2

Cerium Dioxide Nanoparticles Protect Cardiac Progenitor Cells against the Oxidative Stress

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Abstract

In the last decade, the combined applications of nano- and stem cell technology are among the newest approaches in regenerative medicine and Tissue Engineering (TE). In this context, the possibility to fabricate scaffolds with composite materials consisting of a polymer matrix and nanoparticles (NPs) as structural elements could allow to develop a novel generation of bioactive materials, capable of directing and controlling cell behavior. In particular, cerium dioxide (CeO_2) NPs are promising tools to scavenge reactive oxygen species (ROS) and to confer protection to cells from the oxidative stress owing to cerium ability to switch the oxidation state ($Ce^{4+/}Ce^{3+}$). In the present experimental study, 10, 25, or 50 μg/mL CeO_2 powder was administered to murine adult cardiac progenitor cells (CPCs) in complete medium for 24 hours (h) and the effects onto cells evaluated at 1, 3 and 7 days (d) from the ceria powder withdrawal from the culture. After a single 24 h CeO_2

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pulse, CPCs were able to take up the NPs and retain them inside the cytosol, while preserving their stemness phenotype and multipotential capability at all time-points considered. Moreover, when challenged with 50 μM H_2O_2 for 30 min, CeO_2-treated CPCs were protected from the oxidative stress. In particular, after 24 h, only the highest concentration was protective; after 7 d, ROS levels were mitigated with all concentrations. This study demonstrated that internalized CeO_2 NPs can act as a long-term defense against the oxidative insult. NPs were activated only when cells were hit by an external oxidative perturbation, remaining inert in respect to the main CPC characteristics. In conclusion, these results suggest that CeO_2 nanoparticles hold an enormous potential in TE treatments protecting stem cells against the oxidative damage.

Keywords: Cerium dioxide, cardiac precursor cells, tissue engineering, reactive oxygen species.

2.1 Interaction of Cerium Oxide Nanoparticles with Biological Systems

Over the last few years, nanotechnology has made significant strides especially in the field of regenerative medicine, thus enabling the development of a new generation of nanostructured biomaterials for medical applications. In particular, nano-composite hybrid scaffolds, made by incorporating nanoparticles into bio-compatible/erodible polymeric matrices, have gained rising attention. The possibility to fine-tune the properties of these materials to meet a broad range of applications makes them attractive systems for tissue engineering. For example, polymeric scaffolds loaded with hydroxyapatite nanoparticles are already used for bone tissue reconstruction [1]. In this respect, deciphering how cells interact with scaffolds and the mechanisms through which nano-components are internalized without exerting direct effects on cell behavior is particularly intriguing in order to obtain novel biomaterials with promising and controllable properties.

Recently, cerium oxide nanoparticles (CeO_2, nanoceria) have been demonstrated to favor cardiac precursor cell (CPC) adhesion and growth when embedded into PLGA scaffolds [2]. In particular, cerium oxide nanoparticle filling of PLGA films resulted in enhanced mechanical properties and in a change in scaffold nano-rugosity. On these functionalized supports, cells exhibited better adhesion and growth as compared with PLGA alone. CPCs were able to acquire a typical alignment, due to support rugosity, which, combined with that determined by the presence of ceramic nanoparticles, provided

better anchorage sites for cell engraftment. Nevertheless, cardiac-derived cells displayed better growth performance when cultured onto CeO_2-PLGA films, as compared with films loaded with titanium oxide (TiO_2), thus suggesting a potential chemical stimulus can be exerted by ceria nanoparticles on cardiac resident progenitor cells [2].

CeO_2 is a rare earth oxide material of the lanthanide series commonly used in important industrial applications [3, 4], but recent reports highlighted the beneficial effects of cerium oxide in biological systems [5, 6]. In particular, it has been proposed that ceria nanoparticles could exhibit an oxidant scavenging activity reducing the cytotoxic effects of intracellular oxidative stress conditions via changes of the oxidation state: Ce^{4+} / Ce^{3+} [7–9]. Ceria nanoparticles display their unique property to store and release oxygen because of the great mobility of these atoms inside the lattice; each released oxygen atom causes the formation of a vacancy and electron transfer to Ce^{4+} which reduces to Ce^{3+}. This mechanism seems to be greatly facilitated in nanoparticles, where the higher surface area is accompanied by more oxygen vacancies and, thus, higher Ce^{3+} concentration in the lattice, resulting in enhanced catalytic properties [10, 11]. Indeed, reactive oxygen species (ROS), such as superoxides and peroxides, could react on these active sites and be counteracted; as a consequence, Ce^{3+} ions would be oxidized in Ce^{4+} ions in a reversible and autocatalytic way. This is because of the cerium ability to switch between the 3+ state under reducing conditions and 4+ state under oxidizing conditions [6, 12]. This ability, combined with multiple active sites that may be generated on a single nanoparticle, could provide an explanation to ceria antioxidant properties with the ability to scavenge ROS and mostly as a catalyst with superoxide dismutase (SOD) and catalase mimetic activities [13–15]. These properties candidate ceria as a novel long-lasting antioxidant compound with the promise to actively participate in mitigating oxidative stress, which is considered a critical actor in the establishment and progression of several diseases, including cardiovascular dysfunctions [16–19], or after treatments such as chemotherapy [20].

2.2 Cerium Oxide Nanoparticles Shield Cardiac Precursor Cells against the Oxidative Stress

In the last decade, evidence has been acquired that an adult stem cell pool is present in almost every organ of the body. These cells are endowed with self-renewal capability and can be committed to a specific cell lineage. The identification of a cardiac progenitor cell (CPC) population in the adult

mammalian heart has definitively broken the dogma that the adult myocardium is a terminally differentiated tissue [21, 22]. CPCs are believed to control the healthy tissue homeostasis and to repair the diseased tissue in pathological conditions [23]. Cardiovascular diseases, such as the Myocardial Infarction (MI), represent the first cause of mortality and morbidity in both industrialized and developing countries, and the oxidative stress is cause and/or consequence leading to a reduced cardiac functionality [24]. Moreover, ROS plays a crucial role in reducing stem cell lifespan and inducing senescence [25] as well as in maintaining self-renewal [26] into special hypoxic microenvironments, named "niches". Ito K. and co-workers have demonstrated an active role of ROS in inducing loss of Hematopoietic Stem Cell self-renewal capacity via p38 MAPK phosphorylation [27]. In particular, in the post-ischemic myocardium, the release of inflammatory cytokines and ROS production [28–30] generate a hostile microenvironment, which, on one hand, could favour stem cell recruitment, also from other body districts [31], while, on the other hand, might hamper resident progenitor cell proliferation and differentiation [32]. Indeed, recent reports suggested an inductive role for low-levels of oxidant production and cytokines in promoting stem cell differentiation [32–34] and in cardiomyogenesis during the embryonic development [35]. Conversely, the generation of high-ROS levels during pathophysiological conditions contributes to cell damage and remodelling [36]. Thus, the stem cell behavior appears tightly dependent on the microenvironmental niche properties, not only in terms of nutrient and oxygen supply, but also of reactive oxygen species balance [37].

In a recent work, our research group investigated mouse Lin^{neg}/Sca-1^{pos} CPC (CPCs) response following CeO_2 nanoparticle treatment [38]. In particular, the possibility that CeO_2 could confer protection to cells against the oxidative stress was investigated. For this purpose CPCs were grown in the presence of various concentrations of CeO_2 (10 μg/mL, 25 μg/mL and 50 μg/mL) having mean particle size of about 5–8 nm (Figure 2.1).

Ceria antioxidant effects have been already proved in other biological systems, but the interactions between these nanoparticles and cardiac resident progenitor cells has never been analyzed, to the best of our knowledge. In this study, cells were subjected to a single administration for 24 hours; after that, ceria was removed and analyses were conducted. Interestingly, at 7 days after ceria withdrawal, nanoparticles had been internalized and retained as aggregates inside the cell cytoplasm (Figure 2.2).

CPC morphology and undifferentiated phenotype were not affected being Sca-1 expression stable at all time points and preserved at high levels with

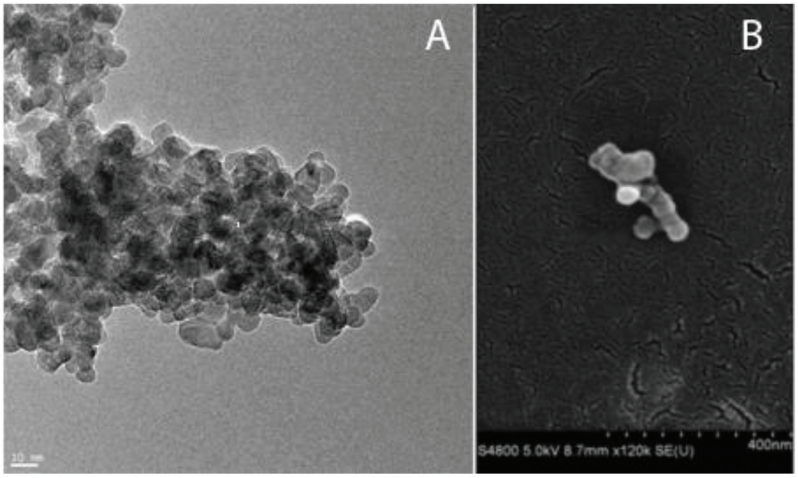


Figure 2.1 Transmission (A) and Scanning (B) electron micrographs of CeO_2 nanoparticles.

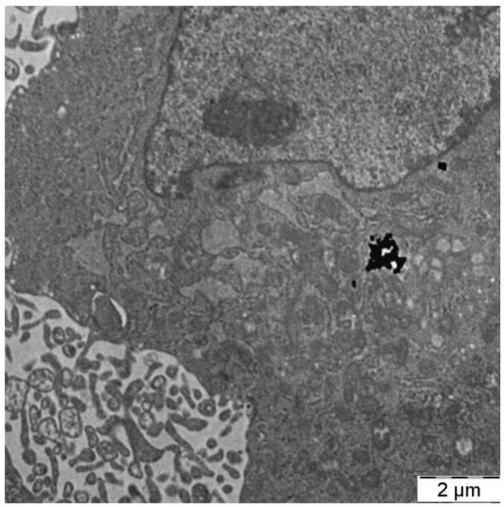
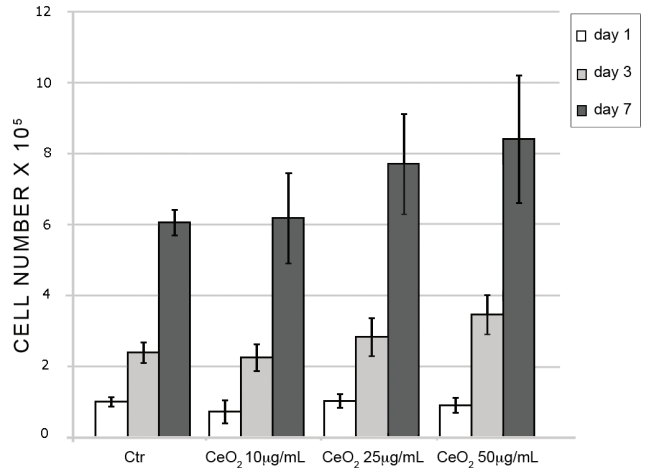
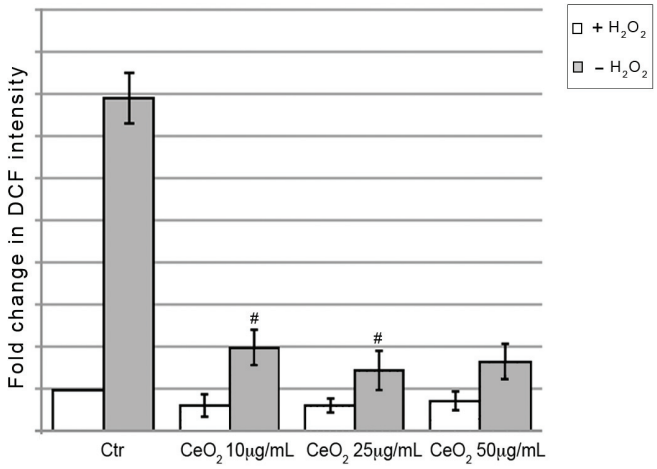


Figure 2.2 Representative TEM micrograph showing CeO_2 NPs inside the cytoplasm of a CPC at 7 days from the administration of 50 µg/mL CeO_2 *in vitro*.

all ceria concentrations. Also self-renewal and multipotency, two important properties defining stem cells, were maintained when CPC were pre-treated with 10, 25 and 50 µg/mL of CeO_2 nanoparticles. Cell counts at 1 d, 3 d and 7 d demonstrated that cells were viable and proliferating without significant differences in respect to untreated controls (Figure 2.3, A). Appropriate



A



B

Figure 2.3 A) CPC proliferation assessed at 1 d, 3 d, and 7 d after 24 h CeO_2 exposure. The values are expressed as means $\pm$ SD of three independent experiments. (#= $p > 0.05$). B) Effect of $H2O_2$ on intracellular ROS levels in Lin- Sca-1pos CPCs at 7 days after CeO_2 treatment. ROS production, measured using a DCFH probe, decreased with all NPs concentrations tested. (#= H_2O_2 treated cells vs. CeO_2-$H2O_2$ treated cells). The values are expressed as means $\pm$ SD of the fold change in DCF fluorescence intensity with respect to H_2O_2-untreated control (ctr-) from three different tests ($p < 0.05$).

differentiating stimuli as well as the presence of neonatal cardiomyocytes in direct co-culture with CeO_2-treated CPCs were able to induce adipogenic, osteogenic and cardiac commitment as demonstrated by the occurrence of lipid droplets, calcium deposits, the up-regulation of GATA-4, the membrane translocation of connexin 43 and the expression of a-sarcomeric actinin, respectively. Furthermore, the incubation with 24 h CeO_2 did not promote a pro-oxidant effect in cells, meaning that, in the absence of any stress stimulus, ROS levels remained unmodified as compared to controls (Figure 2.3, B).

Altogether, these results demonstrated that no toxic effects were exerted by CeO_2 nanoparticles on CPCs at the concentrations tested and NPs were activated only when cells were hit by an external oxidative perturbation, remaining inert in respect to CPC homeostasis and differentiation. In fact, H_2O_2-induced cell injury and subsequent dichlorofluorescein fluorescence assay revealed a strong capability to reduce the oxidative stress in the long run (7 days) with all concentrations, while only the higher dose (50 μg/mL) was protective in the short run (24 hours). In fact, ROS production decreased by an initial 30% with 50 μg/mL NPs to approximately 50% and 75% at 3 and 7 d, respectively, with 25 and 10 μg/mL as well. In agreement with other reports, our data indicated that intracellular nanoparticles worked as a potent scavenger able to protect cells from the oxidative damage and markedly attenuated ROS production over time. Consistently, Das et al. reported that, after 30 d, CeO_2 was still able to protect spinal cord cells from H_2O_2-induced cytotoxicity, showing better survival than their untreated counterpart [12]. Thus, these results suggest that with a single nanoparticle administration, CeO_2 could be able to act over time, limiting the generation of ROS following a tissue damage and, so, favouring the establishment of better conditions for CPC proliferation and differentiation *in vivo*. On the other hand, such findings also indicated that a possible intracellular threshold level could be necessary before the antioxidant effects could appear. It is clear that a number of aspects, such as the synthesis techniques, nanoceria characteristics in terms of size, shape and charge, dosage and exposure time, administration procedures, need to be considered and thoroughly tuned for maximizing the beneficial effects of the nanoceria before these nanoparticles could be effectively used in therapeutic applications [39]. Indeed, differences in each of these parameters could help explaining the conflicting results so far obtained with cerium oxide in biological systems [5, 38–41]. Recently, Park et al. showed that cerium oxide treatment induced oxidative stress with ROS formation and cytotoxicity in some mammalian cells; however, the authors also admitted that these

effects were not detectable in other cell types [41]. Therefore, it could be worth conducting further investigations in order: i) to elucidate the biological mechanisms behind the action of cerium oxide; ii) to understand the interactions between this promising material and tissues (both healthy and damaged) *in vivo*.

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