BASIC SCIENCE

Nanomedicine: Nanotechnology, Biology, and Medicine
12 (2016) 1151–1159

Original Article

nanomedjournal.com

Biocompatibility assessment of fibrous nanomaterials in mammalian embryos

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Received 27 September 2015; accepted 15 January 2016

Abstract

Currently there is a growing interest in the use of nanotechnology in reproductive medicine and reproductive biology. However, their toxic effects on mammalian embryos remain poorly understood. In this work, we evaluate the biocompatibility of two fibrous nanomaterials (NMs): cotton cellulose nanofibers (CNF) and carboxylated multiwalled carbon nanotubes (MWCNT-COOH), by performing an investigation of the embryonic development, gene expression (biomarkers focused on cell stress, apoptosis and totipotency) and *in situ* apoptosis in bovine embryos. Exposure to NMs did not interfere in preimplantation development or in the incidence of apoptosis in the bovine embryo, but they did affect the gene expression. The results presented are important for an understanding of the toxicity of cotton CNF and MWCNT-COOH on mammalian embryos. To our knowledge, we report the first evaluation of biocompatibility between these NMs on preimplantation embryos, which may open a new window for reproductive biomedical applications.

Key words: Fibrous nanomaterials; Biocompatibility; Gene expression; Apoptosis

Nanomaterials (NMs) have garnered increasing interest recently in medicine and biology fields. Among existing NMs, cellulose nanofibers (CNF) and carbon nanotube (CNT) are fibrous NM which have received considerable attention. CNFs have emerged as attractive NMs due to their hydrophilicity, flexibility, mechanical strength, broad chemical-modifying capacity, biodegradability aspect and low cost.[1,2] CNTs have unique characteristics, such as large contact surface, stability, flexibility, stiffness, strength, as well as thermal and electrical conductivities.[3] These NMs can be applied in drug delivery,[4,5] regenerative medicine,[6,7] and diagnostic systems.[8]

In recent years, nanotechnology has been introduced into the fields of reproductive medicine and reproductive biology. Emerging reproductive applications of NMs include treatment of chronic disease, support of assisted reproduction techniques and embryogenesis.[9–11]

Despite their tremendous potential, little is known about the effects of these NMs on mammalian embryos. Further, most of the studies on NMs have focused on their applications in nanotechnology, often overlooking, during their design and fabrication, the toxic effects associated with their use. The unique chemical and physical properties of NMs, such as the small size, shape, and high reactivity, which enable their applications in diverse areas might also render them potentially toxic to cells and tissues.[12] The results found in the literature often show discrepancies and variability depending on the cell type under investigation, surface functionalization, and NM size. Therefore, in-depth studies are needed to better understand the potential deleterious effects of NMs and to optimize the use of nanotechnology in the field of biology and medicine.

In the present study, we used bovine embryos as the experimental model due to the ethical and practical work limitations when it comes to working with human embryos and,

Funding sources: This study was supported by the Rede de Nanotecnologia Aplicada ao Agronegócio (AGRONANO), Research Center for Biotechnological Applications of Carbon Nanotubes under the PRONEX grant (CBB - APQ-04334-10), FAPEMIG, CNPq, CAPES (04/CII-2008-Projet 7 Network Brazil Nanobiotec) and Brazilian Ministry of Agriculture, Livestock and Food Supply (Department of precision livestock).

Conflict of interest: The authors declare that they have no conflict of interest.

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http://dx.doi.org/10.1016/j.nano.2016.01.006
1549-9634/

in many respects, bovine embryos constitute a good substitute model for human embryos. Moreover, bovine animals are normally monovulators just like humans, the embryos are about the same diameter and have a broadly similar pattern of energy metabolism, measured as oxygen, pyruvate, and glucose consumption and lactate production.[13] Approximately the same proportion of *in vitro*-produced bovine zygotes reach the blastocyst stage when *in vitro* and major zygotic genome activation is initiated at closely related stages.[14]

Thus, the aim of the present study was to evaluate *in vitro* biocompatibility of cotton CNF and carboxylated multiwalled carbon nanotubes (MWCNT-COOH), by investigating the developmental competence, gene expression of biomarkers related to cell stress and apoptosis (*PRDX1*, *BAX*, *HSP70.1*) and totipotency (*HAD1*, *OCT4*) and apoptosis in bovine embryos.

This work provides a direct comparison of the impact of cotton CNF and MWCNT-COOH on mammalian embryos. To our knowledge, we report the first *in vitro* assessment of the biocompatibility between these NMs and preimplantation embryos.

Methods

All chemicals were from Sigma Chemical (St. Louis, MO, USA), unless otherwise stated.

Preparation of nanomaterials

CNF was prepared by acid hydrolysis of commercial cotton fibers purchased from the local market. The fibers were finely chopped in a knife mill, passed through a 10-mesh sieve, dewaxed with 1:1 (v/v) ethanol:cyclohexane for 12 h in a soxhlet apparatus and then vigorously washed with tap water. The dewaxed samples were dried for 12 h at 100 °C in an air-circulating oven. About 5 g of fibers were dispersed in 100 mL of 6.5 M sulfuric acid at 45 °C and stirred vigorously for 75 min. After that, 500 mL of cold distilled water was added to stop the reaction. The sulfuric acid was partially removed from the resulting suspension by centrifugation at 8,000 × g for 15 min. The non-reactive sulfate groups were removed by centrifugation followed by dialysis. Then the fibers were resuspended and dialyzed against tap water with a tubing cellulose membrane (76 mm, D9402- Sigma) until the pH reached 6–7. The resulting suspension was sonicated (Branson 450 sonifier, Branson Ultrasonics, Danbury, USA) for 5 min (in ice bath) and stored in a refrigerator.

The MWCNTs were synthesized using a floating catalytic chemical vapor deposition process using ferrocene and ethylene as the transition metal and carbon precursors, respectively. After the synthesis, the MWCNTs were submitted to a simple purification process by washing and filtering several times with isopropyl alcohol in a Millipore filtration system in order to remove any non-reacted ferrocene and other carbon impurities. After the cleaning process, the MWCNTs were dried at 80 °C for 12 h and functionalized with carboxyl through oxidation in nitric/sulfuric acid for 15 min. The MWCNT-COOH were then washed in neutral pH, and dried at 60 °C during 12 h.

Transmission electron microscope (TEM) analysis

An aliquot of cotton CNF suspension was diluted and sonicated for 5 min. A drop of this resultant diluted suspension was deposited on a carbon micro grid net (400 meshes) and the grid was stained with a 1.5% solution of uranyl acetate and dried at room temperature. Samples MWCNT-COOHs were prepared by ultrasonic dispersing in ethanol and dropping on a carbon-coated copper grid. The NMs were characterized by TEM using an FEI Tecnai G2 Spirit electron microscope at 120 kV.

Zeta potential

The Zeta potential of the cotton CNF and MWCNT-COOH were determined by microelectrophoresis laser Doppler technique (Zetasizer Nano ZN; Malvern Instruments Ltd, Malvern, Worcestershire, UK).

In vitro production embryos

The oocytes were obtained from ovaries collected from slaughtered cows. After selection, oocytes were matured *in vitro* in TCM-199 media (Gibco Life Technologies, Inc., Grand Island, NY, USA), supplemented with 10% fetal calf serum (FCS; Nutricell, Campinas, SP, Brazil), 20 μg mL^{-1} follicle stimulating hormone (FSH; Pluset, Serono, Italy) and incubated at 5% CO_2, 38 °C in the air and 95% humidity for 24 h. *In vitro* fertilization was performed in 100-mL drops of Fert-TALP supplemented with 2×10^6 spermatozoa mL^{-1}, 20 mg mL^{-1} heparin and 6 mg mL^{-1} fatty acid-free bovine serum albumin (BSA) Fraction V and covered with mineral oil for 21 h at 38 °C under 5% CO_2 in humidified air. Presumptive zygotes were cultured in Charles Rosenkrans 2 (CR2aa) medium with 10% FCS at 5% CO_2, 38 °C and 95% humidity for 7 days.

Exposure of embryos to nanomaterials

Cotton CNF or MWCNT-COOH were dispersed in 2 μg mL^{-1} in CR2aa medium and treated with ultrasonic agitation under 200 W of power, at 24 kHz working frequency and 50% pulse factors per second (UP200, Hieslcher-Germany) for 1 min at 4 °C. Afterwards, cotton CNF or MWCNTs were diluted in CR2aa medium (final concentration of 0.2 μg mL^{-1}) and 10% FCS; they were subsequently used for embryo culture. The selection of this concentration was based on previous studies that we evaluated the cytotoxicity of cotton CNF on bovine fibroblast cells.[15]

On day 7 post-fertilization embryos at the blastocyst stage were randomly distributed into three culture groups: the control group (without nanomaterials; number of embryos = 43), the cotton CNF group (0.2 μg mL^{-1}; number of embryos = 41) and the MWCNT-COOH group (0.2 μg mL^{-1}; number of embryos = 46). Embryos in all groups were cultured in CR2aa medium, supplemented with 10% FCS and granulosa cell monolayer for 72 h in microdrops covered with mineral oil under 5% CO_2 at 38 °C in the air and 95% humidity. After 72 h of exposure to NMs during *in vitro* culture, the embryos at blastocyst stage (10 days post-fertilization) were recovered and submitted to apoptosis and gene expression analysis.