Original Article

Pilot *in vivo* investigation of cerium oxide nanoparticles as a novel anti-obesity pharmaceutical formulation

Antonella Rocca, MSc[a,b,*], Stefania Moscato, MSc[c], Francesca Ronca, PhD[d], Simone Nitti[e], Virgilio Mattoli, PhD[a], Mario Giorgi, PhD[f], Gianni Ciofani, Ph.D.[a,*]

[a]*Istituto Italiano di Tecnologia, Center for Micro-BioRobotics @SSSA, Pisa, Italy*
[b]*Scuola Superiore Sant'Anna, The BioRobotics Institute, Pisa, Italy*
[c]*University of Pisa, Department of Clinical and Experimental Medicine, Pisa, Italy*
[d]*University of Pisa, Department of Surgical, Medical, Molecular Pathology and Emergency Medicine, Pisa, Italy*
[e]*Istituto Italiano di Tecnologia, Nanochemistry Department, Genova, Italy*
[f]*University of Pisa, Division of Pharmacology & Toxicology, Veterinary Sciences Department, Pisa, Italy*

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Abstract

Obesity is a worldwide pathological condition that strongly impairs human health, and, to date, no effective therapy against excessive fat accumulation has been found yet. Since overweight correlates with an increased oxidative stress, our aim is to investigate the antioxidant effects of cerium oxide nanoparticles (nanoceria) as a potential pharmaceutical approach for the treatment of obesity. Nanoceria were tested both *in vitro* and *in vivo*; they were proven to interfere with the adipogenic pathway by reducing the mRNA transcription of genes involved in adipogenesis, and by hindering the triglycerides accumulation in 3T3-L1 pre-adipocytes. Nanoceria, intraperitonally injected in Wistar rats, did not show appreciable toxic effects, but instead efficiently contributed in reducing the weight gain and in lowering the plasma levels of insulin, leptin, glucose and triglycerides.

From the Clinical Editor: Obesity is now a significant problem worldwide. To date, obesity surgery remains the best treatment for weight reduction. Much research has been conducted to discover an effective pharmacological treatment against obesity. In this article, the authors continued their previous work in studying the anti-adipogenic properties of cerium oxide nanoparticles. The antioxidant effects of nanoceria were studied in in vitro and in vivo experiments. It was shown in animal model that nanoceria could reduce body weight effectively. These promising results may provide a novel treatment in the clinical setting in the future.

Key words: Cerium oxide nanoparticles; Oxidative stress; Adipogenesis; Anti-obesity formulation

Cerium oxide nanoparticles (nanoceria, NC) have demonstrated to own great potential as pharmaceutical agents in nanomedicine.[1] They mimic superoxide dismutase and/or catalase activity, depending on the presence of crystalline defects on their surface (Ce^{3+}/Ce^{4+} ratio) and on the pH of the environment where they accumulate,[2] thus behaving as efficient reactive oxygen species (ROS) scavengers. Additionally, NC can self-regenerate their antioxidant properties thanks to the ability to switch between the two oxidation states of cerium.[3]

*Corresponding authors at: Istituto Italiano di Tecnologia, Center for Micro-BioRobotics @SSSA, Viale Rinaldo Piaggio 34, 56025 Pontedera (Pisa), Italy.
E-mail addresses: antonella.rocca@iit.it (A. Rocca), gianni.ciofani@iit.it (G. Ciofani).

Beneficial effects of nanoceria cover a wide area of applications, ranging from macular degeneration to cancer therapy.[1] Our group reported inhibition of adipogenesis in mesenchymal stem cells induced by nanoceria,[4] correlating ROS to the lipid formation. Several studies, in fact, demonstrated that ROS are necessary for lipid accumulation, and that carbohydrate restriction in obese adults diminished not only the body weight, but also oxidative stress markers.[5] Oxidative stress could be, thus, an effect of obesity, but it could also activate pathways leading to an increased white adipose tissue accumulation, as demonstrated by ROS production in fat tissue, correlated with the reduction of antioxidant enzymes and with the increase of NADPH oxidase activity.[6]

In the latest years, many efforts have been spent to find novel efficient drugs for the treatment of obesity, a pathological status that globally affects 12% of adults (data from World Health

Organization, 2008). The main consequences of obesity are represented by the development of several pathologies, including type 2 diabetes, hypertension, insulin resistance, atherosclerosis, and cancer.[7] At the present, surgery is the most efficient approach for patients with severe obesity, but it could entail severe complications and results into a quick recovery of the lost weight.[8] Among the anti-obesity drugs recently approved by the FDA, Lorcaserin and Qsymia are worth to be mentioned. Lorcaserin is a serotonin 5-HT_{2C} receptor agonist that mimics the effects of serotonin causing an increase of satiety and the reduction of the appetite.[9] Qsymia combines two drugs, phentermine and topiramate, for losing weight by suppressing the appetite and by increasing the sense of satiety.[10] Both of these drugs, however, show considerably side effects like dizziness, headache, insomnia, and risk of teratogenicity.[11] Another strategy to decrease body weight is the assumption of dietary polyphenols (such as green tea, resveratrol, curcumin, *etc.*), that exhibit antioxidant and anti-inflammatory effects related to lipid accumulation,[12] but unfortunately they are rapidly metabolized by enzymes, resulting in very low stability and bioavailability after the ingestion.[13]

Nanoceria could overcome most of the limitations typical of strong traditional anti-oxidant agents because of the previously mentioned self-regenerating catalytic properties. Conversely to commercially available drugs against obesity, NC could in fact own the advantage to strongly scavenge the ROS production for a long-sustained period of time, thus both lowering the needed doses and their assumption, and eventually reducing the adverse side effects typical of other drugs. Starting from our previous results on NC-induced adipogenesis inhibition, in this study we tested the ability of NC to interfere in the lipid accumulation processes both *in vitro* on 3T3-L1 pre-adipocytes and *in vivo* in rats.

Methods

Cell cultures

Embryonic mouse pre-adipocytes (3T3-L1, ATCC® CL173™ from ATCC, Manassas, VA, USA) were grown in expansion medium composed by high glucose Dulbecco's Modified Eagle's Medium (DMEM), 10% calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 200 mM glutamine, and used at the second passage in all the experiments.

Cells were seeded in 24-well plates at a density of 20,000 cells/cm^2 for all the experiments. At the confluence, they were fed with expansion medium for further 48 h before differentiation induction into adipocytes, performed for 7 days in the presence of 0, 20, and 50 μg/ml of NC. The adipogenic differentiation medium was composed by DMEM, 10% fetal bovine serum, 1 μM dexamethasone, 5 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 100 U/ml penicillin, 100 mg/ml streptomycin, and 200 mM glutamine.

All reagents for cell culture were from Gibco (Life Technologies, Waltham, MA, USA).

Nanoceria were purchased from Sigma, Saint Louis, MO, USA (544841), and extensively characterized in a previous work.[14] Nanoparticles were dispersed through a mild sonication in ultrapure MilliQ water (Millipore, Billerica, MA USA) at a concentration of 10 mg/ml. Appropriate dilutions in the cell culture medium were performed just before the experiments. The antioxidant ability of the nanoceria was estimated with a specific assay (Total Antioxidant Capacity Assay Kit, MAK187 from Sigma), as reported in details in the Supplementary Material.

Quantitative real time RT-PCR (qPCR)

RNA isolation was performed through the automated robotic workstation QIAcube (Qiagen, Venlo, the Netherlands), by using the RNeasy® Plus Mini kit (Qiagen) according to the manufacturer's protocol. Cells were pelleted and disrupted with lysis buffer, while for the *in vivo* experiments, 30 mg of adipose tissue, collected through surgical excision and immediately frozen, was homogenized with an Ultra-Turrax (IKA, Staufen, Germany) just before the RNA extraction. The RNA quantity and purity were evaluated with analysis of absorbance at 260/280 nm (Nanodrop, Thermo Scientific, Waltham, MA, USA). To obtain cDNA, 1 μg of RNA was reverse-transcribed by using iScript™ Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA).

For *in vitro* tests, the amplification mix was assembled as follows: 10 μl of SsoAdvanced™ SYBRGreen® Supermix (Bio-Rad), 1 μl of forward and reverse primers (8 μM), 4 μl of MilliQ water, and 5 μl of diluted cDNA. For the tissue samples, the amplification was performed using the RT^2 Profiler PCR Array (Qiagen, PARN-049Z) in order to detect the expression profiles of 84 genes related to adipogenesis. The qPCR reaction was achieved on a thermocycler CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The temperature protocol comprised one cycle at 98 °C for 30 s, 40 cycles at 98 °C for 3 s and 60 °C for 15 s, a temperature ramp from 65 °C to 95 °C, with 0.5 °C/s increments. For the normalization, *B2m* and *Gapdh* (for *in vitro* samples) and *Hprt* and *Rplp1* (for *in vivo* samples) were used as reference genes. The relative quantification of the target genes was calculated through the $\Delta\Delta Ct$ method. Primer sequences for *in vitro* studies were obtained through NCBI/Primer-BLAST and are reported in Table 1. Detailed information of the genes analyzed with the RT^2 Profiler PCR Array is listed in the Table S1 of the Supplementary Material.

Glycerol-3-phosphate dehydrogenase (G3PDH) assay

The employed colorimetric assay (ab174095 from Abcam, Cambridge, UK) provides a substrate that reacts with G3PDH of the samples by forming an intermediate compound strongly absorbing at 450 nm. The cell cultures were pelleted and homogenized in lysis buffer. Adipose tissue (10 mg), harvested from visceral fat, was disrupted in 200 μl of buffer by Ultra-Turrax (IKA) and then sonicated with a Sonoplus Mini 20 (Bandelin, Berlin, Germany) to perform efficient homogenization. In both cases, the collected supernatant was used for the subsequent analysis following the protocol as defined for cell cultures. The reaction was carried out at 37 °C for 30 min, and the reading of absorbance, performed at 450 nm with a microplate reader (Victor3, Perkin Elmer, Waltham, MA, USA), allowed the G3PDH content to be quantified.

Table 1
Sequences of primers for target and housekeeping genes used for *in vitro* study.

Gene	Symbol gene	Sequence (5' – 3')
CCAAT/enhancer binding protein (C/EBP), alpha	*Cebpa*	F – CGTGGTGGTTTCTCCTTGA
		R – GAGTATCCAAGGCACAAGGT
Glycerol-3-phosphate dehydrogenase 1	*Gpd1*	F – CGCTGACATCCTGGTTTTTGT
		R – GCCCTCGTCTACCCCCTTA
Lipoprotein lipase	*Lpl*	F – AGAAGGGAAAGGACTCAGCA
		R – TTTGTTTGTCCAGTGTCAGCC
Peroxisome proliferator-activated receptor gamma	*Pparg*	F – AGACATTCCATTCACAAGAGC
		R – ATCGCACTTTGGTATTCTTGG
Beta-2 microglobulin	*B2m*	F – ATGCTATCCAGAAAACCCCTC
		R – GCAGTTCAGTATGTTCGGCT
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	F – GATGCTGCCCTTACCCCG
		R – TTTTGTCTACGGGACGAGGAAAC

Lipid droplets quantification

Lipid content quantification in cell cultures was performed with the AdipoRed™ Assay (Lonza, Basel, Switzerland). After 7 days of differentiation, cell cultures were incubated with 30 µl of the reagent in 1 ml of PBS at 37 °C. After 10 minutes the fluorescence was measured with the microplate reader with excitation at 485 nm and emission at 572 nm. Qualitative evaluation was moreover performed under fluorescence microscope (Eclipse TI, Nikon, Tokyo, Japan).

Animals and in vivo experimental procedures

Twelve adult male Wistar rats, 10-week-old and weighing 300 ± 25 g, were used in this study. The experimental protocol was approved by the Ethics Committee of the University of Pisa (protocol number 003723/2013, in accordance to EU Directive 2010/63/EU for animal experiments). The experiments started after 7 days of acclimation of the animals, maintained under constant conditions of temperature (25 °C), humidity (50%), and light (12 h cycle dark/light) in laboratory cages (three animals per cage). Food and water were provided *ad libitum* (Teklad Global 18% Protein Rodent Diet, Harlan, Denver, CO, USA).

The animals were divided in two groups: 6 rats were treated with NC and 6 rats were used as control. NC was administrated twice a week for six weeks through intraperitoneal injection at a dose of 0.5 mg/kg in 500 µl of sterile water. Intraperitoneal route was chosen as an optimal compromise between easiness of treatment and nanoparticle distribution. This administration route was in fact proven to provide similar biodistribution with respect to an intravenous injection,[15] but allowing at the same time repetitive injections without any adverse effect for the animals. Since administration of nanomaterials through the intraperitoneal route could be associated to macrophage activation and consequent cytokine release, we have tested effects of acute and high-dose NC treatment on RAW 264.7 cells (50 and 100 µg/ml for 24 h), as widely described in the Supplementary Material, highlighting no activation of macrophages in terms of IL-6, IL-10, and TNF-α release.

All the animals were weighted once a week, and at each week i, the percentage of weight variation Δw for each animal with respect to the initial weight w_0 was determined as $\Delta w = (w_i - w_0)/w_0 \cdot 100$, where w_i is the weight of the animal at week i. After 6 weeks the rats were sacrificed in CO_2 chamber, and blood samples collected through cardiac puncture for biochemical analysis. Tissues were harvested and either fixed in paraformaldehyde for histological sectioning or stored at −80 °C for further analyses.

Inductively coupled plasma spectrometry (ICP-AES)

NC content in main organs (brain, liver, kidney, spleen) was determined through an inductively coupled plasma atomic emission spectrometer (ICP-AES, iCAP 6500, Thermo Scientific). The collected tissues were weighted and placed at −80 °C; thereafter, the digestion process was carried out through a treatment with a HNO_3:H_2O_2 (4:1) solution in a microwave digestion system. The mineralized samples were finally dissolved in 10 ml of water, and the Ce concentration determined at 404.0 nm.

Histology

For tissue analysis, little fragments of visceral fat, liver, kidney and spleen were collected after animal sacrifice and immediately fixed in a 4% paraformaldehyde solution overnight at 4 °C, washed in 1× phosphate saline buffer (PBS, Sigma), and finally rinsed in 70% ethanol until processing. Thereafter, the samples were dehydrated through an increasing ethanol gradient. Subsequently, the samples were clarified in xylene twice for 2 h, rinsed in liquid paraffin at 60 °C for 4 h and wax-embedded. Specimen sections 5-µm thick were obtained, mounted onto slides, stained in hematoxylin and eosin, and finally observed with a DMRB microscope (Leica, Wetzlar, Germany).

Biochemical analysis

Whole blood samples were centrifuged in tubes containing heparin as anti-coagulant, and isolated plasma was used for the analysis of leptin, insulin, glucose and triglycerides. The concentration of insulin and leptin was obtained through ELISA (EZRMI-13K for insulin and EZRL-83K for leptin, both from Millipore), following the manufacturer's protocols. The circulating levels of glucose and triglycerides were determined through colorimetric kits (ab65333 for glucose and ab65336 for triglycerides, from Abcam).

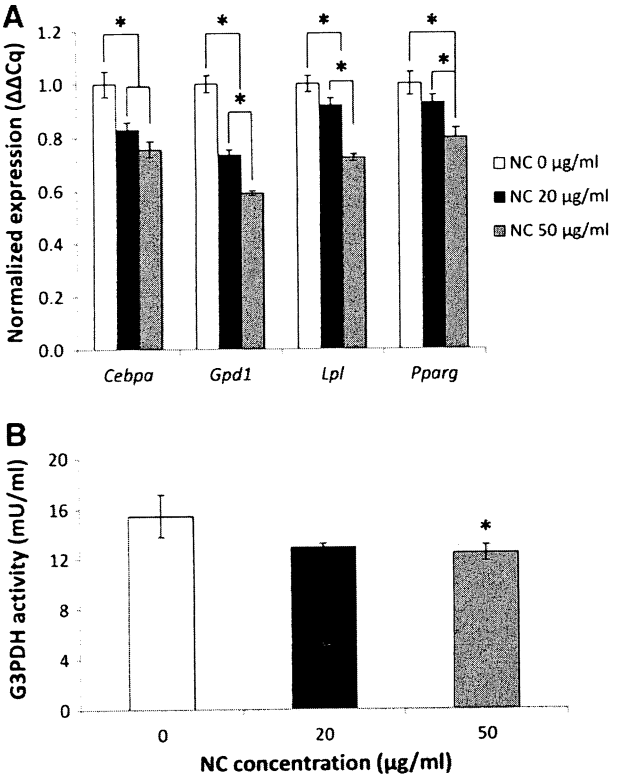

Figure 1. mRNA quantification of adipogenic genes in cell cultures: analysis of *Cebpa*, *Gpd1*, *Lpl*, and *Pparg* in pre-adipocytes after 7 days of differentiation in the presence of NC (**A**). Analysis of triglycerides production: quantification of G3PDH activity in pre-adipocytes treated with NC (**B**). * $P < 0.05$.

Statistical analysis

Data were analyzed with one-way ANOVA followed by Bonferroni's *post-hoc* test or with two-tailed unpaired *t* test through KaleidaGraph (Sinergy Software); qPCR results were evaluated with Bio-Rad CFX Manager software. In all experiments, data with *P*-value < 0.05 were considered statistically significant.

Results

Effects of NC on pre-adipocyte differentiation

The results of qPCR highlighted that NC induced a down-regulation of the main adipogenesis marker genes during differentiation of preadipocyte 3T3-L1 cells (Figure 1, *A*). Treatment with NC at both doses significantly ($P < 0.05$) decreased transcription of *Cebpa* (1.2-fold for NC 20 µg/ml and 1.3-fold for NC 50 µg/ml), *Gpd1* (1.4-fold for NC 20 µg/ml and 1.7-fold for NC 50 µg/ml) and *Lpl* (1.1-fold for NC 20 µg/ml and 1.4-fold for NC 50 µg/ml) with respect to the untreated cells. In the case of *Pparg*, the reduction was statistically significant only at the highest dose of NC (50 µg/ml, 1.3-fold) when compared to the control.

To examine the metabolic changes during the differentiation of 3T3-L1 cells, the activity of G3PDH was determined. Data in Figure 1, *B* show that the activity of this enzyme was reduced by the NC treatment at both doses (12.9 ± 0.3 mU/ml for NC 20 µg/ml and 12.4 ± 0.6 mU/ml for NC 50 µg/ml) with respect to the control (15.4 ± 1.6 mU/ml), but being statistically significant ($P < 0.05$) just at 50 µg/ml.

Fluorescence images of the intracellular lipidic droplet accumulation in the cells (with the respective bright field acquisitions) exposed at different NC doses are reported in Figure 2, *A*. The quantitative evaluation of the fluorescence (Figure 2, *B*) highlights that NC caused a significant decrease of lipid droplets both at 20 µg/ml (−9%) and 50 µg/ml (−12%) with respect to the control cells ($P < 0.05$).

Effects of NC on rats

The percentage of weight variation with respect to the initial weight of NC-treated and control rats is reported in Figure 3, *A*. At the end of the six-week monitoring period, control rats showed an elevated weight gain (13.5%) since the beginning of the experiments, whereas the rats treated with NC showed only a limited and non-statistically significant increment (2%) compared to the initial weight.

The NC biodistribution, assessed through ICP-AES, showed that nanoparticles are mainly retained by spleen (Figure 3, *B*), while low accumulation was found in brain, liver and kidney. Overall, by considering administered doses and elemental analysis results, we have estimated an accumulation of about 3% of the whole injected amount of nanoparticles in the examined organs.

To detect any effect induced by NC injection, tissue organization and cellular morphology of the organs of interest were analyzed by staining with hematoxylin and eosin (Figure 4). Concerning the features of the adipose tissue, adipocytes maintained their large lipid droplets with the nucleus placed at the edge of the cellular cytoplasm. Kidney sections showed that NC did not affect tubular, glomerular and interstitial structures. In the liver slides we can observe, also in the NC-treated rats, the typical lobule architecture characterized by a central vein from which hepatocytes depart, and are separated by sinusoids. Spleen histology indicates that NC did not cause alterations of the normal tissue architecture, composed by lymphoid aggregations and vascular sinuses.

Plasma concentration of insulin and leptin was determined through ELISA at the end of the treatment. The results revealed that NC significantly reduced the insulin (0.4 ± 0.5 ng/ml) and the leptin concentration (8.1 ± 1.7 ng/ml) with respect to the control group (2.8 ± 1.1 ng/ml for insulin and 17 ± 4.5 ng/ml for leptin; $P < 0.05$; Figure 5, *A-B*). Furthermore, the glucose and triglycerides levels were also reduced in rats following the treatment with NC (0.032 ± 0.003 nmol/µl for glucose and 0.9 ± 0.1 nmol/µl for triglycerides) when compared to the control groups (0.039 ± 0.004 nmol/µl for glucose and 1.3 ± 0.1 nmol/µl for triglycerides; $P < 0.05$; Figure 5, *C-D*).

The analysis of the transcribed genes in the samples of adipose tissue (Figure 6, *A*) demonstrated that the transcription of *Angpt2*, *Bmp2*, *Ddit3*, *Lep*, and *Twist1* in rats treated with NC was significantly lower with respect to the control group (*Angpt2* 1.6-fold, *Bmp2* 1.5-fold, *Ddit3* 1.3-fold, *Lep* 2.4-folds,

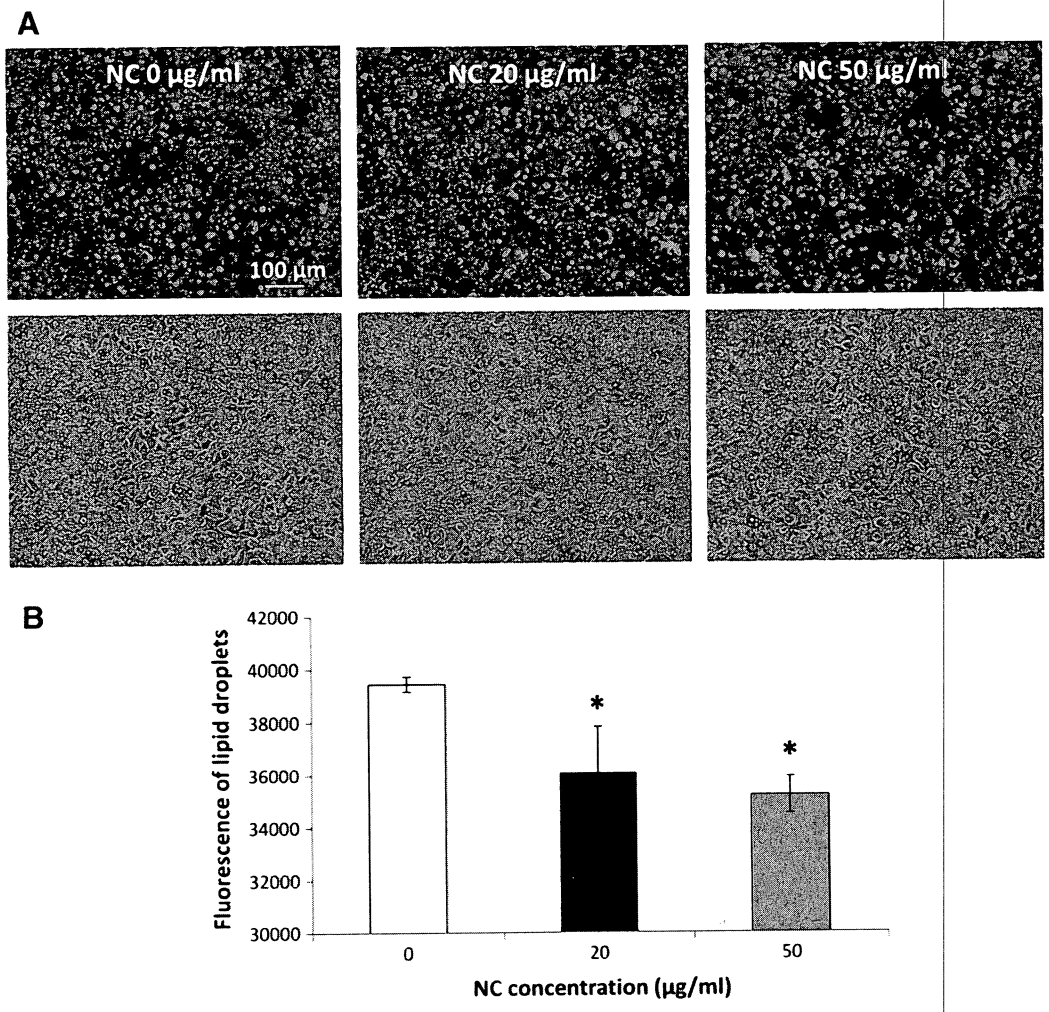

Figure 2. Lipid droplets staining through Adipored™: qualitative (**A**) and quantitative (**B**) assessment of lipid vesicles (green stained) in 3T3-L1 cultures incubated with NC after 7 days of differentiation. * $P < 0.05$.

Twist1 1.4-fold; $P < 0.05$), while the transcription of *Irs1* and *Klf4* was significantly increased (*Irs1* 1.4-fold, *Klf4* 1.5-fold; $P < 0.05$). Furthermore, *Bmp4* and *Ldha* were down-regulated in presence of NC (*Bmp4* 2.1-folds, *Ldha* 2-folds), even if this decrement resulted statistically non-significant.

The measurement of G3PDH activity at the end of the experiment indicated a decrease following NC administration, being the amount of G3PDH 11.3 ± 1.9 mU/ml for the rats treated with NC and 14.7 ± 0.5 for the control rats ($P < 0.05$; Figure 6, *B*).

Discussion

Oxidative stress is a necessary condition for the progression of the adipocyte maturation, but also a fundamental phenomenon during the development of obesity.[16-18]

In this study, the maturation fate of 3T3-L1 following NC treatment was ascertained by analyzing gene transcription, enzymatic activity, and lipid droplet formation. The influence of NC on the adipogenic process was verified through quantitative real time RT-PCR of *Pparg*, *Cebpa*, *Gpd1* and *Lpl*. *Pparg* is a key transcription factor necessary for the adipogenesis, while *Cebpa* drives pre-adipocytes to complete differentiation activating other specific genes.[19] Furthermore, *Pparg* and *Cebpa* are involved in a positive feedback where these molecules stimulate each other's expression.[20] *Gpd1* is the enzyme responsible for the triglyceride biosynthesis, while *Lpl* regulates the hydrolysis of triglycerides.[21] Our results confirmed that NC cause a significant decrement of mRNA transcription of the investigated main adipogenesis marker genes. Similar effects were reported by Jeong et al that observed a down-regulation of *Pparg* and *Cebpa* genes in 3T3-L1 cells after administration of *Gelidium elegans* extract for 8 days of differentiation, because of its inhibitory effects on ROS production.[22]

The characterization of the anti-adipogenic properties of NC comprised the evaluation of the G3PDH activity. The activity of this enzyme, which presents a key role during the triglyceride synthesis, significantly diminished following NC administration. Also the fluorescent staining of lipid droplets proved that NC affect the adipogenic pathway in terms of reduced lipid accumulation. Analogously, a study conducted by Lee et al demonstrated that isorhamnetin, a

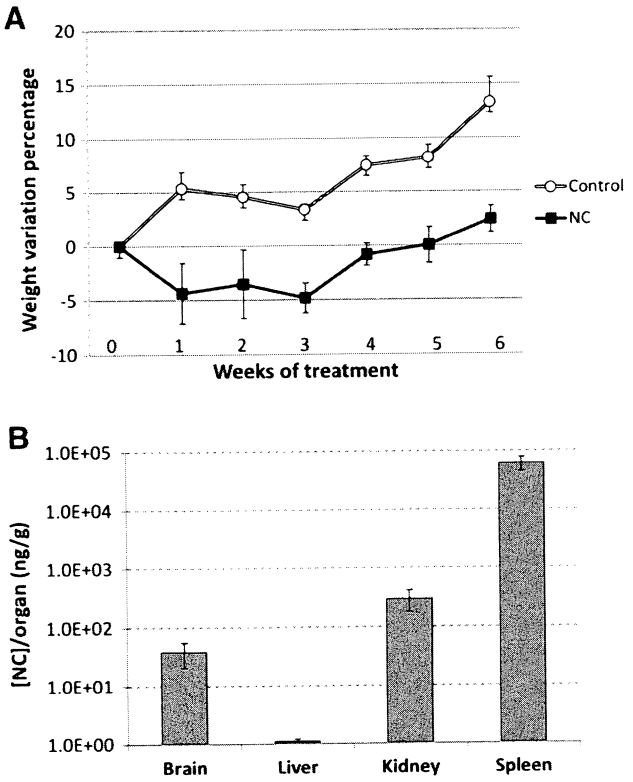

Figure 3. Body weight evaluation: time-course analysis of weight variation of NC-treated and control rats (**A**). ICP-AES for NC organ accumulation assessment: quantification of cerium in brain, liver, kidney and spleen after 6 weeks of NC administration (**B**).

flavonoid with antioxidant properties, is also able to reduce both G3PDH activity and lipid content.[23] These data collectively confirm as NC interfere with the adipogenesis due to their ROS scavenger ability, inhibiting both genes transcription and phenotype development typical of terminally differentiated adipocytes.

In the recent years, the interest toward NC as therapeutic agent has enormously grown up, and many examples can be found where these nanoparticles have been tested *in vivo* for the treatment of several pathologies, where an imbalance of the redox state is involved.[24-26] Starting from previous promising results in the inhibition of adipogenesis *in vitro*, in this study we decided to shift experiments *in vivo* in an attempt to examine the influence of NC on lipogenesis and, consequently, on obesity. For this purpose, we treated normally fed rats with NC injections at a single dose of 0.5 mg/kg body weight twice a week for 6 weeks. Nanoceria were intraperitoneally administrated, basing on a study demonstrating an improved biodistribution with respect to other administration routes.[15] The NC concentration (0.5 mg/kg) was chosen based on the data of Hirst et al, which showed how this dose is well tolerated *in vivo* after 15 days of treatment.[27] The outcomes on the body weight have demonstrated that NC are effective in hindering weight gain in rats exposed to NC just after a 1-week treatment.

Concerning organ accumulation, we observed that NC were mainly accumulated in the spleen at the end of the 6-week treatment. Spleen is the organ involved in the body detoxification and in the clean-up of the blood from aged red cells.[28] The NC up-take by spleen can be attributed to its role of phagocytosis of foreign bodies, thanks to the elevated numbers of resident macrophages, and our finding is consistent with other studies that observed a major accumulation of NC in spleen with respect to other organs.[29-31] Despite the strong accumulation in the spleen, histological analysis with hematoxylin and eosin did not reveal any impairment of the tissues. Furthermore, analysis on other organs (liver, kidney, and adipose tissue) showed the typical morphology of the tissues without difference between treated and control rats. Our findings are in agreement with several other studies, confirming that NC are not toxic and do not alter the organ cytology.[27,32,33] Just in one case, reported by Tseng et al, granulomatous formations were found after 30 days in liver of rats exposed to a single dose of nanoceria[34]; however, this hepatic damage was probably due to the high dose of administered NC (85 mg/kg), which resulted in a difficult nanoparticle clearance.

Given the intraperitoneal administration, a consistent amount of nanoceria is expected to remain in this compartment; however, as mentioned in the previous section, this is not expected to consistently alter the nanoparticle distribution, with respect to an intravenous administration.[15]

Concerning the excretion of NC, here we did not perform analysis on feces/urines. However, several studies pointed out as fecal excretion is the main way of nanoceria removal following administration by injection, ingestion or inhalation most probably because of biliary clearance.[15,28,30] Nanoceria excretion is indeed an important point that deserves future deeper investigations: while we consider the long-lasting effects of nanoceria an advantage for the treatment of obesity with respect to other traditional drugs, because of the chronic oxidative stress that characterizes this pathology, potential NC-based therapies should take into consideration how to stop the pharmacological effects when they are not necessary anymore. This can be achieved by a careful evaluation of the grade of the pathology and of the nanoparticle clearance, and consequently by finding optimal administration doses and modalities able to exert beneficial effects in terms of weigh reduction, without affecting the normal physiological redox state.

The analysis of the plasma profile of typical markers for the assessment of the lipogenesis shows that insulin, leptin, glucose and triglycerides concentrations are strongly reduced in NC-treated rats when compared to the control group. Insulin and leptin are important hormones that stimulate lipogenesis: the first causes a rise of the glucose uptake in the fat cell, the second limits the food intake.[35] Glucose and triglycerides are necessary to produce energy, and their high levels in blood are essential parameters to ascertain obesity.[36] Other findings confirm that the treatment with anti-oxidant agents stems similar effects on the circulating levels of molecules typical of the lipid synthesis: as an example, the orally administration to rats of the Korean traditional drug *Taeeumjowitangkagambang* attenuated the triglycerides and leptin levels, while caused a rise of the antioxidant enzymes with respect to the untreated animals.[37]

The analysis of adipogenesis gene transcription following the *in vivo* NC treatment revealed a significant reduction of five genes (*Lep*, *Bmp2*, *Twist1*, *Angpt2* and *Ddit3*) and a consistent,

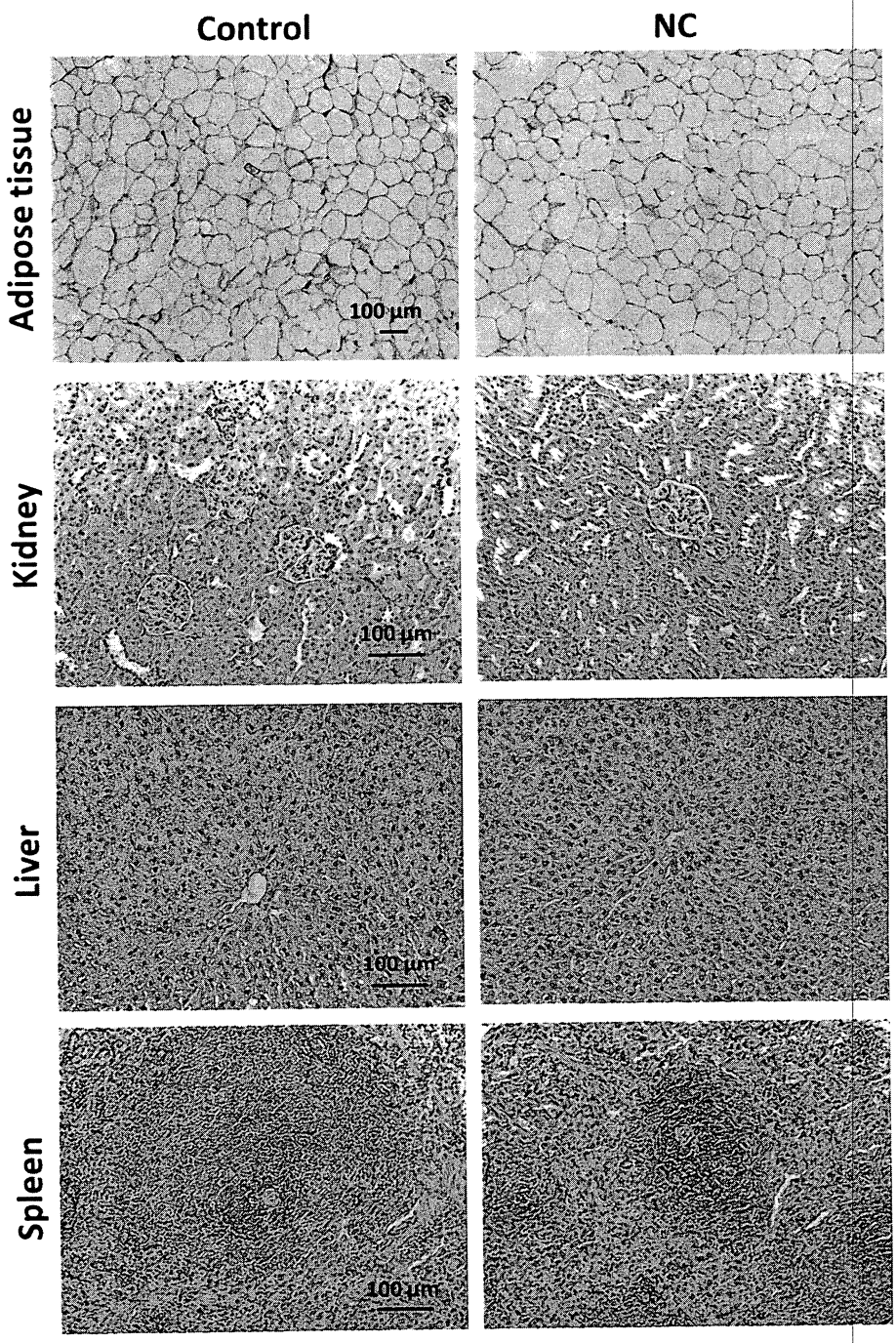

Figure 4. Histology: hematoxylin and eosin staining highlights that the typical aspect of the tissues of the major organs (adipose tissue, kidney, liver, spleen) is maintained in NC-treated animals.

but not-statistically significant, reduction of further two genes (*Bmp4* and *Ldha*). Among these, leptin (*Lep*) is the most down-regulated, coherently to the strong reduction of leptin plasma level we found in NC-treated rats. Leptin is an adipokine produced by adipose tissue, and its mRNA expression positively correlates with obesity.[38] The primary function of leptin is to regulate energy homeostasis and body weight, by acting on the central nervous system.[39] A study reported that the treatment of obese rats with lipoic acid, a micronutrient with antioxidant properties, suppresses oxidative stress and decreases leptin at both plasmatic and gene level.[40] This study is coherent with our hypothesis asserting that the decrement of ROS levels following nanoceria administration implies a lower leptin transcription, although the relevant molecular mechanisms have been not yet deeply investigated.

Bone morphogenetic protein 2 (*Bmp2*) promotes white fat production activating a signaling cascade that induces adipocyte differentiation.[41] The diminished transcription of *Bmp2*, after NC administration, is consistent with a study reporting the inhibition of adipogenesis following *Bmp2* suppression.[42]

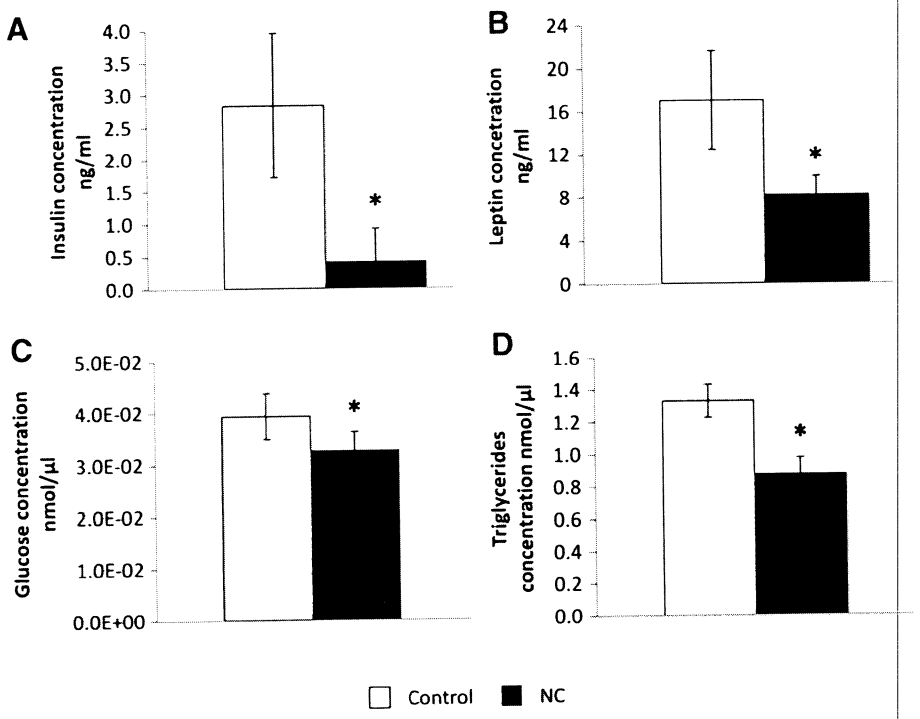

Figure 5. Biochemical analysis: plasma levels of insulin (**A**), leptin (**B**), glucose (**C**) and triglycerides (**D**) after 6 weeks of NC treatment. * $P < 0.05$.

Twist homolog 1 (*Twist1*) is a transcription factor, and its mRNA expression is reduced in adipose tissue of obese subjects, but indeed over-regulated during adipogenic differentiation.[43] The down-regulation of *Twist1* observed in our study can be probably due to the antioxidant activity of *Twist1*, which reduces ROS in several kinds of cells.[44] Our hypothesis is that NC, acting as ROS scavengers, induce a lower transcription of *Twist1* as a compensatory effects due to the decrement of ROS concentrations.

Angiopoietin 2 (*Angpt2*) is a glycoprotein involved in the development of vasculature necessary to sustain adipose tissue.[45] The reduced transcription of *Angpt2* mRNA caused by NC can be ascribed to the weight loss, that negatively affects angiogenic activity.[46]

DNA-damage inducible transcript 3 (*Ddit3*) regulates the endoplasmic reticulum stress, and induces apoptosis in response to cellular stress, including overweight.[47] In accordance with his function, Zhao et al demonstrated that *Ddit3* mRNA expression is elevated in liver of rat fed with high-fat diet, and conversely reduced after moving to low fat-diet.[48] The decrease of *Ddit3* mRNA transcription in rats treated with NC suggests as nanoparticles can attenuate obesity-induced endoplasmatic reticulum stress in adipose tissue, as in case of the before-mentioned switching to a low-fat diet.

Following NC treatment, adipose tissue has a considerable down-regulation of *Bmp4* (2.1-folds) and of *Ldha* (2-folds) compared to the control group, even if this difference was non-statistically significant. Bone morphogenetic protein 4 (*Bmp4*) cooperates with *Bmp2* to activate the signaling cascade involved in the earliest phase of white adipose tissue formation, as corroborated by an *in vivo* study reporting 2-folds increasing of *Bmp4* mRNA in inguinal fat of obese mice with respect to the controls.[49] In our conditions therefore we can ascribe the reduced transcription of *Bmp4* in NC-treated rats to an inhibition of the adipogenesis due to nanoparticle administration.

Further, the down-regulation of lactate dehydrogenase A (*Ldha*) also suggests that glyceroneogenesis, *i.e.*, the alternative pathway to glycolysis for the glyceraldeide-3-phosphate generation,[50] is reduced after 6 weeks of NC treatment, thus also reducing the triglycerides synthesis.

Concerning the up-regulated genes following the NC treatment, we found a significant increment of *Irs1* and *Klf4* expression. Insulin receptor substrate 1 (*Irs1*) is a substrate of the insulin receptor, and several studies sustain that its mRNA expression is reduced in the adipose tissue of obese animals, thus activating a synthesis pathway that leads to fat accumulation.[51] The increased transcription of *Irs1* in NC-treated rats is argued to be ascribable to an improved insulin sensitivity following NC treatment, analogously as observed by Qin et al in the case of chokeberries testing.[52] These authors proved that anti-oxidant chokeberry extract up-regulates *Irs1* mRNA level in rats fed with fructose-rich diet for 6 weeks, thus favoring their weight loss.

Kruppel-like factor 4 (*Klf4*) is induced in the early adipogenesis of 3T3-L1 cells,[53] but less is known concerning adipose tissue. Liao et al observed that adipose tissue of obese patients presents a lower expression of *Klf4* as compared to controls[54]: these data are coherent with our results, thus supporting the hypothesis that *Klf4* is involved in the resolution of inflammation states typical of obesity.

Finally, the evaluation of the G3PDH activity in the adipose tissue confirmed that NC are able to decrease the activity of this enzyme, that reduces the glycolytic intermediate glyceraldehei-de-3P to glycerol-3-phosphate for the triglycerides production. This result is consistent with the observation that an enhanced

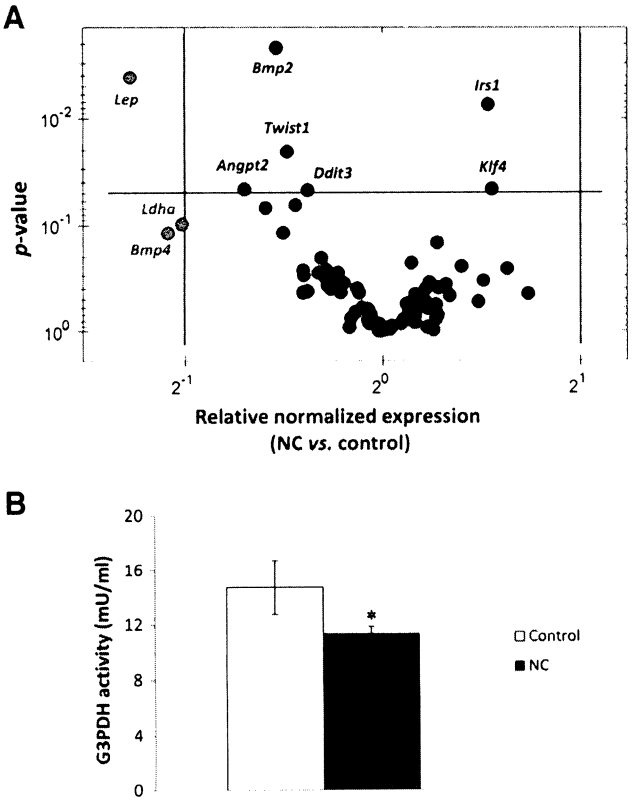

Figure 6. Volcano plot depicting relative normalized expression of the investigated genes in NC-treated rats with respect to the controls: the blue line indicates the *P*-value threshold (0.05), while the green and the red line defines a 2-fold down- and up-regulation, respectively (**A**). Analysis of the enzymatic activity of G3PDH in adipose tissue of rats treated for 6 weeks with respect to the control (**B**). * $P < 0.05$.

activity of this enzyme correlates with an increased fat deposition in human[55] and rats.[56] Since it is widely known that NC exert antioxidant defense, the possible molecular pathway that explains the weight reduction of rats can be elucidated as in the following: once NC have scavenged ROS, leptin is reduced, thus causing a decrease of triglycerides synthesis, confirmed by a reduction of G3PDH activity.

Despite plenty of researchers are focused on the development of anti-obesity drugs, conventional approaches have still several limitations including pathogenic side effects and instability of the compounds. Nanoceria can overcome these important problems due to their optimal tolerability and self-regenerating ability to restore their antioxidant effects. Hence, we can conclude that NC administration could become an effective mean in the clinical therapy of obesity, as demonstrated by a decrement of the *de novo* adipose tissue formation *in vitro* and the inhibition of weight gain *in vivo*, in absence of evident toxicity. Moreover, other beneficial effects of nanoceria on the whole organism, already documented in the literature, should be considered when envisaging their pharmaceutical applications, including increased life-span and protection against external toxic agents.[57]

Further studies are necessary to better elucidate the signaling cascade activated by NC before the application of these nanoparticles as a therapeutic approach against obesity, including testing on obesity animal models, but results are promising and encourage further efforts toward pre-clinical investigations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2015.05.001.

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