Indirect cytotoxicity evaluation of pseudowollastonite

D. DUFRANE*, C. DELLOYE
Orthopaedic Research Laboratory, Université Catholique de Louvain, Avenue Mounier, 53 B 1200 Brussels, Belgium

I. J. MCKAY
Department of Adult Oral Health, St Bartholomew's and the Royal London School of Medicine and Dentistry, Queen Mary and Westfield College, University of London, Mile End Road, London E1 4NS, UK

P. N. DE AZA
Departamento de Ciencia y Tecnología de Materiales, Universidad Miguel Hernandez, Edificio Torrevaillo Avda, Ferrocarril s/n 03202 Elche (Alicante), Spain

S. DE AZA
Instituto de Cerámica y Vidrio ICV-CSIC. Crta de Valencia Km, 24.3.28500 Arganda del Rey, Madrid, Spain

Y. J. SCHNEIDER
Cell Biochemical Laboratory, Université Catholique de Louvain, B 1348 Louvain-La-Neuve, Belgium

M. ANSEAU
Laboratoire de RMN, Université de Mons-Hainaut, Mons, Belgium
E-mail: denis.dufrane@clin.ucl.ac.be

This study aimed to evaluate the cytotoxicity of substances leached by pseudowollastonite ($CaSiO_3$). It has been previously shown that calcium (Ca^{2+}) and silicate (SiO_3^{-}) ions are released from pseudowollastonite into biological solutions. Both of these ions are known to influence the biological metabolism of osteoblastic cells essential in the mineralization process and bone-bonding mechanism.

The indirect toxicity evaluation was performed by extraction method, according to International Standard Organization (ISO). Pseudowollastonite pellets obtained by solid-state reaction were incubated, in culture medium, during 24, 48, 72 or 168 h at different concentrations (5, 10, 15, 50, 100, 200 mg/ml). The cytotoxicity of each extract in presence of human osteoblastic cell line (SaOS-2) was quantitatively assessed by measuring the viability (succinate dehydrogenase activity, MTT), the membrane integrity (the uptake of the neutral red by viable cells, NR) as well as the cell necrosis by measuring the lactate dehydrogenase (LDH) released in the culture medium. No significant alteration of membrane integrity or cell suffering was detectable. However, increased cell metabolism was observed for cells exposed to pseudowollastonite extract with longest extraction time (168 h). In conclusion, mineral elements leached by pseudowollastonite do not significantly affect the metabolism of osteoblastic cells.

Introduction

The essential requirement for an artificial material to bond to living bone is believed to be the formation of a biologically active apatite-like layer on their surfaces [1, 2]. A limited number of ceramic materials called bioactive materials such as Bioglass [3], sintered hydroxyapatite [4], glass–ceramic A-W [5] and more recently chain silicate minerals as pseudowollastonite (psW) ($CaSiO_3$) [6–12] and diopside ($CaMgSi_2O_6$) [13] are generally known to possess the ability to form bone like apatite in the body environment.

Previous studies have demonstrated the formation of a hydroxyapatite-like layer on the surface of psW ceramic both *in vivo* and *in vitro*. Experiments *in vitro* involved

*Author to whom all correspondence should be addressed.

suspension of the material in simulated body fluid or in human parotid saliva [6–10]. *In vivo* experiments consisted of implanting small cylinders of pseudowollastonite into rat tibias [11, 12]. The new bone was growing in direct contact with the psW implants after only 3 weeks. It is thought that the tissue binding of psW seen *in vivo* relies upon the formation of a surface apatite-like layer. The formation of this apatite layer is known to occur rapidly *in vitro*, however the initial interaction between the implanted material with the adjacent osteoblastic cells *in vivo* is more difficult to assess. It is not known exactly how the formation of the apatite-like layer affects the surrounding cells, particularly since this process is known to involve the release of significant amounts of calcium (Ca^{2+}) or silicate (SiO_3^-) ions from the material [3, 4]. Understanding the effects of psW dissolution products have at the cellular level is crucial to optimising the use of psW *in vivo*.

One way to investigate the early interaction between psW and osteoblastic cells is to study the *in vitro* cytotoxicity. Cytotoxicity testing is the initial phase in testing biocompatibility of biomaterials [14]. Using an *in vitro* system, it is possible to assess if the initial dissolution of the material surface might be associated with undesirable cellular effects. Quantitative methods for *in vitro* cytotoxicity have been described and recommended in the literature, although the presently available methods cannot be easily automated [15, 16]. A range of assays based on different aspects of cellular activity can be applied for the assessment of biocompatibility. In this study, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red (NR) and lactate dehydrogenase (LDH) assays for quantitative evaluation which have been adapted for pseudowollastonite are presented [15, 17–20].

The first group of assays measures the ability of viable cells to reduce a water-soluble yellow dye, MTT, to a water-insoluble purple formazan product [17]. These are converted by intracellular dehydrogenase to colored formazans [21, 22]. The second group monitoring the cell membrane integrity, is based on the spectrophotometric determination of NR (3-amino-2-methyl-phenazine hydrochloride) taken up by viable cells and stored in their lysosomes [23]. The third group determines spectrophotometrically the level of lactate dehydrogenase released by cells in necrosis [20].

The aim of this study was to demonstrate, by standardized test methods assays, the influence of pseudowollastonite release on cellular toxicity, although no attempt has been made here to analyze the psW soluble products.

Materials and methods

Pseudowollastonite samples preparation

The starting material was polycrystalline pseudowollastonite ceramic (α-$CaO \cdot SiO_2$) powder of 2–3 μm average particle size, synthesized at 1500 °C for 4 h from a mixture of calcium carbonate (99.5 wt %) and high purity washed Belgian sand (99.9 wt %) with a Ca/SiO_2 molar equal to 1. Pseudowollastonite cylinders were obtained by cold isostatic pressing at 200 MPa, followed by sintering at 1400 °C for 2 h with a heating rate of 5 °C/min (Fig. 1). The specimens for cell study were composed of small cylindrical pellets cut from the cylinders which measured 12–13 mm in diameter, 2 mm thick and 650 mg of weight.

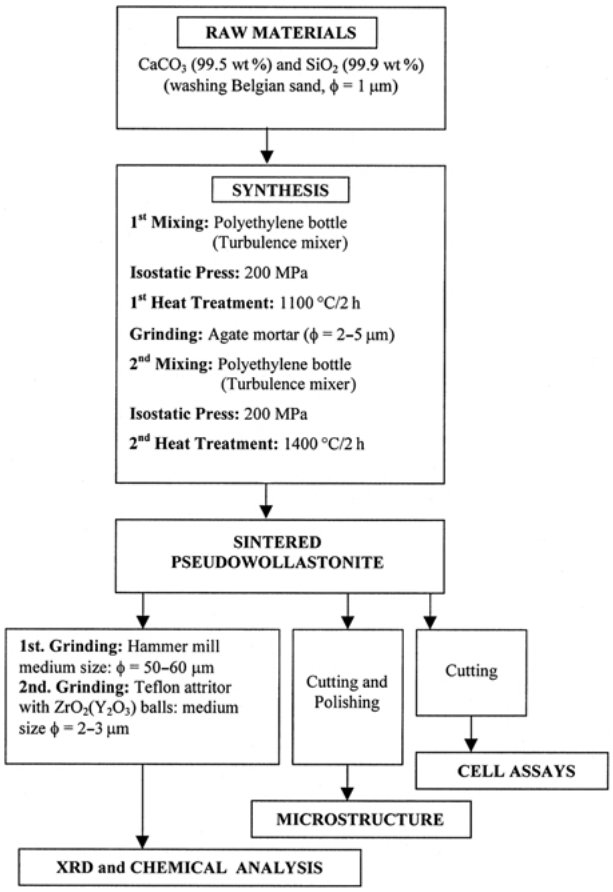

Figure 1 Processing used in the synthesis and subsequent treatment of the pseudowollastonite pellets.

One hundred pellets of pseudowollastonite in total, prepared from the same batch of sintered cylinders, were divided into two groups, one was polished for microstructure characterization and the other used in cell assays.

Sintered pseudowollastonite consisted of nearly equiaxial grains of average diameter ≈ 11.3 μm with round and closed pores of average size ≈ 1.8 μm (Fig. 2). It contained very little vitreous phase mainly located at triple junctions between grains, with a CaO/SiO_2 molar ratio of about 0.98 ± 0.01. The X-ray diffraction analysis confirmed that the pellets consisted of well-crystallized pseudowollastonite.

Establish cell lines

Human osteosarcoma cell line (SaOS-2, ATCC: HTB-85) was used for the cytotoxic evaluation of the psW. Cells were grown in Dulbecco's Modified Eagle's Medium with HAM'S F-12 (50% v/v) supplemented with fetal bovin serum (FBS) (10% v/v) (Bio-Whittaker; Verviers, Belgium), L-glutamine (200 mM), 100 U/ml penicillin G and 100 μg/ml streptomycin (Life Technologies; Merelbeke, Belgium). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air until

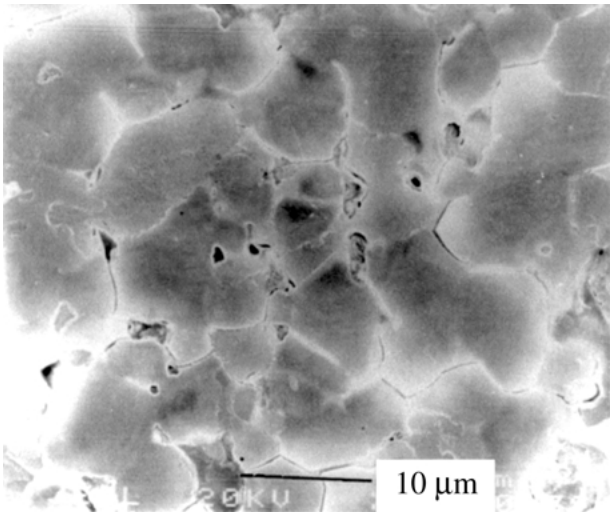

Figure 2 SEM image of the pseudowollastonite before extraction.

about 85–90% confluent. Osteosarcoma cells were then trypsinized (trypsin 0.25%, Sigma Aldrich chemicals, Bornem, Belgium) and were resuspended in medium. Cells were seeded into 96-wells microplates for NR and MTT assays at a density of 1×10^4 cells/wells; into 24-wells microplates for LDH assays at a density of 5×10^4 cells/wells

Preparation of pseudowollastonite extract

The extraction method was carried out according to International Standard Organization (ISO 10993-5) [24]. Under sterile conditions, samples of pseudowollastonite were immersed in the culture medium during 24, 48, 72 and 168 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, without agitation. Ratios between the sample weight (mg) and the medium volume (ml) were 5, 10, 15, 50, 100 and 200 mg/ml.

Cytotoxic assays

Cytotoxicity assays were based on the exposition of human cells to a liquid extract of psW to study the possible toxic substances leached from the biomaterial. Cytotoxicity assays as MTT, NR and LDH assays were then performed.

For each experiment, different groups were tested: positive or toxic control (0.1% Triton X-100), negative or non-toxic control (complete liquid culture medium), different times (24, 48, 72, 168 h) and different concentrations (5, 10, 15, 50, 100, 200 mg/ml) of psW samples [24]. Four samples per group were tested in each experiment. Two independent experiments were performed $(n = 8)$.

Determination of succinate dehydrogenase activity (MTT)

Cells were grown near confluency in culture medium during 72 h at 37 °C and then exposed to the extract of the psW samples during 24 h. Then, cell layers were rinsed in a phosphate buffered saline solution (PBS) and MTT (Sigma Aldrich Chemicals, Bornem, Belgium) was added (125 µl per well of a 5 mg/ml MTT solution in PBS). After 3 h of incubation at 37 °C, the MTT solution was removed and the insoluble formazans crystals were dissolved in 100 µl dimethylsulfoxide (DMSO; Sigma Aldrich Chemicals, Bornem, Belgium). Finally, the absorbance was measured at 540 nm using an ELISA microplate reader (Spectracount; Canberra Packard Benelux, Zellik, Belgium).

Determination of membrane integrity (NR)

Cells were grown near confluency and exposed to the extract as for MTT assay. Neutral red (Sigma Aldrich chemicals, Bornem, Belgium) was added (100 µl per well of a 0.4% (w/v) NR solution in distilled water, then diluted 1 : 80 in culture medium). After 3 h at 37 °C, the NR solution was removed and the dye extraction was performed by adding 100 µl per well of a 1% acetic acid in 50% (v/v) ethanol solution. The plates were agitated and finally the absorbance was measured at 540 nm using an ELISA microplate reader.

Determination of extra-cellular lactate dehydrogenase activity (LDH)

The lactate dehydrogenase activity was assayed in culture medium [20]. Confluent SaOS-2 cultured in 24-multiwells plates were pre-incubated with culture medium containing 10% FBS. After 72 h, medium was replaced and cells were then incubated for 24 h with extract of psW. After incubation, 100 µl aliquot of cell-free supernatant was added to 1 ml of reagent (Sigma Aldrich Chemicals, Bornem, Belgium). NADH conversion to NAD^+ was monitored spectrophotometrically (Vitalab 20, Merck Eurolab, Leuven, Belgium) at 340 nm. Total LDH activity was determined from the supernatant of a sample treated with 0.1% Triton X-100 for 3 min.

Statistical analysis

Mean values $\pm$ standard deviation (SD) of absorbances obtained from cells incubated in the presence of the extracts, either from controls or material extracts, were calculated. Results, for MTT and NR assays, were expressed as the percentage of the corresponding negative control conducted in the same experiment. Statistical significance was determined by one-way analysis of variance (ANOVA) with a Bonferroni post-hoc test. A p-value < 0.05 was considered statistically significant. The statistical tests were carried out using Systat version 8.0 (SPSS Inc., 1998).

Results

Positive controls where SaOS-2 cells were exposed to 0.1% Triton X-100 showed significant decreases in the amount of NR incorporated (respectively -88.35, 89.36, 80 and 85.83%, $p < 0.005$) (Fig. 3) and in the cellular activity measured by MTT (respectively -95.45, 90.32, 93.1, and 95.74%, $p < 0.005$) when compared to control media. Similarly, the positive control caused a highly significant increase in the amount of cell lysis measured by the release of LDH

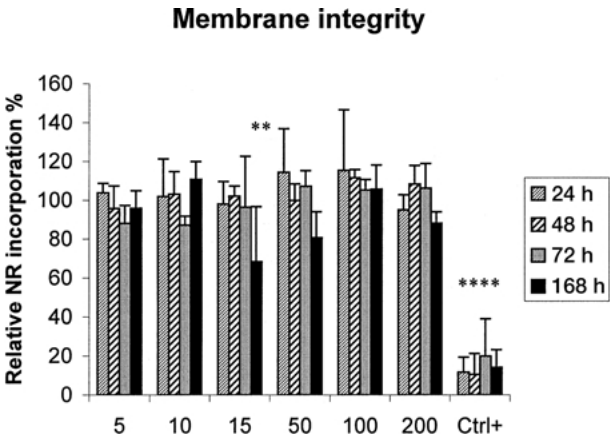

Figure 3 Relative NR intra-lysosomal incorporation by SaOS-2 cells after incubation with negative, positive control and psW extracted during 24, 48, 72 and 168 h with concentrations at 5, 10, 15, 50, 100, 200 mg/ml. Data obtained following incubation of cells with these extracts are expressed as percentages of the values obtained from cells incubated with the negative control and are the means ± SD ($n = 8$) ($* = p < 0.005$;$** = p < 0.05$).

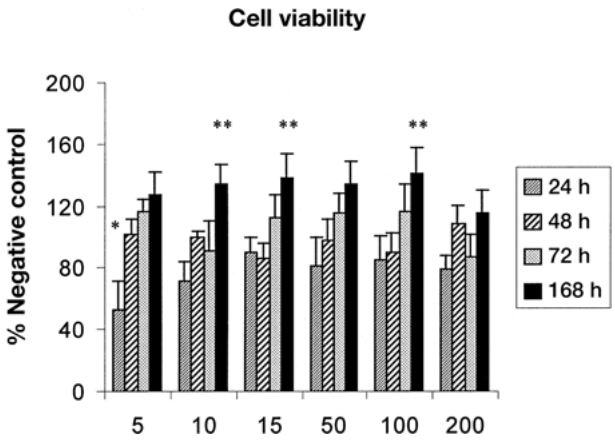

Figure 5 Relative MTT formazan formation by SaOS-2 cells after incubation, during 24 hours, with psW extracted during 24, 48, 72 and 168 h with concentrations at 5, 10, 15, 50, 100, 150, 200 mg/ml. Data obtained following incubation of cells with these extracts are expressed as percentages of the values obtained from cells incubated with the negative control and are the means ± SD ($n = 8$). Two independent experiments were performed and gave similar results ($* = p < 0.005$, $** = p < 0.05$).

(+346.77, 428.89, 314.83 and 379.11 U/l, $p < 0.005$) (Fig. 4).

Overall the extracts of pseudowollastonite ceramic had no significant effects on the membrane integrity as judged by NR incorporation. One exception was the observed decrease of NR incorporation for cells exposed to extract of psW with 168 h of extraction time and 15 mg/ml of extract concentration (−31.67%, $p < 0.05$) (Fig. 3). Whilst statistically significant this one set of parameters does not appear to be biologically important since both lower and higher amounts of psW fail to produce a significant effect. Similarly, no significant increase of lactate dehydrogenase activity was observed for cells exposed to any of the psW extracts (Fig. 4).

Cellular viability measured by the MTT assay revealed some interesting and significant trends. Levels of MTT were generally lower for the media incubated with material for 24 h for all concentrations of material used (Fig. 5). In tests using the lowest concentration of material 5 mg/ml for 24 h, there was a significant reduction in levels of MTT product compared to cells exposed to the negative control (−47.06%, $p < 0.005$) (Fig. 5). Longer extraction times lead to increases in the cellular viability measured. Extracts incubated with material for the longest period (168 h) in all cases give relatively the highest values compared to media alone. In the case of extracts from 10, 15 and 100 mg/ml of psW, there is a significant enhanced level of activity compared to controls with media alone (+34.61, 38.03 and 41.45%, $p < 0.05$) (Fig. 5). Thus, it appears that the extraction time of pseudowollastonite can influence the cellular viability in both a positive and a negative manner.

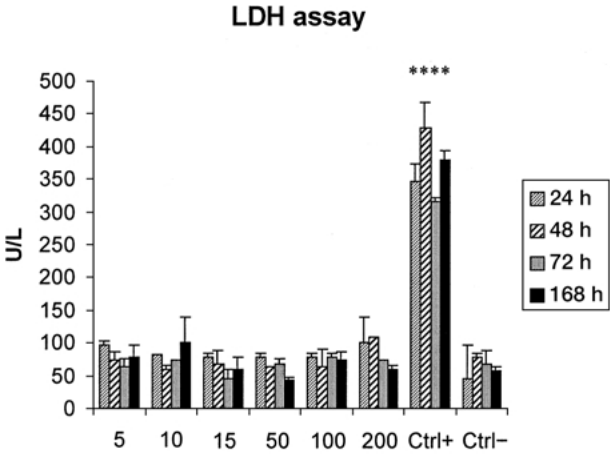

Figure 4 Extracellular level of LDH in SaOS-2 cells after incubation with negative, positive control and psW extracted during 24, 48, 72 and 168 h with a concentration at 5, 10, 15, 50, 100, 200 mg/ml. Each value is the mean ± SD ($n = 8$) ($* = p < 0.005$).

Discussion

Various *in vitro* and *in vivo* studies have demonstrated the capacity of bioactive ceramic to induce an hydroxyapatite-like layer indispensable for bone-biomaterial interaction. However, *in vitro* cytotoxicity studies have been rarely performed. Pseudowollastonite is able to induce several changes, as pH increase, release of calcium (Ca^{2+}) and silicate (SiO_3^-) ions, in the environment leading to the apatite-like layer formation [6–9]. These changes can potentially lead to cellular toxicity. This work was performed to study the impact of pseudowollastonite release on the cellular metabolism.

It is widely accepted that *in vitro* testing procedures are of considerable importance in cytotoxicity investigations of a biomaterial. According to International Standard (ISO 10993-5) qualitative or quantitative methods can be applied for this evaluation [24]. Qualitative evaluation based on the assessment of cell structural changes is a reliable method in the hand of a trained morphologist [25]. However, the literature recommends, when possible, an objective evaluation of cytotoxic effects by quantitative methods [14]. Quantitative methods encompass many tests which can be applied for cytotoxicity evaluation. Literature and International Standards do not specify a single test but attempt to present guidelines for the choice of suitable

tests. In order to obtain an objective evaluation of the cytotoxicity of pseudowollastonite, three different cell properties have been studied in this work. Cell viability was investigated by MTT assay which is by far the most sensitive assay [17, 18]. Cell membrane integrity was investigated by a modified NR assay for a better sensitivity. Indeed, as reported by Zhang SZ *et al*., increase of lysosome proliferation, obtained by cell aging following a culture incubation for a period longer than 24/48 h, could increase the sensitivity of this test. In the present study, the cell incubation time was 72 h [26].

Cell suffering was determined by the lactate dehydrogenase released by cells in the culture medium. The reaction catalyzed by this enzyme and related to cell necrosis is [20]:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \longrightarrow \text{L- Lactate} + \text{NAD}^+$$

To avoid the variation inherent in using primary cells, these experiments used the human osteosarcoma cell line SaOS-2. These cells have been extensively characterized and shown to possess many of the characteristics of normal osteoblastic cells. Indeed, alkaline phosphatase expression, calcifying matrix formation, parathyroid hormone-stimulatable adenylate cyclase, increase of alkaline phosphatase level with $1,25(OH)_2D_3$ have been reported [27, 28].

The results presented in this study demonstrate that extracts of psW show almost no cytotoxic activity when tested on the osteoblastic cell line SaOS-2. In addition, few changes in cell viability were observed in function of the time of extraction. Thus, extracts of the lowest concentration of the material (5 mg/ml) for the shortest time (24 h) are correlated with reduced cell viability whilst extracts prepared for the longest period (168 h) are associated with increase of cell viability. It is important to consider that although the primary aim of the MTT assay is to assess cell viability by measuring the levels of intracellular dehydrogenases, these enzymes can also influenced by cellular metabolism [29]. In order to understand the basis for the observed changes in cellular activity, it is necessary to consider the changes that occur when media incubated with psW. Previous studies have shown that a number of changes when psW is incubated in simulated body fluid. Calcium (Ca^{2+}) or silicate (SiO_3^-) ions are released from the media whilst concentrations of phosphate decline as the calcium-phosphate layer forms on the surface of the material [6, 7, 9, 10]. There is also a simultaneous small rise in pH of the bulk solution that could affect cellular activity. In this study, it is not known exactly how any changes in the media relate to different concentrations of psW or the length of incubation. However, the pattern of MTT activity suggests that incubating material for 168 h rather than 72 h leads to detectable differences. It seems likely that many of the changes can be related to the release of calcium and silicate ions from the material. The stimulatory effect of the longest incubations suggests that cellular activity and possibly cell proliferation are stimulated by released material constituents. There are several instances where extracts of other biomaterials have been shown to modulate cellular activity [30, 31]. The reason why lower concentrations of psW incubated for the shortest period of time are associated with reduced MTT values is more difficult to determine. One possibility is that the release of the different components from the psW may not be exactly co-ordinated. It is notable that the release kinetics of calcium and silicate ions from psW into simulated body fluid are not exactly matched [7]. Thus, there may be a transient period when extracts of psW have a qualitatively different composition. In this set of experiments either increasing the period of extraction or the concentration of the material has the effect of masking this minor cytotoxic effect.

Conclusions

In conclusion, these experiments demonstrate that extracts of psW do not show any significant cytotoxic effects and confirm the biocompatibility of this material. Clearly analysis of soluble factors released by the material can only partly explain the activity of psW *in vivo*. Further studies will aim to examine the interplay between soluble factors and the surface structure of psW and how these together influence cellular behaviors such as adhesion, proliferation and differentiation.

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