CMJ Original Research June 2018, Volume: 40, Number: 2
Cumhuriyet Medical Journal 135-141
http://dx.doi.org/10.7197/223.v40i37154.416738

Microstructural evaluation and cytotoxicity investigations of α-Al_2O_3 bone replacement materials

Kemikle yer değiştirebilir α-Al_2O_3 malzemelerin mikro yapısal değerlendirmesi ve sitotoksik incelemeleri

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Received/Accepted: April 18, 2018 / May 03, 2018
Conflict of interest: There is not a conflict of interest.

SUMMARY

Objective: To evaluate the production of porous α-Al_2O_3 by slip casting method and to investigate the cytotoxicity by MTT and Agar diffusion tests as well as SEM microstructural evaluation.
Method: Ceramic process route includes slip casting for porous osteoconductive α-Al_2O_3 production. For examination of morphology, SEM was employed. To evaluate the cytotoxicity, MTT and Agar diffusion tests were conducted.
Results: The conducted tests have given very close results to positive control groups which mean that the product α-Al_2O_3 is a very good candidate for bone replacement and osteoconductivity.
Conclusions: The porous α-Al_2O_3 production was succeeded and the cytotoxicity tests have been approved that this material can be a good candidate for replacement.
Keywords: α-Al_2O_3, porous ceramics, SEM, cytotoxicity.

ÖZET

Amaç: α-Al_2O_3'nın alçı kalıba döküm yöntemiyle gözenekli olarak üretilebilirliğinin değerlendirilmesi, MTT ve Agar difüzyon testleriyle sitotoksisitesinin ve SEM ile de mikroyapısal incelemesinin yapılması.
Yöntem: Gözenekli ve doku büyüyebilir α-Al_2O_3 üretimi için alçı kalıba döküm yöntemi kullanılmıştır. Morfolojik analiz için SEM kullanılmıştır. Sitotoksisite için MTT canlılık testi ve Agar difüzyon testi kullanılmıştır.
Bulgular: Yapılan testler, α-Al_2O_3'nın positif kontrol grubuna yakın canlılık değerleri verdiğini göstermiştir. Bu da aluminanın kemik ile yer değiştirebilir bir aday malzeme olabileceğini göstermektedir.
Sonuç: Gözenekli alumina üretimi başarılı olmuştur. Özellikle sitotoksisite testleri göstermiştir ki alumina kemikle yer değiştirebilir çok önemli ve uygun bir adaydır.
Anahtar sözcükler: α-Al_2O_3, gözenekli seramikler, SEM, sitotoksisite.

INTRODUCTION

The human health and comfort is the most important factor affecting stable daily life and human effort, so there are currently widespread interests in porous ceramics for body part's regeneration or implantation owing to their specific properties, such as low bulk density, low specific heat, low thermal conductivity and high specific surface area (Carn 2004; Sepulveda 1997; Colombo 2003; Liu 1998; Mear 2007; Zou 2005; Zampieri 2004). Orthopedic surgery and even dental surgery is in need for more strong and resistant materials as well as having better osteoconductive characteristics. The ceramics are the most common materials for improving

mechanical strength of the accompanying tissue and they are also compatible with body that is similar to bone. Bioceramic materials have attracted a great deal of interest to human comfort for numerous applications by scientists, physicians and engineers for the last four decades. In general, bioceramic materials are classified into three broad categories that include bioinert (such as alumina, zirconia) (Ghaemi et al, 2017; Sequeira, 2017), bioresorbable (such as tricalcium phosphate, TCP) (Sarkar et al, 2015) and bioactive (such as HAp bioactive glasses, glass-ceramics) (Lizzi, et al, 2017). A bioinert material is nontoxic whereas a bioactive material is one that elicits a specific biological response at the interface of the biological part and the material, which results in the formation of a bond between and tissues and the material (Ashiku 1997; Gross 1988; Li, 2016). Alumina can be sintered to a high density when small amounts of MgO are added. It is thought that the MgO is segregated to the grain boundaries and inhibits grain growth (Jorgensen 1964; Brook 1969), either by second-phase pinning (Jorgansen 1967) or by a solid-solution-impurity drag mechanism (Brook 1968). In the present investigation, sintering and production of MgO-doped porous alumina has been studied. This study is expected to have high impact among the studies of same type by giving the cell and cytotoxicity information and the overall characterization of the product at once. Porous α-Al_2O_3 – MgO ceramics through a powder metallurgical technique were synthesized and the cytotoxicity of ceramics were evaluated by MTT test. The cell viability, cytotoxicity, and cell distribution on surface were evaluated by SEM.

MATERIAL AND METHODS

In this study, the most common used ceramic implant material examined is high-purity α-Al_2O_3 - 0.5 wt. % MgO. High-purity Al_2O_3 and MgO powders were supplied by *Alumina* Refinery. The raw materials were weighed in weight proportion and ball milled with ethanol for 6 days using zirconia balls at 250 r.p.m. The Al_2O_3- MgO ceramic slurry mixture was well homogenized with 1 mol% *Darvan ® 821A* was added as a binder to ultra-fine powder ($\leq$ 1 μm) to give sufficient strength to the green compact. Darvan ® 821A is a highly effective dispersing agent used to lower the viscosity of particulate slurries and to inhibit the settling of particulate solids. Mixture was dried overnight at 80 °C and then dried and shifted through a 38 μm sieve for granulation. The powders were pressed hydrostatically at 200 MPa and the green density of each pellet was maintained at 60 % of theoretical density. The isothermal sintering was carried out at 1400°C for 2-4-6h. in static air in a closed alumina crucible and cooled to room temperature (5 °C/min).

The following techniques were employed to characterize the produced sintered α-Al_2O_3 – MgO ceramics. The relative density, open porosity and water absorption of the sintered materials were determined using Archimedes' method, as specified by European Standard EN 99 (ISO 10545-3, 1991) (Acchar 2008). The vacuum method (ISO 10545-3) was used in the laboratory to determine water absorption with greater precision. The vacuum method allows all open pores to be filled and the vacuum process was conducted in a chamber in which the air pressure was lowered to a value of 10^{-3} mbar and held at that value for 30 minutes. The crystal structure of the samples was determined from X-ray diffraction (XRD) data. An X-ray Diffractometer (Rigaku D/MAX/2200/PC) with a monochromatic Cu-K_α radiation (λ= 1.5408 Å) was used over a 2θ angle from 20° to 80° to characterize the crystal structure of the sintered compacts. The surface-fracture morphology and analysis of the ceramics was examined by using a scanning electron microscope (FEG-SEM, MIRA3 XMU, Brno, CZ). The cell viability tests and cytotoxicity was evaluated by MTT test.

RESULTS AND DISCUSSION

3.1. Physical Properties and Densification

Figure 1a, 1b presents the relative density versus open porosity and water absorption in samples sintered at 1400°C for 2-4-6 h. Values were determined according to equation (1)

$$E_v = \frac{m_{2v} - m_1}{m_1} \times 100(\%) \quad (1)$$

The foregoing formulas contain the following magnitudes, all expressed in grams (g):

m_1: mass of the dry sample

m_{2v}: mass of the sample impregnated with water after the vacuum process and weighing, also under vacuum.

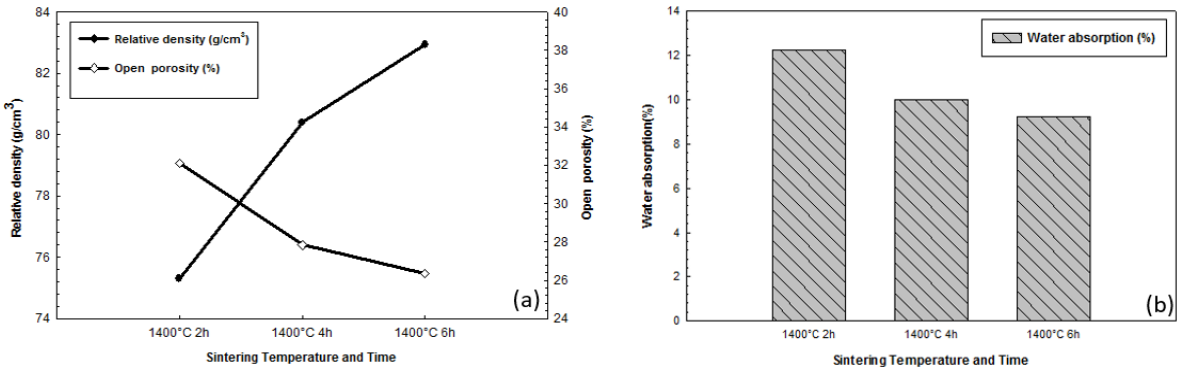

Figure 1. (a) Variations in relative density - open porosity and (b) water absorption in α-Al_2O_3 – MgO ceramics sintered at different sintering time produced by powder metallurgy technique.

In this study, the relative densities of sintered α-Al_2O_3 – MgO ceramics produced by powder metallurgy technique were found as % 80.9 at 1400°C 6h. Figure 1a, 1b reveals that the increasing sintering time increases the bulk density of the composites. It can be seen that for all samples, the sintering time has a positive impact on the sintered density, where longer sintering time gives higher density. This may be attributed to reduction in the amount of porosities during sintering which leads denser materials. An open porosity value (%) of sintered ceramics produced by powder metallurgy technique at 1400°C 6h was found to be 26.34%±0.11. Water absorption (9.22%) value was obtained at 1400°C 6h sintered α-Al_2O_3 – MgO ceramics produced by powder metallurgy technique.

3.2. XRD Analysis

The results of the crystallographic analysis of the produced samples and powders, carried out with X-ray diffraction, are summarized in Figure 2. In all cases, the diffractograms have predominantly registered the corundum phase – (Al_2O_3), matched for every sample (ICSD card no. 71-1128) due to the dissolution of MgO and reacts with Al_2O_3 in the ceramic composite. Figure 2a shows the X-ray diffraction patterns of the original powder mixture after ball milling. After 6 days of milling, the peaks become increasingly broader with long milling time and a number of new broadened peaks appear. As the sintering is occurred, the peaks are sharpened suggesting the increase in the crystallite size (Figure 2b). In order to realize the sub-micron microstructure in the sintered ceramics, it is essential to have the nanocrystalline powders as starting milled powders. The figure proves that the Al_2O_3-MgO composites are successfully produced without noticeable undesirable phases. X-ray diffraction patterns of Al_2O_3-MgO composite powders show that the peaks correspond to corundum phases, confirming that MgO is effectively incorporated into the Al_2O_3 matrix by forming corundum.

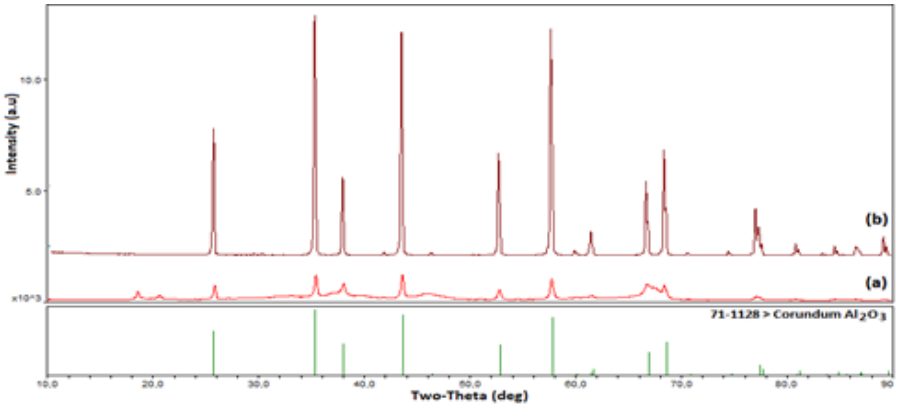

Figure 2. (a) X-ray diffraction of the initial powders after ball milling for 6 days. (b) X-ray diffraction of the sintered sample at 1400°C 6h obtained by powder metallurgy technique.

3.3. SEM Analyses

The surface morphology of the MgO doped alumina-based ceramics was examined by scanning electron microscopy (FEG-SEM, MIRA3 XMU, Brno, CZ). The samples were fixed on aluminum stubs and pre-coated with gold/palladium thin film for 5 min under argon atmosphere using a Quorum ER150S sputter coater. The SEM samples were examined at an accelerating voltage of 20 kV. SEM images of the sintered ceramics were presented in Figure 3 (a- c). SEM images reveal that the homogenous morphology and different grain size distribution were obtained for all compositions after sintering.

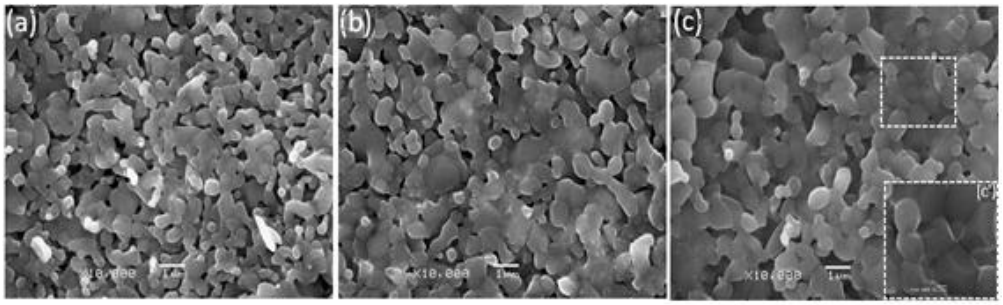

Figure 3. SEM images of the α-Al_2O_3 – MgO ceramics sintered at 1400°C (a)2h, (b) 4h and (c-c') 6h. obtained by powder metallurgy technique.

Figure 3 (a, b, c) also represents the surface morphology of samples become more smooth by increasing sintering time and production method. As observed, especially in Figure 3 (c and c'), the increasing glassy phase and MgO may contribute to the smoothness of the surface and more particles seem to be embedded to the matrix structure. It is found that the average grain size has a slight increase as the sintering time increases.

3.4. MTT Cell Viability and Cytotoxicity Tests

MTT is the most well-known yellow tetrazole, then reduced to purple formazan in living cells. A solubilization solution (dimethyl sulfoxide, an acidified ethanol solution in this study) is added to dissolve the insoluble purple formazan product into a reddish solution (Mosmann, 1983). The absorbance of this solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The degree of light absorption depends on the solvent. Figure 4 represents the MTT reaction in viable cells to form reddish colored solution. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color.

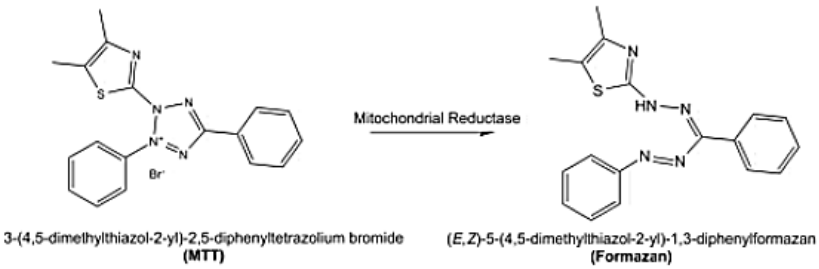

Figure 4. MTT reducing reaction by living cells.

Table 1. MTT Cell Viability Tests and Survival % in accordance with alumina against positive and negative control groups.

Material	Relative Optical Density	Relative Survival (%)
Al_2O_3	0.8236	93
Negative control - DMSO	0.1984	18
Positive control- DMEM	0.8842	100

As seen from Table 1, the positive control group is assumed to have the best viability, while alumina group shows a 0.82 relative optical density and relative survival of 92%, both are very close to positive control group and far from negative control. One can conclude that alumina, produced by powder metallurgy route is a very good candidate bulk material for cell survival and osteoconductive implantable material for this study.

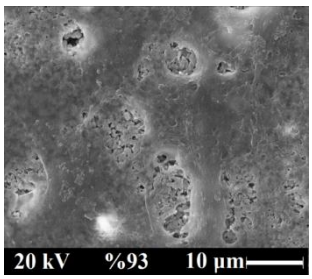

Figure 5. SEM-SE image of alumina sample's (sintered at 1400°C for 6 h, relative density of 83% and water absorption of 9%) surface having a relative optical density of 0.8236 and relative survival of 93%.

Figure 5 stands for alumina material having 93% relative survival. Figure shows the cell network directly penetrated to the surface and a very well distribution of all surface of alumina. The SEM image was taken from the alumina bulk material with the highest density to evaluate the hardest condition for the growth of cells. Cells were spread all over the surface even into the pores to form a surrounding living layer of cells.

As illustrated in Figure 6, the optical images of alumina (a), positive control group (b) and negative control group (c) shows the optical density of cells and viability properties in plates. As known, negative control has DMSO in plate and deteriorates the cell formation while the positive group was incubated in the broth to increase the cell viability up to 100%. Although there is no broth in alumina case, the cell viability is still at about 92% which is very acceptable for cell growth followed by a probable tissue formation.

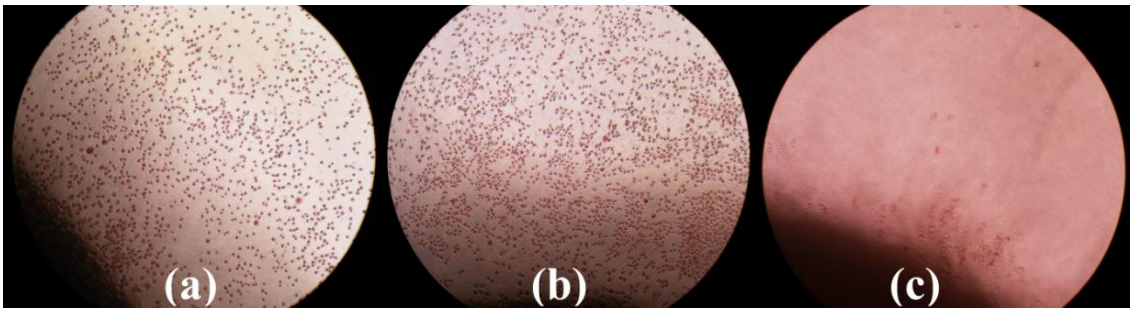

Figure 6. Optical images of (a) alumina (0.8236), (b) positive control group (0.8842), (c) negative control group (0.1984) within cell formation and viability.

CONCLUSION

The present investigation suggests α-Al_2O_3 – MgO ceramics to be used as implant interface. The effects of sintering time on the phase composition, densification and microstructure of MgO doped Al_2O_3 ceramic composites were studied. The main phase of the sintered ceramic composites was corundum (Al_2O_3) which are known biocompatible. The preferable technique for desired porous ceramics should be achieved by powder metallurgy method. Bioceramics must have properties of osteointegration by means of vein growth in the structure for better scaffold and tissue interaction. The porous alumina can be classified as osteoconductive material and the results showed that the viability and survival is almost same as positive control units as seen in MTT cell viability test results. This study may be a new avenue to new mechanical and cell culture studies in vitro and in vivo in the near future.

ACKNOWLEDGMENT

The author would like to thank to CU Advanced Technology Research and Development Lab-SEM Analysis facility for SEM imaging.

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