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Research Article

Potential Role of Cerium Oxide Nanoparticles for Attenuation of Diabetic Nephropathy by Inhibition of Oxidative Damage

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Abstract

Background: Diabetes is the growing health problem worldwide and increasing diabetes prevalence has been reported in the most countries. Several mechanisms are suggested for diabetic nephropathy that oxidative stress is the most important one. In this study efficiency of nanoceria (a potent antioxidant) was evaluated for attenuation of nephropathy and oxidative stress diabetic mice. **Materials and Methods:** Mice were divided into five groups, each comprising of 6 mouse, diabetes was induced by a single dose of streptozocin (65 mg kg^{-1} b.wt., IP) diluted in citrate buffer (pH = 4.6). One week after streptozocin administration, blood glucose was taken using a glucose oxidase method and the mice whose blood glucose values were above 200 mg dL^{-1} accepted as diabetic. All animals were anaesthetized and blood was collected for BUN and creatinine levels assessment in plasma and kidney tissue were excised at 4°C and oxidative stress and pathological changes were assayed. **Results:** The significant increase in BUN and creatinine in plasma in diabetic mice accompanied by pathological changes in kidney tissue confirmed the nephropathy in diabetic mice. Also, increased in reactive oxygen species formation, lipid peroxidation, glutathione oxidation and protein carbonyl concentration were observed in the kidney tissue of diabetic mice. Nanoceria treatment significantly ($p<0.05$) inhibited oxidative damage in kidney tissue and pathological changes in diabetic mice. **Conclusion:** This study showed that nanoceria has protective effects against diabetic nephropathy via inhibition of oxidative stress pathway. Therefore, nanoceria can be considered as a potential complementary therapy beside other blood glucose-lowering drugs for amelioration of diabetic complications.

Key words: Nanoceria, diabetes, nephropathy, oxidative stress, streptozocin

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes mellitus is one of the metabolic disorders which is diagnosed by hyperglycemia and inadequate endogenous insulin secretion or action[1]. Type 2 diabetes involves more than 90% of all cases of diabetes and the social and economic burden of diabetes is a major global problem especially in developing countries. The International Diabetes Federation estimated that 4.8 million people died of diabetes in 2012 and the number of adults with diabetes may increase to 552 million in 2030[2]. More than one-third of diabetic patients experience macrovascular and microvascular complications such as nephropathy[3,4]. Diabetic Nephropathy (DN) is the main cause of end-stage kidney disease in worldwide[5-8]. Which despite major improvement in diabetic care, its overall incidence remain considerable[9]. Acute renal failure has been defined by various criteria, primarily GFR or adjusted creatinine and urine output as proposed by the International Acute Dialysis Quality Initiative[10,11]. Several mechanisms are suggested for DN but oxidative stress is the most important factor in progression of diabetic complications such as nephropathy[12-14]. Diabetes conditions and subsequently high glucose concentration induces the production of oxygen and hydroxyl free radicals customarily[15,16]. Therefore, hyperglycemia directly increases oxidative stress in glomerular mesangial cells, a target cell in DN, so inhibition of oxidative stress could ameliorate all the manifestations associated with DN[12]. Previous studies revealed protective effects of some antioxidant against diabetic complications such as nephropathy[17-19]. Cerium is a lanthanide metal element that nanoparticle form is well-known as catalysts and antioxidant. Cerium oxide nanoparticles (Nanoceria) have oxygen defects in their lattice structure that lead to play as a regenerative free radical and scavenger in a physiological environment[20]. Hence, nanoceria, apparently well tolerated by the organism, might fight chronic inflammation and the pathologies associated with oxidative stress[21]. Previous studies showed that nanoceria increased antioxidant status and detoxification of free radicals in the brain and liver tissues[22,23]. The present study aims to examine the antioxidant effects of nanoceria in streptozocin-induced oxidative stress in the kidney of mice.

MATERIALS AND METHODS

Materials: Stereptozocine (STZ), sodium citrate, cerium oxide nanoparticles, comassie blue, ethylenediamine tetra acetic acid (EDTA), 5,5dithiobis-2-nitrobenzoic acid (DTNB), glutation (GSH), tris-Hcl, 4,2hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), morpholinopropansulfonic acid (MOPS), ethyleneglycol-bis (2-aminoethylether)-N,N,Ń, Ń-tetraacetic acid (EGTA), KCL, $MgCl_2$, KH_2PO_4, succinate, NaoH, ethanol, ethylacetate, 2'7'-dicholorofluoresceindiacetate (DCFH-DA), n-butanol, HCL, thiobarbutiric acid (TBA), phosphoric acid, trichloroacetic acid (TCA), Guanine hydrochloride and 2,4dinitrophenylhydrazine (DNPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and merck chemical Co. (Germany). All chemicals were of analytical grade and of standard biochemical quality.

Animal treatment: Male swiss albino mice, weighing 30-35 g were provided from Laboratory Animals Research Center, Mazandaran University of Medical Sciences, Sari, Iran. Animals were housed in an air-conditioned room with controlled temperature of 22±2°C and maintained on a 12:12 h light cycle with free access to food and water. All experimental procedures were conducted according to the ethical standard and protocols approved by the Committee of Animal Experimentation of Mazandaran University of Medical Sciences, sari, Iran.

Experimental design: Animals were divided into 5 groups, with 6 mouse in each group. Non-diabetic control mice, mice were treated with nanoceria (60 mg kg^{-1}), diabetic mice, diabetic mice treated with nanoceria at concentration of 60 mg kg^{-1} for 4 weeks, diabetic mice treated with vitamin E for 4 weeks, diabetes in male Swiss albino mice was induced by a single dose of intraperitoneal injection of streptozotocin (65 mg kg^{-1} b.wt.) diluted in citrate buffer (pH = 4.6)[24]. One week after STZ administration, blood was taken from the lateral veins of the tail and blood glucose was measured by a glucometer using glucose oxidase method. The mice whose blood glucose values were above 200 mg dL^{-1} were accepted as diabetic, then all animals were anaesthetized and blood was collected from the heart by syringe then were centrifuged at 3000×g for 5 min at 4°C and plasma was frozen at -70°C until use. Kidney tissue were excised on ice and was homogenized in phosphate buffered saline, then centrifuged at 800×g for 10 min at 4°C. The supernatant was collected and oxidative stress markers were assayed.

Measurement of Blood Urea Nitrogen (BUN) and creatinine: The BUN and creatinine are markers of kidney dysfunction that were determined by commercial reagents (obtained from Parsazmoon Co. Iran).

Determination of Reactive Oxygen Species (ROS): To determine the amount of kidney tissue ROS generation, dichlorofluorescin-diacetate (DCFH-DA) was used as indicator. Briefly 2 mL of kidney tissue homogenate (1 mg protein mL^{-1}) loaded with DCFH by incubating with this reagent for 15 min at 37°C. Then was monitored at 480 nm (excitation) and at 520 nm (emission) by Shimadzu RF5000U fluorescence spectrophotometer[25].

Measurement of lipid peroxidation (LPO): The content of MDA was determined using the method of Zhang *et al.*[26], briefly 0.25 mL phosphoric acid (0.05 M) was added to 0.2 mL of kidney tissue homogenate with the addition of 0.3 mL 0.2% TBA. All the samples were placed in a boiling water bath for 30 min. At the end, the tubes were shifted to an ice-bath and 0.4 mL n-butanol was added to each tube. Then, they were centrifuged at 3500 rpm for 10 min. The amount of MDA formed in each of the samples was assessed through measuring the absorbance of the supernatant at 532 nm with an ELISA reader (Tecan, Rainbow Thermo, Austria). Tetramethoxypropane (TEP) was used as standard and MDA content was expressed as nmol mg^{-1} protein[26].

Measurement of glutathione content: Glutathione (GSH) content was determined by DTNB as the indicator and spectrophotometer. Briefly 0.1 mL of kidney tissue was added into 0.1 mol L^{-1} of phosphate buffers and 0.04% DTNB in a total volume of 3.0 mL (pH 7.4). Then developed yellow color and was read at 412 nm on a spectrophotometer (UV-1601 PC, Shimadzu, Japan). The GSH content was expressed as µg mg^{-1} protein[27].

Measurement of protein carbonyle: Determination of protein carbonyl by spectrophotometric method, briefly 200 µL are need of kidney tissue hemogenate. Samples are extracted in 500 µL of 20% (w/v) TCA. Then, samples placed at 4°C for 15 min. The precipitates are treated with 500 µL of 0.2% DNPH and 500 µL of 2 N HCl for control group and samples are incubated at room temperature for 1 h with vortexing at 5 min intervals. Then proteins are precipitated by adding 55 µL of 100% TCA. The micro-tubes are centrifuged and washed three times with 1000 µL of the ethanol-ethyl acetate mixture. And the micro-tubes are dissolved in 200 µL of 6 M guanidine hydrochloride. The carbonyl content is determined by reading the absorbance at 365 nm wavelength[28].

Measurement of catalase content: Catalase activity was assayed by measuring the absorbance decrease at 240 nm in a reaction medium containing H_2O_2 (10 mM), sodium phosphate buffer (50 mM, pH: 7.0). One unit of the enzyme is defined as 1 mol H_2O_2 as substrate consumed per minute and the specific activity is reported as units per milligram protein[29].

Measurement of protein concentration: Protein content was determined in kidney tissue with bradford method[30]. Bovine Serum Albumin (BSA) was used as standard, homogenate samples mixed with coomassie blue and after 10 min, absorbance were determined at 595 nm by spectrophotometer.

Pathological investigation: Firstly animal anesthetized by chloroform, then kidney tissue were removed from the control and tested mice and rinsed with physiologic serum and fixed in formalin for 18 h then dehydrated in a graded series of ethanol and also we used toluene for extracting alcohol, after we used paraffin in oven for tissue and rapidly, tissues saturated by paraffin and after 4 h, blocks samples fixed on microtome and sections with thickness of 13 µm were obtained. Then sections were transferred on slides. Finally for assessment with light microscope were stained with hematoxylin and eosin[31].

Statistical analysis: Results are presented as Mean±SD. All statistical analyses were performed using the SPSS software, version 10. Assays were performed in triplicate and the mean was used for statistical analysis. Statistical significance was determined using the one-way ANOVA test, followed by the *post hoc* Tukey test. Statistical significance was set at $p<0.05$.

RESULTS

Effects of nanoceria on body weight and plasma glucose: Table 1 and 2 shows that diabetic mice had lower weight gain than control group and nanoceria alone had not significant effect on the body weight but inhibited weight loss in diabetic mice when administrated for 4 weeks. But nanoceria injection

Table 1: Effect of *in vivo* administration of cerium oxide nanoparticles on weight in the treated mice before injection of sodium citrate, STZ, nanoceria and vitamin E (day 0) and in the 35 days after of treatment

	Body Weight (g) (Mean±SD)	
Animals groups	Day 0	Day 35
Control	32.0±2	38.0±1
Diabetic	32.5±1	24.7±1
Nanoceria	33.0±2	36.0±1
Diabetic+nanoceria	32.0±1	32.0±2
Diabetic+vitamin E	32.0±1	31.0±2

Values are expressed as Mean±SD for 6 mouse in each group

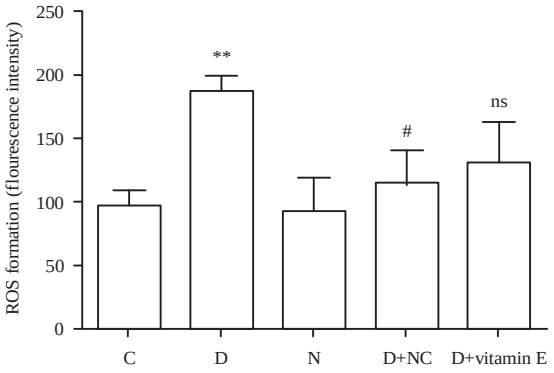

Fig. 1: Effect of nanoceria on ROS formation in kidney tissue. The ROS formation in C: Control mice, D: Diabetic mice, N: Mice that received nanoceria for 4 weeks only, D+Nc: Diabetic mice that received nanoceria for 4 weeks, D+vitamin E: Diabetic mice that received vitamin E for 4 weeks was determined using DCF-DA. Values represented as Mean±SD (n = 6). **p<0.01 compared with control mice, #p<0.01 compared with diabetic mice

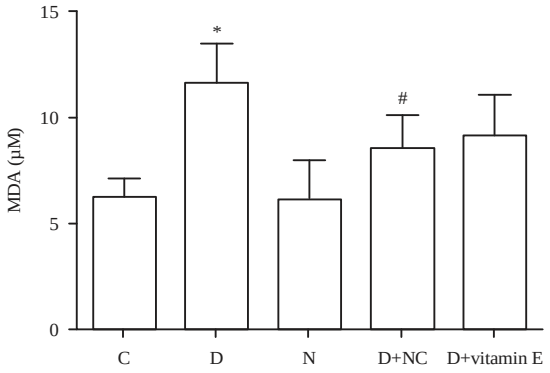

Fig. 2: Effect of nanoceria on lipid peroxidation in kidney tissue, MDA formation in C: Control mice, D: Diabetic mice, N: Mice that received nanoceria for 4 weeks only, D+Nc: Diabetic mice that received nanoceria for 4 weeks, D+vitamin E: Diabetic mice that received vitamin E for 4 weeks was measured using TBA reagent. Values represented as Mean±SD (n = 6), *p<0.05 compared with control mice, #p<0.01 compared with diabetic mice

Table 2: Effect of *in vivo* administration of cerium oxide nanoparticles on blood glucose levels in the treated mice before injection of sodium citrate, STZ, nanoceria and vitamin E (day 0) and in the 35 days after of treatment

	Plasma glucose (mg dL^{-1}) (Mean±SD)	
Animals groups	Before injection (0 day)	After injection (35th day)
Control	88±3	89±3
Diabetic	280±13.2***	300±15***
Nanoceria	90±5	95±3
Diabetic+nanoceria	280±13.2###	283±3###
Diabetic+vitamin E	280±13.2###	290±10###

Values are expressed as Mean±SD for 6 mouse in each group, ***Significantly different when compared to the control (p<0.001), ###Significantly different when compared to the diabetic mice (p<0.001)

Table 3: Effect of *in vivo* administration of nanoceria and vit E on blood urea nitrogen (BUN) and serum creatinine in control and treated mice blood

Animal groups	BUN (mg dL^{-1})	Creatinin (mg dL^{-1})
Control	24±3	0.5±0.1
Diabetic	73±5**	1.2±0.3**
Nanoceria	23±2	0.4±0.01
Diabetic+nanoceria	48±5#	0.74±0.1#
Diabetic+vitamin E	50±5#	0.80±0.2#

Values are expressed as Mean±SD for 6 mouse in each group, **Significantly different when compared to the control (p<0.01), #Significantly different when compared to the diabetic mice (p<0.05)

caused no significant (p<0.05) decrease in blood glucose in diabetic mice and in diabetic mice that received vitamin E has shown similar effect to nanoceria.

Effect of *in vivo* administration of nanoceria on BUN and serum creatinine: Diabetes induction was associated with significant (p<0.05) increase in serum levels of BUN and creatinine which are indicators of kidney damage and nanoceria administration prevented the elevation of BUN and creatinine in diabetic mice which is similar to effect of vitamin E (Table 3).

ROS formation: The ROS formation is indicator of oxidative stress that as shown in Fig. 1, significantly (p<0.05) was increased in diabetic mice and ROS formation markedly (p<0.05) were decreased after nanoceria administration but in diabetic mice treated with vitamin E no significant (p<0.05) change was seen in ROS formation in kidney tissue (Fig. 1).

Lipid peroxidation: One of the end products of LPO is malondialdehyde (MDA) and elevation of MDA is known a marker for oxidative stress. The MDA level was increased in diabetic kidney tissue significantly (p<0.05) as compared to control, as indicated in Fig. 2 diabetes induced LPO significantly was inhibited by nanoceria and nanoceria showed better protective effect against LPO than vitamin E (Fig. 2).

GSH concentration: Generally imbalance between ROS and antioxidants levels such as GSH in kidney caused oxidative stress-induced renal injury in diabetic patients[32]. The GSH levels in diabetic mice decreased to 72.33 μM in kidney tissue as compared to control group (127/3333 μM) and GSH concentration in diabetic mice that received nanoceria for

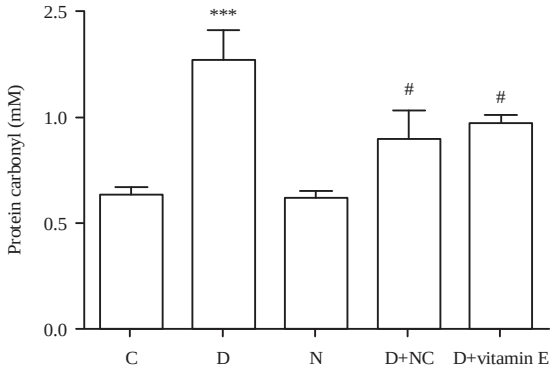

Fig. 3: Effect of nanoceria treatment on GSH levels in kidney tissue, GSH levels in C: Control mice, D: Diabetic mice, N: Mice that received nanoceria for 4 weeks only, D+Nc: Diabetic mice that received nanoceria for 4 weeks, D+vitamin E: Diabetic mice that received vitamin E for 4 weeks was determined using DTNB. Values represented as Mean±SD (n = 6), **p<0.01 compared with control mice, #p<0.01 compared with diabetic mice

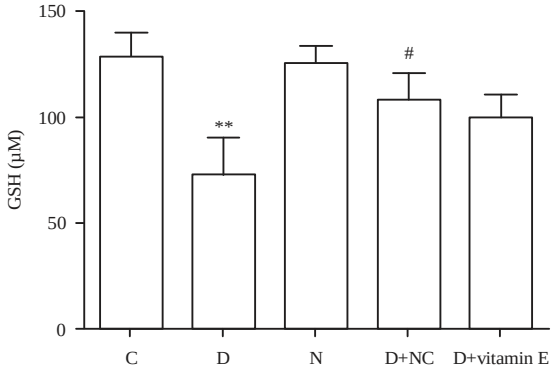

Fig. 4: Effect of nanoceria treatment on catalase levels in kidney tissue, GSH levels in C: Control mice, D: Diabetic mice, N: Mice that received nanoceria for 4 weeks only, D+Nc: Diabetic mice that received nanoceria for 4 weeks, D+vitamin E: Diabetic mice that received vitamin E for 4 weeks was determined. Values represented as Mean±SD (n = 6), **p<0.01 compared with control mice, #p<0.01 compared with diabetic mice

4 weeks was 108/33 µM that significantly (p<0.05) was higher than of diabetic mice and GSH level in mice that treated with vitamin E was 99 µM and overall nanoceria showed the better effect as compare to vitamin E (Fig. 3).

Catalase activity: Figure 4 shows that catalase activity increased in diabetic mice and administration of nanoceria significantly (p<0.05) decreased catalase activity in

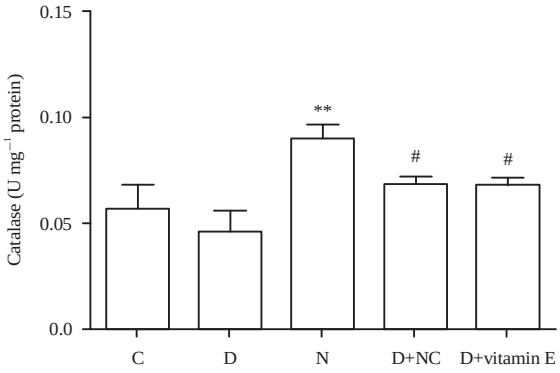

Fig. 5: Effect of nanoceria treatment on protein carbonyl levels in kidney tissue, protein carbonyl levels in C: Control mice, D: Diabetic mice, N: Mice that received nanoceria for 4 weeks only, D+Nc: Diabetic mice that received nanoceria for 4 weeks, D+vitamin E: Diabetic mice that received vitamin E for 4 weeks was determined. Values represented as Mean±SD (n = 6), ***p<0.001 compared with control mice, #p<0.01 compared with diabetic mice

diabetic mice as compared to control group and vitamin E has lower effect than nanoceria (Fig. 4).

Protein carbonyl: Protein carbonyl is an indicator of protein oxidation in diabetic patients that can be monitored by the changes of absorbance at 365 nm. Administration of nanoceria leads to decrease of protein carbonyl as compared to diabetic group (Fig. 5).

Histological examination: Histological studies in kidney of STZ-induced diabetic mice showed glomerular size increase in the tubules of proximal convoluted and this alterations were effectively decreased after treatment with nanoceria after 4 weeks (Fig. 6a-c).

DISCUSSION

In this study, we evaluated the ability of nanoceria in preventing of diabetic nephropathy induced by streptozotocin in male mice. Consistent with the previous studies on the elevation of oxidative damage in diabetic situation[12-14,16,33]. The results of this study showed development of oxidative stress markers in diabetic mice. In addition, nanoceria administration could improve the pathological and biochemical markers of kidney damage in diabetic mice. Prevalence of type 2 diabetes is increasing in through of the world and this disorder is associated with various structural and functional complications[34-36]. Elevation

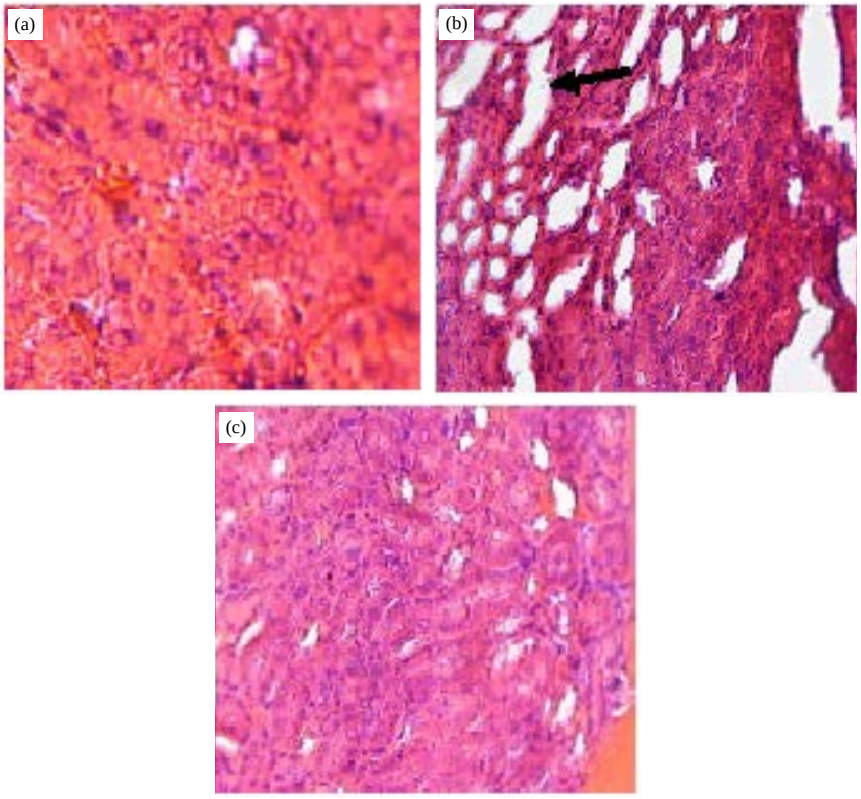

Fig. 6(a-c): Effects of nanoceria on diabetes induced-morphological change in kidney tissue. Pathological examination in mice kidney of control group, nanoceria (Only) group, STZ: Induced diabetic group and STZ+nanoceria group. Tissue sections were stained with hematoxylin and eosin and evaluated with light microscope (200x)

of blood glucose level in diabetic patient could lead to induction of ROS generation in both humans and animal[37]. Previous studies have shown that ROS has the main role in the triggering pathophysiological signaling which leads to development of numerous macrovascular and microvascular complications including nephropathy[38]. Our results demonstrated that induction of diabetes in mice, results in imbalance of the ROS production and antioxidant system in kidney tissue (decreased GSH and increased catalase activity) in comparison to control mice that was parallel to elevation of BUN and creatinine and pathological changes in kidney tissue. These results were in line with the previous reports that showed hyperglycemia is the major cause of oxidative stress and main risk factor for diabetic nephropathy[12]. Indeed, numerous cell types such as endothelial, vascular smooth muscle, mesangial and tubular epithelial cells are able to ROS production under hyperglycemic condition. These observations indicated that oxidative stress could be considered as a major risk factor in progression of diabetic nephropathy. Therefore, in addition to tight glycemic control, using of antioxidant may be another strategy to reduce pathologic consequences of hyperglycemia. Use of antioxidants has increased in the management of diabetes side effects over the last few years[17,18,39-41]. Recent studies have shown protective effects of many natural (plants) and synthetic (vitamins, supplements) antioxidants for improvement of diabetic nephropathy[17,19,31,33,39,42]. Cerium is a metal element of lanthanide family, when oxided and surrounded by lattice of oxygen, could has similar potential antioxidants such as superoxide dismutase and catalase and showed beneficial profile in alleviating pathological situation which induced by oxidative stress[22,43,44]. However, little information is available about the effect of nanoceria on diabetic nephropathy. Therefore, it was hypothesized that the nanoceria administration would ameliorate diabetes-induced damage in kidney tissues. In this study it is observed that high serum levels of creatinine and BUN in diabetic mice that are indicators of kidney damage. Though, these factors improved after treatment with nanoceria that probably is because of free radical scavenging activity of cerium oxide nanoparticles[44]. Oxidative stress promoted peroxidative reactions in lipids and increasing MDA concentration an indicator of LPO[45]. It is found that an increased in LPO in the diabetic group which are

consistent with previous reports that showed increased LPO during diabetic's nephropathy[40]. This parameter significantly ($p<0.05$) decreased in diabetic mice that received nanoceria. Also ROS may damage to proteinsand protein oxidation is an important result of ROS overproduction in biological system and protein carbonyl is considered as a marker of protein damage[46,47]. In present study we observed a significant increase in protein carbonyl levels in diabetic animals on the other hand, in diabetic mice which received nanoceria, a decrease in protein carbonyl levels was observed in kidney tissue. In the physiological state, endogenous antioxidant system such as superoxide dismutase (SOD), glutathione peroxidase, catalase and glutathione prevents oxidative stress mediated kidney damage. In mammals, these molecules are responsible for the detoxification of free radicals that generated during glucose auto-oxidation[48]. So, any disturbances in the antioxidant defense system compromises cellular redox balance and cell viability which finally leads to organ failure. As alteration in antioxidant enzymes activities, impaired glutathione metabolism[49] and decreased ascorbic acid levels in diabetes model were reported in previous studies[39]. Administration of STZ increased catalase activity and decreased GSH content in kidney tissue in diabetic group as compared to control group that were an agreement with previous studies[33]. Nanoceria administration inhibited depletion of antioxidant system in diabetic mice. Infact ROS production is one of the main consequences of abnormal glucose metabolism in diabetic patients and reducing GSH levels was happened in diabetes[18,45]. It is shown that nanoceria reduce ROS production in diabetic mice and balanced antioxidant system. Also, diabetic animals not gained weight as severity as thatof control mice after 4 weeks. But at the end of the experiment, in histological investigation, higher kidney/body weight ratios were observed. This may be due tohypertrophied of glomerular and increasing of thickness the kidney membrane in diabetic mice[50]. Treatment with nanoceria improved histopathological changes in kidney tissue. But, no significant change inplasma glucose concentration was observed in diabetic group that received nanoceria compared to diabetic groups that supposedthe protective effects of nanoceria probably is due to its antioxidant properties and free radical scavenging activity[51]. These effects mediated via reversible ROS binding and destruction of free radicals with shifting between the Ce^{3+} (reduced) and Ce^{4+} (oxidized) forms at the particle surface[21].

CONCLUSION AND FUTURE RECOMMENDATION

In conclusion, this study showed that nanoceria have protective effects against diabetic nephropathy via reducing oxidative stress. Therefore, antioxidant features of nanoceria make it an attractive candidate as complementary therapies beside other blood glucose-lowering drugs for diabetic complications. Future research is required to determine exact dose and duration of supplementation with nanoceria.

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