harbell *et al*., 1997). The testing methods used address different aspects of cytotoxicity and the results taken together give an overview of the cytotoxic potential of the material tested. Cytotoxicity assays are based on the use of various parameters quantifying cell death or measuring the effect of the product on cell metabolism. Among the

most common methods used to determine cytotoxicity is the MTT test (Verhulst *et al*., 1998). The test was proved to be more accurate and time saving than other conventional haemocytometer counting methods (Freimoser *et al*., 1999). The well with the highest absorbance indicates the highest viability in the case of MTT assay.

Cytotoxicity of extracts of dental ceramics

Generally, breakdown products of dental ceramics have not been reported to have known toxic effects and several ions like sodium, potassium, silicon and aluminium in dental ceramics are considered non-toxic (Schuster *et al*., 1996). Bagambisa *et al*. (1994) reported that dental ceramics impaired the overgrowth of an osteoblast cell line and suggested that the toxicity was related to surface structure rather than to degradation products. The basis of the undesirable effects of nanoparticles may stem from their small size (surface area to volume ratio and size distribution), chemical composition (purity, crystallinity, electronic properties, etc.), surface charge, surface structure (surface reactivity, surface groups, inorganic, or organic coatings), solubility, shape, and aggregation behaviour (Nel *et al*., 2006). Although 250 mg/ml of test material produced significant difference in cellular viability compared to the control groups ($p<0.05$), the suppression of SDH activity was lower than 25%, which is accepted to be biocompatible (Wataha, 2001).

Cytotoxicity of unconditioned ceramic discs

In the present study, one sample disc per well resulted roughly in 4/10 coverage, ISO requires about 1/10 of the cell layer shall be covered. However, if a colorimetric assay like MTT is used for evaluation of viability on a “per well basis”, the sensitivity is markedly decreased since a certain percentage of cells (60-90%) are only in direct contact with tissue culture polystyrene, and not with the sample discs. Results obtained from several previous studies confirm our observations (as shown in Table 2) of the low toxic potential of dental ceramics (Li *et al*., 1993; Sjögren *et al*., 2000; Messer *et al*., 2003; Uo *et al*., 2003). According to Tables 3 and 4, cytotoxicity of dental porcelain was tested by direct contact test with MRC-5 cells. In both the results, the cellular viability was lower compared to control, indicative of cytotoxic effect on the cells. In Table 2, the cell viability of tested group is significantly different compared to control ($p<0.05$). The toxicity of material is mainly caused by either released ions and compounds or worn debris from metals, ceramics or polymers (Bearden and Cooke, 1980). Slow releases of ions from zirconia ceramic plate, yttrium in both (saline and lactic acid) solutions and zirconium and yttrium in lactic acid were detected. High calcium release from tri-calcium phosphate (TCP) powders due to its high solubility may change the ion homeostasis in culture medium and inhibit cell viability. Owing to the larger specific surface area of the powder compared to ceramic plate, a higher yttrium release from ZP into the culture medium can be expected, which may be relatively toxic to the cells (Li *et al*., 1993). However, identifying the exact components in the dental porcelain that can cause the cytotoxic effect is difficult, as also reported by Wataha *et al*. (1995) who have debated on the difficulties in correlating cytotoxicity with elements, released from dental materials. This forms the limitation of the present study. It is known that tissues surrounding the site of an implanted prosthetic alloy are exposed to increased concentrations of the metals comprising the alloy. However, the exact identity and concentration of such metallic products are usually unknown, thus limiting the possibilities for quantifying any observed toxicological response to the metals (Bearden and Cooke, 1980).

Cytotoxicity of aged porcelain disc under direct contact with human fibroblasts

To our knowledge, there is no data on the long-term cytotoxicity effect of dental ceramics. Short-term *in vitro* testing results are influenced by initial element release that does not continue to occur at the same cytotoxicity level over a long

period (Wataha and Lockwood, 1998). Previous studies concluded that short-term *in vitro* testing, which is usually less than a week, may not be appropriate to predict long-term elemental release or cytotoxicity and that the idea of an accelerating test is feasible (Nelson *et al*., 1999b). In our study, aging of the discs of the material tested was carried out using a procedure previously published (Nelson *et al*., 1999a; Nelson *et al*., 1999b; Messer *et al*., 2003). The aging procedure mimicked most accurately long-term *in vitro* cytotoxicity tests. BSA was proved to be the most effective accelerating solution in previous studies (Nelson *et al*., 1999a). Short-term (72-168 hours) *in vitro* testing of dental casting alloys for cytotoxicity may not reflect *in vivo* biocompatibility. An accelerated test for evaluation of dental casting alloy cytotoxicity could help screen newly developed alloys more rapidly and accurately. Dental casting alloys were conditioned in either saline, cell-culture medium, or a saline/BSA solution for 168 hours before standard *in vitro* cytotoxicity testing. Overall, the saline/BSA solution was the most effective at changing alloy cytotoxicity from the unconditioned (0 month) toward the 10-month values (Nelson *et al*., 1999a). The result obtained after the aging process showed that the porcelain material tested was not significantly different from the control. This led us to conclude that the aging procedure has removed the most labile elements that are released most easily, which are only relevant to the short-term biological behavior of the materials. Based on Table 4, there was no significant difference in cellular viability of aging dental porcelain compared to control (p=0.05) as opposed to same material which did not undergo aging process with control (p<0.05). This finding is compatible with other findings obtained from previous studies using the preconditioning of other types of dental materials, such as dental alloys (Nelson *et al*., 1999a; Nelson *et al*., 1999b). Cytotoxicity was rated based on cell viability relative to controls as not cytotoxic, slight, moderate or severe where activity relative to controls was less than 30%, between 30 and 60%, between 60 and 90% or greater than 90% respectively (Lönnroth and Dahl, 2001; Lönnroth and Dahl, 2003). In conclusion, the locally produced dental porcelain is ranked non-cytotoxic in terms of *in vitro* cellular response to human osteoblast (HOS) and fibroblast (MRC-5) cell lines under the prevailing test conditions.

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