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Polydimethylsiloxanes biocompatibility in PC12 neuronal cell line

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ABSTRACT

Cochlear implants, the only way to recover from severe/profound hearing loss, may cause adverse effects, among which reactions to silicone materials coating implant electrodes, leading to apoptosis and necrosis of spiral ganglion cells. Our aim was to evaluate whether three polydimethylsiloxane (PDMS) compounds (hexadimethylsiloxane, octamethyltrisiloxane, decamethylcyclopentasiloxane) used in silicone rods could exert toxic effects on an *in vitro* neuronal cell model (PC12). Cell viability, morphology and mRNA expression levels of apoptotic markers were evaluated on PC12 cells at different PDMS dilutions up to 6 days of exposure. The results showed that at the highest concentrations tested cell viability was reduced by hexadimethylsiloxane and octamethyltrisiloxane at all times of exposure, but only from 72 h onwards by decamethylcyclopentasiloxane. The number of neurites per cell was not affected by hexadimethylsiloxane, but was significantly reduced from 24 h onwards by octamethyltrisiloxane and decamethylcyclopentasiloxane. Neurite length was reduced by hexadimethylsiloxane only at 24 h, and by octamethyltrisiloxane and decamethylcyclopentasiloxane at all exposure intervals. In controls exposed to silicone or glass rods cell viability was reduced only after 24 h, but neurite number and length was never reduced at any exposure interval. Biomolecular investigations showed that apoptotic markers did not change in any experimental condition, suggesting that PDMS are biocompatible. The reduction of cell viability and neurite number and length caused by exposure to these compounds was probably caused by a PDMS surface film formed over the cell medium, preventing air exchange, and not by the release of cytotoxic molecules.

1. Introduction

Hearing loss is defined as an impairment of the ability to hear sounds that may deeply impact the quality of life and, according to World Health Organization, affects 328 million adults and 32 million children all over the world [1]. Hearing loss consequences include trouble to understand speech sounds, often affecting the ability to communicate, a delay in development of language skills and several socio-economic difficulties. Adults and children with severe/profound sensorineural hearing loss (SNHL), the most common form of deafness occurring after damage of inner ear hair cells and/or spiral ganglion, routinely undergo cochlear implants (CI), surgically implanted electronic devices that directly stimulate the auditory nerve in the inner ear, allowing the recovery of auditory perception [2–4]. Although surgically and technologically advanced [3,5] these devices may benefit from further advancements aimed to prevent adverse effects associated to surgery, leading to delayed auditory recovery and loss of residual hearing [4,6,7]. Recent studies focus on improving CI efficiency and outcome by increasing biocompatibility and biostability of the materials used in their production.

Electrodes in CI are currently coated by the most common form of silicone, polydimethylsiloxane (PDMS) [8,9], but the use of this polymer is impaired by material aging processes [10–12] and intolerance or allergic reactions [13–15]. Aging degradation processes causes the release of low molecular weight siloxanes in the surrounding tissues [16–18] that may cause damages to cochlear neurons, which are unable to regenerate and whose loss is a permanent event [2,19,20]. Therefore, it is necessary to evaluate the toxicity of possible PDMS degradation products on the cochlear neuronal cells, whose activity and functionality is required for CI efficiency [8]. The *in vitro* biocompatibility of solid PDMS by adhesion assay and differentiating inhibition tests was previously shown on a neuronal cell model, the rat pheochromocytoma cell line PC12 [21–23], while biocompatibility data on PDMS degradation products are still lacking. We therefore tested the toxicity of

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three liquid PDMS compounds on PC12 cell line, following previous protocols employed to test the toxicity of the same PDMS compounds on an inner ear cell line [24]. We chose to test hexadimethylsiloxane (6-DMS), octamethyltrisiloxane (8-DMS) and decamethylcyclopentasiloxane (10-DMS) because they are typical compounds to produce silicone suitable to coat cochlear implant electrodes. Current information on PDMS degradation processes mainly derive from investigation of breast prostheses [25]. Relevant amounts of very low molecular weight compounds have been shown to be released from implants [25]. Accordingly, we selected these PDMS because of their very low molecular weight and because 6-DMS and 10-DMS are reported as PDMS degradation products [25].

2. Materials and methods

2.1. Cell culture

The rat pheochromocytoma cell line PC12 was obtained from Interlab Cell Line Collection (ICLC ATL98004, Genoa, Italy). The cells were cultured in RPMI-1640 medium containing 5% fetal bovine serum (FBS, Gibco, Gaithersburg, MD, USA), 10% horse serum (HS, Gibco), 2 mM L-glutamine (Gibco) and 1% penicillin-streptomycin (Pen-Strep, Gibco) (complete medium). Cells were induced to differentiate into neuron-like cells for 6 days using a differentiation medium composed of RPMI-1640 medium containing 0.5% FBS, 1% HS, 2 mM L-glutamine, 1% Pen-Strep and 50 ng/ml nerve growth factor β (NGF-β, Sigma-Aldrich, Milan, Italy). The PC12 cells were maintained at 37 °C in 5% CO_2. After 6 days the cells reached the differentiation plateau, showing at least one neurite per cell with a length equal to the cell body diameter [26].

2.2. Compounds

The liquid PDMS compounds hexadimethylsiloxane (6-DMS), octamethyltrisiloxane (8-DMS) and decamethylcyclopentasiloxane (10-DMS) were purchased from Sigma-Aldrich (Milan, Italy). Silicone rods, provided by Cochlear Research and Development Ltd (Addlestone, UK), were 20 mm long, 1.10 mm in diameter, flexible, transparent and with density lower than 1.00 g/ml. The glass rods were prepared by cutting 20-mm long pieces from Pasteur pipettes made of soda-lime glass with a diameter of about 1.10 mm; the ends of glass rods were melted to seal them. Glass rods were used as silicone rod controls to verify possible mechanical damages.

2.3. Cell viability assay

A total of 2×10^4 cells/well were seeded in 6-well plates containing 2 ml/well of RPMI medium supplemented as previously described (complete medium). The cells were incubated with the differentiation medium for 6 days and at the end of differentiation time were treated with liquid PDMS compounds for 24 h, 48 h, 72 h and 6 days, at dilutions 1:10 and 1:5 for 6-DMS, 1:50 and 1:10 for 8-DMS, 1:1000 and 1:100 for 10-DMS (Table 1), with matching untreated controls. The PDMS compounds were directly added to the well plates containing the complete culture medium. The dilutions were based on the behaviour of the compounds in the culture medium. The three PDMS compounds had different density and viscosity (10-DMS > 8-DMS > 6-DMS), thus their ability to dissolve in the culture medium was different according to the dilution. As controls, cells were also exposed to silicone and glass rods in the same conditions.

Table 1
Dilutions and concentrations (M) of the PDMS compounds tested.

Compound	Dilutions	
6-DMS	1:10 (0.471 M)	1:5 (0.942 M)
8-DMS	1:50 (0.068 M)	1:10 (0.340 M)
10-DMS	1:1000 (0.003 M)	1:100 (0.026 M)

Cell viability was assessed using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Milan, Italy) following the manufacturer's protocol. Cells grown in each well of the 6-well plate for all time intervals were collected, resuspended in 500 µl, divided into 100-µl aliquots and re-seeded in 96-well plates. To each well 20 µl of a solution 20:1 of 2 mg/ml 3- (4,5-dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl)-2- (4-sulfophenyl)- 2H-tetrazolium (MTS) and 0.92 mg/ml phenazine methosulfate (PMS) were added and each plate was incubated for 3 h at 37 °C. Measures for optical density (OD) at 492 nm were performed by an Elisa microplate reader (SIRIO S, SEAC s.r.l, Florence, Italy). The OD values for each sample were normalized to the mean OD value of the respective untreated controls, considered as 100% viability rate. Data were expressed as the mean value ± standard error of the mean (SEM) of three independent experiments, each performed in triplicate.

2.4. Cell morphology

A total of 5×10^3 cells/well were seeded in 6-well plates containing 2 ml/well of RPMI medium supplemented as previously described (complete medium). The cells were incubated with the differentiation medium for 6 days and at the end of differentiation time were treated with the highest dilutions of liquid PDMS compounds for 24 h, 48 h, 72 h and 6 days. Changes in cell morphology were examined by phase contrast microscopy (Nikon Eclipse TE2000-U, Nikon, Florence, Italy). Images were acquired with Nis Elements D 3.2 software (Nikon). Thirty different fields of view (10 fields/well) were analyzed for the number of neurites and their length using the ImageJ software (https://imagej.nih.gov/ij/). The number of neurites was calculated with data normalized to the related number of neurons, the neurite length was calculated with data normalized to the related number of neurites. Values calculated for each sample were normalized to the mean value of the respective untreated controls, considered as 100% rate. Data were expressed as the mean value ± SEM of three independent experiments, each performed in triplicate.

2.5. Real time PCR

A total of 2×10^4 cells/well were seeded in 6-wells plates containing 2 ml/well of complete medium. The cells were incubated with the differentiation medium for 6 days and at the end of differentiation time were treated with 6-DMS, 8-DMS and 10-DMS for 24 h and 6 days, at the dilutions shown in Table 1, with matching untreated controls. Cells were also exposed to silicone rods and to glass rods in the same conditions. At the end of the incubation time, total RNA was extracted with Trizol ® (Thermo Fisher Scientific, Milan, Italy) and treated with DNase I (New England Biolabs, Hitchin, UK) following manufacturer's protocols. The samples were reverse transcribed using iScript™ Reverse Transcription Supermix (Bio-Rad, Milan, Italy). The quantitative Real Time PCR (RTqPCR) for the genes of interest (*Bax*, *Bcl2, Bcl2l11*, *Bid*, *Casp3*, *Casp8*, *Casp9*, *Tp53*) was performed in a Chromo-4 System thermocycler (Bio-Rad) with Sso-fast™ EvaGreen® Supermix (Bio-Rad). Gene data were analysed with the comparative cycle threshold method, using GenEx 6.1 software (bioMCC, http://www.biomcc.com/genex-software.html) with three housekeeping genes (*Actb*, *B2m* and *Gapdh*) as reference. Primer sequences of investigated genes are reported in (Table 2. The results were represented as mean value ± SEM *versus* untreated cells of at least three independent experiments performed in duplicate. The *Bax/Bcl2* ratio was calculated with data normalized to controls as previously reported [24].

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