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Suppression of renal cell proliferation, induction of apoptosis and cell cycle arrest: Cytotoxicity of vanadium in broilers

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ABSTRACT

The aims of this study were to clarify the effects of high vanadium on the renal cell cycle and apoptosis in broilers. 420 one-day-old avian broilers were divided into six groups and fed on a control diet (vanadium 0.073 mg/kg), and five high vanadium diets (vanadium 5 mg/kg, high vanadium group I; 15 mg/kg, high vanadium group II; 30 mg/kg, high vanadium group III; 45 mg/kg, high vanadium group IV; 60 mg/kg, high vanadium group V) throughout the experimental period of 42 days. As tested by flow cytometry, the percentage of apoptotic renal cells was increased in high vanadium group II, III, IV and V when compared with that of control group. The Proliferating index (PI) of renal cell and the ratio of S, G_2 + M phase cells were markedly decreased and population of G_0/G_1 cells was increased in high vanadium group II, III, IV and V. The results showed that dietary vanadium in excess of 15 mg/kg was toxic to kidney by the renal cells cycle arrest and increased apoptosis, which caused the growth depression of the kidney in broilers.

Keywords: Dietary High Vanadium; Renal Cells; Apoptosis; Cell Cycle; Flow Cytometry (FCM); Broiler

1. INTRODUCTION

Vanadium is an essential trace element for animal species [1] and widely distributed in the environment. Vanadium compounds have been proven to have a great deal of physiological actions, such as insulin-like action [2], intensive inhibition to the Na, K-ATPase [3,4]. At the same time, the toxicity of the vanadium couldn't be neglected. Cytotoxicity induced by vanadium compounds is well documented: *in vivo* [5,6] and *in vitro* [7] in both man and animals [8-10], and vanadium can mediated apoptosis and cell cycle arrest in MCF7 cell line [11].

The ammonium metavanadate widely used in chemical industry is a dietary micronutrient and is also a toxic compound which is involved in several cases of enzyme inhibition. Vanadium has been shown to impede the activities of different ATPases [12], protein kinases [13], ribonuclease [14] and phosphatases [15], and to inhibit or stimulate the activity of many DNA or RNA enzymes inducing several genotoxic and mutagenic effects [16,17].

Kidney has quite a closed relationship to vanadium that kidney is the primary organ involved in accumulation of vanadium [18], and its averages about three times higher than for the liver or spleen [19]. The major vanadic excretion pathway is by the kidney and the excretion of vanadium is rapid with a biological half-life of 20 - 40 hours in the urine [20]. At present, there is a few records about the effect or nephrotoxicity caused by vanadium. Vanadium inhibits renal organic ion accumulation and renal Na^+, K^+-ATPase *in vitro* and *in vivo* [21]. The samples of vanadium-rich oil and of this oil products provoked acute proliferative glomerulonephritis, nephrosclerosis and acute intracapillary glomerulonephritis with partial tubular and glomerular necrosis associated with acute renal failure in hybrid mice [22]. Vanadium-induced morphologic changes in the kidney were also more pronounced with age in rats [23].

Therefore, there is a paucity of data regarding the effect of high vanadium on the development of renal cells, especially in the cell cycle of renal cells in broilers. In the present study, the ammonium metavanadate was used to examine the nephrotoxicity of vanadium in broilers by the methods of flow cytometry (FCM) and histochemistry. Our data indicated that dietary high vanadium intake could cause increased apoptosis and cycle arrest of renal cells in broilers.

2. MATERIALS AND METHODS

2.1. Chickens and Diets

420 one-day-old healthy avian broilers were divided into six groups of 70 each. There were seven replicates in each group and ten broilers in each replicate. Two of seven replicates in each group were used for the clinical observation during the experiment. Broilers were housed in cages with electrically heated units and were provided with water as well as undermentioned diets *ad libitum* for 42 days.

The control diet was a corn-soybean basal diet (vanadium 0.073 mg/kg) formulated by the US National Research Council (NRC 1994). Ammonium metavanadate was mixed into the corn-soybean basal diet to produce five high vanadium diets (5 mg/kg, high vanadium group I; 15 mg/kg, high vanadium group II; 30 mg/kg, high vanadium group III; 45 mg/kg, high vanadium group IV; 60 mg/kg, high vanadium group V).

The use of chickens in our experiments was followed and all experimental procedures involving animals were approved by Sichuan Agricultural University Animal Care and Use Committee.

2.2. Body Weight and Relative Weight of Kidney

At 7, 14, 21, 28, 35 and 42 days of age during the experiment, five broilers in each group were weighted then euthanized and necropsied. Kidney were dissected from each broiler, and weighed after dissecting connective tissue around the organ. Related weight of kidney was calculated by the following formula:

$$\text{Related weight} = \text{organ weight}(\text{g})/\text{body weight}(\text{kg})$$

2.3. TUNEL Detection

Detection of DNA fragments in situ using the terminal deoxyribonucleotidyl transferase(TDT)-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay is increasingly applied to investigate cell apoptosis. At 42 days of age, five broilers were human killed and then immediately necropsied. Kidneys were dissected and then were fixed in 4% paraformaldehyde and routinely processed in paraffin. TUNEL assay was performed in the deparaffinized sections (at a thickness of 4 um) with an apoptosis Detection Kit (Merck) according to the suppliers' instructions, as described [24]. Briefly, tissue sections were rehydrated in a series of xylene and ethanol solutions and then incubated at room temperature for 20 minutes with Proteinase K (catalog no. JA 1477) in a humidified chamber. Slides were then rinsed in Trisbuffered saline (TBS). The entire specimens were coverd with 3% H_2O_2, and then incubated at room temperature for 5 minutes. Slides were rinsed with TBS. TUNEL enzyme (catalog no. JA 1560) and label solution (catalog no. JA 1559) were mixed and applied to the tissues, which were incubated again in the humidified chamber for 1.5 h at 37˚C. Slides were rinsed thoroughly in TBS. Stop solution, block solution and conjugate were applied by turns. DAB solution was applied for 15 min to stain the nuclei of apoptotic cells. The methyl green solution was used to counterstain the nuclei of normal cells. Tissues were dehydrated in a series of three ethanol baths and two xylene baths for 5 min each. The slides were examined on an Olympus light microscope.

2.4. Annexin-V Apoptosis and Cell Cycle Detection

Five broilers in each group were humanely killed at 14, 28, and 42 days of the experiment and kidney were taken from each chicken immediately. As described previously [25], the cell suspension was filtered through a 300-mesh nylon Mesh, washed twice with cold phosphate buffer solution (PH7.4), and then suspended cells in 1 × binding buffer (Cat. No. 51-66121E) at a concentration of 1×10^6 cells/mL. Transfer 100 μL of the solution to a 5 mL culture tube, and then add 5 μL of Annexin V-FITC (Cat. No. 51-65874X) and 5 μL of PI (Cat. No. 51-66211E). Gently vortex the cells and incubate for 15 min at reaction temperature (25˚C) in the dark. Add 400 μL of 1 × binding buffer to each tube and analyzed by flow cytometry (BD FACSCalibur) within one hour. The percentage of cells in each part of the cell apoptosis was determined by counting 3×10^4 cells, using Cell Quest software.

The cell cycle detection was measured by the previously detailed Cui method [26]. The cell suspension at the concentration of 1×10^6 cells/mL aforesaid was used for cell cycle detection follow these procedures below: transfer 500 μL of the solution to a 5 mL culture tube and desert supernatant after brachytely centrifuge (500 rpm). Then add 5 μL 0.25% Tritonx-100 and 5 μL PI (Cat. No. 51-6 6211E). Gently vortex the cells and incubate for 30 min at reaction temperature (25˚C) in the dark. Finally, add 500 μL phosphate buffer solution (PH7.4) to each tube and analyzed by flow cytometry (BD FACSCalibur) within 45 min. The percentage of cells in each stage of the cell cycle was determined by counting 5×10^4 cells, using ModFit LT for Mac V3.0.

2.5. Statistical Analysis

Data were subjected to one-way analysis of variance (ANOVA) using SPSS 12.0 for windows and presented as means ± standard deviation. Differences between means were assessed by Tukey statistical test. A probability value ≤ 0.05 was considered to be significant.

3. RESULTS

3.1. Changes of Body Weight and Relative Weight of Kidney

Body weight of broilers were lower in high vanadium

group III, IV and V ($p < 0.01$) than in control group during the experiment and body weight was lower ($p < 0.01$) in groups II than in control group from 14 days of age to 42 days of age. The body weight was higher ($p < 0.05$ or $p < 0.01$) in group I than in control group from 28 days of age to 42 days of age. The results were showed in **Table 1**.

Relative weight of kidney was significantly higher ($p < 0.01$) in high vanadium groups IV and V than in control group during the experiment except 7 days of age and was significantly higher in high vanadium group IV than in control group at 28 days of age. The results were showed in **Table 2**.

3.2. Result of TUNEL Detection

TUNEL detection reveled that apoptotic cells had brown-stain nuclei, and had morphologic change of condensed and irregular nuclei. Apoptotic cells were principally distributed within the renal tubule (**Figure 1**). There were increased frequencies of occurrence in high vanadium groups III, IV and V.

3.3. Result of Annexin-V Apoptosis and Cell Cycle Detection

Annexin-V-FITC staining assay by flow cytometry: Annexin V-FITC was used to quantitatively determine the percentages of cells that were actively undergoing apoptosis. Our data suggested that percentages of apoptotic cells in the kidney were significantly increased in high vanadium groups II, III, V and IV when compared with those of control group ($p < 0.05$ or $p < 0.01$) at 14, 28, and 42 days of age. The results were shown in **Table 3**.

Cell cycle detection by flow cytometry: To examine the dynamics of cell cycle progression, G_0/G_1, S and G_2 + M phase distribution of the renal cells in high vanadium group I, II, III, V and IV were compared with those of control group. G_0/G_1 phase cell distribution gradually accumulated at 14, 28, and 42 days of age and became significantly increased ($p < 0.05$ or $p < 0.01$) in high vanadium group II, III, V and IV. S phase, G_2 + M phase cell distribution and proliferation index (PI) were decreased in the renal cells in high vanadium groups III, IV and V at 14, 28, and 42 days of age ($p < 0.05$ or $p < 0.01$). The results were showed in **Table 4**.

Table 1. Effect of high vanadium on weight in broilers (g).

Groups	7 days of age	14 days of age	21 days of age	28 days of age	35 days of age	42 days of age
Control group	138.58 ± 13.55	331.32 ± 26.77	664.04 ± 65.58	1131.90 ± 82.67	1716.20 ± 77.66	2220.29 ± 99.6
High vanadium group I	139.38 ± 9.99	336.71 ± 31.25	669.75 ± 60.32	1174.05 ± 78.65*	1842.60 ± 140.2**	2450.14 ± 149.34**
High vanadium group II	134.95 ± 10.7	301.29 ± 31.23**	554.00 ± 52.82**	961.47 ± 62.91**	1508.80 ± 92.27**	1970.57 ± 145.81**
High vanadium group III	126.24 ± 15.39**	233.79 ± 29.49**	431.25 ± 79.75**	767.26 ± 60.38**	1176.50 ± 86.53**	1436.29 ± 98.65**
High vanadium group IV	119.42 ± 16.73**	178.75 ± 42.77**	315.54 ± 45.17**	556.11 ± 50.77**	901.30 ± 98.07**	1242.71 ± 101.59**
High vanadium group V	90.08 ± 18.76**	102.86 ± 28.27**	218.17 ± 39.92**	358.68 ± 36.42**	582.10 ± 99.19**	709.43 ± 52.85**

Data are presented with the means ± standard deviation (n = 5). Compared with the control group, *$p < 0.05$, **$p < 0.01$.

Table 2. Effect of high vanadium on the relative weight (g/kg body weight) of kidney in broilers.

Groups	7 days of age	14 days of age	21 days of age	28 days of age	35 days of age	42 days of age
Control group	0.87 ± 0.13	0.79 ± 0.08	0.71 ± 0.09	0.61 ± 0.07	0.59 ± 0.06	0.55 ± 0.04
High vanadium group I	0.91 ± 0.08	0.77 ± 0.10	0.68 ± 0.05	0.66 ± 0.11	0.62 ± 0.03	0.52 ± 0.07
High vanadium group II	0.95 ± 0.11	0.82 ± 0.13	0.71 ± 0.11	0.60 ± 0.09	0.64 ± 0.05	0.56 ± 0.06
High vanadium group III	0.96 ± 0.06	0.82 ± 0.08	0.81 ± 0.04	0.65 ± 0.10	0.69 ± 0.07**	0.59 ± 0.10
High vanadium group IV	0.97 ± 0.10	0.94 ± 0.06**	0.88 ± 0.10**	0.78 ± 0.08**	0.68 ± 0.08**	0.67 ± 0.09**
High vanadium group V	1.13 ± 0.09**	1.67 ± 0.15**	1.24 ± 0.12**	0.86 ± 0.12**	0.76 ± 0.07**	0.71 ± 0.11**

Data are presented with the means ± standard deviation (n = 5). Compared with the control group, *$p < 0.05$, **$p < 0.01$.

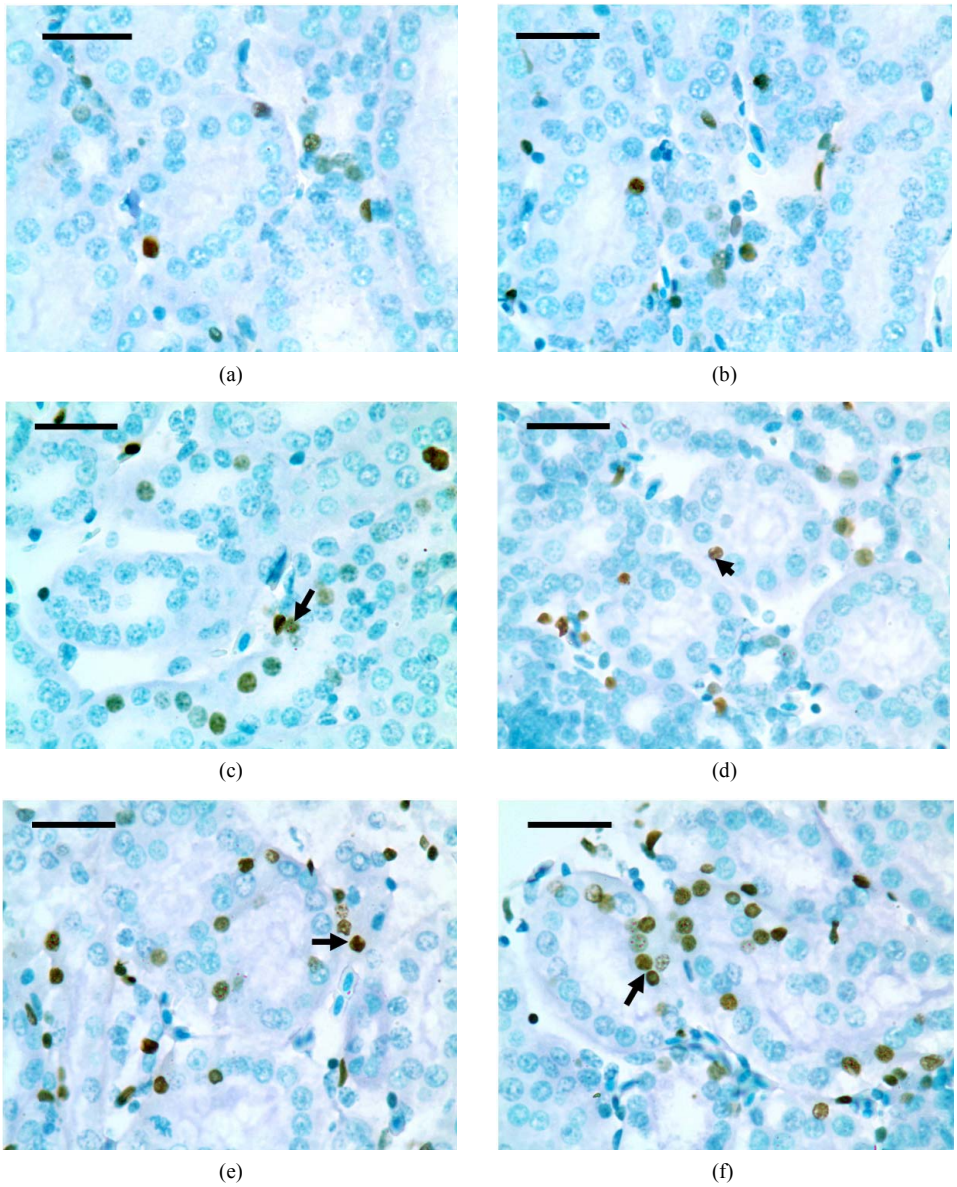

Figure 1. The population of apoptotic cells are increased in high vanadium groups III (d), IV (e), and V (f) when compared with those of control group (a). The arrows point to the apoptosising cells within the renal tubule, TUNEL 1000 × bars = 19.5 μm.

Table 3. The percentages of apoptotic renal cells in broilers.

Groups	14 days of age	28 days of age	42 days of age
Control group	3.06 ± 0.23	3.28 ± 0.40	4.05 ± 0.30
High vanadium group I	3.34 ± 0.44	3.84 ± 0.37	4.03 ± 0.18
High vanadium group II	4.49 ± 0.47*	4.47 ± 0.23**	4.80 ± 0.39**
High vanadium group III	4.74 ± 0.35**	4.85 ± 0.58**	6.04 ± 0.48**
High vanadium group IV	4.89 ± 0.21**	5.33 ± 0.48**	7.19 ± 0.73**
High vanadium group V	5.24 ± 0.56**	6.49 ± 0.72**	7.56 ± 0.59**

Data are presented with the means ± standard deviation (n = 5). Compared with the control group, $^{*}p < 0.05$, $^{**}p < 0.01$.

Table 4. Cycle of the renal cells in broilers (%).

Time	Phases	Control group	High vanadium group I	High vanadium group II	High vanadium group III	High vanadium group IV	High vanadium group V
14 days of age	G_0/G_1	88.07 ± 6.36	85.78 ± 4.23	91.97 ± 2.07**	94.42 ± 1.21**	97.25 ± 1.76**	98.33 ± 0.32**
	S	6.01 ± 1.03	6.94 ± 0.93	3.57 ± 0.21**	2.62 ± 0.51**	1.18 ± 0.65**	0.75 ± 0.06**
	G_2+M	5.92 ± 0.94	6.28 ± 0.90	4.46 ± 0.19**	2.96 ± 0.71**	1.57 ± 1.10**	0.92 ± 0.09**
	PI	11.93	14.22	8.03**	5.58**	2.75**	1.67**
28 days of age	G_0/G_1	85.85 ± 3.06	87.07 ± 5.48	89.46 ± 0.96**	90.81 ± 2.05**	93.81 ± 2.43**	96.63 ± 0.74**
	S	7.43 ± 1.10	6.32 ± 1.09	5.11 ± 0.72**	4.54 ± 0.94**	2.34 ± 1.24**	1.32 ± 0.27**
	G_2+M	6.72 ± 0.47	6.61 ± 0.89	5.43 ± 0.31	4.65 ± 1.11**	3.85 ± 1.19**	2.05 ± 0.17**
	PI	14.15	12.93	10.54*	9.19**	6.19**	3.37**
42 days of age	G_0/G_1	84.95 ± 1.27	84.08 ± 0.66	90.49 ± 0.83*	94.59 ± 1.90**	96.42 ± 0.74**	97.51 ± 0.87**
	S	7.61 ± 0.40	8.21 ± 0.56	5.01 ± 0.42*	2.59 ± 0.15**	1.57 ± 0.17**	1.15 ± 0.11**
	G_2+M	7.44 ± 0.46	7.71 ± 0.33	4.50 ± 0.30	2.82 ± 0.25**	2.01 ± 0.19**	1.34 ± 0.15**
	PI	15.05	15.92	9.51*	5.41**	3.58**	2.49**

$$\text{Proliferating index (PI) value} = \frac{S+(G_2+M)}{(G_0/G_1)+S+(G_2+M)} \times 100\%$$

Data are presented with the means ± standard deviation (n = 5). Compared with the control group, $^{*}p < 0.05$, $^{**}p < 0.01$.

4. DISCUSSION

Vanadium is one of the more toxic trace elements. In the natural state, vanadium occurs with positive valencies of two, three, four and five. The greater toxicity has been attributed to compounds in the higher valence states [10]. Ammonium metavanadate which we used with positive valencies of five have more toxicity to the creature. There have been reported that 10 mg/kg vanadium to the basal diet failed to affect either the growth or mortality of the chicks at six weeks of age, but 25 mg/kg vanadium significantly depressed growth and increased mortality [27] and 20 mg/kg added vanadium for the young chick have shown growth depression of 25% - 30% with a corn-soybean meal [28]. In the present study, broilers in high vanadium groups III, IV and V showed decreased feed intake and depression. 35.31%, 44.03% and 68.05% loss of body weight were in group III, IV and V respectively at the end of the experiment. The results of present study indicated that vanadium overdose 15 mg/kg could inhibit the growth of body and kidney in broilers, which was similar to the results of previous studies. A great quantity of vanadium accumulated (dose-dependently) [29] in the kidney had broken the homeostasis that may be the basic reason for the cytotoxicity. Subcellular distribution studies indicated 90% accumulation of vanadium in the soluble supernatant fraction at varying stages of cytotoxicity [30]. It was concluded that the multifaceted dependency of vanadium cytotoxicity on its cellular content may have resulted from a cellular balancing between proposed regulatory functions for vanadium and the interactions incurred with an excessive content.

Apoptosis is a highly regulated process used to eliminate dysplastic or damaged cells from multicellular organisms [31]. Vanadium could induce apoptosis in mice [32], in male germ cells [33] and in T-lymphocyte Jurkat cells [34]. In the present study, by using flow cytometry, the effect of vanadium on cell population classified by annexin V-FITC and PI was examined. The results showed that vanadium greatly increased the population of cells stained with positive annexin V and negative PI, which represent the increased population of renal cells in early stage of apoptosis. Also, the cells stained with positive annexin V and PI were increased in the high vanadium groups IV and V, which represent the increased population of renal cells in end-stage apoptosis. By statistically analyzing the sum total of early and end-stage apoptotic renal cells in control group and five high vanadium groups, it was easy to confirm from **Table 3** that the populations of apoptotic renal cells were significantly increased in the high vanadium groups II, III, IV and V when compared with those of control group, which was similar to results of TUNEL detection (**Figure 1**).

It is well known there are two major pathways of apoptosis: the mitochondria-dependent and death receptor-mediated. The mitochondria-dependent pathway mainly caused by DNA damage. The TUNEL detection in our study showed that only cell nucleus was stained in positive. This indicated that the renal apoptosis was partially induced by the mitochondria damage according to the basic principle of the TUNEL detection method for detecting the

DNA fragmentation. It claimed that vanadium (IV) mediated free radical generation causes 2-deoxyguanosine hydroxylation, which leads to DNA strand breaks [35]. Meanwhile, the vanadium could lead to the mitochondria damage and inhibit mitochondria respiration or inhibited electron transfer chain [36]. It was postulated that significant amounts of vanadate are accumulated in the intermembrane space of liver mitochondria of the exposed rats [37]. The process of "detoxification" by reduction of vanadate in the tissue may be insufficient to prevent the deleterious action of this compound on mitochondria. According to the abovementioned discussion, we should study further the effect of high vanadium on the mitochondria of the renal cells in the further.

Cell cycle regulation is one of the key regulatory mechanisms of cell growth [38]. Many cytotoxic and genetoxic agents arrest the cell cycle at the different phases and then induce apoptotic cell death [39,40]. In the present study, obviously increased G_0/G_1 renal cell population indicated that the cell cycle of renal cells was arrested at G_0/G_1 phase in high vanadium groups II, III, IV and V. At the same time, markedly decreased renal cell population in S phase, G_2 + M phase and PI (**Table 4**) value showed that the proliferation of renal cells were greatly inhibited, which were consistent with previous studies *in vivo* and *in vitro* [41] and in cancer cells [42]. The cell cycle regulation is much complex and the vanadium induced different phase arrest [43,44].

According to the abovementioned discussion and results in the present study, it is concluded that dietary vanadium in excess of 15 mg/kg is toxic to kidney by the renal cells cycle arrest and increased apoptosis, which causes the growth depression of the kidney in broilers.

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