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Association between VDAC1 mRNA expression and intracellular ATP levels of cultured L-02 hepatocytes during hexavalent chromium toxicity

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One way in which xenobiotics induce apoptotic cell death is to alter the selective permeability of the intracellular voltage-dependent anion channel (VDAC1) in the mitochondrial membrane. In this study, we explored the association between VDAC1 mRNA expression and mitochondrial function during hexavalent chromium compounds [Cr(VI)] treatment. Cultured L-02 hepatocytes were treated with 2, 4, 8, 16, or 32 $\mu\text{mol/L}$ Cr(VI) for 12, 24, or 36 h. Expression of VDAC1 mRNA was measured by qualitative reverse transcription polymerase chain reaction (RT-PCR), whereas intracellular adenosine triphosphate (ATP) levels were determined by an ATP-specific bioluminescence assay. Over this range of Cr(VI) concentrations, average VDAC1 mRNA expression decreased by 84% after 12 h treatment and by 40% after 24 h of treatment, but it increased 2.33-fold at 36 h when compared with the control cultures. Treatment with Cr(VI) disrupted cellular metabolism as evidenced by changes in ATP levels. Cr(VI) treatment caused ATP levels to increase at 12 h, decrease at 24 h, and increase again at 36 h, resulting in a slant V-shaped curve. Correlation analysis revealed a moderate negative relationship between VDAC1 mRNA expression and intracellular ATP levels during Cr(VI) treatment ($r = -0.557$, $P < 0.05$). The negative association seemed to be more obvious in 32 $\mu\text{mol/L}$ Cr(VI) group. Based on these results, heavy metal toxicity may induce over-expression of VDAC1 mRNA, which causes a decrease in ATP levels.

Key words: Heavy metal toxicity, VDAC1 mRNA expression, ATP, relationship.

INTRODUCTION

Hexavalent chromium compounds [Cr(VI)] are toxic heavy metals that readily cross cellular membranes through non-specific anion channels. Intracellular accumulation of Cr(VI) can induce redox reactions, which generate reactive oxygen species (ROS) and oxidative stress. Unchecked oxidative stress can lead to cell damage by opening the mitochondrial permeability transition pore (PTP), collapsing the mitochondrial trans-membrane potential ($\Delta\psi_m$), releasing cytochrome c, and consequently activating the mitochondria-mediated apoptosis

pathway (Valko et al., 2006; Son et al., 2010; Pritchard et al., 2000; Jing-Wei et al., 2006). The mitochondria are the principal organelles for adenosine triphosphate (ATP) synthesis. Mitochondrial damage disrupts the tricarboxylic acid cycle and reduces oxidative phosphorylation (OXPHOS), resulting in a decrease in ATP levels (McFarland et al., 2009). Recently, the voltage-dependent anion channel (VDAC, also known as mitochondrial porin) was shown to localize to the mitochondrial outer membrane (OMM) and mediate the exchange of metabolites across the OMM. Indeed, this permeability is critical for cellular energy metabolism (Shoshan-Barmatz et al., 2010). Under normal physiological conditions, VDAC is permeable to both small ions, such as Cl^- , K^+ , and Na^+ , and to large anions, such as glutamate (Gincel et al., 2000) and ATP (Rostovtseva et al., 1996). Exposure to

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xenobiotics increases VDAC1 expression, which enhances the oligomerization state and enlarges the pore diameter of VDAC1, consequently allowing for the passage of larger molecules such as cytochrome c, and induce apoptosis (Zheng et al., 2004).

The detailed mechanisms of how Cr(VI) disrupts metabolism and induces apoptosis are currently unclear. Furthermore, few studies have investigated the association between VDAC gene expression and dysfunctional energy metabolism. For this reason, we focused on the relationship between VDAC expression and ATP levels during Cr(VI)-mediated toxicity. The VDAC family of proteins includes three protein subunits: VDAC1, VDAC2, and VDAC3. The VDAC1 subunits form the pore of the channel, whereas subunits VDAC2 and VDAC3 play a complementary role in regulating VDAC1 channel activity (Blachly-Dyson et al., 1997; Sampson et al., 1997; Lee et al., 1998). To explore the potential association between VDAC expression and metabolic dysfunction induced by Cr(VI), we analyzed VDAC1 mRNA expression by qualitative reverse transcriptase polymerase chain reaction (RT-PCR) and measured intracellular ATP levels by a bioluminescent assay during Cr(VI) exposure in cultured L-02 hepatocytes.

MATERIALS AND METHODS

Potassium dichromate ($K_2Cr_2O_7$, molecular weight 294.19) was purchased from Sigma (standard grade, content > 99.99%). RPMI-1640 culture medium was obtained from Solarbio (USA), and newborn calf serum was purchased from Shi Ji Qing biology corporation (China). A methyl thiazolyl tetrazolium (MTT) cell death kit was purchased from Amresco (USA). An RNA prep pure kit, Taq PCR Master Mix, SYBR Green-Real Master Mix, and Quantscript RT Kit were obtained from TianGen (China). The ATP assay kit was purchased from Beyotime (China). The human embryonic liver cell line L-02 (L-02 hepatocytes) was obtained from the Shang Hai Center of Cell Culture of the Chinese Academy of Sciences.

L-02 hepatocytes culture

The L-02 hepatocyte cell line was cultured in Roswell Park Memorial Institute (RPMI) medium containing 15% newborn calf serum at 37°C in a 5% CO_2 atmosphere. The culture medium was changed every one to two days. When the cell density had reached 90% confluency, the adherent cells were digested in media containing 0.25% trypsin and was sub-cultured.

Cr(VI) exposure

A 0.1 M Cr(VI) stock solution was made by adding 29.418 g $K_2Cr_2O_7$ to 1000 ml ddH_2O . The stock was then diluted in culture medium to 2, 4, 8, 16, 32, 64, or 128 $\mu\text{mol/L}$. When the cell density had reached 70%, they were exposed to Cr(VI) for different time periods (12, 24, or 36 h) at 37°C. Untreated cultures were used as a control group. Cell survival was analyzed by the MTT method following the manufacturer's instructions. An optimal Cr(VI) dose range that would alter VDAC1 mRNA expression and intracellular ATP levels without causing excessive cell death was determined.

Analysis of VDAC1 mRNA expression by RT-PCR

Design and synthesis of VDAC1 gene primers

The human VDAC1 mRNA sequence (Homo sapiens voltage-dependent anion channel 1, cDNA clone MGC:88007, IMAGE:6023095; Gene bank ID: BC071168) was obtained from the database at <http://www.ncbi.nlm.nih.gov/nucleotide>. VDAC1 primers were designed using the software Primer 3.0. The primers used were as follows: forward 5' ATGTCTTCACCAAGGGCTAT 3' and reverse 5' TCTGGGTCACCTCGGGATT 3'. The PCR product length was 453 bp. For quantitation, β -actin was used as an internal reference standard (Gene bank ID: Human actin beta BC002409, F1379) using the primer pair 5' AGCGAGCATCCCCAAAGTT 3' and 5' GGGCACGAAGGCTCATCATT 3'. The β -actin primers yielded the expected PCR product of 285 bp. Both primers were synthesized, diluted to 10 pmol/ μL , and stored at -20°C until they were used.

Total RNA isolation and reverse transcription

Following 12, 24, or 36 h of treatment of L-02 hepatocytes with various concentrations of Cr(VI), total RNA was isolated using a commercial kit following the manufacturer's instructions. A script cDNA kit was used to carry out reverse transcription in a total reaction volume of 20 μL containing isolated total RNA, oligo(dT)₁₅, dNTPs, DEPC, and H_2O (added in that order). The mixture was heated to 70°C for 5 min followed by the addition of 5 \times First-Strand Buffer, RNasin, and Script M-MLV. The mixture was maintained at 42°C for 50 min in a water bath, the reaction was stopped by heating it to 95°C for 5 min.

PCR and fluorescence quantitative PCR (qPCR)

The VDAC1 and β -actin gene products from the total cellular RNA were amplified by PCR and qPCR in a total reaction volume of 25 μL containing cDNA template (2.0 μL), VDAC1 or β -actin; forward and reverse primers (0.5 μL), 2 \times TaqMaster (12.5 μL), and ddH_2O (9.5 μL). The thermocycle protocol was as follows: 94°C for 4 min followed by 35 cycles of 94°C for 35 s, 55°C for 35 s, and 72°C for 50 s and a final elongation at 72°C for 10 min. After the completion of PCR, the reaction product was analyzed by agarose gel electrophoresis. qPCR was performed in a 25- μL reaction volume with fluorochrome SYBR Green and the same constituents as were used for standard PCR. Each PCR reaction was repeated twice per sample. Relative changes in VDAC1 gene expression were calculated using the formula $2^{-\Delta\Delta CT} \times 100\%$, where CT is the threshold cycle number.

ATP bioluminescence assay

Following Cr(VI) treatment, intracellular ATP levels were determined using a bioluminescence ATP assay kit. Cells were lysed in 200 μL of lysis buffer and centrifuged at 12000 $\times g$ to collect the cell supernatant. ATP detection working solution (100 μL) was added to each well of a 96-well black culture plate and incubated for 3 to 5 min at room temperature, and 40 μL of cell lysate was added to each well. Each lysate sample was examined in four parallel wells. Luminescence was measured immediately, and every sample was measured three times. In addition, the 96-well plates also contained wells with serial dilutions of an ATP standard solution to generate a standard curve. Total ATP levels were corrected for survival rate (as determined by the MTT assay) and normalized against appropriate untreated controls.

Table 1. Survival rates of cells in response to different Cr (VI) doses and treatment times.

| Time(h) | MTT absorbance ($\bar{X} \pm S_d$) / survival rate (%) | | | | | |
|---------|--|------------|------------|------------|------------|-------------|
| | 0 | 2 | 4 | 8 | 16 | 32 |
| 12 | 0.536±0.08 | 0.512±0.02 | 0.491±0.03 | 0.48 ±0.05 | 0.448±0.03 | 0.403±0.03* |
| | 100 | 95.52 | 91.60 | 90.86 | 83.58 | 75.19 |
| 24 | 0.523±0.06 | 0.516±0.03 | 0.527±0.05 | 0.491±0.02 | 0.419±0.06 | 0.346±0.01* |
| | 100 | 98.66 | 100 | 93.88 | 80.11 | 66.16 |
| 36 | 0.517±0.08 | 0.501±0.04 | 0.506±0.07 | 0.456±0.04 | 0.397±0.03 | 0.332±0.04* |
| | 100 | 96.91 | 97.87 | 88.20 | 76.79 | 64.22 |

0, 2, 4, 8, 16, and 32 represent Cr (VI) treatment concentrations in $\mu\text{mol/L}$; *, LSD multiple comparisons follow after the *F* test. *, $P < 0.05$ indicates statistical difference.

Table 2. Changes in VDAC1 mRNA expression induced by Cr(VI) treatment for 12 h.

| Concentration($\mu\text{mol/L}$) | VDAC1 CT | β -actin CT | ΔCT | $\Delta\Delta\text{CT}$ | $2^{-\Delta\Delta\text{CT}}$ | χ^2 test | P-value |
|------------------------------------|----------|-------------------|-------------------|-------------------------|------------------------------|---------------|---------|
| 0 | 27.45 | 22.74 | 4.71 | 0.00 | 1.00 | | |
| 2 | 29.68 | 20.74 | 8.94 | 4.23 | 0.05* | 158.44 | 0.00 |
| 4 | 26.91 | 19.66 | 7.25 | 2.54 | 0.17* | 51.34 | 0.00 |
| 8 | 26.63 | 18.89 | 7.74 | 3.03 | 0.12* | 104.35 | 0.00 |
| 16 | 30.19 | 25.41 | 4.78 | 0.07 | 0.95 | 0.18 | 0.67 |
| 32 | 29.44 | 21.52 | 7.92 | 3.21 | 0.11* | 137.90 | 0.00 |
| \bar{x} | 28.57 | 21.24 | 7.33 | 2.62 | 0.16* | 58.68 | 0.00 |

CT, threshold cycle number in qPCR, ΔCT , difference of VDAC1-CT subtract β -actin-CT, $\Delta\Delta\text{CT}$ represent the difference of 2-32 $\mu\text{mol/L}$ Cr(VI)- ΔCT subtract control- ΔCT ; *, $P < 0.05$ indicates significant difference by comparing each group with the control in χ^2 test; \bar{x} , average level of VDAC1 mRNA after 2-32 $\mu\text{mol/L}$ Cr(VI) treatment.

Data analyses

Data were analyzed in SPSS 13.0. The results of the ATP and MTT assays are expressed as the mean \pm standard deviation ($\bar{X} \pm S_d$). Statistical significance between means were determined by one-way ANOVA followed by LSD post hoc tests. The survival rate of cultured cells (from the MTT assay) and relative VDAC1 mRNA expression are expressed as percent (%) change from control. Statistical significance was determined by Pearson's Chi-square (χ^2) and linear χ^2 tests. For the purpose of χ^2 analysis, both compared groups were divided by the same number to achieve a gain of less than 100%. The association between VDAC1 mRNA expression and ATP levels was analyzed by the Spearman rank correlation coefficient. $P < 0.05$ (two-sided test) was accepted as statistically significant.

RESULTS

Cells survival rate after Cr(VI) treatment

Cr(VI) caused a decrease in survival in L-02 hepatocytes, especially in the high dose group. The high Cr(VI) concentrations tested (64 and 128 $\mu\text{mol/L}$) led to a lower than 60% L-02 hepatocyte survival rates (data not shown). As a result, Cr(VI) doses between 0 and 32 $\mu\text{mol/L}$ were

used to examine the relationship between VDAC1 mRNA expression and ATP levels (Table 1).

Changes in VDAC1 mRNA expression upon treatment with Cr(VI)

In cultures treated with Cr(VI) for 12 h, VDAC1 mRNA expression decreased by as much as 95% at 2 $\mu\text{mol/L}$ and, on average, by 84% over the dose range of 2 to 32 $\mu\text{mol/L}$, relative to untreated control cultures (Table 2, Figure 1A). After 24 h of treatment, VDAC1 mRNA expression decreased by 70% at 2 $\mu\text{mol/L}$ group, and decreased by an average of 40% over the same dosage range (Table 3, Figure 1B). However, after 36 h of Cr(VI) treatment, VDAC1 mRNA expression increased significantly by as much as 8.69 fold in the 2 $\mu\text{mol/L}$ treatment group and by an average of 2.33 over the dose range of 2 to 32 $\mu\text{mol/L}$ relative to untreated control cultures (Table 4, Figure 1C). In summary, treatment with Cr(VI) evoked changes in VDAC1 mRNA expression. Expression increased mildly with Cr(VI) treatment after 12 to 24 h, whereas at 36 h, VDAC1 mRNA expression increased relative to shorter treatments (Figures 1 and 2).

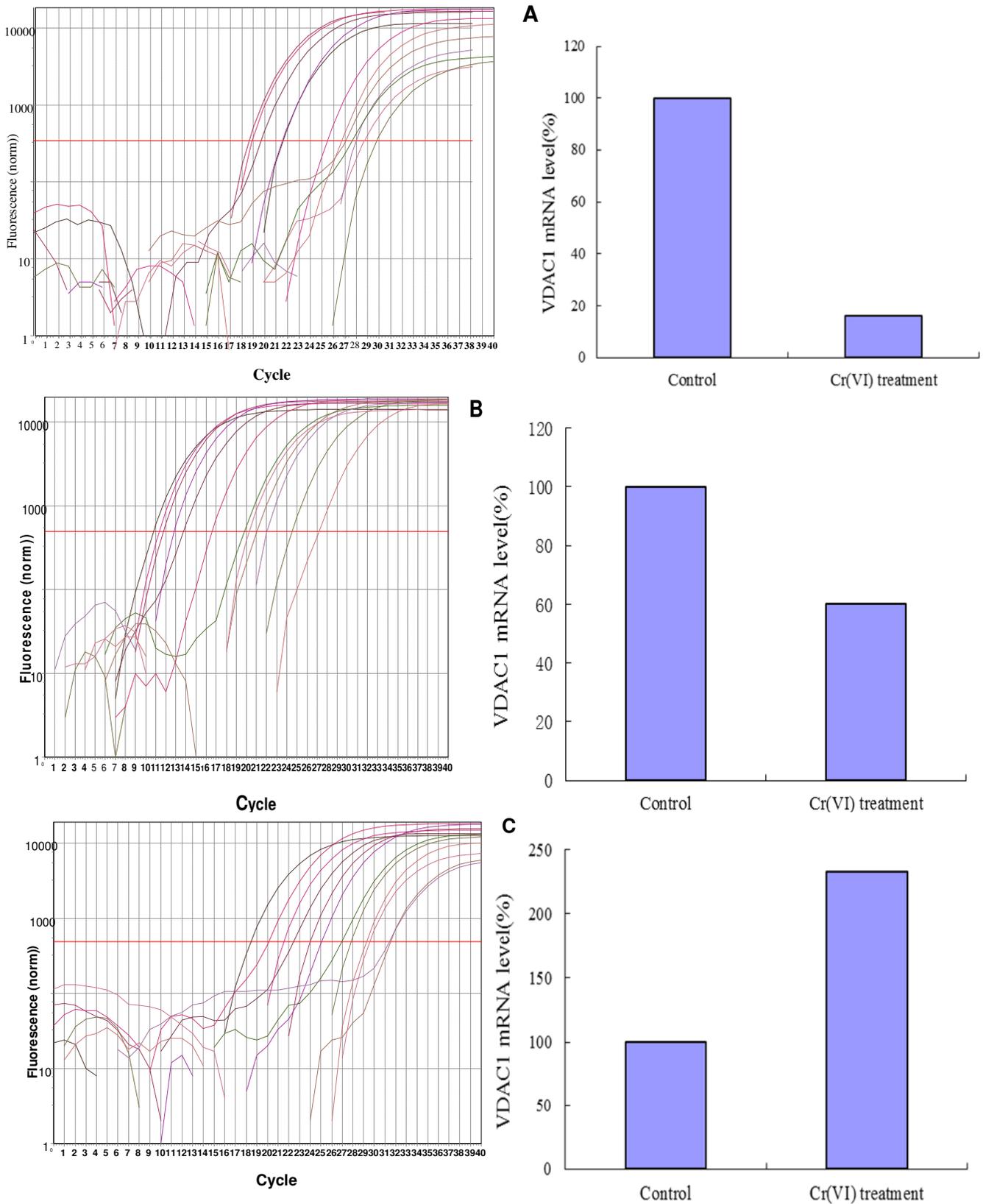


Figure 1. Result of VDAC1 RT-qPCR following Cr(VI) treatment and analysis. A, 12 h; B, 24 h; C, 36 h. The left curve of 1 to 6 represent β -actin, the right curve of 7–12 indicate VDAC1; The height of histogram represented average level of VDAC1 mRNA during Cr(VI) toxicity.

Table 3. Changes in VDAC1 mRNA expression induced by Cr(VI) treatment for 24 h.

| Concentration ($\mu\text{mol/L}$) | VDAC1 CT | β -actin CT | ΔCT | $\Delta\Delta\text{CT}$ | $2^{-\Delta\Delta\text{CT}}$ | χ^2 test | P-value |
|-------------------------------------|----------|-------------------|-------------------|-------------------------|------------------------------|---------------|---------|
| 0 | 19.81 | 10.79 | 9.02 | 0.00 | 1.00 | | |
| 2 | 24.54 | 13.79 | 10.75 | 1.73 | 0.30* | 43.17 | 0.00 |
| 4 | 21.07 | 11.78 | 9.29 | 0.27 | 0.83 | 2.45 | 0.12 |
| 8 | 27.20 | 16.61 | 10.59 | 1.57 | 0.34* | 33.33 | 0.00 |
| 16 | 20.30 | 11.38 | 8.92 | -0.10 | 1.07 | 0.18 | 0.67 |
| 32 | 22.06 | 12.85 | 9.21 | 0.19 | 0.88 | 0.98 | 0.32 |
| \bar{x} | 23.03 | 13.28 | 9.75 | 0.73 | 0.60* | 24.44 | 0.00 |

CT represent the threshold cycle number in qPCR, ΔCT represent the difference of VDAC1-CT subtract β -actin-CT, $\Delta\Delta\text{CT}$ represent the difference of 2-32 $\mu\text{mol/L}$ Cr(VI)- ΔCT subtract control- ΔCT ; *, $P < 0.05$ indicates significant difference by comparing each group with the control in χ^2 test; \bar{x} , average level of VDAC1 mRNA after 2-32 $\mu\text{mol/L}$ Cr(VI) treatment.

Table 4. Changes in VDAC1 mRNA expression induced by Cr(VI) treatment for 36 h.

| Concentration($\mu\text{mol/L}$) | VDAC1 CT | β -actin CT | ΔCT | $\Delta\Delta\text{CT}$ | $2^{-\Delta\Delta\text{CT}}$ | χ^2 test | P-value |
|------------------------------------|----------|-------------------|-------------------|-------------------------|------------------------------|---------------|---------|
| 0 | 26.97 | 18.45 | 8.52 | 0.00 | 1.00 | | |
| 2 | 27.85 | 22.45 | 5.40 | -3.12 | 8.69* | 48.02 | 0.00 |
| 4 | 31.53 | 24.01 | 7.52 | -1.00 | 2.00* | 13.33 | 0.00 |
| 8 | 29.32 | 20.13 | 9.19 | 0.67 | 0.63* | 19.78 | 0.00 |
| 16 | 29.62 | 21.57 | 8.05 | -0.47 | 1.39* | 3.99 | 0.04 |
| 32 | 31.40 | 25.08 | 6.32 | -2.20 | 4.59* | 35.87 | 0.00 |
| \bar{x} | 29.94 | 22.65 | 7.3 | -1.22 | 2.33* | 17.01 | 0.00 |

CT represent the threshold cycle number in qPCR, ΔCT represent the difference of VDAC1-CT subtract β -actin-CT, $\Delta\Delta\text{CT}$ represent the difference of 2-32 $\mu\text{mol/L}$ Cr(VI)- ΔCT subtract control- ΔCT ; *, $P < 0.05$ indicates significant difference by comparing each group with the control in χ^2 test; \bar{x} , average level of VDAC1 mRNA after 2-32 $\mu\text{mol/L}$ Cr(VI) treatment.

Cr(VI)-dependent changes in intracellular ATP levels

After Cr(VI) treatment for 12 h, the ATP levels of L-02 hepatocytes increased significantly compared to untreated controls. This increase in ATP was potentiated at higher Cr(VI) doses. After 24 h of treatment, intracellular ATP levels increased at the lowest Cr(VI) dose but decreased at the other doses. After 36 h, intracellular ATP levels returned to near baseline levels, while the ATP levels in 32 $\mu\text{mol/L}$ Cr(VI) group remained lower than that of the control. In summary, Cr(VI) induced complex changes in ATP levels. For most Cr(VI) doses (4 to 32 $\mu\text{mol/L}$), the relationship could best be described as a slant "V-shaped" curve (Table 5, Figure 3).

Correlation between intracellular VDAC1 mRNA expression and ATP levels

The relationship between intracellular VDAC1 mRNA expression and ATP levels was analyzed by generating scatter plots of ATP vs. VDAC1 mRNA for each Cr(VI) treatment duration and dose. Results of the Spearman's rank correlation test reveal a negative correlation between VDAC1 mRNA expression and ATP levels ($r = -$

0.557, Table 6 and Figure 4).

DISCUSSION

VDAC (MW 30-32 kDa) is expressed on the outer mitochondrial membrane. Its three-dimensional structure is characterized by a β -barrel formed by 19 β -strands and an N-terminal α -helical region located inside the pore (Blachly-Dyson et al., 2001; Hiller et al., 2008; Bayrhuber et al., 2008). Under normal physiological conditions, VDAC is stable in an open state and can allow for the exchange of mitochondrial metabolites and ions. When the structure is altered, however, the channel can allow the passage of larger metabolites, which results in mitochondrial dysfunction and even apoptosis. Consequently, the state of VDAC is critical for mitochondrial metabolism (Shoshan-Barmatz et al., 2008; 2010).

Several lines of evidence have suggested that some xenobiotics mediate cytotoxicity by altering mitochondrial VDAC1 permeability either directly or via ROS generation. Furthermore, expression of VDAC1 mRNA appears to alter the channel's structure. This altered structure increases the permeability of the mitochondrial membrane, causes a collapse in the $\Delta\psi\text{m}$, reduces energy

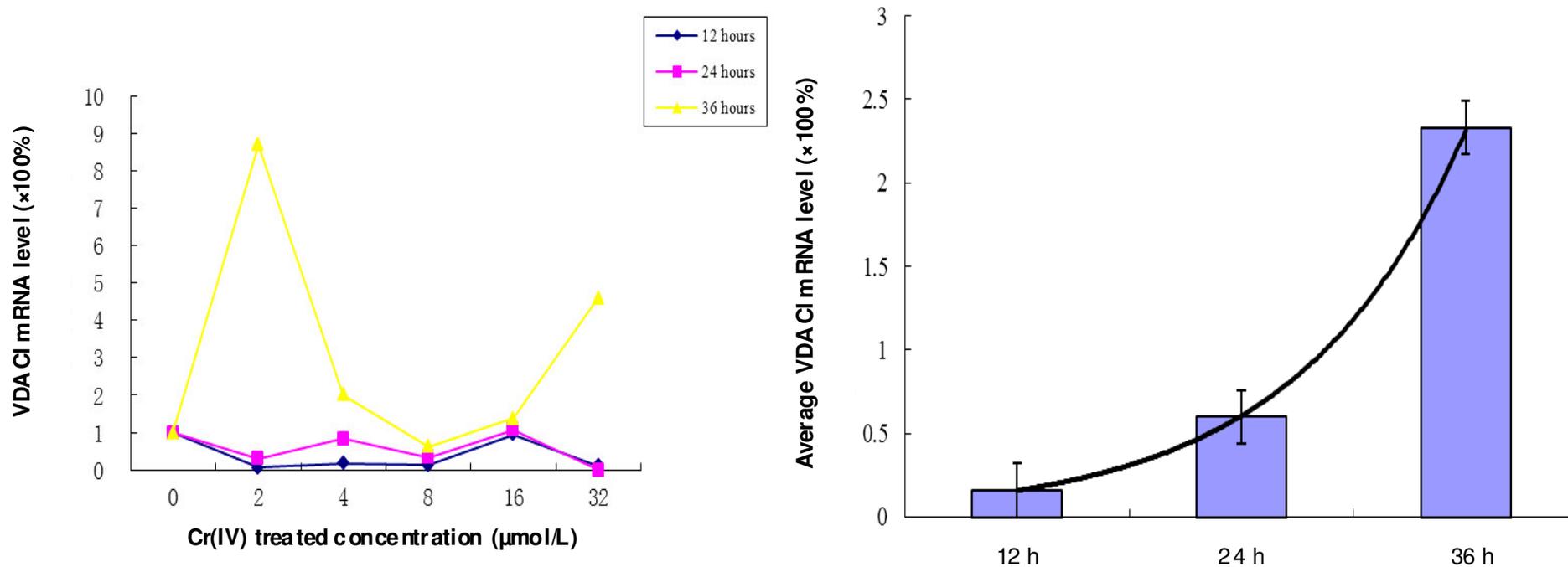


Figure 2. Changes of VDAC1 mRNA levels following Cr(VI) treatment.

Table 5. Dynamic changes in intracellular ATP content during Cr (VI) toxicity.

| Time (h) | ATP content ($\bar{X} \pm S_d$) / relative ratio with control | | | | | |
|----------|---|-------------|-------------|-------------|-------------|-------------|
| | 0 | 2 | 4 | 8 | 16 | 32 |
| 12 | 8.44±0.49 | 10.74±0.63* | 10.47±0.56* | 12.47±0.26* | 13.07±0.42* | 14.38±0.39* |
| | 1.00 | 1.273 | 1.240 | 1.477 | 1.548 | 1.703 |
| 24 | 14.77±0.68 | 17.45±0.83* | 10.10±0.23* | 8.63±0.22* | 6.64±0.54* | 2.45±0.09* |
| | 1.00 | 1.182 | 0.684 | 0.584 | 0.450 | 0.166 |
| 36 | 19.27±0.72 | 19.08±2.04 | 21.51±1.87 | 26.97±0.52* | 20.93±1.61* | 10.47±0.09* |
| | 1.00 | 0.990 | 1.116 | 1.400 | 1.086 | 0.543 |

Note: 0, 2, 4, 8, 16, and 32 represent concentrations of Cr (VI) in µmol/L; *, LSD multiple comparisons follow after the F test. P < 0.05 indicates statistical difference.

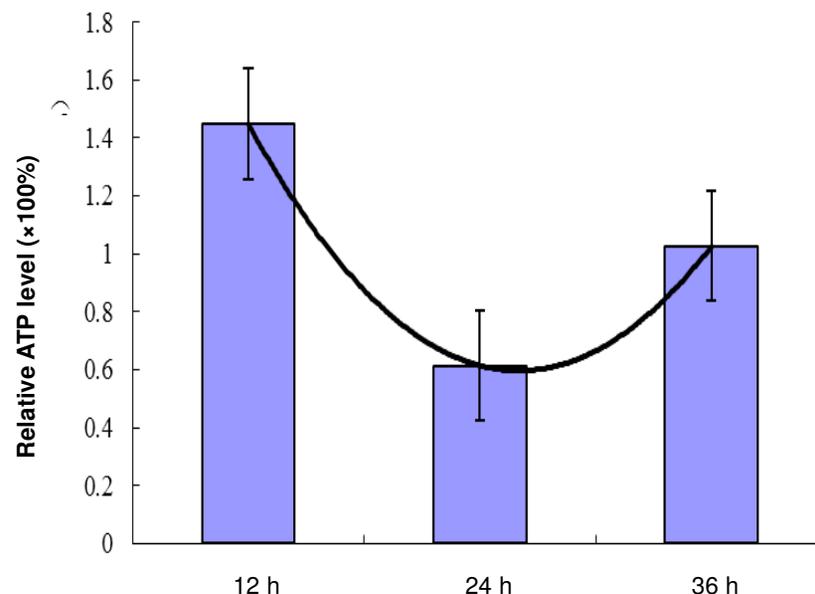
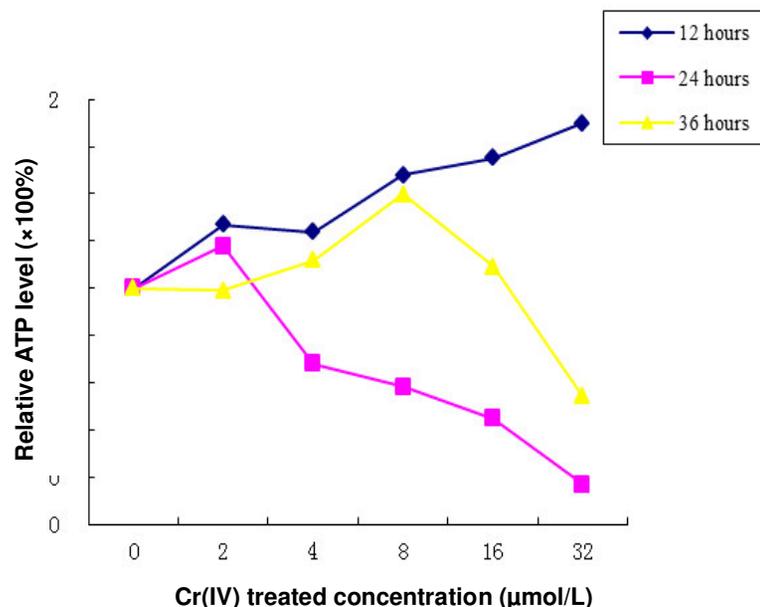


Figure 3. Changes of intracellular ATP levels following Cr(VI) treatment.

metabolism, releases cytochrome c, and leads to apoptosis. VDAC is a biological target of As_2O_3 , and clinically relevant doses of As_2O_3 can induce VDAC1 over-expression and concomitant homodimerization in the multiple myeloma cell line IM-9, resulting in $\Delta\psi_m$ reduction, cytochrome c release, and apoptosis (Zheng et al., 2004). Conversely, acute ethanol exposure decreases respiration and accessibility of mitochondrial adenylate kinase (AK) of permeabilized hepatocyte membranes by 40 and 32%, respectively, suggesting that ethanol decreases mitochondrial outer membrane permeability by inhibiting VDAC1 (Holmuhamedov and Lemastersc, 2009; Hoek et al., 2002). Exposure to 5 and 10 $\mu\text{mol/L}$ Pb reduced VDAC transcription and expression in the neuronal cell lines PC-12 and SH-SY5Y cells, which correlated with decreased cellular ATP levels (Prins et al.,

2010).

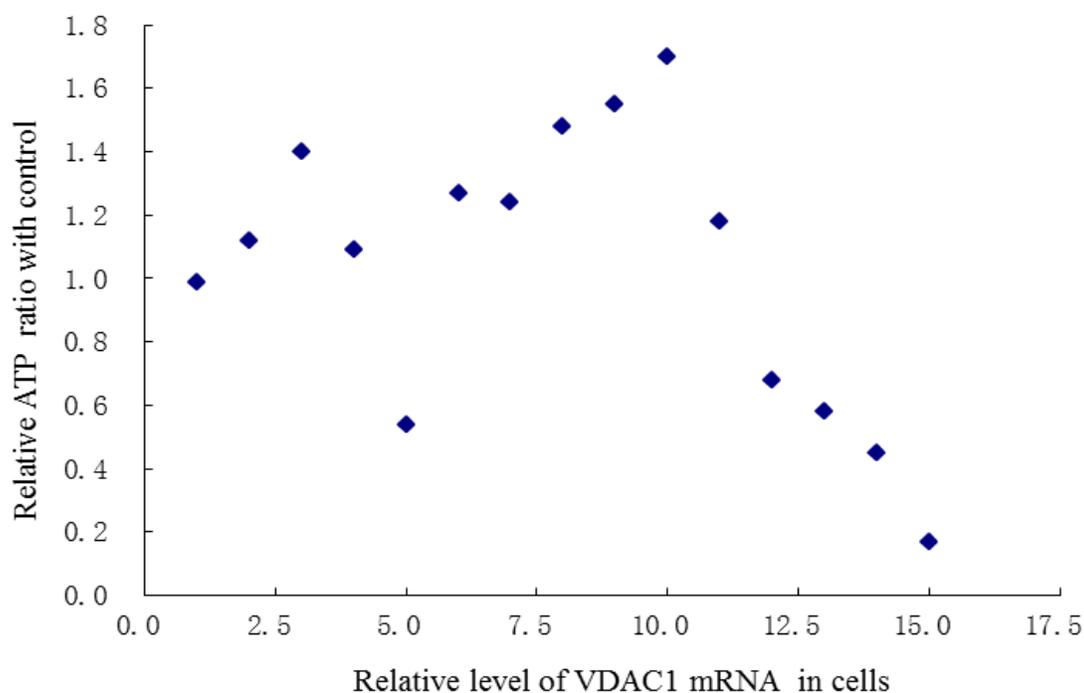
Several studies have suggested that Cr(VI) can enter cells and induced mitochondrial damage and apoptosis (Valko et al., 2006; Son et al., 2010; Pritchard et al., 2000), suggesting a potential association between changes in VDAC1 expression and mitochondrial damage induced by Cr(VI). To test this possibility, we measured ATP levels and VDAC1 mRNA expression during mild Cr(VI)-mediated toxicity. Our results demonstrate that Cr(VI) significantly altered VDAC1 mRNA expression. VDAC1 mRNA expression decreased after 12 to 24 h of Cr(VI) treatment and increased after 36 h. Concomitant with these complex changes in VDAC1 mRNA expression, Cr(VI) also induced metabolic dysfunction as evidenced by changes in ATP levels. Exposure to Cr(VI) caused an early increase in ATP levels at 12 h followed by a

decrease at 24 h and an increase again at 36 h. Specifically, as the concentration of Cr(VI) increased, intracellular ATP tended to increase more at the earlier time point and decrease more at the intermediate time point (24 h). However, the ATP levels returned to near baseline levels at the later time point (36 h), while the ATP levels in 32 $\mu\text{mol/L}$ Cr(VI) group remained lower than that of the control. For the complex changes in ATP levels, the possible reason is that, the increase of ATP level at 12 h indicated activation of mitochondria energy metabolism, the decreased ATP levels at 24 h indicated disruption of mitochondria energy metabolism, interestingly, the increase of ATP levels at 36 h indicated recovery in mitochondrial function, while the continuous decrease of ATP levels in 32 $\mu\text{mol/L}$ Cr(VI) group indicated mitochondrial dysfunction occurrence in L-02

Table 6. Correlation analysis between VDAC1 mRNA expression and ATP levels.

| Cr(VI) concentration ($\mu\text{mol/L}$) | VDAC1 mRNA / Relative ATP levels ($\times 100\%$) | | | | | | |
|---|---|------|-------|------|-------|------|--------|
| | 12 h | | 24 h | | | 36 h | |
| 2 | 0.053 | 1.27 | 0.301 | 1.18 | 8.694 | 0.99 | |
| 4 | 0.172 | 1.24 | 0.829 | 0.68 | 2.000 | 1.12 | |
| 8 | 0.122 | 1.48 | 0.337 | 0.58 | 0.629 | 1.40 | |
| 16 | 0.953 | 1.55 | 1.072 | 0.45 | 1.385 | 1.09 | |
| 32 | 0.108 | 1.70 | 0.877 | 0.17 | 4.595 | 0.54 | |
| Spearman coefficient | | | | | | | -0.557 |
| P-value | | | | | | | 0.031* |

The Spearman grade correlation was used to analyze the data, because the data were indicated by percent (%) and did not belong to a normal distribution. *, $P < 0.05$ indicates a statistical correlation between VDAC1 mRNA expression and ATP content levels.

**Figure 4.** Scatter plot between VDAC1 expression and ATP levels following Cr(VI) treatment.

hepatocytes. So the effects of Cr(VI) treatment time and dose on ATP levels and VDAC1 mRNA expression were highly complex, the correlation analysis revealed a moderate negative relation between expression of VDAC1 mRNA and intracellular ATP levels during Cr(VI) treatment, the negative association seemed to be more obvious in 32 $\mu\text{mol/L}$ Cr(VI) group.

These results suggest that Cr(VI) could lead to metabolism dysfunction by damaging the mitochondria. The negative correlation between ATP and VDAC1 mRNA levels suggest that enhanced expression of VDAC1 leads to mitochondrial dysfunction and a concomitant decrease in ATP, which could be mediated by an increase in OMM permeability. While mechanism of

Cr(VI) alteration of VDAC1 mRNA expression is not known, it is possible that Cr(VI) interacts with the VDAC1 promoter or induces an upstream regulatory gene. In contrast, the changes in VDAC1 expression could be mediated indirectly through oxidative stress. However, the moderate negative correlation between VDAC expression and ATP levels during Cr(VI) treatment suggests that Cr(VI) may inhibit mitochondrial function through other (as yet undefined) mechanisms in addition to regulation of VDAC1 mRNA expression. Analyzing other mitochondria energy metabolism-related genes during Cr(VI) toxicity, such as adenosine transporter 1 (ANT1) and respiratory chain Complexes I-V, could illuminate additional mechanisms of Cr(VI) toxicity.

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