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Expression of heterologous oxalate decarboxylase in HEK293 cells confers protection against oxalate induced oxidative stress as a therapeutic approach for calcium oxalate stone disease

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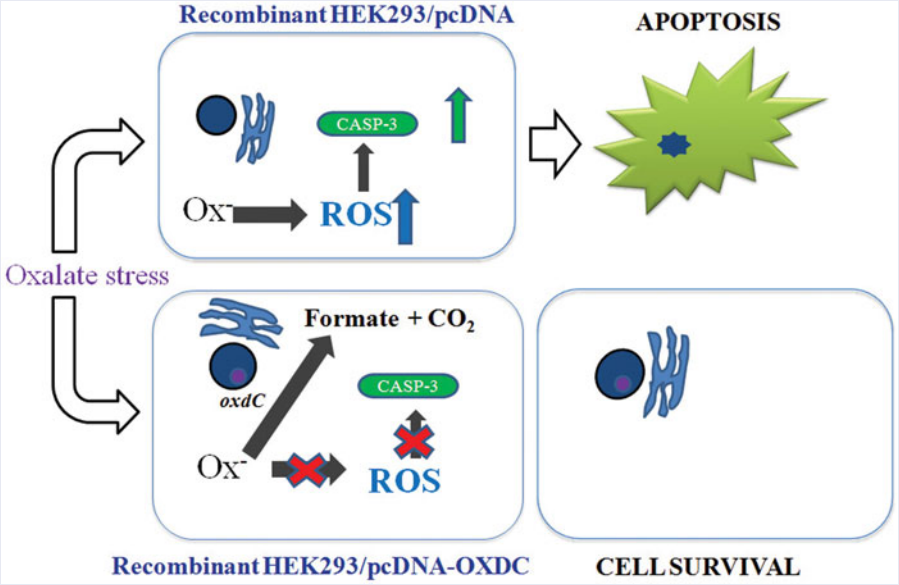
ABSTRACT

Oxalates stimulate alterations in renal epithelial cells and thereby induce calcium oxalate (CaOx) stone formation. *Bacillus subtilis YvrK* gene encodes for oxalate decarboxylase (OxdC) which degrades oxalate to formate and CO_2. The present work is aimed to clone the *oxdC* gene in a mammalian expression vector pcDNA and transfect into Human Embryonic Kidney 293 (HEK293) cells and evaluate the *oxdC* expression, cell survival rate and oxalate degrading efficiency. The results indicate cell survival rate of HEK293/pcDNAOXDC cells pre-incubated with oxalate was enhanced by 28%. HEK293/pcDNAOXDC cells expressing OxdC treated with oxalate, significantly restored antioxidant activity, mitochondrial membrane potential and intracellular reactive oxygen species (ROS) generation compared with HEK293/pcDNA. Apoptotic marker caspase 3 downregulation illustrates HEK293/pcDNAOXDC cells were able to survive under oxalate-mediated oxidative stress. The findings suggest HEK293 cells expressing *oxdC* capable of degrading oxalate protect cells from oxidative damage and thus serve as a therapeutic option for prevention of CaOx stone disease.



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Introduction

Kidney stone disease is a common, painful condition result from the combination of epidemiological, biochemical and genetic risk factors[1]. Calcium oxalate (CaOx) stones are the most prevalent type, accounting for 70–80%. Hyperoxaluria is a condition which is associated with increased excretion of oxalate, a major risk factor for CaOx urolithiasis. Oxalate accumulation in renal tubular cells contributes to the progression of renal injury. The interaction between the oxalate crystals and renal cells result in reactive oxygen species (ROS) generation, and thus play a major role in stone formation. Further, disruption of the cell membrane can lead to mitochondrial dysfunction which initiates the cell to form ROS in abundance. Management of stone disease depends on the size, location and prevailing treatment options have significant drawbacks such as renal damage, renal impairment and stone recurrence. The use of oxalate-degrading microorganisms such as *Oxalobacter formigenes* (*O. formigenes*) in the intestines of animals and human beings was focused as an alternative approach in the prevention of CaOx stone disease. However, the stable colonization efficiency and antibiotic sensitivity of *O. formigenes* in the intestine are still uncertain[2]. Previous report revealed the successful insertion of oxalate degrading genes Oxalyl-CoA decarboxylase (*oxc*) and formyl-CoA transferase (*frc*) from *O. formigenes* into human embryonic kidney 293 cells (HEK293) and suggested that these genes are probable prospects for gene therapy[3]. Zhiqiang et al.[4] have demonstrated the transfer of *oxc* and *frc* into intestinal stem cells to prevent CaOx-related stone formation. Although the transfected cells showed significant degradation of oxalate in the medium, the alterations in oxidative stress and survival efficiency of cells are yet to be analyzed.

The identification of oxalate decarboxylase (*oxdC*) from *Bacillus subtilis* (*B. subtilis*) proved to be a possible therapeutic option for calcium oxalate stone disease in human. OxdC can degrade oxalate into formate and carbon dioxide[5]. Previously, the heterologous expression of OxdC in *Lactobacillus plantarum* (*L. plantarum*) was developed and utilized as a potential probiotic for depletion of intestinal dietary oxalate[6–8]. Since treatment options for patients with primary hyperoxaluria are limited and the use of recombinant bacteria is restricted for degradation of intestinal oxalate, an alternative approach is required. Hence, the present study is focused to investigate oxalate degrading efficiency and cell survival ability of *oxdC* transfected HEK293 in oxalate induced oxidative stress condition.

Materials and methods

Cell culture

HEK293 cells were obtained as a gift from Dr. Giridhara R. Jayandharan, Indian Institute of Technology, Kanpur, India. The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hi-media), 100 U/ml penicillin (Hi-media) and 0.1 mg/ml streptomycin (Hi-media) at 37 °C in a humidified 5% CO_2 atmosphere.

Construction of recombinant vector pcDNAOXDC

The eukaryotic expression vector pcDNA 3.1 (−), Invitrogen, Carlsbad, CA was used for cloning of bacterial gene *oxdC*. PCR amplified His tag fused *oxdC* was cloned in pcDNA vector at *Nhe* I and *Hind* III restriction sites and the resulting recombinant plasmid pcDNAOXDC was confirmed by PCR, restriction digestion and DNA sequencing. To evaluate the protein localization of OxdC in HEK293 cells, eukaryotic expression vector pEGFP-N1 (Clonetech) was employed to subclone the gene of interest *oxdC* and transfected in HEK23 cells. The GFP-tagged OxdC protein expression was visualized using Nikon Eclipse Ti fluorescence microscope (Nikon, Tokyo, Japan).

Stable transfection of HEK293 cells

Transfection was performed using lipofectamine 3000, Invitrogen, Carlsbad, CA according to the manufacturer's instructions. For stable transfection, cells were selected in DMEM medium containing 0.8 mg/ml geneticin (G418, Invitrogen, Carlsbad). The selective medium was changed every 2–3 days till transfectants appeared. The clones were screened by semi-quantitative RT-PCR and confirmed by Western blot analysis using primary mouse monoclonal antibody against 6x-His Epitope Tag Antibody (1:1000, Thermo Fischer Scientific) and a primary rabbit polyclonal antibody against human ß-actin (1:1000, Santa Cruz). Goat anti-mouse IgG (1:1000, Santa Cruz) and Goat anti-rabbit IgG conjugated with HRP (Genei, India) (1:2500) were used as the secondary antibody.

Cytotoxicity assays

Cell viability was evaluated by measuring 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction. Following treatment of oxalate (750 μM) on recombinant HEK293/pcDNA and HEK293/pcDNAOXDC cells for 18 h, MTT was added to the medium (final concentration 0.5 mg/ml) and incubated for 4 h in a humidified atmosphere at 37 °C. The media was removed from wells leaving formazone crystals at the bottom which were dissolved in 200 μl of DMSO. Absorbance was recorded at 570 nm immediately. Optical density values of each well were normalized against the control wells in which no stress was given. Cytotoxic effect of oxalate on recombinant HEK293/pcDNAOXDC cell proliferation was determined by trypan blue exclusion assay by harvesting cells after 18 h. Briefly, the cells were seeded (0.8×10^5/ml) in plates and subjected to oxalate stress. Cells were examined under an optical microscope after trypan blue staining. The percentage of unstained cells was counted and recorded. On staining cells with propidium iodide, live and dead cell population was screened using flow cytometry (BD FACSAria III, BD Biosciences, San Jose)[9]. The data were analyzed using FlowJo v X.0.6 software.

Antioxidant profile

After exposure to oxalate, the cells were washed twice with ice cold PBS and whole cell lysate was prepared by addition of cold lysis buffer (Tris-Cl and sodium fluoride, 50 mM of Tris-Cl; NaCl, 0.15 M; EDTA, 2 mM; sodium pyruvate, 1 mM; PMSF, 10 μg/ml; and triton-X, 0.1%). The cell lysates were centrifuged at 5000 rpm for 10 min and the protein content of the supernatant was estimated using Bradford reagent (Sigma–Aldrich). Catalase activity was assayed by the method of Sinha[10]. The dichromate in the acetic acid is reduced to chromic acetate when heated in the presence of hydrogen peroxide. The chromic acetate thus formed was measured colorimetrically at 570 nm. Results were expressed as μmol of H_2O_2 consumed per mg of protein in one minute. Superoxide dismutase activity was measured by the protocol developed by Kakkar et al.[11]. The enzyme assay was based on the 50% inhibition of formation of nicotinamide adenine dinucleotide-phenazine methosulphate nitroblue tetrazolium formation and the color developed was read at 520 nm. One unit of enzyme activity was defined as the amount of enzyme required to give 50%

inhibition of nitroblue tetrazolium reduction. The lipid peroxidation was determined by estimating malondialdehyde (MDA)[12].

Mitochondrial membrane potential

On exposure of recombinant HEK293/pcDNA and HEK293/pcDNAOXDC cells with oxalate (750 μM for 18 h), alterations in mitochondrial membrane potential were analyzed using 0.5 μM MitoTracker Deep Red, Invitrogen, Carlsbad by flow cytometry[13].

Detection of intracellular ROS generation

In order to detect intracellular ROS generation, recombinant HEK293/pcDNA and HEK293/pcDNAOXDC cells were treated with oxalate (750 μM) for 18 h. Levels of net intracellular ROS production were measured using 2, 7 dichlorodihydrofluorescein diacetate (H_2DCFDA) by flow cytometry[14].

Gene expression analysis

The mRNA levels of β-actin and Caspase 3 in recombinant HEK293/pcDNA and HEK293/pcDNAOXDC cells exposed to oxalate (750 μM for 18 h) were determined with SYBR® green master mix (Fermentas, Inc.) using Real-Time Thermal cycler (Mastercycler® ep realplex; Eppendorf AG, Germany). The primers used in the study were adapted from Tian et al.[15].

Western blot analysis

The whole-cell proteins (20 μg) of recombinant HEK293/pcDNA and HEK293/pcDNAOXDC cells exposed to oxalate (750 μM for 18 h) were solubilized in the sample buffer, separated using SDS-PAGE and transferred to PVDF membrane. The membrane was blocked and exposed to primary rabbit polyclonal antibodies (anti-Caspase 3, 1:500) (Pierce) overnight at 4 °C. The membrane was washed and incubated with the HRP-conjugated anti-rabbit IgG (1:2500) (Genei, India) for 60 min and was detected with Amersham ECL Western blotting reagent (GE healthcare Life Sciences). The blot was scanned using Bio-Rad Gel Doc XR and the intensity of protein bands normalized to β Actin was quantified using Image Lab Software version 5 (Bio-Rad).

Oxalate degrading ability

The enzyme activity of OxdC was assayed by determining the change of oxalate concentration in the medium. Following recombinant HEK293/pcDNA and HEK293/pcDNAOXDC cells cultured for 48 h at 37 °C, the medium was replaced with DMEM supplemented with 750 μM of potassium oxalate. Subsequently, the growth medium was collected at different time points (0, 6, 12, 18, 24, 48 h) and filtered. The cell free extracts were analyzed for oxalate by using a commercially available enzymatic kit (Trinity Biotech).

Statistical analysis

All the grouped data were statistically evaluated with SPSS Statistics Version 19 software. Statistically significant differences between samples were determined by one-way ANOVA followed by Least Significant Difference (LSD) post-hoc analysis. Data were presented as mean ± standard deviation (SD). Results were considered significant if p values <0.05.

Results

Stable transfection of HEK293 cells with pcDNAOXDC

Recombinant plasmid pcDNA-OXDC and vector pcDNA were transfected into HEK293 cells by lipofection. The integration of *oxdC* in the cell was demonstrated by semi-quantitative RT-PCR (Figure 1A) and OxdC protein expression (44 kDa) was confirmed by Western blot (Figure 1B). The recombinant OxdC protein secreted into the supernatant of transfected cell lines was quantified to be 26.3 μg/ml. The fluorescence tagged protein OxdC was observed in the cytoplasm of HEK293 cells (Figure 1C,D)

Heterologous OxdC augment cell viability in oxalate induced toxicity

We investigated the effect of *oxdC* on proliferation and survival of cells. Results illustrated that the cell viability was significantly affected in recombinant HEK293 cells devoid of *oxdC* after 18 h of oxalate treatment. The cytotoxic effect of oxalate was limited in recombinant HEK293/pcDNAOXDC cells. MTT assay revealed HEK293/pcDNAOXDC cells showed enhanced cell viability (28%) (Figure 1E,F) compared with HEK293/pcDNA cells. Figure 1(G,H) shows the cells expressing OxdC exhibited significantly higher levels (∼24%) of survival ability ($p < 0.05$) than cells devoid of OxdC.

Ameliorative effect of OxdC on antioxidant enzyme markers

Catalase and superoxide dismutase activities were significantly reduced in HEK293/pcDNA cells treated with oxalate when compared with non-exposed cells (Table 1). However, no significant alteration in catalase activity was observed in HEK293/pcDNAOXDC cells. Superoxide dismutase activity was significantly increased in recombinant HEK293/pcDNAOXDC cells compared to HEK293/pcDNA cells with oxalate. As a consequence of oxidative stress, the concentration of MDA was significantly increased by 60% in HEK293/pcDNA cells. On the contrary, the MDA level in recombinant HEK293/pcDNAOXDC cells was significantly lower than HEK293/pcDNA cells pre-incubated with oxalate (Table 1).

Assessment of mitochondrial membrane potential

Oxalate exposure to HEK293 cells modulated mitochondrial membrane potential. Recombinant HEK293 cells expressing OxdC showed increased membrane potential when compared to HEK293/pcDNA cells treated with oxalate. Maximum mean fluorescence intensity was observed in HEK293/pcDNA cells not subjected to oxalate (Figure 2A,B).

OxdC expression reduce the ROS generation

On exposure to oxalate, recombinant HEK293 cells expressing OxdC showed significantly decreased ROS generation (45.5%) when compared with HEK293 cells devoid of OxdC (76.33%) ($p < 0.05$) (Figure 2C,D).

OxdC protect cells from apoptosis

HEK293/pcDNA cells pre-incubated with oxalate showed significant upregulation of apoptotic marker caspase 3 when compared with HEK293/pcDNA cells devoid of oxalate stress. The recombinant HEK293/pcDNAOXDC cells pre-incubated with oxalate, exhibited significant reduction of caspase 3 mRNA and protein expression in

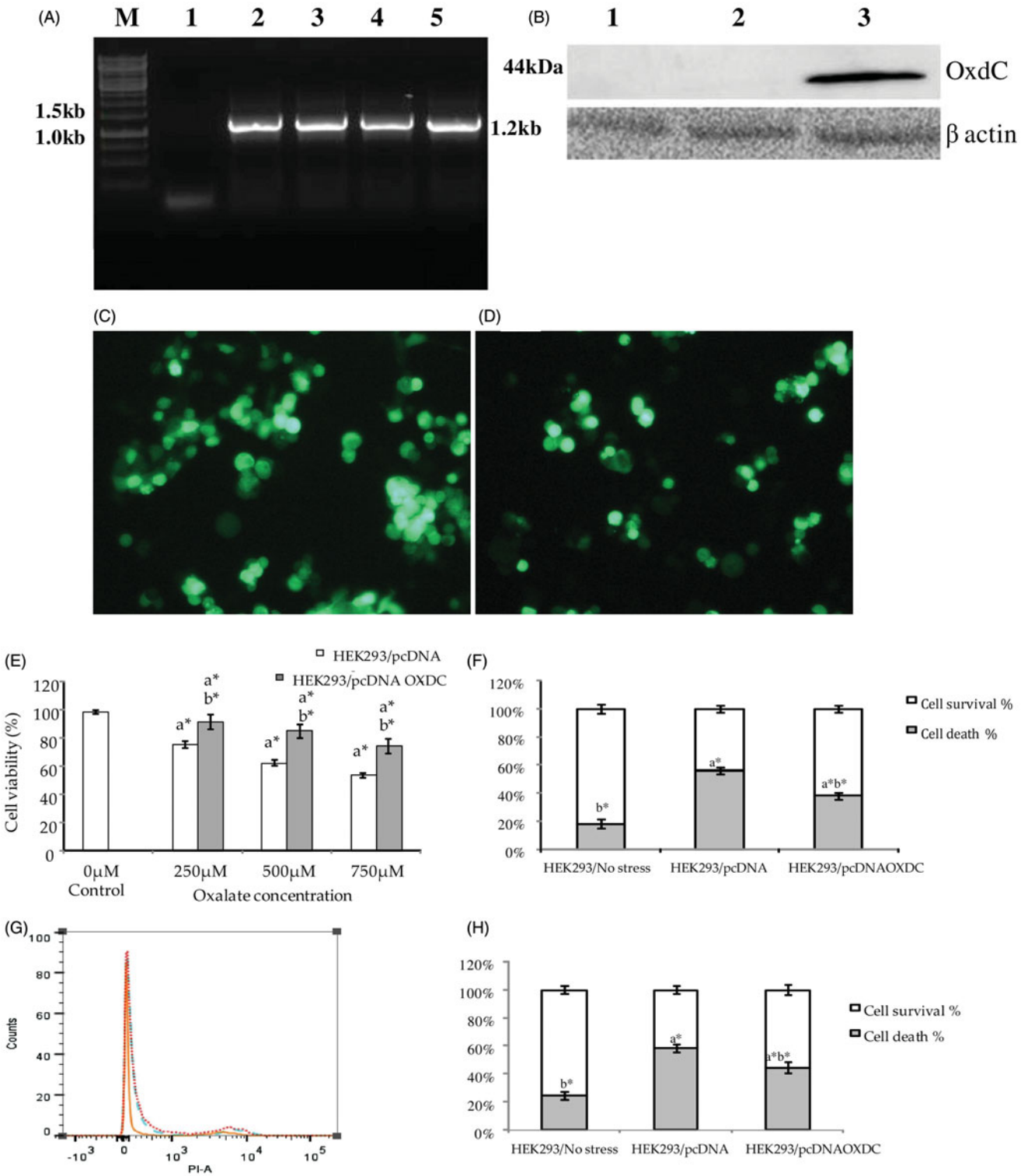

Figure 1. (A) Expression of *oxdC* gene in HEK 293 cells, confirmed by Semi-quantitative PCR. Lane M, 1 kb ladder; lane 1, Empty vector; lanes 2, 3, 4, 5, 1.2 kb amplified product of *oxdC*. (B) The results of protein immunoblot analysis. Lane 1, HEK293 cells, lane 2, HEK293/pcDNA cells, lane 3, HEK293/pcDNAOXDC expressed protein corresponding to size 44 kDa detected in the stably transfected cells. (C) HEK293 cells were transfected with pEGFP-N1 (empty vector) and (D) pOXDC-EGFP. At 48 h, pEGFP-N1 (empty vector) and pOXDC-EGFP transfected cells exhibited bright green fluorescence and fusion proteins (OXDC-EGFP) were located in the cytoplasm. (E) Cell viability of HEK293/pcDNA and HEK293/pcDNAOXDC cells exposed to oxalate (750 μM for 18 h) was determined by MTT assay. (F) Trypan Blue Exclusion assay for determination of cytotoxicity effect of oxalate stress. (G) Live and dead cell population of HEK293/pcDNAOXDC following exposure to oxalate stress by flow cytometry analysis. (H) Bar diagram shows the % of live and dead cells following oxalate treatment. Each experiment was repeated a minimum of three independent times. Continuous line – HEK293/pcDNA (No stress); Dotted line – HEK293/pcDNA (Oxalate stress); Dashed line – HEK293/pcDNAOXDC (Oxalate stress). a* – significant difference from HEK293/pcDNA cells without oxalate stress. b* – significant difference from HEK293/pcDNA cells with oxalate stress. Values are statistically significant at $p < 0.05$.

Table 1. The antioxidant profile of recombinant HEK293/pcDNAOXDC and HEK293/pcDNA cells when exposed to 750 μM of oxalate stress for 18 h.

Antioxidant profile	Recombinant HEK293/ pcDNA without oxalate stress	Recombinant HEK293/ pcDNA with oxalate stress	Recombinant HEK293/ pcDNAOXDC with oxalate stress
Catalase (μmol of H_2O_2 consumed/min/mg)	31.63 ± 2.29	16.39 ± 1.38*	30.97 ± 2.3†
Superoxide dismutase (U/mg protein)	3.31 ± 0.23	1.29 ± 0.09*	2.03 ± 0.4*,†
Lipid peroxidation (nmol/mg protein)	1.45 ± 0.07	3.76 ± 0.16*	2.48 ± 0.16*,†

SOD: one enzyme unit was expressed as inverse of the amount of protein (mg) required for inhibiting reduction rate by 50% in 1 min. Values are statistically significant at $p < 0.05$.
*Significant difference from HEK293/pcDNA cells without oxalate stress.
†Significant difference from HEK293/pcDNA cells with oxalate stress.

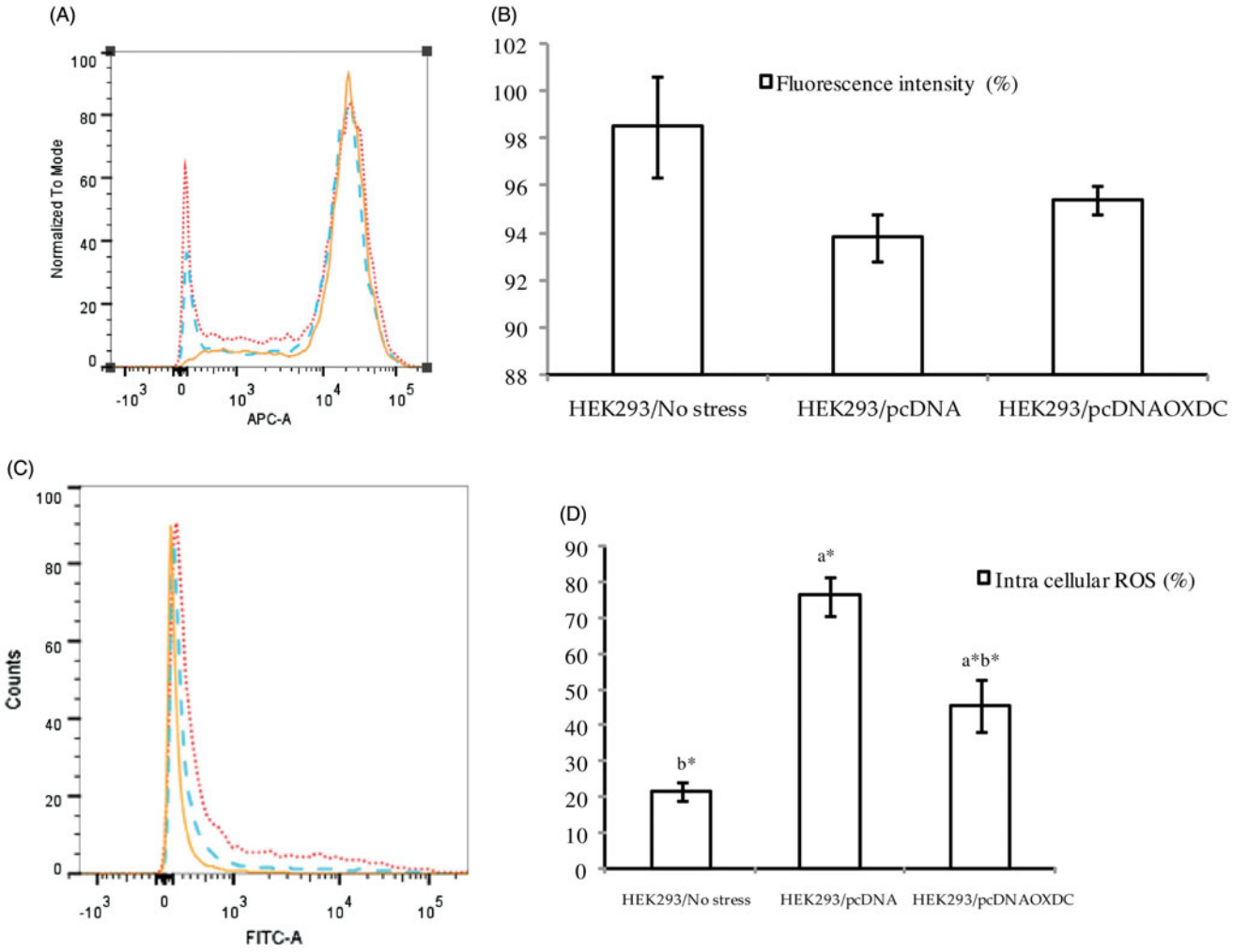

Figure 2. (A, B) – Histogram and bar diagram representation of mitochondrial membrane potential in HEK293/pcDNA and HEK/pcDNAOXDC cells following exposure to oxalate (750 μM) for 18 h. (C, D) – Histogram representation and bar diagram of oxalate on ROS production in HEK293/pcDNA and HEK/pcDNAOXDC cells. Each experiment was repeated a minimum of three independent times. Continuous line – HEK293/pcDNA (No stress); Dotted line – HEK293/pcDNA (Oxalate stress); Dashed line – HEK293/pcDNAOXDC (Oxalate stress). a* – significant difference from HEK293/pcDNA cells without oxalate stress. b* – significant difference from HEK293/pcDNA cells with oxalate stress. Values are statistically significant at $p < 0.05$.

comparison with HEK293/pcDNA cells exposed to oxalate ($p < 0.05$) (Figure 3A–C).

Oxalate degrading ability by recombinant HEK293 cells expressing heterologous OxdC

Figure 4 demonstrates a significant reduction of oxalate level in the medium of recombinant HEK293 cells harboring pcDNAOXDC while oxalate reduction was not observed in recombinant HEK293/pcDNA cells. The oxalate concentration was reduced to 348 μM in the medium of recombinant HEK293/pcDNAOXDC cells. The reduction of oxalate in the medium of HEK293/pcDNAOXDC was a consequence of OxdC.

Discussion

Oxalate is a toxic non-essential end product of metabolism and is excreted unchanged in urine and feces. There is no known naturally occurring enzyme in humans capable of degrading or metabolizing oxalate, nevertheless, it is catabolized by a limited number of bacterial species to formate and carbon dioxide. Evidences

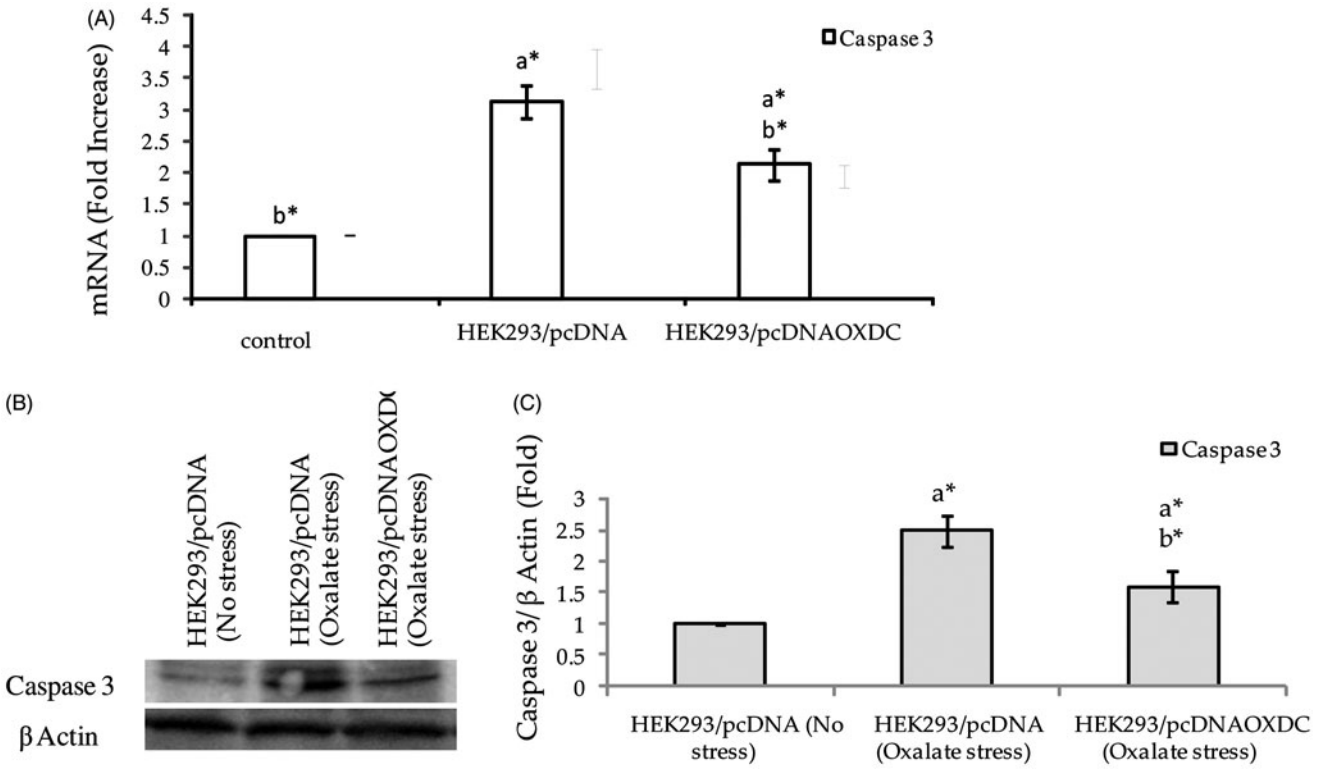

Figure 3. (A) Relative gene expression analysis of caspase 3 in HEK293/pcDNAOXDC and HEK293/pcDNA exposed to oxalate stress (750 μM, 18 h). (B,C) Relative protein expression of caspase 3 in HEK293/pcDNAOXDC and HEK293/pcDNA exposed to oxalate stress (750 μM, 18 h). a* – significant difference from HEK293/pcDNA cells without oxalate stress. b* – significant difference from HEK293/pcDNA cells with oxalate stress. Values are statistically significant at $p < 0.05$.

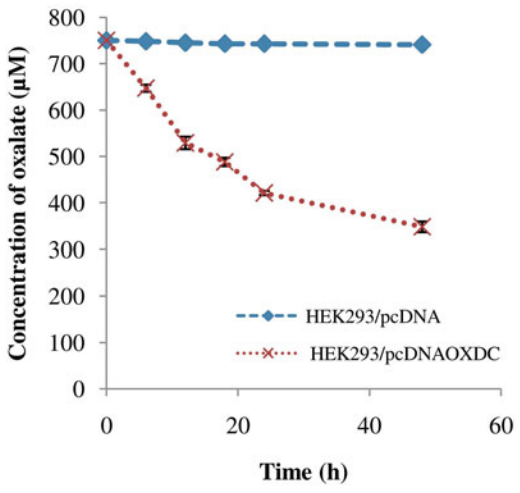

Figure 4. Oxalate degrading ability of recombinant HEK293/pcDNAOXDC and HEK293/pcDNA cells in oxalate rich medium (750 μM) at different time intervals. The represented data are mean value of three independent experiments.

suggested that *O. formigenes* is associated with a decrease in urinary oxalate excretion owing to degradation of oxalate in the gut leading to reduced absorption. However, previous report demonstrated that the intestinal colonization of *O. formigenes* was not successfully accomplished in most patients[16], thus potentially limiting its usage. The identification of OxdC from *B. subtilis* has been proved to be a possible therapeutic option for calcium oxalate stone disease in human. Therefore, the present work is an attempt to analyze the oxalate degrading efficiency of heterologous OxdC in HEK293 cells.

The results of the present study revealed recombinant HEK293 cells expressing OxdC exhibited higher cell viability than cells that were devoid of OxdC when exposed to oxalate stress. The loss in cell viability when exposed to oxalate stress can be attributed to ROS generation. The accumulation of highly reactive oxygen radicals alters enzyme activities and exerts deleterious effect on membrane. The overproduction of ROS leads to the reduction in endogenous antioxidant function that eventually causes development of oxidative stress[17]. Several enzymes play a decisive role in reducing oxidative damage by scavenging ROS as cellular antioxidant system. HEK293/pcDNA cells incubated with oxalate caused decrease in activities of catalase (CAT) and superoxide dismutase (SOD) by about 2 and 2.5 fold respectively, which are concordant with earlier report[18]. The results indicate CAT and SOD activities in HEK293 cells were significantly inhibited by oxalate whereas OxdC presence in HEK293 cells reversed the enzymatic changes suggesting the cytoprotective effect of recombinant HEK293/pcDNAOXDC cells. Zhai et al.[19] and Farooq et al.[20] have revealed that the use of exogenous antioxidants conferred a protective role in oxalate stress. Oxalate provokes ROS generation that in turn causes disruption of cell membranes as a result of lipid peroxidation. Oxalate exposure to HEK293/pcDNA cells showed elevated intracellular MDA levels by 1.7 fold. However, a significant decrease of MDA levels observed in HEK293/pcDNAOXDC cells pre-incubated with oxalate indicates the cells are protected from oxidative damage. Renal injury and inflammation caused by ROS play a major role in stone formation. Previous reports suggested that OxdC isolated and purified from *B. subtilis* exhibited activity in the range of pH 3 to 7 with an optimal activity at pH 5[5,21]. The present results showed that 54% reduction of oxalate in the medium of recombinant HEK293 cells harboring pcDNAOXDC. This suggest that heterologous enzyme OxdC to be biologically active in HEK293 cells. Since OxdC localization occurs in the cytoplasm, oxalates enter the cell and are

effectively degraded to formate and CO_2, thereby protect the cell from oxalate-mediated oxidative stress induced damage.

Earlier studies have elucidated the abrasive effects of both oxalate and CaOx crystals in renal epithelial cell injury through free radicals[22,23]. ROS generation increases in response to renin angiotensin system activation and eventually causes mitochondrial damage[24]. Zhai et al.[19] demonstrated that ROS generation from the intramembrane compartment of mitochondria promotes injury to renal tubular cells. Cytochrome C released from the mitochondria binds to cytosolic apoptosis protease activating factor 1 and thereby activates caspase 9 and caspase 3, which results in the initiation of apoptosis. In concordant with earlier reports, our results indicate that oxalate stress led to decrease in mitochondrial membrane potential that may incite the release of Cytochrome C from the mitochondria resulting in the activation of caspase cascade and subsequently apoptosis[20,24,25]. Caspases are crucial mediators for programmed cell death and caspase 3 plays a vital role in both intrinsic and extrinsic apoptotic pathway. In comparison to earlier reports, the present study revealed caspase 3 upregulation on exposure of HEK293/pcDNA cells to oxalate[19,20]. On the contrary, Patel et al.[26] had reported no significant change in caspase 3 activity on exposure of calcium oxalate monohydrate crystals in renal epithelium cells. Interestingly in our study, HEK293/pcDNAOXDC cells showed significant decrease in caspase 3 expression. These results provide evidence that oxalate-mediated apoptosis is prevented in HEK293/pcDNAOXDC cells. Although the administration of bioflavonoids restored antioxidant activity, subdued ROS generation and decreased caspase 3 expression in NRK-52E cells[19], the degradation of oxalate can prove to be an alternative therapeutic possibility for patients exhibiting hyperoxaluria.

In our study, the heterologous OxdC protein expression enabled in reduction of oxalate stress thus provided a favorable environment for cell survival. The presence of OxdC in recombinant HEK293 cells confers protection against oxidative stress through the reduction of oxalate concentration in the medium that consequently decreases ROS generation. Poljsak[27] suggested that the first-line of defense in quenching ROS-mediated apoptosis is by eliminating exogenous ROS generating pollutants from the milieu. The interaction involving CaOx crystals and oxalate ions with renal tubular epithelium may enhance urinary calculi formation[28]. Therefore, the reduction of oxalate ions and oxalate-mediated oxidative stress are possible therapeutic options for the prevention and treatment of CaOx stone disease. The present study indicates recombinant HEK293 cells expressing OxdC can notably contribute in protecting the cell from apoptosis by degrading the oxalate in the medium. The transfection of *oxdC* enabled the HEK293 cell lines to acquire oxalate degrading ability. The presence of *oxdC* gene can mitigate the oxalate stress; thereby protect the cell from oxalate ion-mediated oxidative damage. The development of stable transfectant with oxalate degrading efficacy can provide new insights into the treatment of hyperoxalauria and CaOx kidney stone disease.

Conclusions

Oxalate-mediated renal injury may promote stone formation by providing cellular debris for CaOx crystal nucleation and aggregation. Our results demonstrate that recombinant HEK293/pcDNAOXDC cells can ameliorate oxalate stress and thereby confer protection against oxidative damage resulting from oxalate ions. Hence, the reduction of oxalate induced oxidative stress can prove to be a possible therapeutic option for the prevention of CaOx stone disease. Despite recombinant HEK293/pcDNAOXDC cells showed significant efficiency in degradation of oxalate *in vitro*, experimental based *in vivo* model is required to demonstrate the feasibility of our approach.

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Disclosure statement

The authors report no declarations of interest.

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