

In Vitro Cell Study of Possible Anti-inflammatory and Pain Relief Mechanism of Far-infrared Ray-emitting Ceramic Material

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Received 26 Sep 2011; Accepted 26 Apr 2012; doi: 10.5405/jmbe.1029

Abstract

Inflammation and pain are the major chronic symptoms in geriatric medicine. This study examines the possible mechanism of a far-infrared ray-emitting ceramic material (bioceramic) on these symptoms using cell models. Effective doses of lipopolysaccharides (LPS) were added to induce acute episodes of inflammation in murine macrophage (RAW 264.7) and human chondrosarcoma (SW1353) cells. The inducible nitric oxide synthetase (iNOS), cyclo-oxygenase-2 (COX-2), and prostaglandin E2 (PGE2) levels were determined for the cell lines. Bioceramic treatment was found to have significant inhibitory effects on COX-2 and PGE2 and a probable effect on iNOS in the cell models of LPS-mediated inflammation. Bioceramic treatment may be an alternative method for palliative pain control to reduce chemical drug dependence for the protection of renal functions in the chronic pain disease population.

Keywords: Bone marrow stromal cells (BMSCs), Osteogenic differentiation, Collagen I nanospheres

1. Introduction

Inflammation is characterized by the release of pro-inflammatory cytokines and inflammatory mediators, free radicals, and prostaglandin E2 (PGE2), which are synthesized by inducible nitric oxide synthase (iNOS) and cyclo-oxygenase (COX-2). These inflammatory mediators and cytokines are involved in numerous human diseases, including rheumatoid arthritis, asthma, atherosclerosis, and endotoxin-induced multiple organ injury. Anti-inflammatory agents reduce the inflammatory response by suppressing the production of inflammatory cytokines and mediators. People who often take anti-inflammatory agents such as acetaminophen or non-steroidal anti-inflammatory drugs (NSAIDs) are at increased risk of end-stage renal diseases (ESRDs), which is in dose-dependent risk [1]. ESRDs are becoming an increasing burden in Taiwan. Since 2000, Taiwan has had the highest incidence rate and prevalence of ESRDs with ESRDs affecting approximately 400 people for every million. The over 40,000

ESRD patients comprise 7% of the National Health Insurance budget for dialysis treatment, but represent only 0.17% of the population. Drug abuse for pain relief is one of the major causes of ESRDs [2-6]. Overuse of anesthetic drugs has been increasing steadily in Taiwan and other countries [7]. Incidences of renal complications are becoming more frequent due to abuse of pain relief drugs. It is thus desirable to find non-pharmacological alternative therapies to reduce drug dependency in pain control. Far-infrared (FIR) ray treatment is an alternative pain relief method, but there has been no published evidence of its pain relief and anti-inflammation properties.

In our earlier publications on FIR ray-emitting ceramic materials (bioceramics), bioceramics were shown to promote microcirculation by up-regulating calcium-dependent nitric oxide and calmodulin in various cell lines [8,9]. Bioceramics were also shown to exert antioxidant effects by increasing hydrogen peroxide scavenging ability [10-16]. Other studies on FIR rays have also indicated beneficial effects, including the elevation of regional skin temperature and increased skin blood flow [17-20]. The present study investigates the effects of bioceramic irradiation on the intracellular levels of iNOS, COX-2, and PGE2 in an *in vitro* cell model under

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lipopolysaccharide (LPS)-induced inflammation. A possible pain relief mechanism is proposed.

2. Materials and methods

2.1 Bioceramic powder

Ceramic powder is composed of micro-sized particles produced from several ingredients, mainly elemental components (namely Ca, Zr, S, Si, Al, Mg, Fe, O, and C) obtained from the Department of Radiology, Taipei Medical University Hospital (Fig. 1). The average emissivity of the ceramic powder was 0.98 at wavelengths of 6 to 14 μm , as determined using a physical, chemical, and CI SR5000 spectroradiometer (CI systems Ltd., Migdal Ha'Emek, Israel), which represents an extremely high ratio of FIR intensity (Fig. 1). Numerous biological effects can be induced by this ceramic powder at room temperature without direct contact by employing a method previously used by the authors [8-16].

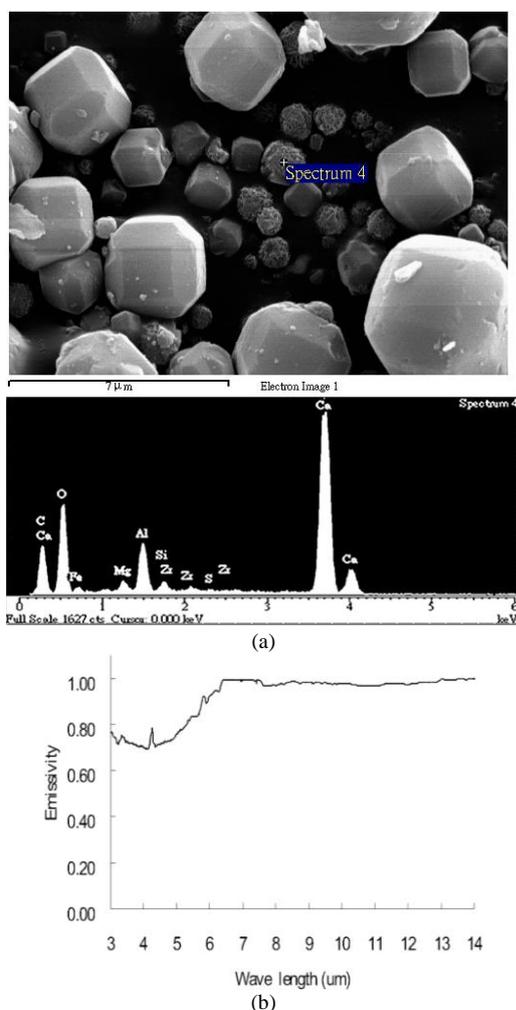


Figure 1. (a) Elemental analysis of ceramic powder of the bioceramic conducted using electron microscopy equipment with electron beam processing on the selected spectrum. The relative concentration of these main elements (originally as elemental oxides) is shown. (b) Range of wavelengths representing FIR rays is from 8-14 μm . Average emissivity of the bioceramic powder is averaged over 0.90.

2.2 Cell culture

RAW 264.7 cells were obtained from a mouse macrophage cell line from the Bioresource Collection and Research Center (BCRC). Cells were cultured in Dulbecco's modified eagle medium (DMEM), supplemented with 2 mM glutamine, antibiotics (100 U/mL penicillin A and 100 U/mL streptomycin), and 10% heat-inactivated fetal bovine serum (FBS; Gibco/BRL, Gaithersburg, MD, USA), and maintained in a 37 $^{\circ}\text{C}$ humidified incubator containing 5% CO_2 . Cells were seeded until they were 80% confluent on the bottom of the dishes (Fig. 2).

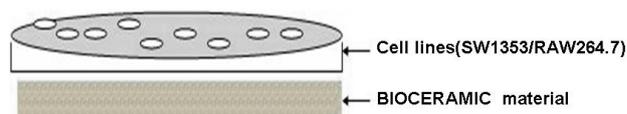


Figure 2. Bioceramic material inserted beneath the dishes of cell lines.

Chondrosarcoma (SW1353) cells were seeded into a 24-well cell culture plate (GeneDireX, Inc., Flint Place Poway, CA, USA) one day before the experiment. The SW1353 cells were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (all obtained from PAA Laboratories, Pasching, Austria). The cells were grown under standard conditions in a humidified incubator at 37 $^{\circ}\text{C}$ and 5% CO_2 . The cells were seeded at an initial density of 5×10^3 cells/ cm^2 for 24 h before the experiments were commenced.

2.3 Western blot

After the cells were seeded into a 24-well cell culture plate (GeneDireX, Inc.) one day before the experiment, they were stimulated with 1 $\mu\text{g}/\text{mL}$ LPS for 24 h, with 100 g of bioceramic powder or a control (non-functional powder, namely regular milk powder) enclosed in a plastic bag (10 \times 20 cm) as the bioceramic irradiation source.

The effect of bioceramic treatment on the iNOS expression for the RAW 264.7 cell line was determined by Western blot analysis. At the end of the incubation period, the cells were washed with phosphate-buffered saline (PBS), scraped with a rubber policeman, and sonicated for 2 min in ice. Proteins (50 $\mu\text{g}/\text{lane}$) were separated by electrophoresis on an 8% acrylamide gel and transferred to nitrocellulose, which was then incubated with an anti-iNOS antibody at a dilution of 1/200. The bands corresponding to iNOS were visualized by enhanced chemiluminescence (ECL).

The effect of bioceramic treatment on COX-2 production for RAW 264.7 and SW1353 cell lines was determined using Western blot analysis. Equal amounts of whole cellular extracts were analyzed using 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to polyvinylidene difluoride-nylon membranes (1.5 h). The membranes were subsequently blocked with phosphate-buffered saline with Tween 20 (PBST) containing 3% bovine serum albumin at 4 $^{\circ}\text{C}$ overnight. After blocking, the membranes were incubated with primary antibodies (anti-COX-2) (Cell Signaling Technology, Beverly,

MA), diluted to 1:1000 in anti-GAPDH and 1:10000 in PBST, at 4 °C for 24 h, and washed with PBST for 10 min four times. The membranes were then incubated with secondary antibodies (anti-COX-2 antibodies) (The Jackson Laboratory, Bar Harbor, ME), diluted to 1:20000 in PBST (phosphate buffered saline tween-20) Recipe, at room temperature for 2 h, before being washed with PBST for 15 min four times. After washing, the membranes were visualized with ECL detection reagents and autoradiographic film.

2.4 ELISA

The human chondrosarcoma cell line SW1353 was cultured in Leibovitz's L-15 medium (Invitrogen, Taichung City, Taiwan), 100 U/mL penicillin, 100 mg/mL streptomycin (Invitrogen), and 10% FBS (Invitrogen). Cells were seeded into a 24-well cell culture plate (GeneDireX, Inc.) one day before the experiment. They were then stimulated with 20 ng/mL LPS for 48 h, with and without bioceramic material placed beneath the culture medium discs. The supernatant was harvested and used to measure PGE2 production by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA).

2.5 Statistical analysis

Statistical evaluations of data from the bioceramic and control groups were performed using the paired *t*-test. Results with a *p* value of < 0.05 were considered significant.

3. Results

3.1 Effect of bioceramic on iNOS level in RAW 264.7 cell line under LPS induction

Figure 3 shows the normalized production means of iNOS protein (iNOS/GAPDH) in the control and bioceramic groups. The results may reflect the ability of bioceramic to suppress iNOS expression in LPS-induced iNOS in RAW 264.7 cells. However, the results did not have statistical significance.

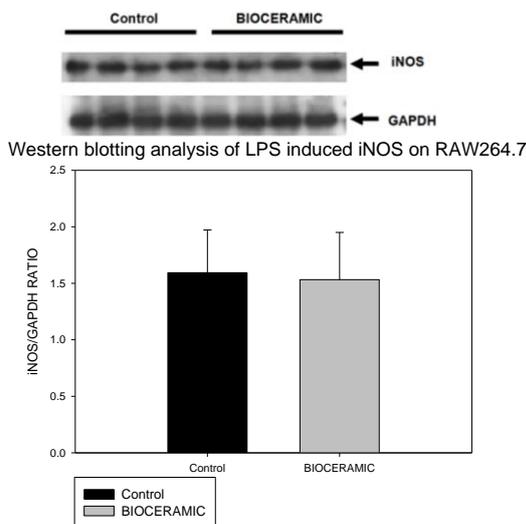


Figure 3. Effects of bioceramic on the expression of iNOS in RAW 264.7 cells.

3.2 Effect of bioceramic on COX-2 production in RAW 264.7 cell line

Western blot analysis shows that the expression levels of COX-2 protein were significantly lower in the bioceramic group than those in the control group (Fig. 4). The results show a significant suppression of LPS-induced COX-2 in RAW 264.7 cells by the bioceramic material.

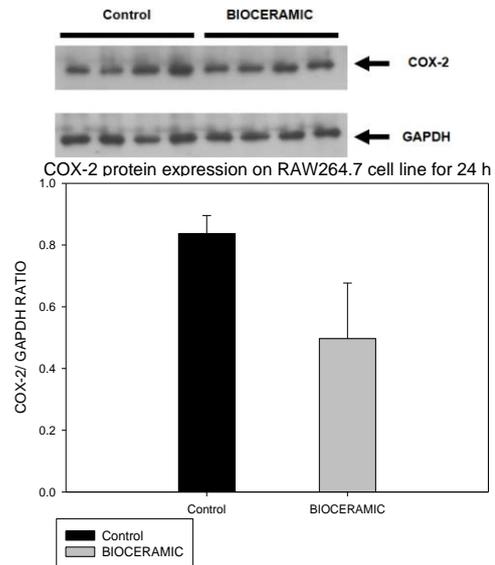


Figure 4. Effects of bioceramic on the expression of COX-2 in RAW 264.7 cells. **p* < 0.05 indicates significant difference compared with the control group.

3.3 Effect of bioceramic on COX-2 production in SW1353 cell line

Western blot analysis of the culture disc of the bioceramic group indicates that LPS treatment resulted in a significantly lower COX-2 protein level than that in the LPS-treated control group (Fig. 5). The results show a significant suppression of LPS-induced COX-2 in SW1353 cells by the bioceramic material.

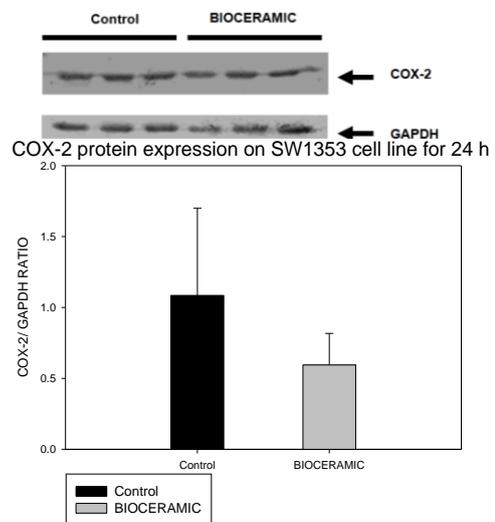


Figure 5. Effects of bioceramic on the expression of COX-2 in SW1353 cells. **p* < 0.05 indicates significant difference compared with the control group.

3.4 Effect of bioceramic on PGE₂ production in SW1353 cell line

LPS-induced PGE₂ production in the bioceramic group was significantly lower than that in the control group according to the ELISA (Fig. 6). The results show a significant suppression of LPS-induced PGE₂ in cells by the bioceramic material.

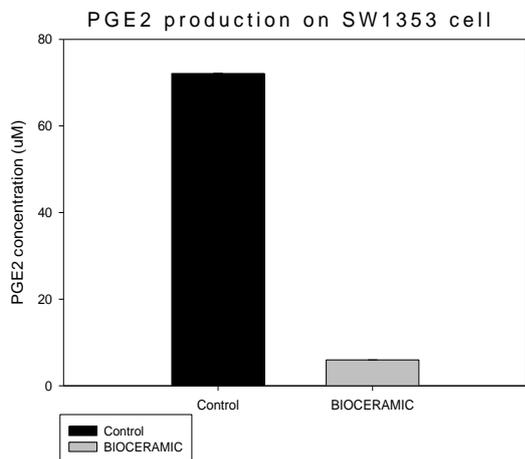


Figure 6. Effects of bioceramic on the expression of PGE₂ in SW1353 cells. * $p < 0.05$ indicates significant difference compared with the control group.

4. Discussion

Under normal conditions, fine afferent fibers of the peripheral nervous system are activated by brief or high intensity stimuli. These stimuli do not usually induce tissue damage, causing transient pain sensations, which only serve as a physiological warning. Under pathological conditions, such as major inflammation produced by mild tissue damage or infection, stimuli activate afferent fibers with a lower intensity, and the resultant pain may be more persistent. Chronic pain is symptomatic of numerous conditions, including rheumatoid arthritis, osteoarthritis, lower back pain, neuropathic pain, migraines, and cancer pain. The majority of pain conditions, whether inflammatory or neuropathic, are associated phases of inflammation in which a variety of chemical mediators are able to alter the functions of peripheral afferent fibers [21].

Inflammation is the major process of the development of chronic joint disease, and LPS-induced inflammation is the most widely used biological model for arthritis [22-24]. LPS is derived from Gram-negative bacteria, which are responsible for activating a series of non-specific immune reactions. LPS has been found to increase total IgG and other biomolecules production, one of known to be a rheumatoid factor (RF), which is an autoantibody that is most relevant in rheumatic arthritis (RA). RF is an antibody that joins IgG to form immunocomplexes that contribute to RA autoimmune pathogenesis [25-30]. To examine the possible anti-inflammatory and pain relief mechanism of bioceramic material irradiation, this study focused on the biomolecules iNOS, PGE₂, and COX-2, which contribute to the pathogenesis of

inflammatory joint disease. Nitric oxide (NO) and cyclooxygenase are the rate-limiting enzyme in prostaglandin biosynthesis, but the outcome of this interaction varies from model to model. NO either promotes prostaglandin release through iNOS activation or decreases prostaglandin production during its slow release [31-35]. The affective role of NO in inflammatory pain sensations is biphasic or dual, relatively complex, and is dependent on the amount and rate of production. In contrast, PGE₂ and COX-2 undoubtedly act as critical inflammatory mediators. A direct link exists between the specific concentrations of PGE₂ and COX-2 at the sight of local inflammation and the pain experienced by a patient [36-45]. PGE₂ is produced during inflammatory responses, and increased levels of PGE₂ mediate certain features of inflammation, including pain, edema, and fever [46-48]. Peripherally and regionally, inflammation also causes COX-2 induction. In the central nervous system, COX-2 is induced by nociceptor signaling through inflammatory cytokines [49-51]. COX-2 catalyzes the conversion from free arachidonic acid to the prostanoid precursor, which is then converted to PGE₂. Consequently, prostanoids and PGE₂ lead to increased excitability and a reduced pain threshold. Blocking COX-2 is thus one of the best treatments for pain sensitivity. The present study established an *in vitro* model using cell lines to determine the potential anti-inflammatory ability of bioceramic by clarifying its relationship with iNOS, PGE₂, and COX-2 levels (Fig. 7). A notable inhibitory effect on both PGE₂ and COX-2 expression by LPS-stimulated cell lines was found. The results suggest an anti-inflammatory effect of bioceramic on inflammatory tissue for pain relief. A previous study demonstrated that aspirin treatment reduced LPS-induced PGE₂ formation and abolished the COX-2 protein and its mRNA expression [52-55]. Aspirin and other NSAIDs are inhibitors of PGE₂ and COX-2 synthesis. The drugs are also used in the treatment of other related inflammatory diseases that cause PGE₂ and COX-2 overproduction [36-39,42,43].

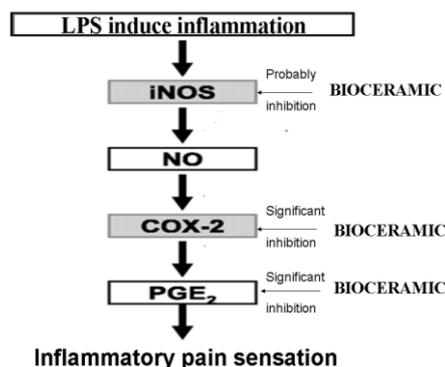


Figure 7. Proposed model of bioceramic interaction with the signaling pathways of LPS inducing inflammation through iNOS, COX-2, and PGE₂. Bioceramic exhibits significant inhibitory effects on COX-2 and PGE₂, and a probable effect on iNOS.

5. Conclusion

Using *in vitro* cell studies, this paper examined possible mechanisms of the beneficial effect of bioceramic materials against inflammation. Significant inhibitory effects on both

PGE2 and COX-2 and potential inhibitory effect on iNOS expression under LPS stimulation were found. Bioceramic appears to have a retrogressive effect on LPS-induced inflammation, and may ameliorate pain sensation. Prospective clinical applications of bioceramic are currently being explored. Because the biological effects of bioceramic occur through physical induction, bioceramic exhibits similar effects to those of NSAIDs and other pain relief remedies, whether by oral intake or injection, though with no risk of producing renal damage. Bioceramic can be used for relieving acute inflammatory joint disease to reduce drug dependency. Future investigations on the effects of bioceramic on inflammatory pain or inflammatory arthritis will focus on the biomolecular level.

Acknowledgments

The authors gratefully acknowledge the support from Mr. Gary Lu (Grand Textile Corp.), Miss Francis Chou (Hou-Kon Biotech. Corp.), Mr. Tai-Lin Ping (Health Control Corp.), Mr. Blitz Sung, Tien-Fu Huang, and Tzy-Chin Day (Hua Mao Nano-Tech.) for their contributions to this research.

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