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Camel urine inhibits the cytochrome P450 1a1 gene expression through an AhR-dependent mechanism in Hepa 1c1c7 cell line

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ABSTRACT

Aim of the study: Drinking camel urine has been used traditionally to treat numerous cases of cancer yet, the exact mechanism was not investigated. Therefore, we examined the ability of three different camel urines (virgin, lactating, and pregnant source) to modulate a well-known cancer-activating enzyme, the cytochrome P450 1a1 (Cyp1a1) in murine hepatoma Hepa 1c1c7 cell line.
Materials and methods: The effect of different camel urines, compared to bovine urines, on Cyp1a1 mRNA was determined using real-time polymerase chain reaction. Cyp1a1 protein and catalytic activity levels were determined using Western blot analysis and 7-ethoxyresorufin as a substrate, respectively. The role of aryl hydrocarbon receptor (AhR)-dependent mechanism was determined using electrophoretic mobility shift assay (EMSA) and the AhR-dependent luciferase reporter gene.
Results: All types of camel, but not bovine, urines differentially inhibited the induction of *Cyp1a1* gene expression by TCDD, the most potent Cyp1a1 inducer and known carcinogenic chemical. Importantly, virgin camel urine showed the highest degree of inhibition at the activity level, followed by lactating and pregnant camel urines. Furthermore, we have shown that virgin camel urine significantly inhibited the TCDD-mediated induction of Cyp1a1 at the mRNA and protein expression levels. Mechanistically, the ability of virgin camel urine to inhibit Cyp1a1 was strongly correlated with its ability to inhibit AhR-dependent luciferase activity and DNA binding as determined by EMSA, suggesting that AhR-dependent mechanism is involved.
Conclusions: The present work provides the first evidence that camel urine but not that of bovine inhibits the TCDD-mediated toxic effect by inhibiting the expression of *Cyp1a1*, at both transcriptional and post-transcriptional levels through an AhR-dependent mechanism.

1. Introduction

The Arabian (or one-humped) camel (*Camelus dromedarius*) is exceptionally well-adapted to drought and heat, and is able to survive and reproduce in conditions not tolerated by other domestic animals (Abdalla et al., 1988). The camel has played a crucial role in desert dwellers for thousands of years. Not only the camel has provided transportation and food, but also its milk and urine have been used traditionally for the maintenance of good health and in the treatment of diverse diseases (Redwan el and Tabll, 2007; Conesa et al., 2008; Agrawal et al., 2009). The medicinal use of camel urine is dated back to the time of the famous Persian scholar known as Avicenna (980-1037), author of al-Qanoon (The Canon). For the Bedouin people, camel urine remains an important natural remedy for different diseases.

Until recently, it is traditionally claimed that drinking camel urine has cured and treated numerous cases of cancer, but this claim has never been exposed to scientific scrutiny investigation. A very few studies have been published in the literature regarding the medicinal properties of camel urine, with just one report describing a possible anti-carcinogenic activity (al-Harbi et al., 1996). Furthermore, Khorshid and Moshref (2006) have recently reported the anti-carcinogenic effect of camel urine in different cancer types in rats. However, these studies did not investigate the mechanisms by which camel urines exhibit anti-carcinogenic effect.

Abbreviations: AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; 7ER, 7-ethoxyresorufin; EROD, 7-ethoxyresorufin O-deethylase; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide); PAHs, polycyclic aromatic hydrocarbons; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive element.

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Strategies for protecting human cells and tissues from the toxic effects of carcinogenic and cytotoxic metabolites generally include attenuation of the carcinogen-activating genes signaling pathways (Korashy et al., 2007a). Among those genes, the cytochrome P450 *1A1* (*CYP1A1*) is strongly correlated with increased incidence of several human cancers such as colon, rectal, and lung cancers (Slattery et al., 2004; Shah et al., 2009). In this context, studies on the carcinogenicity and mutagenicity of the polycyclic aromatic hydrocarbons (PAHs) have demonstrated a significant role for the induction of CYP1A1 in bio-activating these environmental toxicants into their ultimate carcinogenic forms (Korashy and El-Kadi, 2005). It is well established that CYP1A1 bio-activates PAHs to epoxide and diol-epoxide intermediates that subsequently lead to DNA and protein adducts formation which eventually causes different types of cancers (Shimada and Fujii-Kuriyama, 2004). Therefore, the expression level of CYP1A1 is considered to be a useful biomarker of exposure to carcinogenic substances (Williams et al., 2000).

The current knowledge of the mechanism of CYP1A1 induction by PAHs such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent CYP1A1 inducer tested to date (Wei et al., 2002), clearly suggests a transcriptional regulation, in which the binding of TCDD to a cytosolic transcription factor, the aryl hydrocarbon receptor (AhR), is the first step in a series of cellular events leading to carcinogenesis and mutagenesis (Whitlock, 1999). TCDD–AhR complex thereafter translocates to the nucleus where it heterodimerizes with another transcription factor, the AhR nuclear translocator (ARNT). This complex then binds to xenobiotic responsive element (XRE) located in the enhancer region of *CYP1A1 gene* to activate its transcription (Whitlock, 1999; Song and Pollenz, 2002).

The possibility that the claimed anti-carcinogenic effect of camel urine is attributed to inhibiting the expression of *CYP1A1* gene has not been examined before. Therefore, we hypothesize that camel urine prevents the toxic effect of TCDD through inhibiting the expression of *Cyp1a1* gene at the activity, mRNA and protein levels using the murine hepatoma (Hepa 1c1c7) cell line as a model.

2. Materials and methods

2.1. Materials

7-Ethoxyresorufin (7ER), Dulbecco's Modified Eagle's Medium (DMEM), protease inhibitor cocktail, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rabbit anti-goat IgG secondary antibody, and resveratrol (99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). Resorufin and 100× vitamin supplements were purchased from ICN Biomedicals Canada (Montreal, QC). TRIzol and T4 polynucleotide kinase reagents were purchased from Invitrogen Co. (Grand Island, NY). [γ-^{32}P]-ATP (3000 Ci/mmol) was supplied by DNA Core Services Laboratory University of Alberta (Edmonton, AB). The High-Capacity cDNA reverse transcription kit and SYBR® Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA, USA). Chemiluminescence Western blotting detection reagents were from GE Healthcare Life Sciences (Piscataway, NJ, USA). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Cyp1a1 goat polyclonal primary antibody, glyceraldehyde-3-phosphate dehydrogenase (Gapdh) rabbit polyclonal antibody, anti-rabbit IgG peroxidase secondary antibody and goat anti-ARNT antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Luciferase assay reagents were obtained from Promega (Madison, WI, USA). All other chemicals were purchased from Fisher Scientific Co. (Toronto, ON).

2.2. Urine sample collections

Urine was collected aseptically from female virgin, pregnant and lactating healthy domestic camels (*Camelus dromedaries*) or bovines (*Bos primigenius*), five of each. The urine was collected from farm and desert living animals. The collection of urine was usually conducted during the feeding time and was performed by experienced attendants. Urine was allowed to flow directly into stainless steel containers and then transferred to glass vials. Urine samples are transported to the laboratory as soon as practical (<4 h) and were frozen at −80 °C. Camel and bovine urines were collected and kept in the frozen state in a similar manner. Frozen urine samples are shipped from Riyadh, Saudi Arabia, to Edmonton, Alberta, Canada on dry ice.

2.3. Cell culture and treatments

Murine hepatoma Hepa 1c1c7 cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM, without phenol red supplemented with 10% heat-inactivated fetal bovine serum, 20 μM L-glutamine, 100 IU/ml penicillin G, 10 μg/ml streptomycin, 0.1 mM non-essential amino acids, and vitamin supplement solution. Cells were grown in 75 cm^2 tissue culture flasks at 37 °C under a 5% CO_2 humidified environment.

Hepa 1c1c7 cells were plated onto 96- and 6-well cell culture plates in DMEM culture media for Cyp1a1 enzyme activity, and RNA and protein assays, respectively. In all experiments, the cells were pretreated for indicated time interval in serum-free media with various volumes of pregnant, lactating and virgin camel urine in the presence of TCDD as indicated. Stock solutions of TCDD were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C, in which the concentration of DMSO did not exceed 0.05% (v/v).

2.4. Cytotoxicity of camel urine

The effects of different urines on Hepa 1c1c7 cell viability were determined by measuring the capacity of reducing enzymes present in viable cells to convert MTT salt to formazan crystals as described previously (Korashy and El-Kadi, 2006). Twenty-four hours after incubating the cells with the tested urines in a 96-well cell culture plate at 37 °C under a 5% CO_2 humidified incubator, the media were removed and a 100 μl of serum-free medium containing 1.2 mM of MTT dissolved in phosphate-buffered-saline (PBS), pH 7.4, was added to each well. The plate was then incubated in a CO_2 incubator at 37 °C for 2 h. The media were then decanted off by inverting the plate; and a 100 μl of isopropyl alcohol was added to each well, with shaking for 1 h to dissolve the formazan crystals. The color intensity in each well was measured at wavelength of 550 using BIO-TEK Instruments EL 312e microplate reader, Bio-Tek Instruments (Winooski, VT). The percentage of cell viability was calculated relative to control wells designated as 100% viable cells.

2.5. Determination of Cyp1a1 enzymatic activity

Cyp1a1-dependent 7-ethoxyresorufin (7ER) O-deethylase (EROD) activity was performed on intact living Hepa 1c1c7 cells using 7ER as a substrate (Kennedy et al., 1993). After incubation of the cells with different urines and TCDD for 24 h, media were aspirated and the cell monolayers were rinsed with PBS. Thereafter, 100 μl of 2 μM 7ER in assay buffer (0.05 M Tris, 0.1 M NaCl, pH 7.8) was then added to each well. Immediately, an initial fluorescence measurement ($t = 0$) at excitation/emission (545 nm/575 nm) was recorded from each well using Baxter 96-well fluorometer (Deerfield, IL). The plates were then replaced in the incubator, and additional set of fluorescence measurements of the wells

were recorded every 5 min for 20 min interval. The amount of resorufin formed in each well was determined by comparison with a standard curve of known concentrations. The working solution was then aspirated, the cells were rinsed twice with PBS, and 50 μl of double de-ionized water was added to lyse the cells. After placing of the cell plates at −80 °C for 30 min, the cell lysates were allowed to thaw, and protein levels were determined using a modified fluorescent assay (Lorenzen and Kennedy, 1993). The rate of resorufin formation was expressed as pmol/min/mg protein.

2.6. RNA extraction and cDNA synthesis

Total RNA was isolated using TRIzol reagent (Invitrogen®) according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio. Thereafter, first strand cDNA was synthesized using the High-Capacity cDNA reverse transcription kit (Applied Biosystems®) according to the manufacturer's instructions. Briefly, 1 μg of total RNA from each sample was added to a mix of 2.0 μl of 10× reverse transcriptase buffer, 0.8 μl of 25× dNTP mix (100 mM), 2.0 μl of 10× reverse transcriptase random primers, 1.0 μl of MultiScribe reverse transcriptase, and 3.2 μl of nuclease-free water. The final reaction mix was kept at 25 °C for 10 min, heated to 37 °C for 120 min, heated for 85 °C for 5 s, and finally cooled to 4 °C (Zordoky et al., 2008).

2.7. Quantification of mRNA expression by real-time polymerase chain reaction (RT-PCR)

Quantitative analysis of specific mRNA expression was performed by RT-PCR by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems®). The 25-μl reaction mix contained 0.1 μl of 10 μM forward primer and 0.1 μl of 10 μM reverse primer (40 nM final concentration of each primer), 12.5 μl of SYBR Green Universal Master Mix, 11.05 μl of nuclease-free water, and 1.25 μl of cDNA sample. The primers used in the current study were chosen from previously published study (El Gendy et al., 2010) and were purchased from Integrated DNA Technologies (IDT, Coralville, IA). Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. The RT-PCR data were analyzed using the relative gene expression (i.e. ΔΔCt) method, as described in Applied Biosystems User Bulletin No. 2 (Livak and Schmittgen, 2001). Briefly, the data are presented as the fold change in gene expression normalized to the endogenous housekeeping gene (β-actin) and was determined using the equation fold change = $2^{-\Delta(\Delta Ct)}$, where ΔCt = Ct (target) − Ct (β-actin) and Δ(ΔCt) = ΔCt (treated) − ΔCt (untreated).

2.8. Protein extraction and Western blot analysis

Twenty-four hours after incubating the cells with different urines and TCDD, the cells were washed once with cold PBS and collected by scraping in 100 μl of lysis buffer (50 mM HEPES, 0.5 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 μl/ml of protease inhibitor cocktail). The lysates were incubated on ice for 1 h with intermittent vortexing every 10 min, followed by centrifugation at 12,000 × *g* for 10 min at 4 °C. The supernatant was then stored at a −80 °C freezer for later use in the Western blot analysis. Western blot analysis was performed as described previously (Sambrook et al., 1989). For Cyp1a1 immunodetection, 30 μg of proteins from each treatment group were diluted with same amount (1:1) of 2× loading buffer (0.1 M Tris–HCl, pH 6.8, 4% SDS, 1.5% bromophenol blue, 20% glycerol, 5% β-mercaptoethanol), boiled and loaded onto a 10% SDS-polyacrylamide gel. Samples were electrophoresed at 120 V for 2 h, and the separated proteins were transferred to Trans-Blot nitrocellulose membrane (0.45 μm) in a buffer containing 25 mM Tris–HCl, 192 mM glycine, and 20% (v/v) methanol. Protein blots were blocked overnight at 4 °C in a solution containing 5% skim milk powder, 2% bovine serum albumin and 0.5% Tween 20 in TBS solution (0.15 M NaCl, 3 mM KCl, 25 mM Tris-base). Thereafter, the blocking solution was removed and the blots were rinsed three times in a wash buffer (0.1% Tween 20 in TBS). Proteins were detected by incubation with a primary polyclonal goat anti-mouse Cyp1a1 antibody for 2 h at 4 °C in TBS containing 0.01% sodium azide and 0.05% Tween 20. The primary antibody solution was removed and blots were rinsed three times with a wash buffer, followed by incubation with horseradish peroxidase-conjugate rabbit anti-goat secondary antibody for 1 h at room temperature followed by washing as previously described. Antibody detection was performed using the enhanced chemiluminescence method. The intensity of Cyp1a1 bands was quantified, relative to the signals obtained for Gapdh, using Java-based image-processing software, ImageJ® (W. Rasband [2005] National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij).

2.9. Electrophoretic mobility shift assay (EMSA)

XRE complementary oligonucleotides, 5'-GAT CTG GCT CTT CTC ACG CAA CTC CG-3' and 5'-GAT CCG GAG TTG CGT GAG AAG AGC CA-3', were synthesized, then annealed by heating to 70 °C for 7 min, then allowed to cool to room temperature. The double-stranded XRE was then labelled with [γ-^{32}P]-ATP at the 5'-end using T4 polynucleotide kinase (Invitrogen®), according to the manufacturer's instructions, and used as a probe for EMSA reactions. EMSA was performed as described previously (Rogers and Denison, 2002). Briefly, aliquots of guinea pig cytosolic protein (2 mg) were incubated for 15 min at room temperature in a reaction mixture (20 μl) containing 25 mM HEPES, pH 7.9, 80 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol (v/v), and 400 ng poly(dI.dC). Thereafter, ∼1 ng (100,000 cpm) [γ-^{32}P]-labelled XRE was incubated with the mixture for another 15 min before being separated through a 4% non-denaturing PAGE. The specificity of binding was confirmed by competition experiments; cytosolic extracts were pre-incubated at room temperature for 20 min with a 100-fold molar excess of unlabelled XRE or 0.6 μg of anti-ARNT antibody (Santa Cruz Biotechnology, Inc.) before the addition of the labelled XRE. The gel was dried at 80 °C for 1 h, and AhR-XRE complexes formed are visualized by autoradiography (Gharavi and El-Kadi, 2005).

2.10. Transient transfection and luciferase assay

Hepa 1c1c7 cells were plated onto 12-well cell culture plates. Each well of cells was transfected with 1.6 μg of the XRE-driven luciferase reporter plasmid pGudLuc 1.1, generously provided by Dr. M.S. Denison (University of California at Davis), using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen®). Luciferase assay was performed according to the manufacturer's instructions (Promega®) as described previously (Korashy et al., 2007b). Briefly, after incubation with urine and TCDD for 24 h, the cells were washed with PBS and 200 μl of 1× lysis buffer was added to each well with continuous shaking for at least 20 min, then the content of each well was collected separately in 1.5-ml micro-centrifuge tubes. The tubes were then centrifuged to precipitate cellular waste, and 100 μl of cell lysate was incubated with 100 μl of luciferase assay buffer. The luciferase activity was quantified using a TD-20/20 luminometer (Turner BioSystems), and was reported as relative light unit.

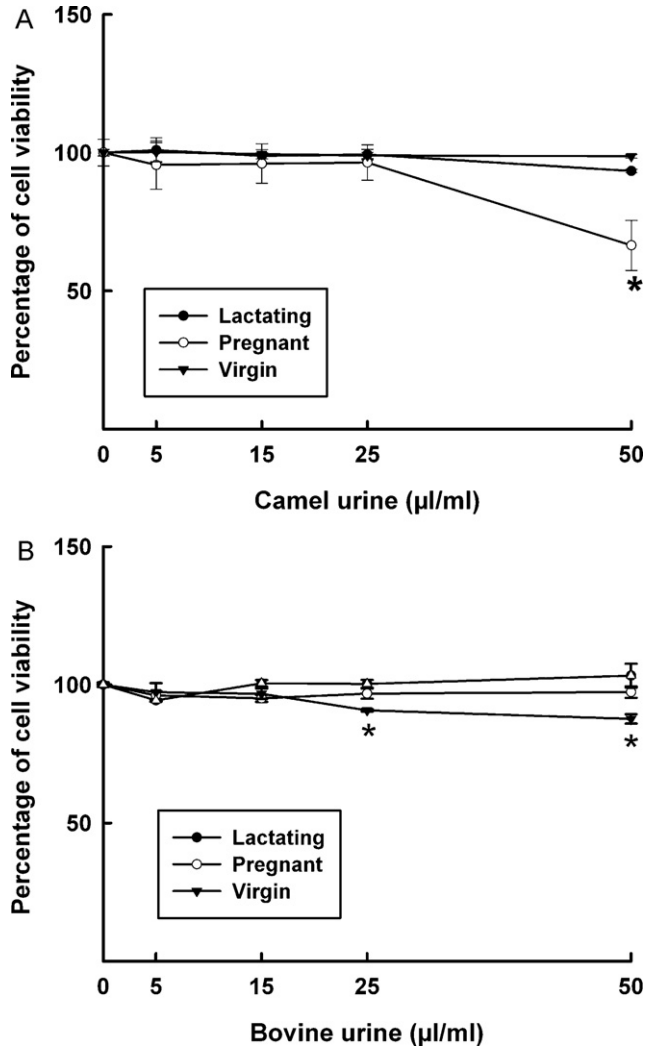

Fig. 1. Effect of camel and bovine urines on Hepa 1c1c7 cell viability. Cells were incubated with various volumes of camel (lactating, pregnant, and virgin) (A) or bovine (B) urines for 24 h, and cell viability was assessed using the MTT assay. Values are presented as percentage of the control (mean ± SEM, $n = 8$). $^{*}p < 0.05$ compared with control (volume = 0 µl).

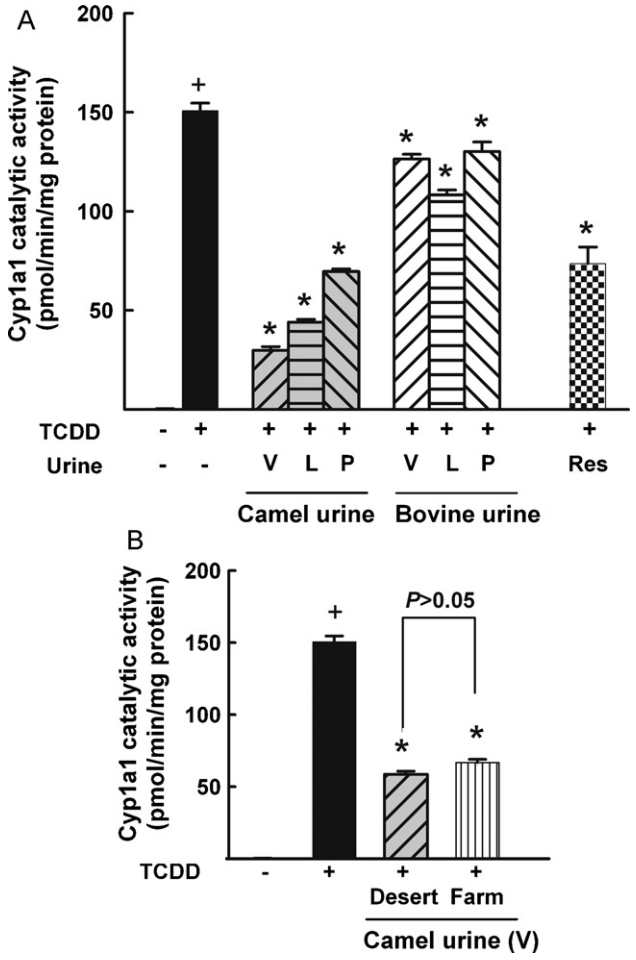

Fig. 2. Effect of camel and bovine urines on the TCDD-induced Cyp1a1 activity. Hepa 1c1c7 cells were treated with 15 µl/ml of lactating (L), pregnant (P), or virgin (V) camel and bovine urines or the positive control, resveratrol (Res, 25 µM) for 30 min before the incubation with TCDD (1 nM) for an additional 24 h. Cyp1a1 activity was measured in intact living cells using EROD assay. Values are presented as mean ± SEM ($n = 8$). $^{+}p < 0.05$ compared with DMSO-treated cells, $^{*}p < 0.05$ compared with TCDD-treated cells.

2.11. Statistical analysis

All results are presented as mean ± SEM. The comparative analysis of the results from various experimental groups with their corresponding controls was performed using SigmaStat® for Windows, Systat Software Inc., (San Jose, CA). One-way analysis of variance (ANOVA) followed by Student–Newman–Keul's test was carried out to assess which treatment groups showed a significant difference from the control group. The differences were considered significant when $p < 0.05$.

3. Results

3.1. Effect of different camel urines on Hepa 1c1c7 cell viability

To determine the cellular toxicity effects of lactating, pregnant, and virgin camel and bovine urines, Hepa 1c1c7 cells were treated for 24 h with increasing volumes of camel or bovine urine (0, 5, 15, 25, and 50 µl/ml) and the cell viability and proliferation were determined by MTT assay. Fig. 1 shows that neither camel nor bovine urines were toxic to Hepa 1c1c7 cells up to 15 µl/ml. However, cell viability was decreased by high volumes of the urine used. Pregnant camel urine (50 µl/ml) significantly decreased cell viability by 35% (Fig. 1A), whereas virgin bovine urine significantly decreased cell viability by 12% and 15% at 25 and 50 µl/ml, respectively (Fig. 1B). Based on these results, a volume of 15 µl/ml of both camel and bovine urines was chosen to be used in the subsequent experiments.

3.2. Effect of camel urines on the TCDD-induced Cyp1a1 catalytic activity in Hepa 1c1c7 cells

To determine the capacity of camel urine, in comparison with bovines, to alter the induction of Cyp1a1 catalytic activity by TCDD, Hepa 1c1c7 cells were pre-incubated with different types of camel and bovine urines (15 µl/ml) for 30 min before the incubation with 1 nM TCDD for additional 24 h. Fig. 2 shows that TCDD alone markedly induced Cyp1a1 enzymatic activity level by 150-fold. Furthermore, lactating, virgin, and pregnant camel urines tested significantly inhibited the TCDD-induced Cyp1a1 activity as compared to their corresponding bovine urine. In this context, virgin camel urine showed the highest inhibitory effect (80%), followed by lactating camel urine (70%), whereas the minimum inhibitory effect was reported with pregnant camel urine (54%) (Fig. 2A). The obtained inhibition was in a manner similar to what was observed with the AhR antagonist, resveratrol (25 µM, positive control),

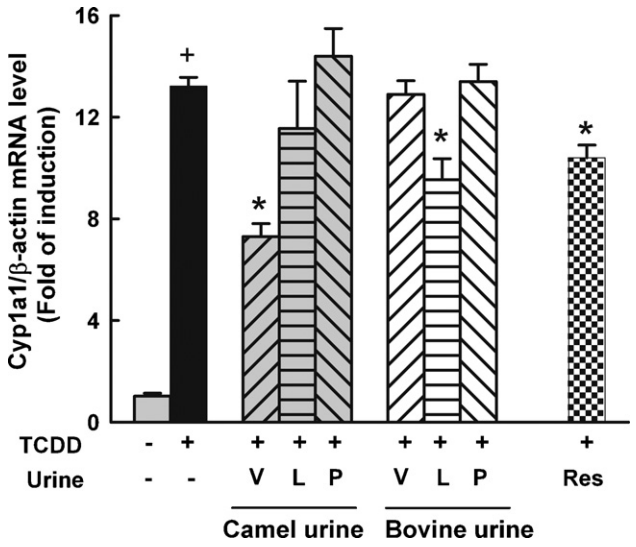

Fig. 3. Effect of camel and bovine urines on the TCDD-induced Cyp1a1 mRNA expression. Hepa 1c1c7 cells were treated with 15 μl/ml of lactating (L), pregnant (P), or virgin (V) camel and bovine urines or the positive control, resveratrol (Res, 25 μM) for 30 min before the incubation with TCDD (1 nM) for an additional 6 h. The amount of Cyp1a1 mRNA was quantified using real-time PCR and normalized to β-actin housekeeping gene. Values represent mean of fold change ± SEM. ($n = 4$). $^{+}p < 0.05$ compared with DMSO-treated cells, $^{*}p < 0.05$ compared with TCDD-treated cells.

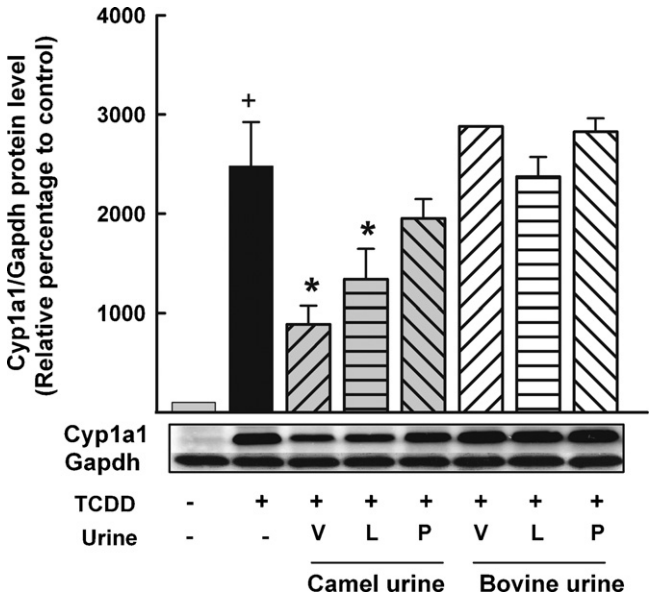

Fig. 4. Effect of camel and bovine urines on the TCDD-induced Cyp1a1 protein. Hepa 1c1c7 cells were treated with 15 μl/ml of lactating (L), pregnant (P), or virgin (V) camel and bovine urines for 30 min before the incubation with TCDD (1 nM) for and additional 24 h. Protein (30 μg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4 °C and then incubated with a primary Cyp1a1 antibody for 2 h at 4 °C, followed by 1 h incubation with secondary antibody at room temperature. Cyp1a1 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to Gapdh signals, which was used as loading control. One of three representative experiments is shown. Values represent mean of fold change ± SEM. ($n = 3$). $^{+}p < 0.05$ compared with DMSO-treated cells, $^{*}p < 0.05$ compared with TCDD-treated cells.

which significantly reduced TCDD-induced Cyp1a1 catalytic activity by 50% (Fig. 2A). Taken together, virgin, lactating and pregnant camel urines are novel potent Cyp1a1 inhibitors.

Furthermore, we investigated whether the inhibition of Cyp1a1 by camel urine is a diet-related effect, therefore, we have tested the effect of virgin camel urine, the urine showed highest inhibitory effect, from different facilities, particularly farm and desert, on TCDD-induced Cyp1a1 catalytic activity. Our results showed that virgin camel urines from both desert and farm facilities inhibited the TCDD-induced Cyp1a1 activity by approximately 60% and 54%, respectively (Fig. 2B). In addition, the magnitude of inhibition observed in farm camel urine was not significantly different from those obtained with desert camel data. These results ruled out any possible effect of diet in virgin camel-mediated effect.

3.3. Effect of camel urines on Cyp1a1 mRNA levels in Hepa 1c1c7 cells

To further explore whether the inhibition of Cyp1a1 by camel urine is a transcriptional mechanism, we have determined the effect of camel urines on the expression of Cyp1a1 mRNA levels. For this purposes, Hepa 1c1c7 cells (10^5 cells per well) were plated onto 6-well tissue culture plates until 70–80% confluence, thereafter, 15 μl/ml of different types of urines was added to the cells 30 min before the addition of 1 nM TCDD for 6 h. The amount of Cyp1a1 mRNA was quantified by real-time PCR and normalized to β-actin, a housekeeping gene. Our results showed that virgin camel urine caused a significant inhibition of TCDD-induced Cyp1a1 mRNA by approximately 45% (Fig. 3) in a manner similar to what observed at the catalytic activity levels (Fig. 2A). In contrast, both lactating and pregnant camel urines did not cause a significant inhibition of the TCDD-mediated induction of Cyp1a1 mRNA (Fig. 3). The positive control, resveratrol (25 μM) significantly inhibited the TCDD-induced Cyp1a1 mRNA by 20% (Fig. 3).

3.4. Effect of camel urines on the expression of Cyp1a1 protein level in Hepa 1c1c7 cells

Western blot analysis was carried out to examine whether the obtained inhibition on TCDD-induced Cyp1a1 mRNA levels is translated into a functional Cyp1a1 protein. Hepa 1c1c7 cells were incubated for 30 min with camel urines before the addition of 1 nM TCDD for 24 h. Fig. 4 shows that both virgin and lactating, but not pregnant, camel urines caused a significant inhibition of the TCDD-induced Cyp1a1 protein levels by approximately 65% and 45%, respectively (Fig. 4). Taken together, these results showed that virgin camel urine stands prominently in its inhibition of the induction of *Cyp1a1* gene expression at the activity, mRNA and protein levels.

3.5. Inhibition of AhR transformation and XRE Binding by virgin camel urine

To further examine the effect of camel urines on AhR transformation and hence binding to the XRE of the *Cyp1a1* gene, virgin camel urine, that showed the maximum inhibition of the *Cyp1a1* gene expression at the activity, mRNA and protein levels, was utilized. For this purpose, EMSA was performed on guinea pig hepatic cytosol pre-incubated for 30 min with virgin camel urine (15 μl/ml) before the incubation with 20 nM TCDD for an additional 2 h, a positive control for AhR transformation. Fig. 5 shows that TCDD significantly activated the AhR through direct transformation of the AhR/ARNT/XRE complex, as determined by the shifted band (lane 2) compared to DMSO (lane 1). However, pre-incubation with virgin camel urine significantly inhibited the TCDD-induced activation of AhR and hence the transformation of the AhR/ARNT/XRE complex (lane 3) as shown by the intensity of the band compared to TCDD. The specificity of virgin camel urine-mediated effects on AhR/ARNT heterodimer binding to XRE was confirmed by compe-

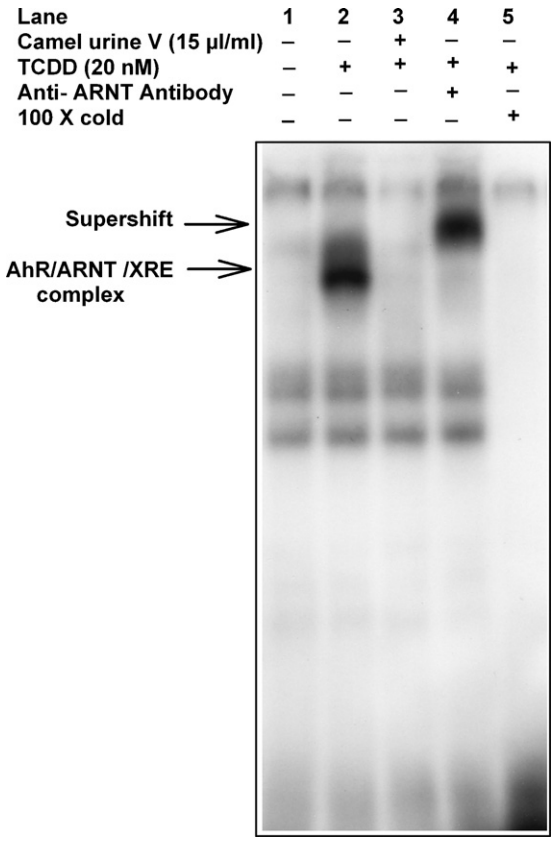

Fig. 5. Effect of virgin camel urine on AhR/ARNT/XRE binding. Cytosolic extracts (2 mg) from untreated guinea pig liver were incubated with DMSO and virgin (V) camel urine for 30 min before another incubation with TCDD (1 nM) for 2 h. The cytosolic proteins were mixed with [γ-^{32}P]-labelled XRE, and the formation of AhR/ARNT/XRE complexes was analyzed by EMSA. The specificity of binding was determined by incubating TCDD-treated cytosolic extracts with 100-fold molar excess of cold XRE or anti-ARNT antibody. AhR/ARNT/XRE complex formed on the gel was visualized by autoradiography. This pattern of AhR activation was observed in three separate experiments, and only one is shown.

tition assays using anti-ARNT antibody (lane 4) or 100-fold molar excess of unlabelled XRE (lane 5).

3.6. Inhibition of AhR-dependent reporter gene expression by virgin camel urine

The ability of virgin camel urine to inhibit the AhR-dependent gene expression was assessed using Hepa 1c1c7 cells transiently transfected with the XRE-driven luciferase reporter gene. Cells were pre-incubated with virgin camel urine (15 µl/ml) for 30 min before the incubation with 1 nM TCDD for 24 h. Fig. 6 shows that treatment of transfected Hepa 1c1c7 cells with virgin camel urine caused a significant inhibition of the TCDD-induced AhR-dependent reporter gene expression.

4. Discussion

The current study provides the first mechanistic evidence, to our knowledge, that camel urine significantly inhibited the induction of *Cyp1a1*, a cancer-activating gene, by TCDD at the transcriptional and post-transcriptional levels through an AhR-dependent mechanism.

One of the strategies for protecting human cells and tissues from the toxic effects of carcinogenic and cytotoxic metabolites include attenuation of the carcinogen-activating genes signaling pathways and/or enhancing the adaptive mechanisms by increasing the

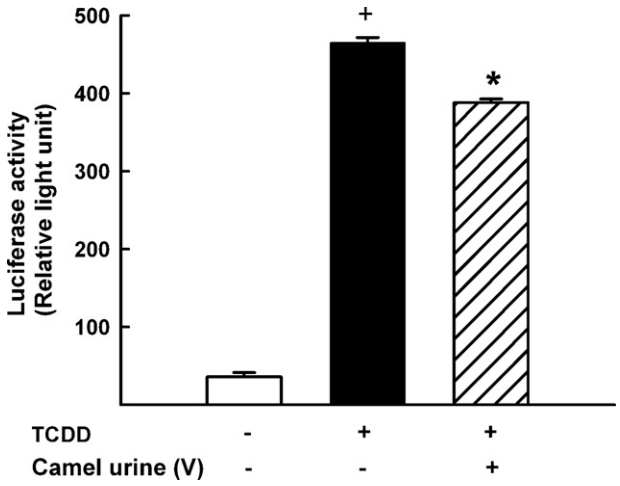

Fig. 6. Effect of virgin camel urine on luciferase activity. Hepa 1c1c7 cells transiently transfected with luciferase reporter gene were grown onto 12-well cell culture plates for 24 h. Thereafter, cells were incubated with DMSO and virgin (V) camel urine for 30 min before the incubation with TCDD (1 nM) for an additional 24 h. Cells were lysed and luciferase activity was measured according to the manufacture's instructions. The graph represents the mean ± SEM ($n = 4$). $^{+}p < 0.05$ compared with DMSO-treated cells, $^{*}p < 0.05$ compared with TCDD-treated cells.

expression of detoxification and antioxidant genes. Therefore, we have tested the capacity of three different camel urines to alter the expression of *Cyp1a1*, a well-known cancer-activating gene. We hypothesize that camel urine induces its anti-cancer effects by inhibiting the expression of *Cyp1a1* gene.

To test our hypothesis, we have first assessed the potential effect of three camel urines, obtained from, lactating, pregnant and virgin camels, on the induction of Cyp1a1 by TCDD using EROD as a probe for Cyp1a1 activity in Hepa 1c1c7 cells (Hasspieler et al., 2006). Our results showed that all the three camel urines tested altered EROD activity to varying extents in a urine-dependent fashion. For example, TCDD-mediated induction of Cyp1a1-dependent EROD activity was markedly reduced by both lactating and virgin camel urines. Surprisingly, the highest inhibition of the TCDD-induced Cyp1a1 activity levels was observed with virgin camel urine. In addition, such effect was not attributed to the diet, as virgin camel urines from independent facilities showed approximately similar inhibitory effect on the TCDD-induced Cyp1a1 catalytic activity.

Modulation of Cyp1a1 activity by camel urine could be attributed, at least in part, to a transcriptional and/or translational mechanism, in which camel urine could alter the expression of Cyp1a1 mRNA and/or protein. The transcriptional regulation of Cyp1a1 gene by camel urine was demonstrated by the ability of camel but not bovine urines, particularly virgin camel urine, to inhibit the Cyp1a1 mRNA expression, in a manner similar to what was observed at the activity levels. Surprisingly, neither lactating nor pregnant camel urine altered Cyp1a1 mRNA expression level. On the other hand, the translational regulation of *Cyp1a1* gene expression was confirmed by the ability of both camel virgin and lactating urines, but not pregnant urine to significantly down-regulate Cyp1a1 protein. These differential effects of camel urines on Cyp1a1 protein level was not attributed to altered cell viability since the expression of Gapdh protein, which was used as a loading control, was not significantly altered among the different treatments. Taken together, the results obtained strongly suggest that the inhibition of Cyp1a1 by camel urine, particularly virgin, is mediated at least in part at the transcriptional and the translational levels. Furthermore, the ability of pregnant camel urine to significantly decrease the TCDD-induced Cyp1a1 at the activity but not at the mRNA or protein levels suggests that a post-translational mechanism is involved.

Perhaps the finding of greatest interest in the current study is the observation that virgin camel urine, the one that exhibited the highest inhibitory effects on *Cyp1a1* gene expression, directly binds to and inhibits the transformation of cytosolic AhR to a DNA-binding form *in vitro*, which is extensively used to assess binding and affinity of ligand to the AhR (Jeuken et al., 2003). This indicates that virgin camel urine could be a novel AhR antagonist that inhibits the binding of the AhR ligand such as TCDD to the AhR. Importantly, the ability of virgin camel urine to inhibit AhR transformation and hence XRE binding is strongly correlated with their ability to inhibit the AhR-dependent gene expression in intact cells. Surprisingly, the inhibitory effect of virgin camel urine on the AhR transformation into its DNA-binding form *in vitro* was greater than its ability to inhibit the AhR-dependent gene expression. This finding could be explained by the inability of virgin camel urine to recruit proper co-repressor to inhibit the gene transcription (Jeuken et al., 2003). Although the potential mediators in camel urines involved in the down-regulation of Cyp1a1 were not examined in this study, ongoing research in our laboratory has shown the presence of several compounds in camel urine. In this regard, using Liquid chromatography–tandem mass spectrometry (LC–MS/MS) and one-dimensional gel electrophoresis, we have shown that several proteins are relatively abundant in camel urine, specifically lysozyme, immunoglobulin heavy chain, albumin, dermatopontin, a CD44 antigen-like protein, prothrombin, alpha-1-antichymotrypsin, CD44E-like protein, and lactoferrin (unpublished data). Among these mediators, lactoferrin, an iron-binding glycoprotein, is abundant in camel urine as compared to bovine urine (unpublished data). In this context, lactoferrin is known to exert *in vitro* and *in vivo* anti-tumor activity (Roseanu et al., 2010). Importantly, it has been recently reported that lactoferrin inhibits the development of cancer through inhibiting CYP1A1 activation in 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch carcinoma model. Taken together the results obtained from our laboratory and previously published reports, we speculate that lactoferrin could be responsible for camel urine-mediated effect. However, further studies are required to confirm the role of camel lactoferrin in the inhibition of Cyp1a1.

In conclusion, the present work provides the first evidence that camel urine inhibits the TCDD-mediated effect, at least in part by inhibiting the expression of Cyp1a1, a cancer-activating gene, at both the transcriptional and the post-transcriptional levels through an AhR-dependent mechanism. These results are of potential clinical significance to humans in that it uncovers the molecular mechanism involved and could explain the anecdotal evidence for the successful use of camel urine in the treatment of various medical conditions.

Conflict of interest

There is no conflict of interest.

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