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

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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]

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

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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.