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Chi-square analysis of the reduction of ATP levels in L-02 hepatocytes by hexavalent chromium

Yang Yuan[1,2], Li Peng[1], Hu Gong-Hua[1], Dai Lu[1], Zhong Xia-Li[1], Zhou Yu[1] and Zhong Cai-Gao[1]

[1]School of Public Health, Central South University, Changsha Hunan, China
[2]Department of Clinical Laboratory, Huaihua Medical College, Huaihua Hunan, China

Abstract

This study explored the reduction of adenosine triphosphate (ATP) levels in L-02 hepatocytes by hexavalent chromium (Cr(VI)) using chi-square analysis. Cells were treated with 2, 4, 8, 16, or 32 µM Cr(VI) for 12, 24, or 36 h. Methyl thiazolyl tetrazolium (MTT) experiments and measurements of intracellular ATP levels were performed by spectrophotometry or bioluminescence assays following Cr(VI) treatment. The chi-square test was used to determine the difference between cell survival rate and ATP levels. For the chi-square analysis, the results of the MTT or ATP experiments were transformed into a relative ratio with respect to the control (%). The relative ATP levels increased at 12 h, decreased at 24 h, and increased slightly again at 36 h following 4, 8, 16, 32 µM Cr(VI) treatment, corresponding to a "V-shaped" curve. Furthermore, the results of the chi-square analysis demonstrated a significant difference of the ATP level in the 32-µM Cr(VI) group ($P < 0.05$). The results suggest that the chi-square test can be applied to analyze the interference effects of Cr(VI) on ATP levels in L-02 hepatocytes. The decreased ATP levels at 24 h indicated disruption of mitochondrial energy metabolism and the slight increase of ATP levels at 36 h indicated partial recovery of mitochondrial function or activated glycolysis in L-02 hepatocytes.

Key words: Hexavalent chromium; ATP level; Interference effect; Chi-square test

Introduction

Hexavalent chromium, Cr(VI), is a well-documented human carcinogen and is widely found in human living environments as the result of industrial production or discharge (1). Recently, Cr(VI) pollution has been found in crops due to the uptake of Cr(VI) from the soil and in rivers in some regions of China (2,3). The toxicity caused by oral Cr(VI) ingestion is thought to be due to toxicity to the liver, which is the main organ of biological metabolism, and liver damage (or hepatotoxicity) from Cr(VI) exposure has been confirmed in animal experiments and in cultured L-02 hepatocytes, which showed hepatocyte ultrastructure disruption, mitochondrial damage and apoptosis (4-6).

Mitochondria are the main site of ATP synthesis, which is produced by the tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OXPHOS) in the inner mitochondrial membrane (7). At present, the detailed interference effect of Cr(VI) on the cellular ATP levels is not known. Generally, Cr(VI) can induce apoptosis and lead to a decrease in cell survival or cell number, and differences in cell number result in the different cellular ATP levels. And in this case manual adjustment of cell number is commonly applied to balance differences in cell number in each group. Small sample *t*-tests or one-way ANOVA were applied to compare the differences between the Cr(VI) treatment groups and control. However, in our view, adjusting the cell number is not the only way to analyze the toxic effects of Cr(VI) *in vitro*. The chi-square statistical test (χ^2) can also be used to analyze the physiological or toxicological effects of Cr(VI) *in vitro*.

The χ^2 test is a commonly used statistical method and consists of the Pearson chi-square, linear-by-linear chi-square, McNemar and Mantel-Haenszel tests, among others. Currently, χ^2 analysis is widely applied to compare the difference of a relative ratio existing between two or more groups. It is frequently used in the fields of clinical and experimental epidemiology to explore etiological

Correspondence: Zhong Cai-Gao, School of Public Health, Central South University, Changsha Hunan, 410078 China.
E-mail: zcg54@xysm.net

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factors, to assess risk, and to predict trends of disease development. However, the χ^2 test is rarely applied in the field of *in vitro* cytotoxicity. For this reason, after cultured L-02 hepatocytes were exposed to 0, 2, 4, 8, 16, and 32 µM Cr(VI) for 12, 24, or 36 h, a χ^2 test was applied to analyze the interference effect by comparing the difference between cell survival rate and intracellular ATP levels to establish a novel method of analyzing the cytotoxicity induced by toxic chemicals *in vitro*.

Material and Methods

Material

Potassium dichromate [$K_2Cr_2O_7$, abbreviated as Cr(VI)] was purchased from Sigma (USA). Roswell Park Memorial Institute (RPMI-1640) culture medium was purchased from Solarbio (USA), and newborn calf serum was purchased from Shi Ji Qing (China). A methyl thiazolyl tetrazolium (MTT) cell death kit was purchased from Amresco (USA). The ATP assay kit-S0026 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 Chinese Academy of Sciences. A 0.1 M Cr(VI) stock solution was prepared 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 µM.

L-02 hepatocyte culture and Cr(VI) exposure

L-02 hepatocytes were cultured on a six-well plate with RPMI-1640 medium containing 15% newborn calf serum at 37°C in a 5% CO_2 atmosphere. The culture medium was changed every 1-2 days. When the cell density reached 60% confluence, the cells were exposed to Cr(VI) for different periods of time (12, 24, or 36 h) at 37°C. Untreated cultures were used as a control group. Cell survival was analyzed by the MTT method according to manufacturer instructions.

MTT reduction assay

The MTT assay was performed according to manufacturer instructions. The growing cells were collected by 0.25% Trypsin digestion, centrifugation, and supernatant removal. Two milliliters of RPMI-1640 culture medium containing 15% newborn calf serum was added to resuspend the cells as a single cell suspension. The cell suspension was then inoculated in a 96-well culture plate at a density of 1.0 x 10^4 cells/well. The following day, the cells were grown in medium containing 0, 2, 4, 8, 16, or 32 µM Cr(VI) for 12, 24, or 36 h at 37°C. Following Cr(VI) treatment, MTT was added at a volume of 10 µL/well and cultured for 4 h at 37°C, then 100 µL formazan lysate was added, and the cells were cultured for 6 h at 37°C. Finally, the 96-well culture plate was removed from the incubator and continually shaken for 5 min on a micro-oscillator to completely dissolve the formazan. Immediately, cell vitality was analyzed by measuring absorbance at 492 nm with a multifunction microplate reader (Thermo Varioskan Flash 3001, USA).

ATP bioluminescence assay

L-02 hepatocytes were seeded at a density of 2.5 x 10^5 cells/well on three six-well plates. When the cells reached 60% confluence, they were exposed to Cr(VI) for 12, 24, or 36 h. Following Cr(VI) treatment, intracellular ATP levels were determined using a bioluminescent ATP assay kit. The cells were disrupted in 200 µL lysis buffer by mechanical disruption, and centrifuged at 12,000 *g* to collect the cell supernatant. Meanwhile, an aliquot (100 µL) of an ATP detection working solution was added to each well of a black 96-well culture plate and incubated for 3 min at room temperature. Then, four replicates of 40-µL samples of the cell lysate from each group were added to the wells. After allowing the reaction to take place for a few seconds, the luminescence value was measured. In addition, the 96-well plates also contained serial dilutions of an ATP standard solution to generate a standard curve, and the ATP levels in L-02 hepatocytes were calculated by comparison with the ATP standard curve.

Data analysis

Data were analyzed statistically with Microsoft Office Excel 2003 and SPSS 13.5. The results of the ATP and MTT assays are reported as means ± SD. The statistical significance of differences between means was determined by an *F*-test (ANOVA analysis) followed by least significant difference (LSD) *post hoc* tests. The survival rate of the cultured cells (from the MTT assay) and the relative ATP levels are reported as percent (%) change from control. Statistical significance was determined by Pearson chi-square or linear χ^2 tests. For the purpose of χ^2 analysis, the compared groups were divided by the same number to achieve a gain of less than 100%. A $P < 0.05$ values (two-sided test) was accepted as statistically significant.

Results

Cell viability

Following treatment with 2, 4, 8, 16, and 32 µM Cr(VI), L-02 hepatocyte viability decreased progressively over 12, 24, or 36 h ($P < 0.05$). The survival rates ranged from 88.20 to 100% after treatment with low concentrations of Cr(VI) (2, 4, and 8 µM), and the high Cr(VI) concentrations (16 and 32 µM) led to lower cell survival rates (64.22 to 83.58%). Further details from this experiment are shown in Table 1.

ATP level in L-02 hepatocytes

Following 12 h of Cr(VI) treatment, the ATP levels of L-02 hepatocytes were increased. However, after 24 h of treatment, intracellular ATP levels decreased significantly with Cr(VI) exposure, except for a slight increase in the 2-

Table 1. Effect of Cr(VI) on the viability of L-02 hepatocytes.

Time	MTT absorbance/survival rate (%)						χ^2 test (P value)
	Cr(VI) (μM)						
	0	2	4	8	16	32	
12 h	0.536 ± 0.08	0.512 ± 0.02	0.491 ± 0.03	0.487 ± 0.05	0.448 ± 0.03	0.403 ± 0.03*	
	(100)	(95.52)	(91.60)	(90.86)	(83.58)	(75.19)	0.00#
24 h	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)	0.01#
36 h	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)	0.01#

Cr(VI) = hexavalent chromium; MTT = methyl thiazolyl tetrazolium. Data are reported as means ± SD and percent within parentheses, N = 4. *P < 0.05 (least significant difference multiple comparisons following the *F*-test); #P < 0.05 for linearity (linear χ^2 test).

Table 2. ATP levels in L-02 hepatocytes following hexavalent chromium (Cr(VI)) treatment.

Time	ATP content/relative level (%)						χ^2 test (P value)
	Cr(VI) (μM)						
	0	2	4	8	16	32	
12 h	8.44 ± 0.49	10.26 ± 0.63*	9.59 ± 0.56*	11.33 ± 0.26*	10.92 ± 0.42*	11.71 ± 0.39*	
	(100)	(121.56)	(113.62)	(134.24)	(129.38)	(138.74)	0.03#
24 h	14.77 ± 0.68	17.22 ± 0.83*	10.10 ± 0.23*	8.10 ± 0.22*	5.32 ± 0.54*	1.62 ± 0.09*	
	(100)	(116.58)	(68.38)	(54.84)	(36.02)	(10.96)	0.00#
36 h	19.27 ± 0.72	18.49 ± 2.04	21.05 ± 1.87	23.79 ± 0.52*	16.07 ± 1.61*	6.72 ± 0.09*	
	(100)	(95.96)	(109.24)	(123.46)	(83.40)	(34.88)	0.15

Data are reported as means ± SD and percent within parentheses, N = 4. *P < 0.05 (least significant difference multiple comparisons following the *F*-test); #P < 0.05 for linearity (linear χ^2 test).

μM Cr(VI) group. Following 36 h of Cr(VI) treatment, the low ATP levels showed a slight up-regulation, while the ATP levels in the 16 and 32 μM Cr(VI) groups remained lower than control. The graphic change of relative ATP levels was described as a "V-shaped" curve (Table 2, Figure 1).

χ^2 analyses comparing cell survival rate and relative ATP levels

Following 12 h of Cr(VI) treatment, the χ^2 test showed a significant difference in ATP levels in the 8, 16, and 32 μM groups (P < 0.05). Following 24 h of Cr(VI) treatment, the χ^2 test showed a significant difference in ATP levels in the 4, 8, 16, and 32 μM groups (P < 0.05). Following 36 h of Cr(VI) treatment, the χ^2 test showed a significant difference in ATP levels in the 8 and 32 μM Cr(VI) groups (P < 0.05) (Table 3).

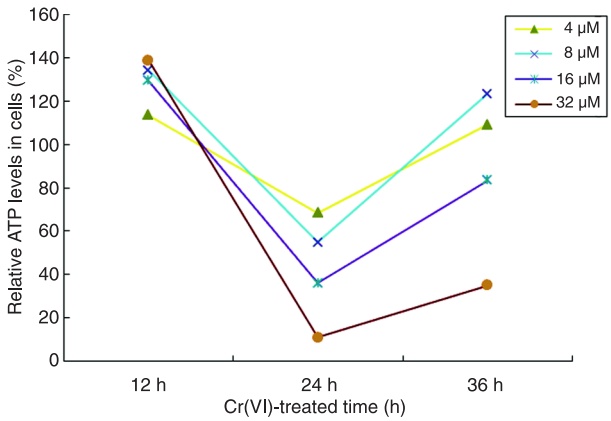

Figure 1. Relative ATP levels in L-02 hepatocytes following hexavalent chromium (Cr(VI)) treatment.

Table 3. χ^2 analysis of ATP level and cell viability of L-02 hepatocytes after treatment with hexavalent chromium (Cr(VI)) for 12, 24, and 36 h.

Concentration of Cr(VI) (μM)	Cell survival rate (%)	Relative level of ATP in cells (%)					
12 h		100.00	121.56	113.62	134.24	129.38	138.74
0	100.00	1.000					
2	95.52		0.065				
4	91.60			0.120			
8	90.86				0.002*		
16	83.58					0.001*	
32	75.19						0.000*
24 h		100.00	116.58	68.38	54.84	36.02	10.96
0	100.00	1.000					
2	98.66		0.202				
4	100.00			0.022*			
8	93.88				0.003*		
16	80.11					0.000*	
32	66.16						0.000*
36 h		100.00	95.96	109.24	123.46	83.40	34.88
0	100.00	1.000					
2	96.91		0.887				
4	97.87			0.396			
8	88.20				0.011*		
16	76.79					0.564	
32	64.22						0.014*

*P < 0.05 between ATP relative level and survival rate.

Discussion

Cr(VI) is a common environmental pollutant that is widely used in electroplating, metal refining, printing, dyeing, tanning, and other industrial and agricultural processes, and its carcinogenicity has been documented by the International Research Agency of Cancer (IRAC) (1). In China, epidemiological studies suggested that occupational Cr(VI) exposure led to chronic damage of liver, lung, nasal mucosa, skin and other organs, and an increased risk of cancer incidence (8-10). Furthermore, the study of Cr(VI) cytotoxicity revealed that Cr(VI) could readily cross the cell membrane through nonspecific anion channels, resulting in excessive generation of reactive oxygen species. Consequently, induced oxidative stress, genetic damage, mitochondrial dysfunction, activation of apoptosis-related caspases, and mitochondrial-mediated apoptosis were observed (11-13), while chromium-induced genotoxicity and apoptosis were closely associated with Cr(VI) carcinogenesis (14).

Mitochondria are the main site of ATP synthesis, which is produced mainly through the TCA cycle and OXPHOS, named as mitochondrial aerobic respiration (7). Under normal physiological conditions, mitochondrial aerobic respiration is the main way of energy provision, while glycolysis in the cytoplasm is negligible due to the low effectiveness of ATP production (7). Interestingly, the glycolysis metabolism is activated as a compensatory means of energy production existing in many cancer cells (15-17). At present, it is unclear whether toxic chemicals cause also the activation of glycolysis in the process of toxicity. In an adverse environment of exposure to toxic chemicals, several studies have shown that the disorder of energy metabolism induced by toxic chemicals was closely associated with mitochondrial dysfunction. For example, acute ethanol exposure led to suppression of mitochondrial ATP generation and fatty acid oxidation and decreased respiration and accessibility of mitochondrial adenylate kinase in permeabilized hepatocytes (18,19). Exposure to 5 and 10 μM Pb reduced decreased cellular ATP levels in the neuronal cell lines PC-12 and SH-SY5Y, which correlated with voltage-dependent anion channel (VDAC) transcription and expression (20). VDAC is an important protein located on the outer mitochondrial membrane, which controls mitochondrial life and death (21). At present, the effect of Cr(VI) hepatotoxicity on cellular ATP levels remains ambiguous; therefore, it is important to elucidate the interference effect of Cr(VI) on ATP levels

in L-02 hepatocytes.

Different doses of Cr(VI) can lead to differences in cell survival rates and cell number from control and consequently alter intracellular ATP levels. Therefore, it was interesting to scientifically evaluate the interference effect of Cr(VI) on ATP level in cells. For the first time, a chi-square test was used to analyze experimental data on the toxicity of Cr(VI), which is a novel method of analysis of the toxicological effects induced by Cr(VI). Chi-square testing was applied to compare differences between cell survival rates and ATP levels. If there were significant differences between the variables, this would indicate that the change in intracellular ATP levels is not related to changes in cellular survival rates, which could indicate that Cr(VI) interferes with ATP synthesis in L-02 hepatocytes.

The experimental results showed that Cr(VI) led to a gradual decrease of cell survival rate in L-02 hepatocytes at 12, 24, or 36 h of exposure, and the 32 µM Cr(VI) treatment was able to significantly decrease the cell survival rate. Meanwhile, the relative ATP level showed a pattern of Cr(VI) interference with ATP levels described as an increase at 12 h, a decrease at 24 h, and a new slight increase at 36 h, looking like a "V-shaped" curve. Furthermore, the results of the Pearson χ^2 test showed that doses of 8, 16, and 32 µM Cr(VI) induced a significant increase of ATP levels at 12 h, while 4, 8, 16, and 32 µM Cr(VI) doses induced a significant decrease of ATP at 24 h. However, after Cr(VI) treatment for 36 h, the ATP levels increassed slightly again, but the ATP levels in the 16 and 32 µM Cr(VI) groups were still lower than control. In our view, the increase of ATP level at 12 h indicated activation of mitochondrial aerobic respiration, the decreased ATP levels at 24 h indicated disruption of mitochondrial energy metabolism and, interestingly, the slight increase of ATP levels at 36 h indicated partial recovery of mitochondrial function or activated glycolysis in L-02 hepatocytes.

In summary, the χ^2 test enabled us to distinguish the confounding effects of decreased cell survival rate from changes in intracellular ATP content. This study is the first to demonstrate that exposure to 32 µM Cr(VI) leads to a significant increase in cellular ATP at 12 h, a decrease at 24 h, and a slight increase again at 36 h. Furthermore, in future studies, the χ^2 statistical test could also be considered as a reference for exploring cytotoxicity or pharmacological mechanisms of other chemicals. It would be interesting to further explore the molecular mechanism of mitochondrial energy metabolism- or glycolysis-related genes by the χ^2 method during Cr(VI) toxicity.

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