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Microelectrode Array-evaluation of Neurotoxic Effects of Magnesium as an Implantable Biomaterial

Ting Huang [1], Zhonghai Wang [2], Lina Wei [1], Mark Kindy [2,3,4], Yufeng Zheng [1,5], Tingfei Xi [1,6,*], Bruce Z. Gao [2,**]

[1] Center for Biomedical Materials and Tissue Engineering, Academy for Advanced Interdisciplinary Studies, Peking University, Beijing 100871, China
[2] Department of Bioengineering, Clemson University, Clemson, SC 29634, USA
[3] Departments of Neurosciences and Regenerative Medicine and Cell Biology, Medical University of South Carolina, Charleston, SC 29466, USA
[4] Ralph H. Johnson VA Medical Center, Charleston, SC 29403, USA
[5] Department of Materials Science and Engineering, College of Engineering, Peking University, Beijing 100871, China
[6] Shenzhen Key Laboratory of Human Tissue Regeneration and Repair, Shenzhen Institute, Peking University, Shenzhen 518057, China

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Magnesium (Mg)-based biomaterials have shown great potential in clinical applications. However, the cytotoxic effects of excessive Mg^{2+} and the corrosion products from Mg-based biomaterials, particularly their effects on neurons, have been little studied. Although viability tests are most commonly used, a functional evaluation is critically needed. Here, both methyl thiazolyl tetrazolium (MTT) and lactate dehydrogenase (LDH) assays were used to test the effect of Mg^{2+} and Mg-extract solution on neuronal viability. Microelectrode arrays (MEAs), which provide long-term, real-time recording of extracellular electrophysiological signals of in vitro neuronal networks, were used to test for toxic effects. The minimum effective concentrations (EC_{min}) of Mg^{2+} from the MTT and LDH assays were 3 mmol/L and 100 mmol/L, respectively, while the EC_{min} obtained from the MEA assay was 0.1 mmol/L. MEA data revealed significant loss of neuronal network activity when the culture was exposed to 25% Mg-extract solution, a concentration that did not affect neuronal viability. For evaluating the biocompatibility of Mg-based biomaterials with neurons, MEA electrophysiological testing is a more precise method than basic cell-viability testing.

1. Introduction

In recent years, magnesium (Mg) and its alloys have been investigated for applications as implantable biomedical materials because of their excellent mechanical properties and complete biodegradability[1]. Clinical application of Mg-based biomaterials has shown great potential in orthopedic implants and cardiovascular stents[2,3], and suitability for applications in nerve regeneration is currently being studied. Vennemeyer[4] created a pure Mg microfilament to serve as a temporary scaffold within a biodegradable poly-caprolactone nerve conduit. Although the range of clinical applications continues to increase, the cytotoxic effects of excessive magnesium ions (Mg^{2+}, a more highly concentrated than normal physiological level) and corrosion products from Mg-based biomaterials have been less studied. Recent reports on Mg-related cytotoxicity have focused mainly on bone and vessel cells and fibroblasts[5]. Few studies have focused on neuronal cytotoxicity.

Cytocompatiblity of Mg-based biomaterials is typically studied using cell-viability tests, such as the methyl thiazolyl tetrazolium (MTT) and lactate dehydrogenase (LDH) assays. However, such tests do not provide information on nonlethal damage to cells. Mg^{2+}, the main corrosion product of Mg-based biomaterial, plays an important role in neuroelectrophysiological function: A voltage-dependent blocker of glutamate N-methyl-D-aspartate (NMDA) receptors, Mg^{2+} is associated with the entry of calcium ions into neurons and the initiation of action potentials[6]. Although increases in extracellular Mg^{2+} concentration caused by biomaterial implantation may not affect cell viability, they can elevate the risk of suppression of neuronal excitability[7]. Therefore, a more precise functional method is required to evaluate the biocompatibility of Mg-based implantable biomaterials.

The microelectrode array (MEA) has been recently used in in vitro electrophysiological testing to supplement patch clamp testing, the traditional method of intracellular electrophysiological recording. MEA assessment provides noninvasive, long-term, real-time extracellular-signal recording with high-throughput and

* Corresponding author. Prof., Ph.D.; Tel.: +86 10 62753404; Fax: +86 10 62753404.
E-mail address: xitingfei@pku.edu.cn (T. Xi).
** Corresponding author. Tel.: +1 864 6560185; Fax:+1 864 6564466.
E-mail address: zgao@clemson.edu (B.Z. Gao).

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multiparameter outputs. In vitro neuronal networks on MEAs, having been developed as biosensors, are widely used in neuropharmacological tests and are now viewed as the physiologically based neurotoxicity-testing platform of the 21st century[8]. However, few studies report electrophysiological testing of biomaterials on the MEA.

Here we studied the effect of the corrosion products of pure Mg and defined concentrations of Mg^{2+} ([Mg^{2+}]) on neurons. First, we studied the effects on neuronal viability by conducting MTT and LDH assays using our chick forebrain neuron (CFBN) culture model. Then, we used our recently established CFBN network–microelectrode array (CFBNN–MEA) biosensor to study neuronal electrophysiology effects. Our CFBNN has been proven to have a comparative development process and firing pattern with rodent cortical neurons that is widely used in MEA study, but with the advantages of abundant resources, cost-effective cultures, and an easy dissection process[9].

2. Materials and Methods

2.1. Material preparation

2.1.1. Preparation of defined concentrations of standard Mg^{2+} solutions

2.1.1.1. Viability test. Magnesium chloride hexahydrate (M2670, Sigma) was dissolved at 100 mmol/L in serum-free neurobasal (NB) culture media (Neurobasal Medium; 21103-049, Gibco, Invitrogen) supplemented with 2% B27 (17504-044, Gibco, Invitrogen), 1% GluMax (35050-061, Gibco, Invitrogen), and 0.5% penicillin/streptomycin (P/S, 10378-016, Gibco, Invitrogen). The medium was then diluted to 30, 10, 3, 1, 0.3, and 0.1 mmol/L [Mg^{2+}] for use in the viability test as standard Mg^{2+} solutions. Because cell-culture medium contains Mg^{2+} at approximately physiological level, [Mg^{2+}] specified in this paper is given as a relative concentration; that is, the amount is our addition to the original [Mg^{2+}] in the media.

2.1.1.2. Electrophysiological test. Magnesium chloride hexahydrate was dissolved in phosphate buffered saline (PBS) at 2 mol/L and was serially diluted to 200, 20, and 2 mmol/L [Mg^{2+}] for use in electrophysiological testing as stock solutions.

2.1.2. Preparation of Mg-extract solutions

Pure Mg (99.95% in purity, Hunan Rare-Earth Metal Research Institute of China) was cut into 1.5 cm square specimens (1.0 mm height). They were polished successively with 400#, 1000#, and 2000# grit paper, ultrasonically cleaned in pure ethanol for 20 min, sterilized in ultraviolet light for 30 min, and dried in a laminar hood. The Mg-extract solutions were prepared according to ISO 10993:12: The Mg specimens were immersed in serum-free NB culture media for 24 h in a standard cell-culture incubator (37 °C, 5% CO_2). The volume of the medium was determined by the specimen's surface area-to-media-volume ratio of 3 cm^2/mL. The medium was then collected, its pH value was measured, and its [Mg^{2+}] was tested using inductively coupled plasma atomic emission spectrometry (ICP-AES, Optima5300DV, USA). Then the collected medium was filtered through a Ø0.22 μm membrane and diluted with untreated culture media to 50% and 25% concentrations. The original and diluted media were then stored as Mg-extract solutions at 4 °C.

2.2. Neuron viability test

2.2.1. Primary neuronal cultures for viability testing

Forebrain tissues were harvested from Day 7 White Leghorn chick embryos as described by Heidemann[10]. The tissues were dissociated enzymatically in 0.25% trypsin with EDTA (T4049, Sigma) for 5 min at 37 °C and were mechanically dissociated by trituration through a 1 mL pipette tip. After centrifugation at 1000 r/min for 5 min, the cells were suspended in M199 media (M4530, Sigma) supplemented with 2% B27 and 1% P/S. Prior to seeding, the 96-well culture plates were coated with 0.05% w/v polyethylenimine (P3143, Sigma) for 2 h and then washed 3 times in deionized water. Cells were plated onto coated culture plates at a density of 6.4×10^4 cells/well (2000 cells/mm^2) and cultured for 24 h in a standard incubator (37 °C, 95% humidity, and 5% CO_2). Then the medium was changed to NB culture media for maintenance. After 3 days, half of the media was replaced with fresh culture media with 2 μmol/L cytosine arabinoside (Ar-AC, C1768, Sigma) to prevent glial proliferation. After 5 days, the medium was completely replaced with 150 μL of standard solution with defined Mg^{2+} concentrations or Mg-extract solutions; fresh culture medium was used as the negative control. The cells were subsequently used for MTT and LDH tests at Day 1, Day 3, and Day 5. Five identical wells were prepared for statistical analysis.

2.2.2. MTT and LDH measurements

At Day 1, Day 3, and Day 5, 50 μL of supernatant was transferred from each well of the culture plates to the corresponding well of new 96-well culture plates for LDH tests. Then, 10 μL of 5 mg/mL MTT (M5655, Sigma) in PBS was added to each well, and the culture plates were incubated for 4 h. The supernatant in each well was carefully removed by aspiration, and 150 μL of dimethylsulfoxide (DMSO, D8418, Sigma) was added. The plates were shaken for 10 min to thoroughly resolve purple formazan and placed on a multidetection microplate reader (BioTek, Synergy™ 4) to measure optical density (OD) at 570 nm; the reference wavelength was 630 nm. The results were expressed as:

$$\text{MTT reduction\%} = \text{OD (test)}/\text{OD (control)} \times 100\%$$

LDH leakage was measured using a commercial LDH Kit (88953, Pierson) according to the manufacturer's protocol. In brief, the 50 μL of medium that was removed before adding MTT was mixed with 50 μL of reaction buffer for 30 min at room temperature in the dark, followed by the addition of 50 μL of stop solution. Using a multidetection microplate reader (BioTek, Synergy™ 4) and a reference wavelength of 680 nm, the OD of the mixtures was measured at 490 nm. The results were expressed as:

$$\text{LDH release\%} = \text{OD (test)}/\text{OD (control)} \times 100\%$$

2.3. Neuronal electrophysiology test

2.3.1. Primary neuronal cultures for electrophysiological testing

For the MEA test, forebrain tissues that contained a high proportion of glial cells were harvested from Day 9 chick embryos. The dissection and dissociation methods were the same as those described in Section 2.2.1. Cells were suspended in M199 media with 2% B27 at a density of approximately 3×10^6 cells/mL. A 20 μL of cell suspension was plated at the center of a standard MEA (60MEA200/30iR-Ti, Multi-Channel Systems, Germany, Fig. 1(a)) according to Hales's protocol[11]. Prior to cell plating, the MEA surface was activated by oxygen plasma treatment (PDC-32G, Harrick) for 3 min and coated with 0.05% w/v polyethylenimine (P3143, Sigma) for 30 min at 37 °C; it was then washed 3 times in deionized water. After plating, the MEA chip was covered with a Teflon® lid (MEA-MEM5, ALA Scientific) that was permeable to gases, but not water and bacteria, to maintain proper culture osmolality and avoid contamination[12] (Fig. 1(b)). Then, the chip was placed in a 100 mm Petri dish and cultured in a humidified incubator at 37 °C with 5% CO_2. 1 mL of M199 media was added to the chip after cells were attached. After 24 h in the culture, the cells were maintained in the