Comparative whole genome transcriptome analysis and fenugreek leaf extract modulation on cadmium-induced toxicity in liver cells

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Abstract. Cadmium (Cd), an economically valuable metal, is widely used in various industrial processes. Although it is of economic value, it is hazardous to human health. Cd accumulates in vital organs where it causes various diseases. Natural compounds with chelating or antioxidant properties have been tested to reduce the toxic effect of Cd. The anti-oxidant, anti-diabetic and hypocholesterolemic properties of fenugreek (*Trigonella foenum-graecum*) leaves make it a candidate for investigation as protective agent against Cd-induced toxicity. In the present study, the protective effects of fenugreek leaf extract (FLE) on cell viability, morphology, and whole genomic transcription in cadmium chloride ($CdCl_2$)-treated rat liver cells were analyzed. The cells were treated with 25 μM $CdCl_2$ alone, or co-treated with 5 μg/ml FLE for 48 h. The co-treated cells were pretreated with FLE for 2 or 4 h, followed by $CdCl_2$ treatment. Genomic transcription analysis was performed in the $CdCl_2$-treated cells following treatment for 6 h. The $CdCl_2$ caused a significant decrease in viability (35.8±4.1%) and morphological distortion of the cells, compared with the untreated control cells; whereas 4 h pretreatment with FLE (5 μg/ml) reversed the Cd-induced morphology alteration and increased the cell viability to 102±3.8%. Genomic transcription analysis of the $CdCl_2$ only-treated cells showed 61 upregulated and 124 downregulated genes, compared with 180 upregulated and 162 downregulated genes in the FLE pretreated cells. Furthermore, 37 and 26% of the affected total genomic genes in the $CdCl_2$ only-treated cells were involved in binding and catalytic activities, respectively, whereas 50 and 20% of the genes in the FLE pretreated cells were involved in binding and catalytic activities, respectively. In conclusion, these results suggested that genome transcriptome modula-

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tion may be important in the protective effect of FLE against Cd-induced toxicity in normal rat liver cells.

Introduction

Cadmium (Cd) is a biologically non-essential heavy metal, however, it is one of the most toxic environmental and industrial pollutants due to its abuse in agricultural (fertilizers) and industrial products, including nickel-cadmium batteries, pigments, chemical stabilizers, metal coatings and alloys. Unfortunately, it is present almost ubiquitously in food (1-3), with the general population being exposed to Cd through drinking water and food (4). Approximately, two-thirds of Cd exposure through the diet is attributed to contaminated vegetables and one-third is attributed to animal products (5). In the United States, it is estimated that the average person consumes ~30 μg of Cd per day through food (6). It has also been classified as a type I carcinogen by the International Agency for Cancer Research (7).

Cd is a cumulative toxicant, which accumulates throughout life due to its long biological half-life (15-30 years) and poor excretion (8,4). Acute and chronic Cd exposure leads to its accumulation predominantly in the liver and kidneys, in addition to other tissues and organs, causing several metabolic and histological disorders in humans and animals (9,10). The molecular mechanisms of Cd toxicity are diverse and complex. It has been demonstrated that Cd causes damage to cells indirectly by the generation of reactive oxygen species, which leads to the unspecific oxidation of proteins and membrane lipids; and consequently causes DNA damage by depleting free radical scavengers, including glutathione, metallothioneins and protein-bound sulfhydryl groups (11,12).

Various plants are important in traditional folk medicine and in pharmacological preparations for the treatment of various diseases. *Trigonella foenum-graecum* is an important spice used in various regions of the Asian, African and European continents. It is commonly called fenugreek. The seeds are commonly used as a spice in food preparations due to their potent flavor and aroma (13,14). Fenugreek leaves are consumed widely in India as a green, leafy vegetable, and are a rich source of calcium, iron, β-carotene and vitamin K (15). The leaves and seeds are used to prepare extracts or powders for medicinal use (16). It has been shown that fenugreek

extract can lower kidney/body weight ratio, blood glucose and blood lipid levels, and improve hemorheological properties in experimental diabetic rats following repeated treatment for 6 weeks (17). The medicinal efficacy of fenugreek has been shown experimentally in diabetic humans and rats (18-20). In type 1 diabetic animals, it has been shown that the supplementation of fenugreek in the diet lowers lipid peroxidation (20). In humans, it has been reported that treatment with fenugreek induces hypocholesterolemia and hypoglycemia (15,18). Fenugreek seeds have also been experimentally shown to protect against breast and colon cancer (21,22). Although hepatoprotective and antioxidant properties of fenugreek in different experimental models have been reported (13,23,24), the protective role of fenugreek leaves against Cd toxicity has not been investigated in animal models or cell lines. The present study investigated the protective effect of fenugreek leaf extract (FLE) against Cd-induced cytotoxicity and whole genome transcription (transcriptome) in cadmium chloride ($CdCl_2$)-treated normal rat liver cells.

Materials and methods

Chemicals. F12K medium, penicillin-streptomycin antibiotic solution (100X), fetal bovine serum (FBS), 0.25% Trypsin-EDTA solution, phosphate buffer solution (PBS), 0.25% Trypsin-EDTA solution and $CdCl_2$ were obtained from Sigma-Aldrich (St. Louis, MO, USA). The dried fenugreek leaf powder was purchased from a local Indian store (Tallahassee, FL, USA). The 3'IVT Express kit and RG230 PM whole genome microarray analysis kit were purchased from Affymetrix (Thermo Fisher Scientific, Inc., Santa Clara, CA, USA). The RNeasy kit was purchased from Qiagen, Inc. (Germantown, MD, USA). Crystal violet, 25% glutaraldehyde, sodium monophosphate and 95% ethanol were purchased from VWR International (Suwanee, GA, USA).

Maintenance of the cell line. The CRL1439 rat normal liver epithelial cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The supplied frozen cells were cultured according to ATCC protocols. The cells were grown in F12K medium containing 2 mM L-glutamine, supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin in T-75 cm^2 flasks (Corning Incorporated, Corning, NY, USA), in a humidified 5% CO_2 incubator at 37°C (NuAire Laboratory Equipment, Plymouth, MN, USA).

FLE preparation. The FLE was prepared by mixing 0.5 g of fenugreek leaf powder with 10 ml of DMSO on a shaker overnight, followed by centrifugation (Hettich Universal 320R) at 1,180 x g for 10 min at room temperature. The supernatant was transferred to a new tube and used for the subsequent assays.

Cell treatment. The cell viability assay was performed by plating cells at an initial density of 5x10^4 cells in polystyrene flat-bottomed 24-well plates and allowed to stabilize overnight in a 5% CO_2 incubator at 37°C. The following day, the cells were treated with either 0 or 25 μM $CdCl_2$ alone, or 5 or 50 μg/ml FLE alone, or were co-treated with FLE and 25 μM $CdCl_2$ at a final volume of 1 ml in triplicate wells for 48 h at 37°C. In the co-treated group, the cells were pretreated with 5 or 50 μg/ml of FLE for 2 or 4 h prior to adding 25 μM $CdCl_2$.

Morphology. At the end of the treatment, the morphology of the untreated control cells, the cells treated with $CdCl_2$ alone or FLE alone, and the FLE + $CdCl_2$ co-treated liver cells were observed under a phase-contrast microscope, and images were captured with a Kodak digital camera (Kodak, Rochester, NY, USA) with 3x under the Nikon Diaphot phase contrast microscope (Nikon Corporation, Tokyo, Japan) with a 10X objective.

Evaluation of cell viability. The cell viability assay was performed according to a previous study (25) using a crystal violet assy. The cell plates were read in a plate reader (BioTek EL800; BioTek Instruments, Inc., Winooski, VT, USA) at 450 nm.

RNA isolation, quantification and analysis. The transcriptome array analysis was performed by plating cells at an initial density of 1.95x10^6 cells in polystyrene flat-bottomed T-75 cm^2 culture flask, with the cells allowed to stabilize overnight in a 5% CO_2 incubator at 37°C. The following day, the cells were treated with 0 or 25 μM $CdCl_2$ alone, or 5 μg/ml FLE alone, or were co-treated with 5 μg/ml FLE and 25 μM $CdCl_2$ at a final volume of 10 ml, in triplicate flasks for each treatment, for 6 h. The co-treated group flasks were pretreated with FLE for 4 h prior to treatment with $CdCl_2$. At the end of the treatment, the cells were washed with PBS and trypsinized. The cells were centrifuged at 500 x g for 5 min at room temperature and the cell pellets were stored at -80°C until further use in transcriptome (microarray) analysis.

Total RNA was isolated from the cells according to the Qiagen RNeasy kit protocol. The concentration of RNA was measured with a Nanodrop 2000 spectrophotometer (Nanodrop; Thermo Fisher Scientific, Inc.). A 250 ng sample of total RNA from each treatment group was used for cDNA synthesis (Bio-Rad MyiQ PCR machine; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and converted to aRNA according to the Affymetrix 3'IVT Express kit protocol. The aRNA was synthesized, purified and hybridized with the Affymetrix Gene array strip (RG230 PM). The array contained 31,139 rat genes and was used for the transcriptome analysis. The RG230 PM rat whole genome array was blocked, hybridized, washed, stained, and images were captured according to the GeneAtlas System (Affymetrix; Thermo Fisher Scientific, Inc.) protocol. GeneAtlas system uses an array strip containing 4 microarrays and enables analysis of four samples simultaneously. This system enables whole genome transcription analysis within 2 days with 1 μg total RNA. Two microarrays were used for each sample. The array strip was hybridized for 16 h. Partek® Express™ (version 1.13.0508; Partek Inc., St. Louis, MO, USA) and Pathway Studio (version 1.1; Ariadne Genomics, Inc., Rockville, MD, USA) software were used to quantify the differentially expressed genes and metabolic pathways affected by each treatment.

Results

FLE has a protective effect on the viability of $CdCl_2$-treated cells. Over previous years, several investigators have used various cell lines and animal models to ascertain the cytotoxicity

and carcinogenicity of Cd as an environmental pollutant. Our previous investigations expanded on the scientific interest on the investigation of Cd-induced toxicity to the ameliorative effects of natural and synthetic compounds against the toxic effects of Cd in various cell lines (26-28). In the present study, the protective effects of FLE against Cd-induced toxicity in CRL 1439 normal rat liver cells were examined. The liver cells treated with 25 μM CdCl$_2$ alone for 48 h exhibited a significant (P<0.01) decrease in cell viability (35.8±4.1%), compared with that of the control cells (100%; Fig. 1). The cells pretreated with FLE prior to CdCl$_2$ treatment had viabilities of 80.0±1.3% (50 μg/ml; 2 h pre), 81.6±1.4% (5 μg/ml, 2 h pre), 88.5±0.9% (50 μg/ml, 4 h pre), and 102±3.8% (5 μg/ml, 4 h pre), respectively, compared with that in the 25 μM CdCl$_2$ alone-treated cells (35.8±4.1%), as shown in Fig. 1. Treatment of the cells with either 5 or 50 μg/ml FLE alone had no significant effect on the cell viability (Fig. 1). These results suggested that FLE had a protective effect against Cd-induced toxicity in liver cells.

FLE has a protective effect against morphological changes of cells induced by CdCl$_2$. The morphological changes in the CdCl$_2$-treated cells were examined and compared with those in the untreated control cells. The control cells were diamond in shape with extensions (Fig. 2A). By contrast, the cells treated with CdCl$_2$ alone exhibited a loss of extensions and the cells became round in shape (Fig. 2B). The cells pretreated with FLE followed by CdCl$_2$ treatment exhibited a morphology similar to that of the control cells (Fig. 2C). The FLE alone-treated cells were diamond in shape with extensions (Fig. 2D). These results indicated that FLE protected the cells from Cd-induced morphological alterations.

RG230 PM whole genome microarray analysis
Differentially expressed genes. The whole genome microarray was performed using Affymetrix RG230 PM and the data were analyzed using Partek Express software to quantify differentially expressed genes. The differential expression of genes in each treatment group is shown in Fig. 3. The differential expression of genes was based on a 2-fold change, compared with the expression in the control cells. The numbers of upregulated and downregulated genes in each treatment group are shown in Table I. In the cells treated with Cd alone, 185 genes of the 31,139 total genes in the rat genome array were differentially expressed. Of these differentially expressed genes, 61 were upregulated and 124 genes were downregulated, respectively, compared with the control cells (Table I). In the cells pretreated with FLE for 4 h followed by CdCl$_2$, 342 genes were modulated, of which 180 genes were upregulated and 162 genes were downregulated, compared with the control cells (Table I). In the FLE alone-treated cells, 108 genes were modulated, of which 77 genes were upregulated and 31 genes were downregulated, compared with the control cells (Table I).

The percentage of affected genes and their ascribed functions in the Cd alone-treated cells are shown in Fig. 4. In total, 37% of these genes have been shown to have binding activity, whereas 26% genes have catalytic activity. These two functions accounted for >50% of the total affected genes present in the CdCl$_2$ alone-treated cells. The remaining 37% of the genes were involved in transporter activity (9%), antioxidant activity (7%), protein binding transcription factor activity
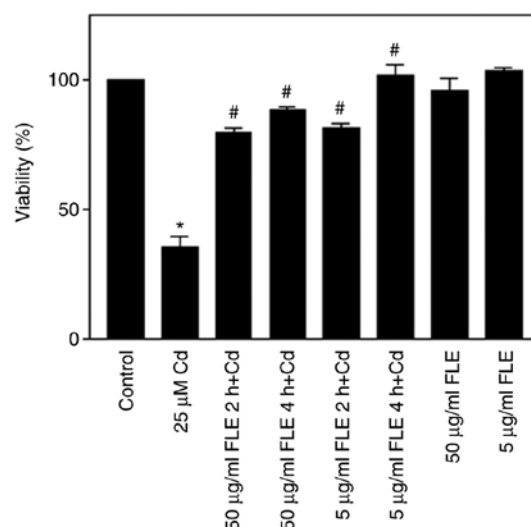

Figure 1. Protective effect of FLE on the viability of CdCl$_2$-treated cells. The cells were treated with CdCl$_2$ for 48 h with or without FLE pretreatment and viability was measured using a crystal violet assay (n=3). *P<0.05, compared with the control; $^#$P<0.05, compared with CdCl$_2$ alone. Statistical analysis performed using Dunnett's multiple comparison test. FLE, fenugreek leaf extract; CdCl$_2$, cadmium chloride.
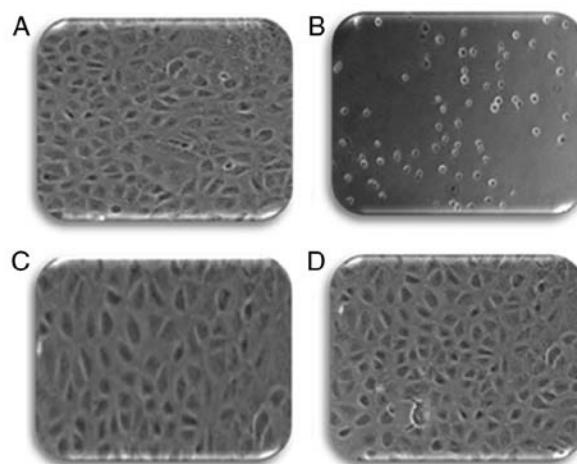

Figure 2. Protective effect of FLE on the morphological changes of CdCl$_2$-treated cells. The cells were treated (A) without CdCl$_2$, or treated with (B) 25 μM CdCl$_2$ for 48 h, (C) pretreated with FLE (5 μg/ml) for 4 h followed by CdCl$_2$ for 48 h, or (D) treated with FLE (5 μg/ml) alone. Images were captured with a Kodak digital camera under the Nikon Diaphot phase contrast microscope (magnification, x10). FLE, fenugreek leaf extract; CdCl$_2$, cadmium chloride.

(7%), molecular transducer activity (5%), nucleic acid binding transcription factor activity (5%), electron carrier activity (2%) and receptor activity (2%).

The percentage of affected genes and their function in the cells pretreated with FLE for 4 h followed by CdCl$_2$ are shown in Fig. 5. Specifically, 50% of the genes have been shown to have binding activity, whereas 20% have catalytic activity. The remaining 30% of the genes were involved in several functional activities, including molecular transducer (7%), transporter (7%), and electron carrier (4%), and enzyme regulator (3%), nucleic acid binding transcription factor (3%), receptor (3%) and structural molecule (3%).
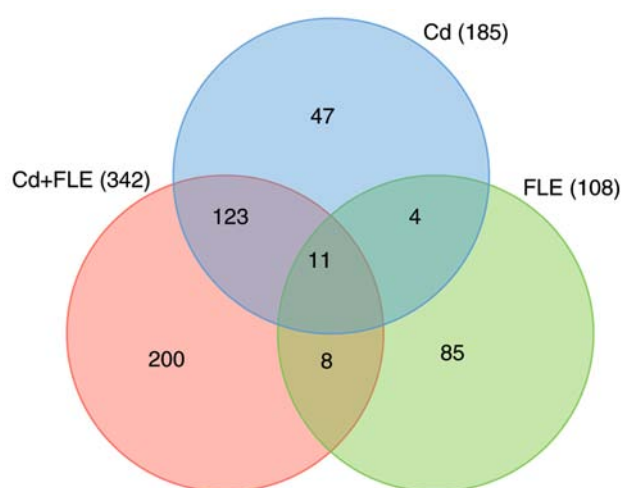
Figure 3. Differentially expressed genes in different treatment groups. The cells were treated with CdCl₂ alone, FLE alone, or pretreated with FLE for 4 h followed by CdCl₂ for 6 h. The total RNA isolated from these treatment groups was subjected to whole genome microarray analysis using an Affymetrix RG230 PM array and the data were analyzed using Partek Express software to quantify differentially expressed genes with a fold change range of >2 and <-2, compared with the control. The total number of differentially expressed genes in each treatment group is indicated in parentheses. FLE, fenugreek leaf extract; CdCl₂, cadmium chloride.

The names of the top 10 upregulated genes, percentage fold upregulation, and function of each of the upregulated genes in the Cd alone-treated cells are shown in Table II. The genes were γ-2a immunoglobulin heavy chain, similar to RIKEN cDNA 1700016G05, transmembrane protein 106B, YY1 transcription factor glycosyltransferase 25 domain containing 1, prefoldin subunit 5, transcription factor AP-2α, phosphatidic acid phosphatase type 2B, selenoprotein W1 (SelW), and spermatogenesis-associated, serine-rich 2-like.

In the cells pretreated with FLE for 4 h followed by CdCl₂, the top 10 upregulated gene names, percentage fold upregulation and function of each gene are shown in Table III. The upregulated genes were γ-2a immunoglobulin heavy chain, BMP-binding endothelial regulator, similar to RIKEN cDNA 1700016G05, periostin, osteoblast specific factor, chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1), fibromodulin, activating transcription factor 5, ribosomal L24 domain containing 1, calpastatin, and SH3 domain binding glutamic acid-rich protein like.

The percentage fold repression and functions of the genes in Cd alone-treated cells are shown in Table IV. The top 10 genes observed were aldehyde oxidase 1, solute carrier family 7 (cationic amino acid transporter, y⁺ system), member 11, heme oxygenase (decycling) 1, ATP-binding cassette, sub-family C (CFTR/MRP), member 4, solute carrier family 39 (iron-regulated transporter), member 1, chemokine (C-C motif) ligand 7, sulfiredoxin 1 homolog (*S. cerevisiae*), monoamine oxidase A, catalase, and adenosine monophosphate deaminase 3.

In the cells pretreated with FLE for 4 h, followed by CdCl₂ treatment, the top 10 downregulated gene names, fold downregulation and function of each gene are shown in Table V. The genes with the highest levels of downregulation were metallothionein 1a, metallothionein 2A, aldehyde oxidase 1, heme oxygenase (decycling) 1, solute carrier family 7

Table I. Number of differentially expressed genes in the treatment groups.

Fold change range	CdCl₂	CdCl₂ + FLE	FLE
1-2	61	180	77
<-15	1	1	0
>-14-<-10	2	2	0
>-10-<-2	121	159	31

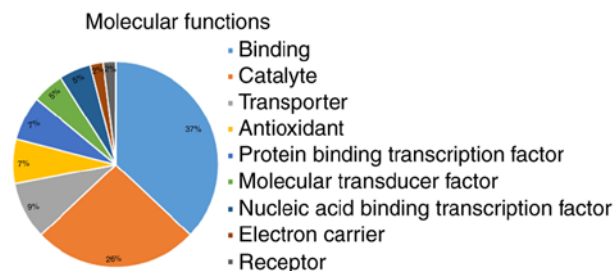CdCl₂, cadmium chloride; FLE, fenugreek leaf extract.

Figure 4. Percentages of genes for each molecular function in CdCl₂ alone-treated cells. The cells were treated with CdCl₂ alone for 6 h and isolated total RNA was subjected to whole genome microarray analysis using an Affymetrix RG230 PM array, with data analyzed using Partek Express software to show the percentage of genes for each molecular function. CdCl₂, cadmium chloride.
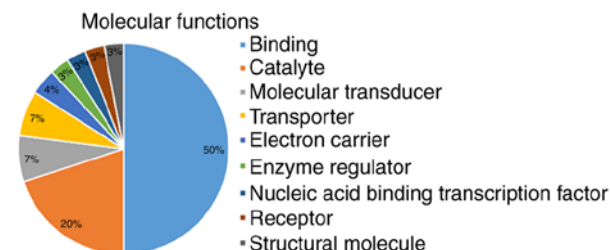

Figure 5. Percentage of genes for each molecular function in cells pretreated with FLE followed by treatment with CdCl₂. The cells were pretreated with FLE for 4 h followed by CdCl₂ for 6 h, and total RNA was isolated and subjected to whole genome microarray analysis using an Affymetrix RG230 PM array. The data were analyzed using Partek Express software to show the percentage of genes for each molecular function. FLE, fenugreek leaf extract; CdCl₂, cadmium chloride.

(cationic amino acid transporter, y⁺ system), member 11, ectonucleoside triphosphate diphosphohydrolase 5, ATP-binding cassette, sub-family C (CFTR/MRP), member 4, Coenzyme Q6 homolog (yeast), zinc finger, AN1-type domain 2A, and glutamate cysteine ligase, modifier subunit. Therefore, the Affymetrix RG230 PM whole genome microarray data analysis clearly showed the differential genes expression in cells treated with Cd alone and in those co-treated with FLE and CdCl₂.

Pathways affected by CdCl₂ and modulation by FLE in rat liver cells. Ariadne software was used to establish the metabolic pathways affected in each treatment group. The most prominent upregulated pathways and percentage of genes

Table II. Prominently upregulated genes in cadmium chloride-treated cells.

Gene	Fold change	Function
γ-2a immunoglobulin heavy chain	7.34	Antigen binding
Similar to RIKEN cDNA 1700016G05	4.28	Serine-type endopeptidase activity
Transmembrane protein 106B	3.1	Uncharacterized
YY1 transcription factor	2.76	DNA binding, metal ion binding
Glycosyltransferase 25 domain containing 1	2.53	Transferase activity, procollagen galactosyltransferase activity
Prefoldin subunit 5	2.46	Unfolded protein binding
Transcription factor activator protein-2, α	2.37	RNA polymerase II core promoter sequence-specific, DNA binding, chromatin binding, RNA polymerase II core promoter proximal region sequence-specific, DNA binding transcription factor activity involved in negative regulation of transcription
Phosphatidic acid phosphatase type 2B	2.32	Integrin binding, lipid phosphatase activity
Selenoprotein W1	2.28	Antioxidant activity, selenium binding
Spermatogenesis associated, serine-rich 2-like	2.27	Uncharacterized

Table III. Prominently upregulated genes in cells pretreated with fenugreek leaf extract followed by cadmium chloride treatment.

Gene	Fold change	Function
γ-2a immunoglobulin heavy chain	6.76	Antigen binding
BMP-binding endothelial regulator	5.01	Inhibitor of bone morphogenetic protein function
Similar to RIKEN cDNA 1700016G05	4.41	Serine-type endopeptidase activity
Periostin, osteoblast specific factor	3.69	Heparin binding
Chemokine (C-X-C motif) ligand 12	3.57	Chemokine receptor binding, chemokine activity
Fibromodulin	3.48	Collagen binding
Activating transcription factor 5	3.41	Heat shock protein binding
Ribosomal L24 domain containing 1	3.3	Ribosome biogenesis
Calpastatin	3.16	Calcium-dependent cysteine-type endopeptidase inhibitor activity, protease binding
SH3 domain binding glutamic acid-rich protein like	3.12	Electron carrier activity, protein disulfide oxidoreductase activity

affected in each pathway from the $CdCl_2$ alone-treated cells and the cells pretreated with FLE for 4 h followed by $CdCl_2$ are shown in Table VI. In the $CdCl_2$ alone-treated cells, the most upregulated pathways were ribosome synthesis, valine, leucine and isoleucine biosynthesis pathway, DNA replication pathway, mismatch repair pathway, ubiquinone and terpenoid-quinone biosynthesis pathway, TCA cycle pathway, Parkinson's disease pathway, sulfur metabolism pathway, RNA transport pathway, and vitamin B6 metabolism (Table VI). The following pathways were the most affected in the cells treated with FLE for 4 h followed by $CdCl_2$: Ribosome synthesis, Valine, leucine and isoleucine biosynthesis, DNA replication, Ubiquinone and terpenoid-quinone biosynthesis, TCA cycle, Parkinson's disease, RNA transport, thiamine metabolism, pantothenate and CoA biosynthesis, and sulfur relay system (Table VI).

The most markedly downregulated pathways and percentage of genes affected in each pathway in the $CdCl_2$

alone-treated cells and in the cells pretreated with FLE for 4 h followed by $CdCl_2$ are shown in Table VII. In the $CdCl_2$ alone-treated cells, the most downregulated pathways were D-arginine and D-ornithine metabolism, bile acid biosynthesis, acid secretion, amino sugars metabolism, butirosin and neomycin biosynthesis, caffeine metabolism, amino sugars and nucleotide sugar metabolism, pentose and glucuronate, ascorbate and alderate metabolism, and drug metabolism. In the cells pretreated with FLE for 4 h followed by $CdCl_2$ treatment, genes involved in the metabolism of xenobiotics by cytochrome P450, steroid hormone biosynthesis and retinol metabolism pathways were affected (Table VII).

Discussion

The marked increase of Cd in the environment is partially due to natural and anthropogenic activities. Exposure to this metal

Table IV. Prominently downregulated genes in cadmium chloride-treated cells.

Gene name	Fold change	Function
Aldehyde oxidase 1	-10.48	NAD binding, electron carrier activity, iron ion binding
Solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	-9.2	Amino acid transmembrane transporter activity
Heme oxygenase (decycling) 1	-6.24	Enzyme binding, heme binding, metal ion binding, protein binding, heme oxygenase activity, oxidoreductase activity
ATP-binding cassette, sub-family C (CFTR/MRP), member 4	-6.12	ATP binding, enzyme binding, chloride channel inhibitor activity, transmembrane transporter activity
Solute carrier family 39 (iron-regulated transporter), member 1	-4.7	Iron ion transmembrane transporter activity
Chemokine (C-C motif) ligand 7	-4.14	C-C chemokine receptor activity
Sulfiredoxin 1 homolog (*S. cerevisiae*)	-4.1	ATP binding, antioxidant activity, oxidoreductase activity, sulfiredoxin activity
Monoamine oxidase A	-4.07	Flavin adenine dinucleotide binding, oxidoreductase activity, serotonin binding
Catalase	-3.58	NADP binding, aminoacylase activityaminoacylase activity, antioxidant activity, heme binding, oxidoreductase activity, receptor binding
Adenosine monophosphate deaminase 3	-3.46	AMP deaminase activity, metal ion binding

Table V. Prominently downregulated genes in cells pretreated with fenugreek leaf extract followed by cadmium chloride treatment.

Gene	Fold change	Function
Metallothionein 1a	-59.21	Cadmium ion binding, zinc ion binding
Metallothionein 2A	-11.63	Cadmium ion binding, zinc ion binding
Aldehyde oxidase 1	-10.03	NAD binding, electron carrier activity, iron ion binding
Heme oxygenase (decycling) 1	-9.72	Enzyme binding, heme binding, metal ion binding, protein binding, heme oxygenase activity, oxidoreductase activity
Solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	-7.28	Amino acid transmembrane transporter activity
Ectonucleoside triphosphate diphosphohydrolase 5	-6.53	Guanosine-diphosphatase activity, uridine-diphosphatase activity
ATP-binding cassette, sub-family C (CFTR/MRP), member 4	-6.42	ATP binding
Coenzyme Q6 homolog (yeast)	-5.05	Flavin adenine dinucleotide binding, oxidoreductase activity, monooxygenase activity
Zinc finger, AN1-type domain 2A	-5	Zinc ion binding
Glutamate cysteine ligase, modifier subunit	-4.97	Glutamate-cysteine ligase activity, protein heterodimerization activity

poses a serious threat to human health. Cd passes through alveolar cells to enter the blood and is eventually deposited into the liver and kidneys due to their high content of metal-binding metallothionein proteins (29,30). As Cd cannot be metabolically degraded or transform to lesser toxic species, this leads to its long-term storage and accumulation in the higher micromolar range in tissues of occupational and non-occupationally exposed populations. Although, various mechanisms for Cd toxicity have been suggested, the manifestations Cd toxicity are mostly due to oxidative stress (11,31). Oxidative stress leads to the damage of important biomolecules, including DNA, protein and lipids, in the organs with potential effects on the whole organism (11,12,32). This damage to biomolecules may be a key pathophysiological factor in liver diseases, including chronic liver injury, hepatic inflammation, fibrosis, and hepatocellular carcinoma (33,34). Various natural plant compounds

Table VI. Percentage of altered genes in the most prominently upregulated pathways.

Name of pathway	$CdCl_2$ alone-treated cells (%)	Cells pretreated with FLE followed by $CdCl_2$ treatment (%)
Ribosome	87	92
Valine, leucine and isoleucine biosynthesis	85	71
DNA replication	83	66
Mismatch repair	78	-
Ubiquinone and other terpenoid-quinone biosynthesis	75	75
TCA cycle	75	75
Parkinson's disease	71	74
Sulfur metabolism	71	-
RNA transport	70	64
Vitamin B6 metabolism	67	-
Thiamine metabolism	-	67
Pantothenate and CoA biosynthesis	-	65
Sulfur relay system	-	63

$CdCl_2$, cadmium chloride; FLE, fenugreek leaf extract.

Table VII. Percentage of altered genes in most prominently downregulated pathways.

Name of pathway	$CdCl_2$ alone-treated cells (%)	Cells pretreated with FLE followed by $CdCl_2$ treatment (%)
D-Arginine and D-Ornithine metabolism	100	100
Primary bile acid biosynthesis	89	89
Collecting duct acid secretion	88	88
Amino sugars metabolism	86	-
Butirosin and neomycin biosynthesis	86	86
Caffeine metabolism	80	-
Amino sugars and nucleotide amino sugars and nucleotide sugar metabolism	79	-
Pentose and glucuronate interconversions	78	83
Ascorbate and alderate metabolism	65	75
Drug metabolism	27	31
Metabolism of xenobiotics by cytochrome p450	-	84
Steroid hormone biosynthesis	-	80
Retinol metabolism	-	80

$CdCl_2$, cadmium chloride; FLE, fenugreek leaf extract.

and synthetic compounds have been tested to reduce the toxic effect of Cd in cell lines or animal models (27,28,35,36). In the present study, the protective effect of FLE on the viability, morphology and whole genome expression were investigated in $CdCl_2$-treated CRL1439 normal rat liver cells.

In the *in vitro* experiments performed in the present study, the cells were treated with 25 μM $CdCl_2$ for 48 h. This treatment caused a significant decrease (35.8±4.1%) in cell viability (Fig. 1) and altered the morphology of the liver cells (Fig. 2B), compared with the untreated control cells (Figs. 1 and 2A). However, FLE pretreatment significantly reversed the altered morphology caused by Cd and increased the viability of the $CdCl_2$-treated rat liver cells (Fig. 1). Pretreatment with 5 μg/ml of FLE for 4 h led to the highest increase in viability and reduction in altered morphology caused by $CdCl_2$ treatment in the rat liver cells (Figs. 1 and 2C). Treatment with 50 μg/ml FLE alone showed marginal toxicity towards the cells (Fig. 1). However, 5 μg/ml FLE alone had no adverse effects on the cell viability and/or morphology (Figs. 1 and 2B). Therefore, pretreatment of cells with 5 μg/ml of FLE for 4 h, followed by 25 μM $CdCl_2$ for 48 h was used for whole genome expression analysis. Fenugreek plant extracts

have been reported to contain alkaloids (37), flavonoids (38) and salicylate (39), which exhibit antioxidant properties. The active components and the biological activities of the isolates from fenugreek with antioxidant and anti-inflammatory activities have been reported (40). A previous study showed that fenugreek leaves contain alkaloids, cardiac glycosides and phenols (41). It has also been shown that fenugreek leaves exhibit anti-oxidant properties against H_2O_2- and CCl_4-induced hepatotoxicity (23). It is possible that the FLE exerts its protective effects against the oxidative stress caused by Cd and provides protection by the increase in cell viability and inhibition of altered morphology.

To discern the genes and pathways affected by 25 μM $CdCl_2$ treatment and modulation by FLE pretreatment, analysis of the whole genome expression on the Affymetrix Gene array strip RG230 PM was performed using GeneAtlas system. It was observed that, in cells treated with $CdCl_2$ alone, more genes in the rat genome RG230 PM Array were downregulated (124 genes) than upregulated (61 genes), whereas in cells pretreated with FLE followed by $CdCl_2$, more genes were upregulated (180 genes) than downregulated (162 genes), which showed the modulation of gene expression by FLE (Table I). It was also observed in the Cd alone-treated cells, that 47 unique genes were expressed, whereas 200 unique genes were expressed in the FLE co-treated cells (Fig. 3). In addition, as shown in Fig. 3, 123 genes were expressed in the cells treated with Cd alone and the cells pretreated with FLE followed by $CdCl_2$ (Fig. 3).

The percentage of differentially regulated genes responsible for each important metabolic function indicated that, in the Cd alone-treated cells, 37 and 26% of the genes were involved in the binding and catalytic activities, respectively (Fig. 4). In the cells pretreated with FLE followed by $CdCl_2$, 50 and 20% of genes were involved in the binding and catalytic activity functions, respectively (Fig. 5). The induced expression of genes with binding function by FLE pretreatment may be as a result of a reduction in oxidative stress caused by Cd, which was reflected in the observed improved viability of the Cd-treated cells (Fig. 2). This observation is in agreement with an earlier report, which also showed that FLE exhibited anti-oxidant properties against H_2O_2- and CCl_4-induced hepatotoxicity (23).

The top 10 upregulated genes in the Cd-treated cells (Table II) were γ-2a immunoglobulin heavy chain, similar to RIKEN cDNA 1700016G05, transmembrane protein 106B, YY1 transcription factor, glycosyltransferase 25 domain containing 1, prefoldin subunit 5, transcription factor AP-2,α, phosphatidic acid phosphatase type 2B, SelW1, and spermatogenesis associated, serine-rich 2-like. The common γ-chain (γc) is central in signaling by interleukin-2 and other γc-dependent cytokines (42). YY1 is known to inhibit the activation of P53 in response to genotoxic stress (43). Previously, it was shown that primary normal human bronchial epithelial cells incubated with 15 μg/l Cd (II) affected YY1-stress response-related transcription factor (44). SelW is a selenocysteine-containing protein with a low molecular weight, indicating the redox activity involved in the anti-oxidant response. Previously, it was reported that the mRNA expression level of SelW was sharply increased by 4.98-fold in the liver of 0.5 ppm cadmium-treated goldfish, compared with that in the control (45). The oxidative stress induced by

metal exposure leads to the activation of transcription factors, including activator protein (AP)-1 and nuclear factor-κB. Previously, it was also shown that cells exposed to cadmium led to a significant activation of AP-1 and all three members of the mitogen-activated protein kinase family (46,47). Unconventional prefoldin RPB5 interactor has been identified as a transcriptional repressor, which binds RNA polymerase II through interaction with the RPB5/POLR2E subunit (48). In the present study, the activation of γ-2a immunoglobulin heavy chain, YY1, SelW, AP-2, and prefoldin RPB5 interactor clearly indicated that $CdCl_2$ treatment induced oxidative stress in the rat liver cells.

In the present study, the genes that were downregulated in Cd-treated cells are listed in Table IV. Specifically, they were aldehyde oxidase 1, solute carrier family 7 (cationic amino acid transporter, y$^+$ system), member 11, heme oxygenase (decycling) 1, ATP-binding cassette, sub-family C (CFTR/MRP), member 4, solute carrier family 39 (iron-regulated transporter), member 1, chemokine (C-C motif) ligand 7, sulfiredoxin 1 homolog (*S. cerevisiae*), monoamine oxidase A, catalase, and adenosine monophosphate deaminase 3. A previous *in vivo* study indicated that the heme oxygenase-1 and monoamine oxidase enzyme activities were decreased in the liver and kidneys of male Wistar albino rats exposed to 1, 2 and 4 mg Cd(2+)/kg body weight for 1 and 3 months (49). The decrease in the observed enzyme activities may be attributed to the downregulation of the enzymes coding gene expression. In contrast to the finding in the present study of downregulated expression of chemokine (C-C motif) ligand 7, the expression of the same gene has been reported to be upregulated in the HepG2 human hepatoma cell line following exposure to 2 and 10 μM Cd using an Agilent microarray, the chemokine, C-C motif, receptor 7 (50). The difference in expression compared with the present result may be due to the Cd concentration used and/or to the cell type (normal vs. tumor cell line). The downregulation of catalase in Cd-treated cells in the present study was consistent with previous studies that catalase levels were markedly decreased (P<0.001) (11,51).

In the cells pretreated with FLE followed by Cd, the expression of γ-2a immunoglobulin heavy chain (6.76-fold; Table II), which has antigen-binding function, was reduced compared with that in the Cd alone-treated cells (7.34-fold; Table II). A higher number of genes coding for binding and catalytic activities (50 and 20%; Fig. 5) were expressed in the FLE pretreatment followed by Cd-treated cells, compared with the number in the Cd alone-treated cells (37 and 26%; Fig. 4). It was also observed that the main metabolic pathways, including amino acid synthesis and DNA replication, were affected by $CdCl_2$ treatment, and that FLE pretreatment modulated these pathway genes.

In conclusion, the results of the present study showed that FLE pretreatment conferred protection against Cd toxicity, as shown by the increased viability and inhibition of altered morphology of the normal rat liver cells. The protective potential of FLE may be attributed to modulation of the whole transcriptome, which prevented Cd-induced toxicity in the normal rat cells. Therefore, the findings from the present study suggested that the unique pharmacological properties of fenugreek leaves can be used to prevent Cd-induced pathophysiological anomalies.

Acknowledgements

Funding

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

CO, LL and VLB designed the experiments. RLL II and CAA carried out the research. CO, VLB, LL and MKF worked on the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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