Effects of cerium oxide nanoparticles on hemostasis: coagulation, platelets and vascular endothelial cells

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ABSTRACT

Cerium oxide nanoparticles (nanoceria, NC) have attracted much attention in biomedicine due to their surface composition that confers interesting redox activities and regenerative properties. Studies have demonstrated that the application of nanoparticles in biomedicine can influence components of hemostatic system, inducing blood clotting, alterations of blood cells, and endothelial cell functions. NC were tested *in vitro* to assess their hemocompatibility and anti-coagulant, anti-inflammatory and anti-senescence activity in human endothelial cells. Hemocompatibility has been evaluated in vitro looking at the impact of NC on coagulation times, fibrinogen and platelet aggregation. The effect of NC on vascular endothelial cells were assayed by testing cell viability, antioxidant activity, anticoagulant (tissue factor, TF-mRNA expression) and anti-inflammatory properties (VCAM-1 exposure, cytokine release) and senescence (telomere shortening). Nanoceria did not show significant effects on coagulation process, hemolysis or platelet aggregation. In endothelial cells, NC did not affect cell viability, reduced oxidative stress, inhibited mRNA-TF expression, VCAM-1 expression and cytokine release. Moreover, NC reduce telomere shortening, possibly counteracting premature senescence. The hemocompatibility combined with anticoagulant and anti-inflammatory phenotype and the ability of counteract the premature senescence in vascular cells make NC a promising therapeutic tool in oxidative stress-related conditions.

Key words Nanoparticles, hemocompatibility, coagulation, endothelial cells, senescence

INTRODUCTION

The most promising application of nanotechnology in biomedical field is the development of new nanomaterials, such as nanoparticles (NPs), useful for early diagnosis and therapy of diseases, associated to careful monitoring for their potential toxicity.[1]

Recently, nanoparticles of cerium oxide (nanoceria, NC), a rare earth element in the lanthanide series, have attracted great interest owing to their regenerative antioxidant properties. Nanoceria show interesting redox activities and regenerative properties owing to the presence of a fraction of Ce^{3+} ions and oxygen vacancies on NC surface that lead to the coexistence of both Ce^{3+}/Ce^{4+} ions in a stable form.[2] The presence of a mixed valance state plays an important role in scavenging reactive oxygen species (ROS) and nitrogen species in self-regenerating activity. The use of NC is of particular relevance in those disorders where the oxidative stress plays a key role and that, if insufficiently buffered by intracellular antioxidant enzyme such as superoxide dismutase (SOD), contribute to the beginning and the progression of chronic diseases.[3] Studies have shown that NC, acting as ROS scavengers, are able to mimic the activity of SOD[2] and catalase.[4] According to their important intrinsic antioxidant and auto-regenerative properties, NC applications have attracted increasingly interest in different fields of medicine.[5, 6] Nanoceria have been evaluated *in vivo* for their efficacy as treatment in a variety of disease states, with applications ranging from reproductive[7], to gastrointestinal[8], to ophthalmologic [9, 10], to neurological [11] and cardiovascular health[12]. In anti-cancer treatment, data have shown NC to be toxic to cancer cells sensitizing them to radiation therapy, while displaying minimal toxicity to normal tissues[13, 14]. Studies have demonstrated the utility of NC in tissue engineering and regenerative medicine, since an adequate presence of NC embedded into the scaffold or into cells could create a favorable milieu for cell migration and proliferation, and promote stem cell differentiation.[15-17].

The design and development of therapeutic nanosystems in medicine require in vitro biocompatibility studies in order to evaluate the impact on hemostatic balance, achieved by the

interaction of coagulation factors, platelets and vascular endothelial cells.[18] In fact, NPs could induce toxicity through the interaction with each component of hemostatic system, inducing blood clotting, platelet aggregation, red cell damage and promoting inflammation and thrombotic complications, thus limiting their biomedical application.[18, 19] To date, studies of NC hemocompatibility on human blood have not yet been performed.

The vascular endothelium is the first barrier that circulating NPs meet after their administration before reaching the target cells. Alterations of endothelial balance towards a pro-inflammatory and pro-thrombotic phenotype lead to endothelial activation, characterized by increased generation of ROS, cytokines, adhesion molecule expression as well as tissue factor (TF), the trigger of the extrinsic pathway of the coagulation.[20] The endothelial activation elicits pathophysiological conditions such as atherosclerosis, thrombosis, and myocardial infarction.[20].

Therefore, the aim of this investigation was to examine the effects of NC: 1) on blood clotting, platelet aggregation and integrity of red cells; 2) on human endothelial cells in physiological and inflammatory conditions, evaluating cell viability, procoagulant and pro-inflammatory phenotype and premature senescence.

MATERIALS AND METHODS

Preparation and characterization of cerium oxide nanoparticle

Cerium oxide nanoparticles (544841, Sigma-Aldrich, St. Louis, MO, USA) extensively characterized in previous works[21] appear as a powder of quite dispersed size distribution (5-80 nm), with a cubic crystalline structure, high purity, and a Ce^{3+} content of a 23%, suitable for an optimal NC redox activity.[22] Moreover, as the Ce^{4+} fraction is greater (about 77%) than the Ce^{3+} fraction, a more pronounced catalase activity is expected.[5, 23] Nanoceria were dispersed in ultrapure MilliQ water (Millipore) at a concentration of 10 mg/mL through a mild sonication, and then diluted in cell culture

medium before the experiments. Particle size distribution and Z-potential of NC in cell culture medium were characterized as previously described.[24]

For the characterization of NC suspension, samples were prepared by putting a drop of suspension onto a 300 mesh carbon coated copper grids and analyzed with a Zeiss LIBRA 120 plus transmission electron microscope (TEM), equipped with an in column omega filter.

Rough estimation of effective exposed surface area of the NC dispersions can be obtained by considering cubical shape of particles, and calculating the respective surface area. Being l the cube edge length, the respective surface area of the particle is simply $A_{NP} = 6\ l^2$. Considering a nominal dimension of 25 nm (dispersion of dimensions is quite high, but this is a reasonable value for calculating bottom limit of active surface area) and density of NC d_{NC}=7.13 g/cm^3, it is possible obtain the nominal active surface for unit of mass $A_{act} = 6 / (d_{NC} * l) = 3.36*10^5$ cm^2/g.

Coagulation tests and platelet aggregometry

Intrinsic, extrinsic and common final pathway of blood coagulation were evaluated by activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT), respectively. All of the clotting time-based assays are dependent on the conversion of fibrinogen to fibrin. Sodium citrate blood was collected from five healthy donors in accordance with the ethical guidelines after informed consent. The samples were centrifuged at 3500 rpm to obtain platelet-poor plasma (PPP). Different concentrations of NC (10, 25, 50 μg/mL) were mixed with 500 μL of PPP and incubated at 37°C for 30 min under agitation. Nominal exposed surface area of the NC in the prepared samples can be calculated as 3.36 cm^2/ml, 8.4 cm^2/ml and 16.8 cm^2/ml, respectively. These values cover the suggested range for reliable estimation of blood-material interactions in coagulation tests. The APTT test was performed with Pathromtin* SL (Siemens Healthcare Diagnostics, Products GmbH, Marburg, Germany) incubating plasma with the optimal quantity of phospholipids and a surface activator for factors of the

intrinsic coagulation system. Thromborel S Reagent (Siemens Healthcare Diagnostics) was used for PT determination, by incubating plasma with optimal amount of human placental thromboplastin that triggers the coagulation extrinsic pathway after addition of calcium ions. The TT test was performed incubating plasma with thrombin (BC thrombin reagent, Siemens Healthcare Diagnostics) that converts the fibrinogen into fibrin. All times of fibrin clot were monitored in an automated BCS –XP analyzer (Siemens Healthcare Diagnostics). Coagulation times were compared to standard controls for each assay.

Since the routinely APTT and PT tests use activators that could mask little changes in clotting times in order to trigger the blood coagulation, we have also investigated plasma recalcification time (PRT). Different concentrations of NC were incubated with 200 µL of PPP, obtained as described above, and the solutions were added into siliconized glass tubes. Then, 200 µL of $CaCl_2$ solution (0.25 mol/L) pre-heated at 37°C were added to trigger the coagulation at the start of sampling time. The time until the PPP clouded and fibrin clot formation initially began was recorded as the PRT. Glass tube was taken as the positive control and siliconized glass tube (without NC) as negative control.

Fibrinogen concentration, a critical component of the blood clotting cascade, was measured using the Clauss method with some modifications (Multifibren® U, Siemens Health car).

Platelet aggregation was evaluated by impedance method using a CHRONO-log® Whole Blood aggregometer (Model 591/592) (Mascia Brunelli, SpA Milan, Italy). Briefly, a 1:1 dilution with 0.5 mL of physiological saline and the specimen of whole blood was performed in a sample cuvette and placed in the device reaction well, warmed for 5 min at 37°C with stirring. A baseline was established and aggregation initiated by adenosine diphosphate (ADP, 20 µmol/L). In order to evaluate their effects, NC were added to blood for 5 minutes before adding ADP. Platelet aggregability was expressed as the change of in electrical impedance (Ω). Aggregation curves were recorded for 6 min and analyzed using AGGROLINK software.

Hemolysis test and blood film staining

To assess the effect of NC on erythrocyte integrity, hemolysis test was performed by spectrophotometric measurement of hemoglobin release, after erythrocyte lysis. Aliquots of NC (0.2 mL) in PBS at different concentrations (10-25-50 μg/mL) were added to 0.8 mL of sodium-citrate whole blood and incubated at 37°C for 1 and 4 h at constant vortexing. Then, plasma was separated by centrifugation at 1000g for 10 min and optical density of the supernatant was measured at 540 nm in a plate reader. Control samples were prepared incubating whole blood with PBS, without NP suspension, at the ratio corresponding to NP sample dilution. As positive control, we have used a detergent, Triton X-10 (1%) that induces hemolysis of red cells. Sample absorbance was corrected for background interference due to NP suspension in PBS without blood.

Smears of peripheral blood stained with May Grunwald and Giemsa dye (Sigma-Aldrich) were obtained from EDTA-blood samples incubated without and with NC and analyzed by optical microscopy.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were harvested and isolated as previously described.[25] Human cells were obtained from discarded umbilical cords and treated anonymously. As such, approval from the University Ethics Review Board was not necessary. All experiments were performed with low-serum culture medium (2%) (Promocell GmbH, Heidelberg Germany)

TEM analysis

Endothelial cells were grown to confluence in 35-mm dishes and treated with/without NC (25 μg/mL). After 3-6-24-48-72 h, HUVEC monolayers were fixed in a 1.5% glutaraldehyde solution in Na

cacodylate buffer, for 30 min at RT. For the ultrastructural analysis of NC uptake, cells were treated as previously described.[26] Thin 90-nm sections were cut using a UC7 (Leica Microsystems, Vienna, Austria) for TEM analysis.

Cell viability, apoptosis and intracellular reactive oxygen species assay

The viability of HUVECs was assayed in presence of NC at 10-100 μg/mL by colorimetric assay using WST-1 (Biovision, San Francisco, USA) as previously described.[27]

Apoptosis was evaluated in HUVECs incubated for 72 h with the highest concentration of NC (50 μg/mL) compatible with cell viability, and then carried out with annexin-V FITC/propidium iodide (PI) apoptosis detection kit (Sigma) according to the manufacturer's instructions. After staining, samples were diluted with binding buffer and assayed by flow cytometry (BD Accuri™ C6) for the quantification of apoptotic cells. The assay allowed viable (negative for both annexin-V FITC and PI staining), necrotic (negative for annexin-V FITC and positive for PI), early apoptotic (positive for annexin-V FITC and negative for PI) and late apoptotic cells (positive for both annexin-V FITC and PI) to be quantified.

The antioxidant activity of NC was tested in HUVECs pretreated with NC for 24-48 h and activated by 30 min of treatment with H_2O_2 (25 μmol/L). Intracellular ROS generation was assessed incubating the cells with 10 μmol/L of the fluorescent dye 6-carboxy-2′,7′-di-chlorodihydro-fluorescein-di-acetate-bis(acetoxymethyl)-ester (C-DCF-DA) (Molecular Probes, Inc., Eugene, OR) as described. [28] Intracellular ROS analysis was performed at the same time as nanoparticle uptake analysis. Afterwards, cells were washed and scraped off into 1 mL of distilled water, sonicated and centrifuged. The supernatant fluorescence, expressed in fluorescence arbitrary units, was measured with a spectrofluorometer at 485-nm excitation and 525-nm emission.

Quantitative real time PCR analysis of mRNA-TF

Cells seeded in 6-well plates were pretreated with NC (25-50 μg/mL) and stimulated with TNF-α (20 ng/mL) for 45 min. Total mRNA was extracted using miRNeasy Mini Kit (Quiagen, S.p.A, Milano, Italy), according to manufacturer's instructions and cDNA was obtained with reverse-transcription utilizing IScript cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA). The quantitative real-time PCR on human TF was performed with QuantiNova™ SYBR® Green PCR kit (Qiagen). The sequences of TF primers are: hTF-FWD 5'-TGTTCAAATAAGCACTAAGTCAGGA-3' and hTF-REV 5'-TAGGAGAAGACCCGTGCCAA-3'; As normalizing gene we used the ribosomal protein L13a RPL13a-FWD 5'-CGCCCTACGACAAGAAAAAG-3' and RPL13a-REV 5'-CCGTAGCCTCATGAGCTGTT-3'. The real time PCR was executed with the Rotor-Gene Q (Qiagen) with the following cycling conditions: 95◦C, 2 min; 95◦C, 5 s and 60◦C, 10s for 40 cycles. The mRNA levels of TF were expressed with the relative quantity method as ΔΔCt.

Detection of endothelial adhesion molecule expression and cytokine release

Enzyme immunoassay of cell surface VCAM-1 was performed as described.[26] To estimate interleukin-6 (IL-6) and interleukin-8 (IL-8) release, HUVECs were plated in 24-well plates (0.5 mL of medium), pretreated with NC (10-25-50 μg/mL) and then stimulated with TNF-α (20 ng/mL) for 24 h. Conditioned medium was collected and centrifuged to remove cell debris and particles. Concentrations of IL-8 and IL-6 were measured by MILLIPLEX MAP High Sensitivity Human Cytokine Magnetic Bead Panel Kit (Millipore, Billerica, MA) based on the Luminex xMAP technology (Luminex Corporation, Austin, Tex). Data were read on the Luminex MAGPIX instrument (Luminex Corporation) and analyzed using MILLIPLEX Analyst 5.1 software (Millipore). Concentrations of cytokines were calculated using a standard curve and normalized to the number of cells.

Evaluation of telomere length

Cells were treated with 25-50 µg/mL of NC for 24 h and then stimulated with 25 µM of H_2O_2 diluted in medium culture for 45 min. After the treatment, cells were washed and kept at 37°C in complete medium overnight. Extraction of total DNA was performed with 5PRIME-PerfectPure DNA Cultured Cell (5PRIME), following manufacturer's instructions. To detect relative Telomere Length (TL), Monochrome Multiplex Quantitative PCR (MMQPCR) was performed according to the method previously described [29] into a 384-well CFX RT-PCR System (Bio-Rad) in a 10 µL reaction mix containing 10-20 ng of genomic DNA in 1x iQ SYBR Green Supermix (Bio-Rad). Forward and reverse primer sequences (Sigma) for telomere and β-globin gene (defined as single copy gene = SCG) and their relative optimal concentrations used in the reaction have been previously described.[29] Each sample was assayed in triplicates, with negative and positive controls included. A standard curve for telomere and β-globin (or single copy gene, SCG) combination was also evaluated in each assay as control of amplification efficiency and linearity.

Statistical analysis

Two-group comparisons were performed by the unpaired Student's *t*-test. Multiple comparisons were performed by one-way ANOVA followed by a multiple comparison test (Bonferroni test). Values of $P < 0.05$ were considered statistically significant. TEM analysis was realized with Graphpad PRISM.

RESULTS

Characterization of NC

As shown in Figure 1, A NC (25 µg/mL) are polydispersed with a size comprised between 5 and 40 nm. Electron diffraction patterns collected on an area of 4 μm^2 confirm the polycrystalline nature of the

sample. The diffraction rings can be indexed with a cubic face centered lattice having a= 5.5 Å which is compatible with the CeO_2 crystal structure. The indexing of the innermost rings of the pattern accordingly to the CeO_2 structure is reported in Figure 1, B.

Blood biocompatibility of NC

Coagulation time for the three main pathways − intrinsic, extrinsic and final common pathway − were evaluated by APTT, PT and TT respectively. Laboratory normal reference range for APTT and PT were 26-36 s and 10-14 s, respectively, and normal values of TT were below 21 s.

The results of the clotting time revealed that NC, in the range of tested concentrations, did not affect APTT, PT and TT clotting times of human plasma, that were within our laboratory reference range (Figure 2, A). Moreover, NC 10-25-50 µg/mL did not affect clotting times, as evaluated by PRT (308.0±3.0 s, 309.0±2.5 s, 295.0±3.5 s *vs* negative control: 307.0±1.0 sec, respectively) (Figure 2, C). However, NC at 50 µg/mL would seem to induce a shortening of PRT, although not statistically significant. For this purpose we have also test a higher concentration of NC (100 µg/mL), demonstrating that the recalcification time was significantly shortened to 231.0±10.0 s. The result of PRT suggested that the maximum concentrations of NC recommended for safe application is 50 µg/mL.

In presence of NC, the fibrinogen levels remained within normal range (180-350 mg/dL) even if a not significant trend towards a decrease was observed at higher NC concentration (Figure 2, B).

Whole blood treated with NC at 10 µg/mL, 25 µg/mL and 50 µg/mL did not show any hemolytic activity after 1 h or 4 h of incubation, as tested by the absence of hemoglobin released in plasma after blood centrifugation (Figure 3, A).

In whole blood, baseline impedance in response to ADP 20 µmol/L was 6.7±1 Ω. ADP at 20 µmol/L caused more platelet aggregation within the concentration range studied (data not shown).

Incubation of whole blood with only NC did not induce platelet aggregation and it had no significant effect on ADP-induced platelet aggregation (Figure 3, B).

Cellular uptake of NC

The lack of detectable cytotoxicity in the range of concentrations (10-50 μg/mL) raised the question whether the NC were capable of being taken up into the cells. Moreover, in order to evaluate the intracellular effects of NC, we have monitored, by TEM analysis, the time of endothelial cell uptake of NC, so to perform subsequent experiments when almost all NC are detected inside the endothelial cells. The treatment with NC does not affect cell morphology and subcellular compartment, compared with control untreated samples (Figure 4, A-B).

Ultrastructural analysis also led us to interpret the endocytosis as a mechanism for particle uptake. We observed the overall process starting with clathrin-coated vesicles (CCV) that undergo fusion with one endosome (3h-inset in Figure 4, B). In Figure 4, B it is possible to observe clathrin-mediated endocytosis both in the early stage of membrane curvature (box) and in the late phase of mature CCV containing NC (arrow heads). Subsequently (6h-24h – Figure 4, C-D), endosomes fuse together in greater structures. Finally, starting at 48 h, we observed a further swelling of endosomes related with breaking of endosome membrane and with the release of particles in the cytosol (arrow heads in Figure 4, E) which we interpreted as a mechanism of particle escaping. Between 6 and 48 h of treatment with NC, we observed a preferential localization in the close proximity of mitochondria (M) while at 72 h this effect is masked by the great portion of cytosolic compartment occupied by particle-containing endosomes (Figure 4, F).

NC cytocompatibility and antioxidant activity

Cytocompatibility tests were performed to evaluate NC effects on proliferation and viability of endothelial cells after 24, 48 and 72 h of treatment with increasing concentrations of nanoparticles (0, 10, 25, 50, 100 μg/mL). The results from the WST-1 assay showed no cytotoxic effects of NC at 10, 25 and 50 μg/mL, after 24-48 or 72 h of treatment (Figure 5, A) , while a reduction in cell viability by 22% was noted in presence of NC 100 μg/mL after 24 h of incubation (data not shown). No significant increase of apoptotic cells was found after 72h of NC treatment (50 μg/mL) (77% viable cells; 17% apoptotic cells) compared with control (83% viable cells; 16% apoptotic cells), confirming that NC at the maximal concentration compatible with cell viability, did not trigger apoptotic process. Figure 5B shows representative flow-cytometry plots of HUVEC treated with NC (50 μg/mL).

Intracellular antioxidant activity of NC on endothelial ROS generation was assessed pretreating cells with NC for 24 h (Figure 6, A) and 48 h (Figure 6, B) under oxidative stress conditions. NC reduced ROS generation induced by H_2O_2 (25 μmol/L) in a concentration-dependent manner (Figure 6). In absence of H_2O_2, NC decreased ROS to a minimum below the physiological level at 25 and 50 μg/mL, after 48 h of treatment, while at 24 h this effect was observed at 50 μg/mL of NC.

NC reduce VCAM-1 surface protein expression, IL-6/IL-8 release and mRNA-TF

Since oxidative stress induces a pro-inflammatory state in endothelial cells, modulation of the inflammatory response by NC was studied by measuring VCAM-1 cell exposure, IL-6 and IL-8 release. NC significantly reduced TNF-α-induced VCAM-1 exposure in a concentration-dependent manner (Figure 7, A). This result was supported by the effect of NC on cytokine release. In fact, NC reduced IL-6 and IL-8 release in supernatants, highlighting that NC are able to modulate the inflammatory response in endothelial cells (Figure 7, B-C). Tissue factor is not expressed in endothelial cells in physiological conditions, but its expression increases after stimulation with pro-inflammatory cues, leading to endothelial procoagulant phenotype. Our results demonstrated that NC are able to

reduce the TF-mRNA after stimulation with TNF-α, suggesting an antithrombotic effect of NC (Figure 7, D).

NC counteract premature senescence

Since NC have powerful antioxidant activity, we have also evaluated their effects on premature cell senescence that is characterized by increased oxidative stress and that may be cause of endothelial damage. Telomere length analysis (telomere/single-copy gene, T/S ratio) on HUVEC total DNA was performed 24 h after H_2O_2 treatment (25 μmol/L, 30 min) in HUVECs pretreated with 25 μg/mL and 50 μg/mL of NC for 24 hours. H_2O_2 treatment induced a telomere shortening of 63% with respect to the untreated control. The telomere length was not shortened by H_2O_2 in HUVECs pre-treated with NC (Figure 8).

DISCUSSION

Nanotechnology can find multiple applications in early diagnosis and therapy of many diseases. Nevertheless, a main concern about the use of NPs in medical applications is the onset of possible adverse effects on human health.[30] A relevant challenge of nanomedicine is to design and to develop compatible nanomaterials that, after administration, do not induce blood coagulation and vascular endothelial activation.

Although there are studies on NC compatibility in cell and animal models, [6, 31] no data exist about the effects of NC on human hemostatic system. A recent study, performed in mice, showed that intratracheal instillation of NC promoted thrombosis *in vivo*, probably as consequence of increased oxidative stress and inflammation.[32] Hemostasis is a complex process orchestrated by endothelial cells, the coagulation cascade and platelets and, as shown, NPs can act on every single player.[33] In fact, the contact between NPs and blood components can result into blood clotting, platelet aggregation and

endothelial activation, leading to a hypercoagulable state that characterize common diseases, such as diabetes mellitus, arteriosclerosis, cancer and obstructive pulmonary disease.[19, 34]

If a NP has not been engineered to specifically interact with the coagulation system or intended to induce or to inhibit it, the first step is to assess its hemocompatibility. Our results demonstrated that the exposure of human blood with different concentrations of NC did not affect the extrinsic and intrinsic pathways of the coagulation, evaluated by aPTT, PT and TT tests. Since plasma recalcification test showed a little statistically non-significant decrease in coagulation time, at NC 50 μg/mL, we have thought to assay a higher concentration of NC, demonstrating 100 μg/mL of NC induced a shortening of clotting time. This result suggests that, in our experimental conditions, the maximum concentration of NC recommended for safe application is 50 μg/mL. A recent study demonstrates that, although NC did not significantly perturb the native conformation of hemoglobin, they can stimulate some adverse effects on circulating lymphocytes at concentrations higher 50 μg/mL that may limit their biomedical application.[35] Moreover, the fibrinogen levels were within normal limits even if lower levels were observed at higher NC concentrations. This slight decrease, that did not influence blood coagulation, as showed by APTT and PT tests, could be due to processes of absorption and/or binding of fibrinogen on NC surface, as observed in other studies investigating the affinity of plasma proteins for different NP surfaces.[36, 37]

Many studies showed that physicochemical properties of NPs can also induce destruction of erythrocytes with release of hemoglobin,[38, 39] leading to anemia and other pathological conditions.[40] One of the mechanisms that plays a crucial role in hemolysis is the oxidative damage to erythrocyte membrane, rich of polyunsaturated fatty acids.[41] Since erythrocytes do not produce SOD and catalase − two key enzymes involved in ROS detoxification − increased oxidative stress leads to cell damage and/or death. Our results showed that NC did not induce hemolysis and changes in erythrocyte shape. Since NC act as ROS scavengers, mimicking the activity of SOD^2 and $catalase^4$, their antioxidant

activity could preserve the membrane integrity after contact with erythrocytes. This result seems important if we consider that some hemolytic disorders are treated with natural antioxidant compounds but their applications have shown some limitations, such as enzymatic degradation, difficulty to reach the target and short half-life. Conversely, NC may overcome these restrictions, thanks to ROS scavenging properties and the ability to reach specifically the target, constituting a promising tool in those disorders characterized by a hemolytic process.

The platelets play an important role in the maintenance of hemostasis and in thrombus formation.[42] NPs can lead to platelet activation directly or interfere with platelet agonist, causing blood-clotting disorders.[19, 43] The mechanisms underlying platelet activation/aggregation differ with the various types of nanoparticles.[44] Moreover, conflicting results were obtained testing NP with different size and surface charge.[43] Our results demonstrated that the preincubation of whole blood with NC did not induce any detectable platelet aggregation both prior and after ADP addition, suggesting that physical properties of NC do not influence the platelet biology.

The vascular endothelium controls hemostasis, vascular tone, angiogenesis and trafficking molecules. Since altered endothelial phenotype is closely related to many cardiovascular diseases,[45] we have performed a detailed analysis of the biological response of human endothelial cells to NC exposure in physiological and inflammatory conditions, so to provide useful information about biocompatibility and to a potential NC use in the preventing or treating cardiovascular diseases.

As showed by TEM images, NC uptake in endothelial cells is dependent on the incubation time, increasing gradually with the incubation and occurring through clathrin-mediated endocytosis, as showed in microvascular endothelial cells.[46] After 3 h, NC are already incorporated in endothelial cells and, after 24 h, their uptake is total. Into the cells, NC are mostly localized in the cytosol, aggregated in endosomes and, starting at 48 h, are released in the cytosol after breaking of endosome's membrane, like a possible mechanism of particles escaping.

Our results showed that NC are able to preserve endothelial cell viability, counteracting oxidative stress, endothelial activation and finally premature senescence. As also showed by TEM images, NC did not affect endothelial cell viability according also to previous studies on cell lines[21, 24, 47] and did not induce apoptosis, reinforcing the conclusion that NC were well tolerated by endothelial cells. Our results provide clear evidence on ability of NC to counteract the induced oxidative burst in endothelial cells. The antioxidant property of NC is widely documented by many experimental studies, both in vitro[8, 15, 21, 47] and in vivo[7, 10-12, 16, 48-50] and can be explained taking into account the unique regenerative surface attributable the coexistence of both $Ce(3^+)/Ce(4^+)$.

The antioxidant capacity of NC represents an important aspect considering that an increased oxidative stress can alter endothelial balance toward a pro-inflammatory and prothrombotic phenotype.

The endothelial expression of TF, the trigger of the extrinsic pathway of the coagulation, may account for thrombotic events associated with cardiovascular diseases.[51, 52] TF is a protein that forms a high-affinity complex with coagulative factors VII/VIIa, activating factors IX and X, thus leading to thrombin generation, fibrin deposition, and platelet activation. In vitro, many inflammatory stimuli can induce TF expression and shift the endothelial balance to a procoagulant state.[25, 53] We have demonstrated that in inflammatory conditions NC reduce TF mRNA expression in endothelial cells, limiting TF protein expression and, consequently TF-procoagulant activity.

The exposure to NP is often associated to inflammation,[54] activating redox-sensitive transcription factors involved in the expression of pro-inflammatory genes such as adhesion molecules and cytokines.[55] This inflammatory response may depend on surface coating of nanoparticles, as demonstrated after exposure of endothelial cells to NPs with different metal composition.[54, 56] Our results showed that NC modulate the inflammatory response, decreasing VCAM-1 surface exposure, involved in the leukocyte recruitment, and the release of cytokines, IL-6 and IL-8. These findings are

consistent with those of previous studies that have shown a protective effect of NC on oxidative and inflammatory response.[57-61]

Previous studies demonstrated that a disturbance of the oxidative balance may lead to premature senescence associated to telomere shortening.[62, 63] There is little information on the effects of nanomaterials on oxidative stress-induced senescence in human cells. To the best of our knowledge, this is the first *in vitro* study showing a suppression of H_2O_2-induced telomere shortening in endothelial cells, pre-exposed to NC. Some studies showed that single-walled carbon nanotubes could inhibit telomerase activity stabilizing selectively human telomeric i-motif DNA, so demonstrating a potential use for cancer therapy.[64, 65] On the other hand, multiple well carbon nanotubes seem to accelerate cellular senescence by telomere shortening while an increasing DNA repair activity is contextually observed.[66]

We instead show that NC protect telomere caps from oxidative damage and this effect is primarily due to their intrinsic antioxidant activity. This result underlies the beneficial use of NC in counteracting the ageing phenotype under conditions of oxidative stress where it is relevant to maintain cell survival.

Our findings indicate that NC, in a range of concentrations of 10 to 50 μg/mL, did not induce blood coagulation and vascular endothelial activation, underlining their safety in biomedical applications. Moreover, in inflammatory conditions, NC modulate the expression of endothelial proteins involved in vascular pathology, as thrombosis, inflammation and premature senescence, suggesting their possible use as therapeutic tool for several cardiovascular disorders.

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FIGURE LEGENDS

Figure 1. Electron microscopy characterization of NC suspension. A) Bright-field image of particles suspension; B) Electron diffraction pattern corresponding to CeO_2 cubic face centered lattice and pattern indexing.

Figure 2. Effect of NC on coagulation system. A**)** APTT, PT and TT were performed incubating PPP with different concentrations of NC (10, 25, 50 μg/mL) for 30 min or with PBS as negative control were tested. Data are expressed in sec as mean of four independent experiments (mean ± SD). B) Plasma recalcification test (PRT) was performed incubating different concentrations of NC (10, 25, 50 μg/mL) with PPP in siliand adding $CaCl_2$, to trigger the coagulation. Glass tube was taken as the positive control and siliconized glass tube (without NC) as negative control. Data are expressed in s as mean of three independent experiments (mean ± SD). (C) Fibrinogen (mg/dL) was determined by the Clauss method with some modifications. Data are expressed as mean of four independent experiments (mean ± SD).

Figure 3. Effect of NC on hemolysis and platelet aggregation. A) After 1 or 4 h of incubation at 37°C with NC (10, 25, 50 μg/mL), the amount of hemoglobin released into the supernatant was quantified using spettrophotometric method as explained in Method section. PBS and Triton X-100 were used as negative or positive controls. Results are expressed as mean ± SD of arbitrary optical density milliunits (OD, mU). Above the histogram, microscopic pictures of the blood smears of the blood without and with NC 50 μg/mL, after 4h of incubation. B**)** Platelet aggregation was measured by impedance aggregometry incubating whole blood with NC (10, 25, 50 μg/mL). ADP at 20 μg/mL was used to induce platelet aggregation. ADP without NC was the positive control Results are expressed as impedance values (ohms, Ω) and are the mean of three independent experiments (mean ± SD).

Figure **4.** TEM images of HUVECs untreated (a) or treated with NC (25 µg/mL) for 3h (b), 6h (c), 24h (d), 48h (e) and 72h (f). Ultrastructure of cells as well as the architecture of cytosolic organelles are unaffected. We could identify nucleus, endoplasmic reticulum, mitochondria and Golgi's apparatus (N, ER, M and G respectively). Highlighted by harrow heads in d some NC escaped from the endosomes.

Figure 5. (A) Cell viability assessment of HUVECs untreated or treated with increasing concentrations of NC for 24-48-72 h, evaluated by WST-1 assay. Data are expressed as mean ± SD of optical density arbitrary units (AU) at 405 nm, and are representative of three independent experiments *P<0.05 vs. control**. (B)** Flow-cytometry evaluation of apoptosis in HUVECs treated for 72 h with 50 µg/mL of NC.

Figure 6. Effects of increasing NC concentrations (0-50 µg/mL) on ROS production in HUVECs in presence or absence of H_2O_2 stimulation. Quantitative evaluation of ROS levels was performed after 24 h (a) and 48 h (b) of incubation with NC. *P<0.05 vs. control. §P<0.05 vs. treatment with H_2O_2 alone.

Figure **7**. Effects of NC on endothelial VCAM-1 surface expression, IL-6/IL-8 release and mRNA-TF expression. (A) Confluent HUVECs were treated with NC (0-50 µg/mL) for 24 h and then stimulated with TNF-α (20 ng/mL) overnight. At the end of the incubation time, VCAM-1 surface expression was quantified by EIA. Values are mean ± SD of optical density arbitrary units (AU) at 405 nm, and are representative of three independent experiments *=P < 0.05 vs TNF-α. (B-C) The cells were pretreated with NC for 24 h and stimulated with TNF-α for 24 h. The culture supernatants were collected and analyzed for IL-6 and IL-8 by ELISA. The data shown are representative of three independent experiments, and values are expressed as mean ± SD *P<0.05 vs TNF-α. (D) HUVECs treated with NC (25-50 µg/mL) for 24 h were stimulated with TNF-α (20 ng/mL) for 45 min. At the end of the incubation time, total mRNA was extracted and RT-PCR was performed with specific primers for TF and RPL13a. The results are expressed as mean ± SEM. *=P < 0.05 vs TNF-α

Figure 8. Effects of NC on relative telomere length (T/S). T/S relative fluorescence is evaluated in the DNA of cells treated with 25 µmol/L H_2O_2 with or without the addition of NC (25 µg/mL or 50 µg/mL). Data are expressed as percent of T/S expression with respect to untreated cells.