



Original Article

Protective Effect of Ginkgo Biloba Extract on Gentamicin-Induced Structural Changes of Calcite–Gelatin Composite

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OBJECTIVE: To evaluate the protective effect of Ginkgo biloba extract (GBE) on gentamicin (GM)-induced morphological damage of an artificial otoconia.

MATERIALS and METHODS: An artificial otoconia powder was placed in a 12-well culture plate containing artificial endolymph. GM (500 μ L; 40 mg/mL) was added to the first four wells. GM (500 μ L; 40 mg/mL) with GBE (500 μ L) was added to the next four wells. PBS (500 μ L) was added to the remaining four wells as a control. The levels of nitric oxide (NO) synthesis were determined in the supernatants of each group. Alteration in surface morphology and calcium content were determined by scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX), respectively.

RESULTS: NO concentration increased in the GM-treated group compared with that in the control group. When the calcite–gelatin composite was treated with GBE, GM-stimulated NO production significantly decreased. The surface of the calcite–gelatin composite exposed to GM showed erosive fissures and had a honeycomb-like appearance. GBE showed a protective effect against dissolution. EDX showed that the calcium content of the GM-treated group significantly decreased. However, the GBE-treated group demonstrated an insignificant change in the calcium concentration compared with the control.

CONCLUSION: From these results, we can conclude that GBE may have a protective effect against GM-induced NO production and superficial structural changes.

KEYWORDS: Calcite–gelatin composite, gentamicin, Ginkgo biloba extract, artificial otoconia

INTRODUCTION

The otoconia are partially embedded in the gelatinous matrix and are linked to one another by filamentous crosslinks^[1]. Two main components of the otoconia are calcite crystals and organic glycosylate proteins. Previous studies have suggested that the underlying core of glycosaminoglycan fibrils serves as a framework for calcite deposition during otoconial development^[2,3]. Since the discovery of the biomimetic growth of artificial otoconia in gelatin gel matrices (calcite–gelatin nanocomposite), which show the same chemical and structural characteristics as human otoconia, it has become possible to detect morphological changes in greater detail, as described in recent studies^[4–6].

Gentamicin (GM) is an aminoglycoside antibiotic, which is effective against gram-negative bacterial infection^[7]. GM ototoxicity is a common cause of drug-induced hearing loss^[8]. Recently, clinical evidence revealed that the degree of GM-induced vestibular damage to the semicircular canal ampullae, saccule, and utricle varied among patients^[9,10]. In addition, morphological damage in combination with increased nitric oxide (NO) production was found in the vestibular organ after GM administration^[11–13].

Ginkgo, a dietary supplement derived from the deciduous tree, Ginkgo biloba, which is considered the world's oldest tree species, contains unique constituents^[14]. Extracts and infusions made from Ginkgo leaves have been used in traditional Chinese medicine for thousands of years^[15]. The protective effect of G. biloba extract (GBE) against GM-induced cochlear ototoxicity has been reported^[16,17]; however, its protective effect on otoconia has been not been extensively reported.

In this study, we evaluated the protective effects of GBE on GM-induced morphological damage of artificial otoconia and NO production *in vitro*.

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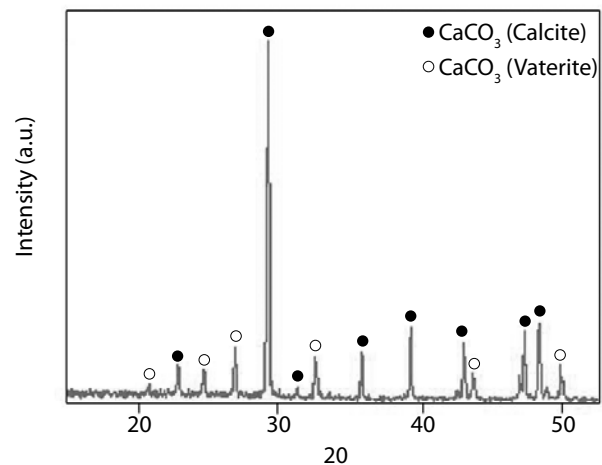
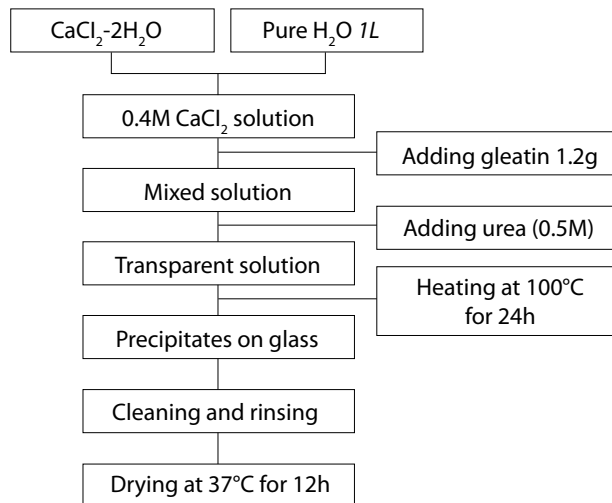


Figure 1. a, b. Schematic of the preparation of a CaCO₃–gelatin biocomposite (a) and X-ray diffraction patterns of a CaCO₃–gelatin biocomposite (b)

MATERIALS and METHODS

Fabrication of Artificial Otoconia

To obtain an artificial otoconia (CaCO₃–gelatin nanocomposite), we modified the precipitation method previously reported by Tas^[18]. First, CaCl₂·2H₂O (0.4 M) solution was prepared in 1 L of deionized water under stirring at room temperature. Gelatin powder (1.2 g) followed by 1 mole of urea were then added to the above solution. A transparent solution was obtained. This solution was transferred to a Pyrex glass flask containing few cover glasses at the bottom. The flask was tightly capped and heated to 100°C for 24 h. CaCO₃ particles were precipitated by the reaction of CaCl₂ and NH₃ gas, resulting from the thermal decomposition of urea. The white-coated cover glass was removed from the flask and washed with an ample supply of deionized water, followed by rinsing with ethanol. The cover glass was then dried in an oven at 37°C overnight. The white powder coating from the cover glass was collected by gentle scraping using a clean and sharp razor blade.

Samples were characterized by powder X-ray diffraction (XRD), which showed the biphasic phases of vaterite–calcite microtablets (Figure 1).

Exposure of Artificial Otoconia to Gentamicin and GBE

The artificial otoconia powder was placed in a 12-well culture plate using an analytical microbalance. Each well was filled with 500 µl of artificial endolymph (AE), which was fabricated by Salt et al^[19]. KCl (140 mM), KHCO₃ (25 mM; 295–300 mosm), and GM (500 µL; 40 mg/mL) were added to the first four wells. GM (500 µL; 40 mg/mL) with GBE (500 µL; 17.5 mg/5 mL, EGb 76, Tanamin injection, YuYu Pharma Inc.; Seoul, Korea) were added to the next four wells. PBS was added to the remaining wells as a control. After incubation for 24 h, the levels of NO synthesis were determined by assaying the supernatants of each group for nitrite using the Griess reagent^[20] (1% sulfanilamide, 0.1% N-1-naphthylenediamine dihydrochloride, and 2.5% phosphoric acid; Sigma-Aldrich, G4410, Seoul, Korea). Nitrate is the stable product of the reaction between NO and molecular oxygen. The absorbance was measured at 540 nm with a Synergy Micro-plate Reader after incubation for 10 min.

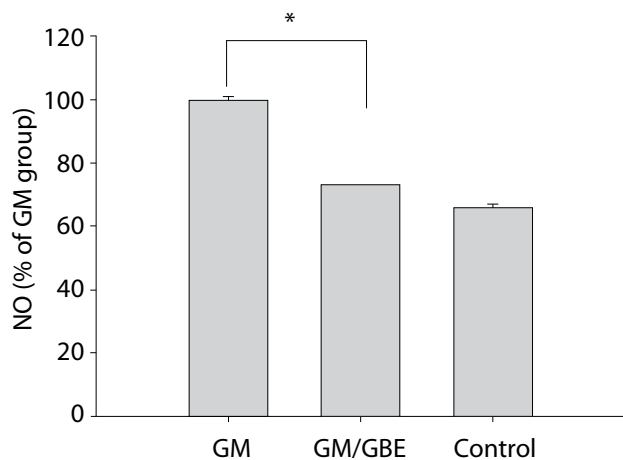


Figure 2. Nitric oxide (NO) levels; GBE protected NO release from GM-exposed calcite–gelatin composite
GBE: Ginkgo biloba extract; GM: gentamicin
Asterisk indicates statistical significance

For the morphological examination, each well was fixed in 2.5% glutaraldehyde at 4°C for 8 h. The specimens were washed three times in PBS and then post-fixed in 1% osmium tetroxide for 1 h at 4°C. They were dehydrated through a graded series of ethanol solutions, critical-point dried, and placed on a stub for sputter coating with gold-palladium (Korea Basic Science Institute). The change in surface morphology and component were examined by scanning electron microscopy (SEM; Korea Basic Science Institute, FE-SEM; Hitachi, Tokyo, Japan). Further evidence of the carbonate deposits as calcite crystals was provided by energy-dispersive X-ray spectroscopy (EDX) analysis.

Statistical analysis was performed by one-way analysis of variance (ANOVA) and the Statistical Package for the Social Sciences (SPSS, version 17, SPSS Inc.; Chicago, Illinois, United States). A p value of <0.05 was considered statistically significant.

RESULTS

The amount of nitrite accumulated in the AE was estimated using the Griess reagent as an indicator of NO release. As shown in Figure 2, NO

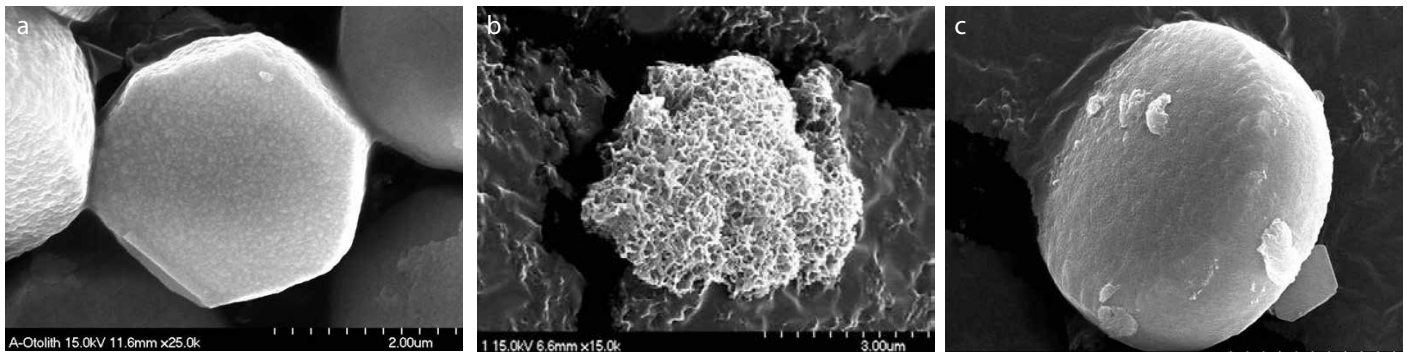


Figure 3. a-c. Scanning electron microscopic findings of the fabricated calcite-gelatin composite (a), the dissolved surface structure of the calcite-gelatin composite upon GM exposure (b) and the GM-stimulated structural change was attenuated by GBE (c)
GBE: Ginkgo biloba extract; GM: gentamicin

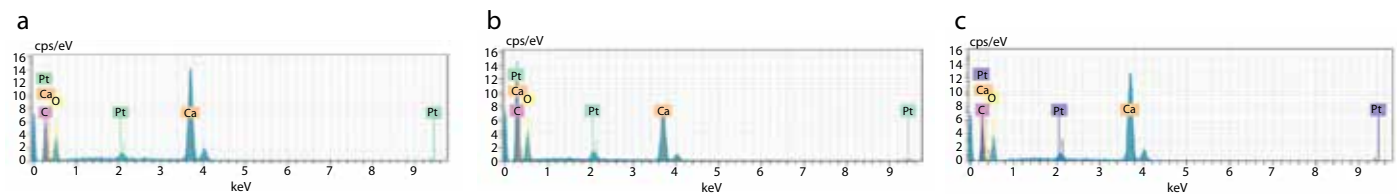


Figure 4. a-c. Energy dispersive X-ray analysis of calcium content in the non-treated group (a), GM-treated group (b), and GBE-treated group (c)
GBE: Ginkgo biloba extract, GM: gentamicin

concentration increased in the GM-treated calcite-gelatin composite group compared with that in the control group. However, treatment with GBE significantly reduced the GM-stimulated nitrite production ($p < 0.05$). The fabricated calcite-gelatin composite had a hexagonal microtablet appearance, as confirmed by SEM (Figure 3a). The surface of the single calcite-gelatin crystal structure dissolved upon GM exposure. The surface also showed erosive fissures and a honeycomb-like appearance (Figure 3b).

Ginkgo biloba extract treatment inhibited the dissolution of calcium-gelatin crystals (Figure 3c). Figure 4 shows the EDX analysis of the calcite-gelatin composite. The resulting spectrum confirmed the presence of elements from the calcite-gelatin composite exposed to GM and a significantly reduced amount of calcium ($p < 0.05$). However, the GBE-treated group demonstrated an insignificant change with respect to the calcium content compared with the control ($p > 0.05$).

DISCUSSION

Since the discovery of the biomimetic growth of artificial otoconia in gelatin gel matrices (calcite-gelatin nanocomposites), which show the same chemical and structural characteristics as human otoconia, it has become possible to detect morphological changes in greater detail, as described in recent studies [4-6]. Aminoglycoside antibiotics have been used for the treatment of gram-negative bacterial infection. However, these drugs have significant risks of nephrotoxicity and ototoxicity [21]. In the inner ear, systemic aminoglycosides cross the blood-labyrinth barrier (BLB) and can damage cochlear hair cells through several mechanisms such as apoptosis or release of reactive oxygen species (ROS), leading to permanent sensorineural hearing loss [22, 23]. GM not only damages the vestibular hair cell but also otoconia [24, 6].

Vestibular toxicity can be prevented by ROS scavengers, such as superoxide anions and glutathione [25]. Studies have shown the *in vitro* and *in vivo* protective effects of GBE against oxidative stress induced by ROS [26].

Gentamicin-induced hair cell damage in cochlear cultures can be prevented by GBE. It acts as an antioxidant and has been shown to significantly reduce GM-induced NO production in cochlear cultures [16, 17, 24]. Our previous report showed that GBE significantly minimizes cochlear damage against endotoxin-induced otitis media with labyrinthitis in a guinea pig model [28]. In the present study, GM-induced NO production was reduced by GBE treatment, and the surface structure was protected. The limitation of this study was the lack of the classical appearance of biologic otoconia. Compared with the artificial otoconia fabricated by Walther et al. [6] or Huang et al. [4], our fabricated artificial otoconia is less sophisticated. This is the first study to evaluate the *in vitro* protective effect of GBE against GM-induced damage of artificial calcite-gelatin composite. Taken together, GM-exposed calcite-gelatin composite showed dissolution of the superficial structure with increased NO production. GBE protected against the GM-induced NO production and superficial structural changes. Further *in vivo* studies are necessary.

Ethics Committee Approval: This study is an *in vitro* study, which does not require ethics committee approval.

Informed Consent: Informed consent is not required for the current *in vitro* study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - C.H.J., J.K.L.; Design - C.H.J.; Supervision - J.K.L.; Resources - C.H.J.; Materials - C.H.J., J.K.L.; Data Collection and/or Processing - C.H.J.; Analysis and/or Interpretation - C.H.J.; Literature Search - J.K.L., C.H.J.; Writing Manuscript - J.K.L., C.H.J.; Critical Review - Y.B.C.

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