

Effect of Vanadium on Splenocyte Apoptosis in Broilers

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Abstract

A total of 420 one-day-old healthy broilers were divided into six groups. There were 70 broilers in each group. The broilers were fed on a corn-soybean basal diet as a control diet (vanadium 0.073 mg/kg) or the same diet amended to contain 5 mg/kg, 15 mg/kg, 30 mg/kg, 45 mg/kg and 60 mg/kg vanadium supplied as ammonium metavanadate for 42 days. When compared with those of the control group, the mitochondrial injury of splenocytes and more apoptotic splenocytes with condensed chromatin with C-shaped, horseshoe-like, petal-shaped or crescent were ultrastructurally observed in the spleen in the 30 mg/kg, 45 mg/kg and 60 mg/kg groups. As measured by flow cytometry (FCM), the number of apoptotic splenocytes was significantly increased in the 15 mg/kg, 30 mg/kg, 45 mg/kg and 60 mg/kg groups. Immunohistochemical tests showed that number of positive splenocytes containing Bax and caspase-3 protein was increased, and number of positive splenocytes containing Bcl-2 protein was decreased in the 15 mg/kg, 30 mg/kg, 45 mg/kg and 60 mg/kg groups. It was concluded that dietary vanadium in excess of 15 mg/kg could result in splenocyte apoptosis in broilers. Splenocyte apoptosis was closely related to mitochondrial injury and changed expression of apoptogenic proteins induced by vanadium.

Keywords: Dietary vanadium; Apoptosis; Flow cytometry (FCM); Spleen; Broilers

Introduction

It has been proven that vanadium is an activator of adenylyl cyclase, a potent anti-carcinogenic agent and potent inhibitors of several phosphohydrolases, such as Na^+K^+ -ATPase and Ca^{2+} -ATPase [1-5]. Also, as an antidiabetic agent, vanadium plays role of insulin [6-8]. At the same time, several reports have shown that vanadium can cause toxic effects on cells or organs, such as oxidative damage (or stress) in rats [9-12] and broilers [13,14], and immunotoxicity in broilers [15-19].

Spleen is the principal peripheral lymphoid organ and plays an important role in protective immune reactions. There are no reports regarding effect of vanadium on splenocyte apoptosis in broilers. In our recent study, dietary vanadium in the range of 30 mg/kg – 60 mg/kg can cause lymphocyte apoptosis in bursa of Fabricius [20], and splenic lesions, inhibition of the splenic growth [16]. As a part of our study on effect of vanadium on spleen, the same broilers were used in the present study to investigate changes of percentages of the splenocyte apoptosis by the methods of pathology, flow cytometry (FCM) and immunohistochemistry, and to evaluate changes of the splenic function in broilers.

Materials and Methods

Chickens and diets

Four hundred and twenty one-day-old healthy broilers were divided into six groups. There were 70 broilers in each group. The broilers were housed in cages with electrically heated units and were provided with water as well as experimental diets *ad libitum* for 42 days. A corn-soybean basal diet formulated by the National Research Council (NRC, 1994) was the control diet (vanadium 0.073 mg/kg). Ammonium metavanadate (NH_4VO_3) was mixed into the corn-soybean basal diet to produce 5 mg/kg, 15 mg/kg, 30 mg/kg, 45 mg/kg and 60 mg/kg vanadium diets.

All experimental procedures involving animals were approved by Sichuan Agricultural University Animal Care and Use Committee.

Ultrastructural observation

At the end of the experiment, three chickens in each group were

ethanized and immediately necropsied. Spleens were dissected and fixed in 2.5% glutaraldehyde and postfixed in 2% veronal acetate-buffered OsO_4 . After dehydration in graded alcohol, the fixtures were embedded in araldite. The blocks were sectioned in a microtome with a glass knife. The sections, 65-75 nm thick, were placed in uncoated copper grids. They were then stained with uranyl acetate, poststained with 0.2% lead citrate, and examined with an electron microscope.

Annexin V apoptosis detection by flow cytometry

Five chickens in each group were humanely killed when the birds were 14, 28, and 42 days old, and the spleens were immediately taken and ground to form a cell suspension. This suspension was filtered through a 300-mesh nylon screen. The cells were washed twice with cold PBS (phosphate buffer solution, pH 7.2-7.4) and then suspended in 1x binding buffer (Cat. No. 51-66121E) at a concentration of 1×10^6 cells/mL. One-hundred- μL portions of the cell suspension were transferred 5-mL culture tubes, and 5 μL of Annexin V-FITC (Cat. No. 51-65874X) and 5 μL of PI (Cat. No. 51-66211E) were added. The mixture was gently vortexed and incubated for 15 minutes at 25°C in the dark. 400 μL of 1x binding buffer was added to each tube. Analysis by flow cytometry (BD FACSCalibur) was conducted within 1 hour.

Bax/Bcl-2, and caspase-3 protein detection

At 14, 28 and 42 days of age, five broilers in each group were humanely killed. Spleens were taken for Bax/Bcl-2 and caspase-3 detection by the immunohistochemical methods (SABC) and stained with DAB as described by Wang et al [21]. Anti-Bax (BA0315), anti-Bcl-2 (BA0412) and anti-Caspase-3 (BA0588), and DAB were purchased from Wuhan Boster Biological Technology Co., Ltd., China.

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Image analysis

Images from five slices per spleen were taken 200 μm apart. Five visions per slice were randomly chosen for assessment of positive cells using image analysis software (JID801D). The average grayscale of the positive cells was automatically calculated. Immunoreactive intensity were expressed by average grayscale. Values < 160 was considered high, 160-170 medium and 170-180 low.

Statistical analysis

The significance of difference among the six groups was analyzed by variance analysis, and results were presented as means \pm standard deviation ($\bar{X} \pm S$). The analysis was done using SPSS 12.0 for Windows.

Results

Clinical observation

Results showed as in the reference [22].

Ultrastructural changes

No obvious abnormal ultrastructural changes were observed in the 5 mg/kg and 15 mg/kg groups compared to the control group (Figure 1A). The mitochondria of splenocytes were enlarged and vacuolated with degenerating cristae (Figure 1B-C) in the 30 mg/kg, 45 mg/kg, and 60 mg/kg groups. Also, apoptotic splenocytes were found to be increased in the 30 mg/kg, 45 mg/kg, and 60 mg/kg groups. These apoptotic cells are characterized as the typical condensed chromatin with C-shaped, horseshoe-like, petal-shaped or crescent (Figures 1B-D).

Results of Annexin V apoptosis detection by flow cytometry

The results in Table 1 showed that the percentage of apoptotic cells in the spleen was increased as dietary vanadium level increased. The percentage of apoptotic splenocytes was significantly higher ($P < 0.01$) in the 15 mg/kg, 30 mg/kg, 45 mg/kg and 60 mg/kg groups than that in the control group.

Results of Bax, Bcl-2 and caspase-3 protein detection

The population of positive splenocytes containing Bax protein (brown-stain) was increased in the 15 mg/kg, 30 mg/kg, 45 mg/kg and 60 mg/kg groups when compared with that of the control group. Average gray scales of the positive cells were significantly lower ($P < 0.01$) in the 30 mg/kg, 45 mg/kg and 60 mg/kg groups than those in the control group from 14 to 42 days of age, and significantly lower ($P < 0.01$) in the 15 mg/kg group than those in the control group from 28 to 42 days of age.

The frequencies of positive splenocytes containing Bcl-2 protein (brown-stain) were decreased in the 15 mg/kg, 30 mg/kg, 45 mg/kg and 60 mg/kg groups in comparison with those of the control group. Meanwhile, average gray scales of positive cells were significantly higher ($P < 0.01$) in the 30 mg/kg, 45 mg/kg and 60 mg/kg groups than those in the control group during the experiment, and were significantly increased ($P < 0.01$) in 15 mg/kg group at 28 and 42 days of age.

Changes of the positive cells containing caspase-3 protein (brown-stained) were the same with the positive splenocytes containing Bax protein.

The results were showed in Tables 2-4 and Figures 2-4.

Discussion

Apoptosis has been characterized as a fundamental cellular activity occurring under a wide range of physiological and pathological conditions [23]. Vanadate-containing compounds exert potent toxicity,

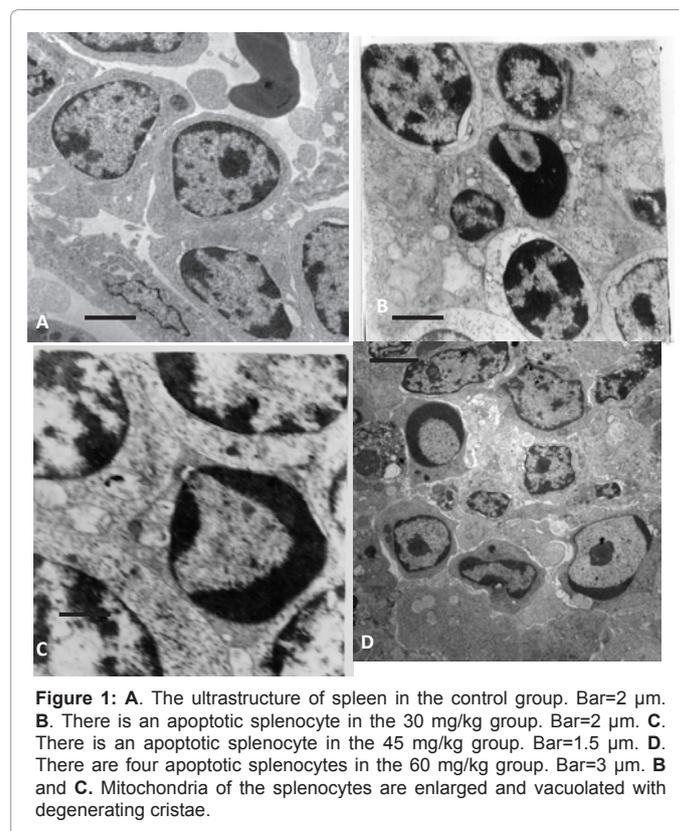


Figure 1: A. The ultrastructure of spleen in the control group. Bar=2 μm . B. There is an apoptotic splenocyte in the 30 mg/kg group. Bar=2 μm . C. There is an apoptotic splenocyte in the 45 mg/kg group. Bar=1.5 μm . D. There are four apoptotic splenocytes in the 60 mg/kg group. Bar=3 μm . B and C. Mitochondria of the splenocytes are enlarged and vacuolated with degenerating cristae.

	14 days	28 days	42 days
control	4.92 \pm 0.46	5.67 \pm 0.57	5.80 \pm 0.70
5 mg/kg group	5.61 \pm 0.56	6.74 \pm 0.70	7.31 \pm 0.81
15 mg/kg group	8.63 \pm 0.95**	9.17 \pm 1.00**	10.14 \pm 1.10**
30 mg/kg group	10.49 \pm 0.84**	11.86 \pm 1.07**	12.84 \pm 1.07**
45 mg/kg group	12.27 \pm 1.04**	11.92 \pm 0.81**	13.26 \pm 1.07**
60 mg/kg group	14.82 \pm 1.03**	14.64 \pm 1.07**	16.90 \pm 1.04**

Data are the means \pm standard deviation (n=5)

* $p < 0.05$, compared with the control group

** $p < 0.01$, compared with the control group

Table 1: The changes on percentage of apoptotic cells of spleen.

	14 days	28 days	42 days
control	184.22 \pm 11.29	185.50 \pm 12.24	187.52 \pm 11.29
5 mg/kg group	180.52 \pm 11.25	179.94 \pm 12.03	180.58 \pm 12.48
15 mg/kg group	177.06 \pm 10.91	166.59 \pm 11.49**	167.36 \pm 10.90**
30 mg/kg group	166.36 \pm 10.69**	166.27 \pm 10.07**	166.26 \pm 10.65**
45 mg/kg group	164.59 \pm 10.32**	157.86 \pm 10.12**	157.99 \pm 10.78**
60 mg/kg group	153.94 \pm 9.39**	154.89 \pm 10.61**	144.59 \pm 10.87**

Data are the means \pm standard deviation (n=5 \times 5)

* $p < 0.05$, compared with the control group

** $p < 0.01$, compared with the control group

Table 2: The average gray scales of Bax positive cells in the spleen.

causing DNA damage, which results in DNA damage that is one of major incentives initiating apoptosis [24]. Chromatin condensation in the apoptotic splenocytes was ultrastructurally observed in the present study. Also, the results determined by FCM showed that the percentages of apoptotic splenocytes were significantly increased in the 15 mg/kg, 30 mg/kg, 45 mg/kg and 60 mg/kg groups. It was further testified from the results in the present study that dietary high vanadium can cause cellular apoptosis in the lymphoid organs in broilers.

	14 days	28 days	42 days
control	148.59±10.55	153.94±13.05	150.26±12.42
5 mg/kg group	150.27±10.05	157.69±13.12	154.10±13.21
15 mg/kg group	153.94±11.03	166.23±12.03*	166.13±13.80*
30 mg/kg group	157.58±11.25*	166.85±12.82*	168.89±12.30**
45 mg/kg group	166.13±13.36**	177.35±14.17**	178.00±15.54**
60 mg/kg group	178.77±14.13**	187.25±13.15**	186.17±16.51**

Data are the means ± standard deviation (n=5×5)

*p<0.05, compared with the control group

**p<0.01, compared with the control group

Table 3: The average gray scales of Bcl-2 positive cells in the spleen.

	14 days	28 days	42 days
control	178.68±8.19	174.27±12.92	174.96±13.92
5 mg/kg group	176.98±10.35	169.85±13.06	172.41±12.29
15 mg/kg group	172.11±12.20	162.41±13.96*	166.40±8.80
30 mg/kg group	162.41±9.70**	153.98±9.99**	156.40±9.67**
45 mg/kg group	156.26±9.26**	149.84±9.26**	152.11±9.26**
60 mg/kg group	149.42±10.09**	144.14±9.56**	144.27±9.68**

Data are the means ± standard deviation (n=5×5)

*p<0.05, compared with the control group

**p<0.01, compared with the control group

Table 4: The average gray scales of Caspase-3 positive cells in the spleen.

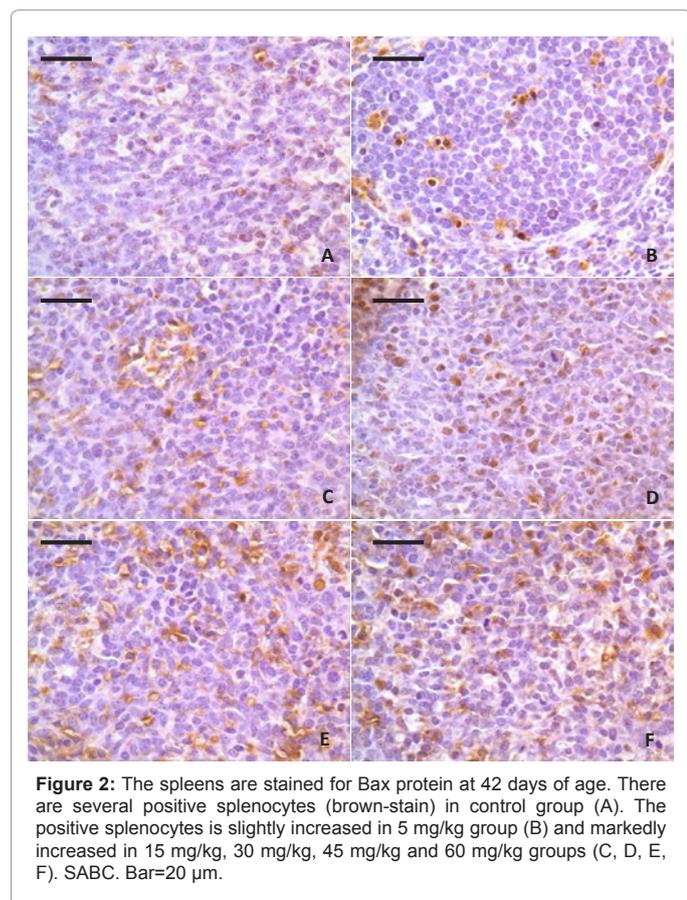


Figure 2: The spleens are stained for Bax protein at 42 days of age. There are several positive splenocytes (brown-stain) in control group (A). The positive splenocytes is slightly increased in 5 mg/kg group (B) and markedly increased in 15 mg/kg, 30 mg/kg, 45 mg/kg and 60 mg/kg groups (C, D, E, F). SABC. Bar=20 µm.

Vanadium-induced apoptosis may be related to mitochondrial injury [25]. Vacuolated mitochondria with degenerating cristae were ultrastructurally observed in the present study. Because Bcl-2 is expressed in the inner mitochondrial membrane [26], mitochondrial injury can cause mitochondrial apoptogenic proteins, such as those of the Bcl-2 family, from mitochondria to the cytoplasm [25] that initiates

apoptotic process. Meanwhile, the Bcl-2 protein family regulates the release of apoptosis-activating factors and the ratio of Bcl-2 to Bax determines cell survival or death [27]. In the present study, positive cell population of the Bcl-2 protein expression was decreased while positive cell population of the Bax protein expression was increased in the 15 mg/kg, 30 mg/kg, 45 mg/kg and 60 mg/kg groups, which is consistent with the results observed in the bursa of Fabricius of our recent report [20]. At present, there are no systematic studies on effect of vanadium on expression of Bcl-2 protein family except our report [20]. The broken balance between expression of Bcl-2 and Bax proteins not only induces apoptotic process, but also activates caspase-3 protein [28]. Positive cell population of the caspase-3 protein expression was increased in the 15 mg/kg, 30 mg/kg, 45 mg/kg, and 60 mg/kg groups in the present study, the above mentioned changes of apoptogenic protein expression finally resulted in an increase in apoptotic splenocytes.

It has been shown that mitochondrial injury and expression of pro-apoptotic proteins are related to the production of free radicals. In our another report [20], the results have shown that dietary vanadium in the range of 30 mg/kg – 60 mg/kg can decrease activities of SOD and GSH-P_x, and ability to inhibit hydroxyl radical, and increase MDA content, which induces oxidative damage and lipid peroxidation in the spleen of broilers. Decreased activities of antioxidant enzymes, increased lipid peroxidation and accumulated free radicals in the spleen induce mitochondrial injury and alter expression of apoptotic proteins, which finally results in splenocyte apoptosis.

In conclusion, dietary vanadium in excess of 15 mg/kg can cause splenocyte apoptosis and impact splenic function in broilers. Splenocyte apoptosis is closely related to mitochondrial injury and changed expression of apoptogenic proteins induced by vanadium.

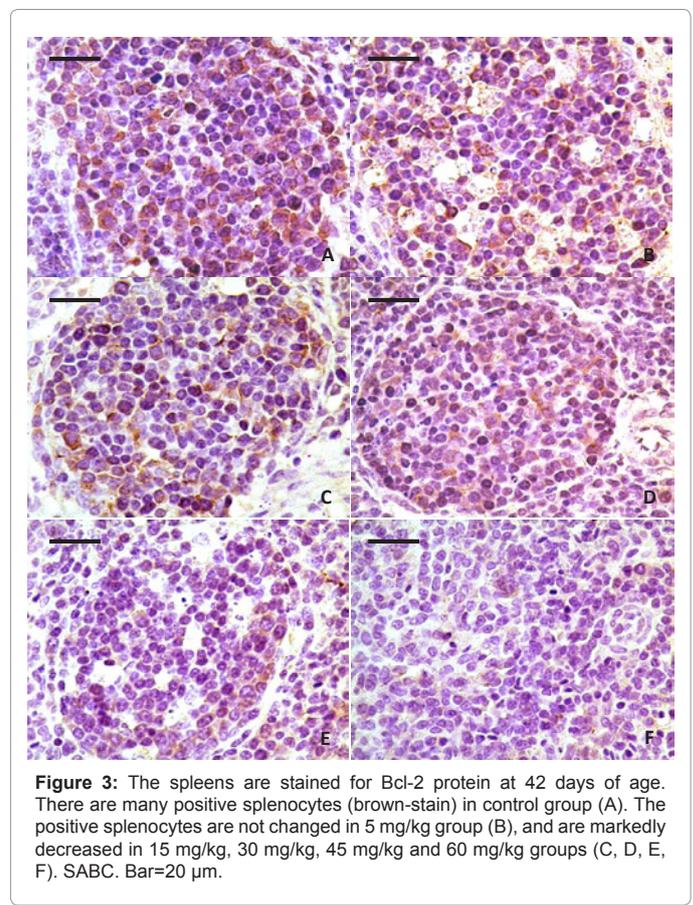


Figure 3: The spleens are stained for Bcl-2 protein at 42 days of age. There are many positive splenocytes (brown-stain) in control group (A). The positive splenocytes are not changed in 5 mg/kg group (B), and are markedly decreased in 15 mg/kg, 30 mg/kg, 45 mg/kg and 60 mg/kg groups (C, D, E, F). SABC. Bar=20 µm.

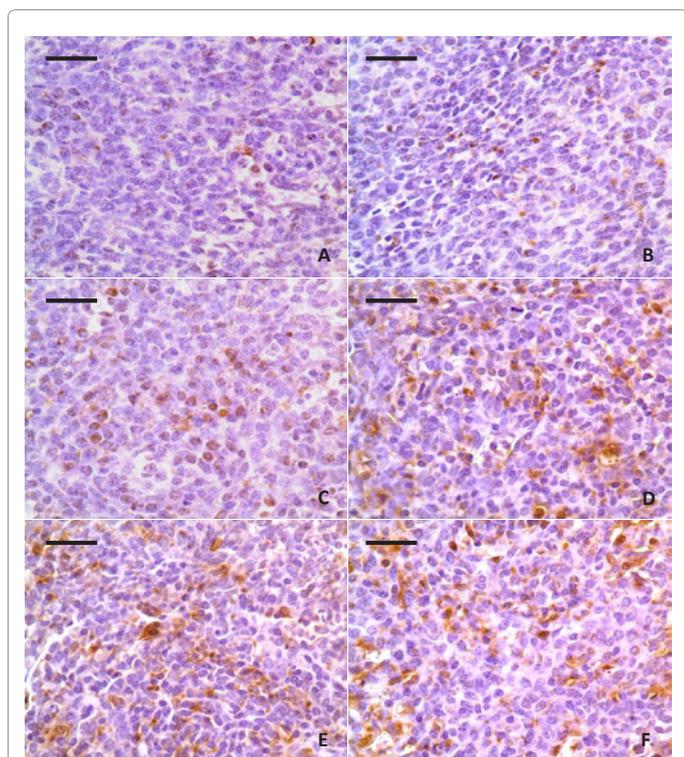


Figure 4: The spleens are stained for Caspase-3 protein at 42 days of age. There are several positive splenocytes (brown-stain) in control group (A). The positive splenocytes are slightly increased in 5 mg/kg group (B) and markedly increased in 15 mg/kg, 30 mg/kg, 45 mg/kg and 60 mg/kg groups (C, D, E, F). SABC. Bar=20 μ m.

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