

## PROTECTIVE EFFECT OF ANTHOCYANINS FROM BLUEBERRY ON FLUORIDE-INDUCED IMMUNE SYSTEM INJURY IN WISTAR RATS

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**ABSTRACT:** This study aimed to clarify the repairing effect of anthocyanins on immune system damage induced by fluoride. Animal experiments have shown that fluoride is immunotoxic, and excessive exposure to fluoride can cause immune injury, which may be related to oxidative stress. Anthocyanins are water-soluble natural pigments that are found in edible plants. They belong to the flavonoids family and are powerful antioxidants with physiological activities that can remove free radicals and resist oxidation. In this study, we selected four-week-old healthy male Wistar rats and established a fluorosis animal model caused by subchronic fluoride in drinking water and the animals were then fed anthocyanins. By calculating immune organ indices and detecting the apoptosis of immune cells in rats, in addition to measuring the content of immunoglobulins, cytokines and oxidative stress related indicators in each group, we examined the protective effect of anthocyanins on fluoride-induced immune system injury and the role of oxidative stress in the injury process. The results showed that anthocyanins alleviated the decrease in immune organ indices, apoptosis of immune cells, the level of immunoglobulins and cytokines and oxidative stress in fluoride-treated rats. In conclusion, anthocyanins can alleviate fluoride-induced immune system injury by inhibiting oxidative stress.

Key words: Anthocyanins; Fluoride; Immune system injury; Oxidative stress.

### INTRODUCTION

Fluoride is widely dispersed in nature, and humans consume excess fluoride through water, air and/or food, causing it to accumulate in the human body, eventually leading to systemic fluorosis.<sup>1</sup> Fluorosis can not only cause skeletal and dental fluorosis,<sup>2,3</sup> but also injury to the nervous, reproductive, cardiovascular and immune systems.<sup>4-11</sup> Animal studies have confirmed that fluoride can cause widespread adverse effects on immune system function.<sup>12-15</sup>

Immune function can be regarded as the body's resistance to disease, which plays an important role in maintaining the stability of the body's internal environment. Fluoride exposure can not only inhibit the development of immune organs, destroy their normal morphological structure and induce apoptosis of lymphocytes in

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immune organs in experimental animals,<sup>16-19</sup> but also affect the immune response of macrophages and B cells in the spleen, and thus affect immune function.<sup>20-22</sup> In addition, it was found that sodium fluoride can reduce the percentage of peripheral blood immunoglobulin G (IgG) and the level of cytokines in the serum of mice.<sup>23</sup> Recent research indicated that arsenic and fluoride exposure can affect children's immune function,<sup>24</sup> but the results of a population study on the effect of fluoride exposure alone on immune function are unclear.

Many studies have shown that fluoride can cause oxidative stress in the body, inhibit the antioxidant system *in vivo* and thus affect the body's function.<sup>25,26</sup> A population survey and cell experiments also confirmed that long-term exposure to low levels of fluoride may cause oxidative stress through the production of reactive oxygen species, as apoptosis of lymphocytes was observed in aluminum smelter workers and human lymphocytes *in vitro*.<sup>27,28</sup> If we can identify an effective antioxidant to antagonize the immune system injury caused by fluoride, this will have a positive role in the prevention and treatment of fluorosis. Anthocyanins are water-soluble natural pigments found in edible plants, which belong to the flavonoids family,<sup>29</sup> and have the physiological function of removing free radicals and antioxidation.<sup>30</sup> It has been reported that the consumption of fruit juices rich in anthocyanins can improve the body's antioxidant enzyme activity and plasma antioxidant capacity in healthy young females, thus protecting the body from oxidative stress.<sup>31</sup> Animal and cell experiments have confirmed that blueberry extract rich in anthocyanins can reduce ROS levels, alleviate oxidative stress, and reverse oxidative damage and apoptosis of human keratinocytes.<sup>32,33</sup> In addition, studies have shown that blueberry-anthocyanins can inhibit pro-inflammatory signal transduction in human THP-1 monocytes, and increase the level of serum immunoregulatory cytokines in patients with ulcerative colitis.<sup>34,35</sup> All of these results show that anthocyanins have strong anti-oxidation ability, which can improve the oxidation state of the body, enhance its anti-oxidation ability, and increase the level of immune regulatory cytokines, in order to protect the body from injury.

The present study determined the protective effect of anthocyanins on immune system injury in Wistar rats exposed to fluoride by treating them with anthocyanins extracted from *Vaccinium uliginosum*. This research was carried out to provide a new direction for the prevention and treatment of immunotoxicity caused by fluoride and to delay complications in fluorosis patients.

## MATERIALS AND METHODS

**Experimental animals:** Forty eight four-week-old healthy male Wistar rats ( $140 \pm 15$  g) were purchased from Vital River Laboratory Animal Technology Co., Ltd., Beijing, China. During establishment of the animal model, rats were kept in a controlled environment at  $20 \pm 2^\circ\text{C}$ , with  $50 \pm 15\%$  relative humidity and a 12 h / 12 h light / dark cycle. All animal procedures were approved by the Ethics Committee of Harbin Medical University, and the experimental methods and purposes were in accordance with animal ethical standards and international practices.

*Experimental protocol:* After seven days of adaptive feeding, the rats were randomly divided into 8 groups according to body weight and were treated with fluoride alone or fluoride (Sodium fluoride, CAS: 7681-49-4, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and anthocyanins (Blueberry extract freeze-dried powder anthocyanins purity~51%. obtained from Heilongjiang Ivzhidu Biotechnology Development Co., Ltd., Harbin, Heilongjiang Province, China), respectively. The control group (F0) was fed distilled water and the fluoride-treated groups received 50 mg/L F<sup>-</sup> (F1), 100 mg/L F<sup>-</sup> (F2), and 150 mg/L F<sup>-</sup> (F3) in drinking water freely. The anthocyanins only treated group (FA0) was fed distilled water freely and anthocyanins by gavage (100 mg/kg.body weight/day). The fluoride and anthocyanins combination groups (FA1, FA2, and FA3 groups) received anthocyanins and were treated with fluoride at the same dose as the fluoride only-treated groups, respectively.

*Determination of urinary fluoride in rats by the fluoride ion selective electrode method:*

Urinary fluoride was measured by the fluoride ion selective electrode method. The urine sample was diluted with water and fixed with the same volume of buffer. The potential value of the samples was measured one by one. Finally, the concentration of fluoride in urine was calculated according to the regression equation and the measured potential value.

*Calculation of immune organ indices:* After 90 days, the spleen and thymus were immediately removed from the rats to calculate the immune organ indices. After removal of the connective tissue and fat, the blood and mucus on the surface were dried with filter paper and the organ was weighed. The immune organ index was calculated according to the formula:

$$\text{Immune organ index} = \frac{\text{Immune organ weight (mg)}}{\text{Body weight (g)}}$$

*Detection of monocyte and lymphocyte apoptosis in rat blood by flow cytometry:*

Ficoll solution was shaken and 3 ml was obtained with a syringe, and then 4 ml of the blood sample was added slowly along the wall of the tube into the Ficoll solution. After centrifugation at 100 g for 10 min at 20 °C, the solution separated into layers and the middle lymphocyte layer was drawn to adjust the cell concentration to 1×10<sup>6</sup> cells/ml. The freshly extracted blood mononuclear cells were centrifuged at 400 g for 5 min at 4 °C and the cells were collected. The cells were resuspended in 100 μl

binding buffer, and 5  $\mu\text{l}$  annexin V-FITC and propidium iodide (PI) solution were added. After gentle mixing, the cells were reacted at room temperature for 10 min under dark conditions, added to 400  $\mu\text{l}$  binding buffer, and then detected by flow cytometry.

#### *Detection of IgG, IL-1, IL-2 and TNF- $\alpha$ in serum by ELISA:*

The serum samples were diluted with standard and specimen universal dilutions according to the kit instructions. Standard and specimen universal dilutions were added to the blank wells, and different standard concentrations and diluted samples were added to the other wells (100  $\mu\text{l}$ /well). The wells were sealed with sealing tape and incubated for 120 min at 37  $^{\circ}\text{C}$ . After washing the plate 5 times, the enzyme-labeled antibody dilution was added to the blank wells, and the enzyme-labeled antibody working solution (100  $\mu\text{l}$ /well) was added to the remaining wells, then sealed with new sealing tape and incubated for 60 min at 37  $^{\circ}\text{C}$  in the dark. After washing the plate five times, chromogenic substrate (TMB; 100  $\mu\text{l}$ /well) was added to the blank wells and incubated at room temperature for 15 min, and then stop solution (100  $\mu\text{l}$ /well) was added. Finally, the optical density (OD)<sub>450</sub> values were immediately measured after mixing.

#### *Detection of oxidative stress in rat blood by colorimetry:*

The superoxide dismutase (SOD) detection kit, glutathione peroxidase (GSH-Px) assay kit, micro-reduced glutathione (GSH) detection kit, and malondialdehyde (MDA) detection kit were purchased from Nanjing JianCheng Bioengineering Institute (Nanjing, Jiangsu Province, China). All experimental operations are carried out according to the appropriate instructions. The activity of SOD (450 nm), GSH-Px (412 nm), GSH (405 nm) and MDA (532 nm) were then calculated based on the different absorbance values. The following equations were used:

$$\text{SOD inhibition rate} = \frac{(A_{\text{Control}} - A_{\text{Control blank}}) - (A_{\text{Assay}} - A_{\text{Assay blank}})}{(A_{\text{Control}} - A_{\text{Control blank}})}$$

$$\text{SOD activity} = \frac{\text{SOD inhibition rate}}{50\% \times (0.24\text{ml}/0.02\text{ml}) \times 10}$$

$$\text{GSH-Px enzyme activity} = \frac{(\text{OD}_{\text{Non-enzymatic tube value}} - \text{OD}_{\text{Enzyme tube value}})}{(\text{OD}_{\text{Standard tube value}} - \text{OD}_{\text{Blank tube value}}) \times 20 \mu\text{mol/L} \times 5 \times (1+149) / (1+49)}$$



$$\text{GSH content (mol/L)} = \frac{(\text{OD}_{\text{Assay value}} - \text{OD}_{\text{Blank value}})}{(\text{OD}_{\text{Standard value}} - \text{OD}_{\text{Blank value}}) \times 20 \text{ mol / L} \times 5}$$

$$\left( \text{MDA content (nmol/ml)} = \frac{\text{OD}_{\text{Assay value}} - \text{OD}_{\text{Blank value}}}{\text{OD}_{\text{Standard value}} - \text{OD}_{\text{Blank value}}} \times 10 \text{ nmol/ml} \right)$$

*Statistical analysis:* Statistical analysis was performed using SPSS 13.0 software. All statistical results in this study are expressed as mean  $\pm$  standard deviation (SD). One-way ANOVA was used for statistical inference. The LSD and Duncan tests were used to determine significance between groups. A value of  $P < 0.05$  was considered to indicate a statistically significant difference.

## RESULTS

*The fluoride content in fluoride-treated rat urine:* After 90 days of treatment with different concentrations of fluoride, the urinary fluoride content in rats increased gradually as the dose of fluoride increased. Statistical analysis showed that there was a statistically significant difference between the different doses of fluoride. At the same dose of fluoride, there was no statistically significant difference between the fluoride groups and the fluoride combined with anthocyanins groups (Figure 5 in the supplement after the references).

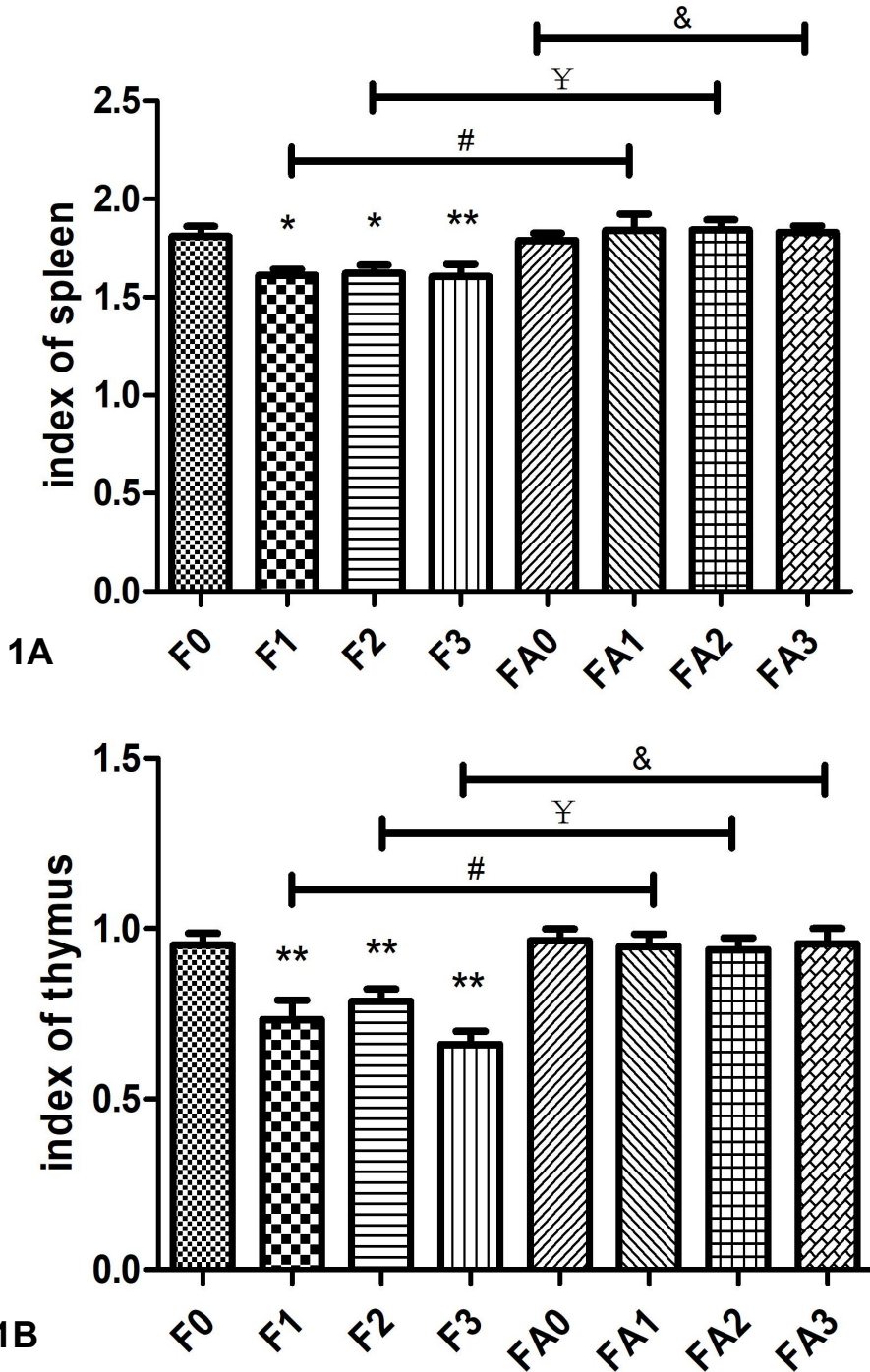
*Effect of anthocyanins on the immune organ indices in fluoride-treated rats:* The weight of immune organs is often related to the body's immune function. When the body's immune function is injured, the weight of the immune organs is reduced.<sup>16,36-38</sup> The spleen is the largest peripheral immune organ in the body and the thymus is the central lymphoid organ of the immune system. These organs are an important part of the body's immune system. Therefore, our study compared the spleen and thymus indices in each group, respectively. The results showed that the spleen and thymus indices in the fluoride-treated groups (F1, F2 and F3) were significantly lower than those in the control group (F0;  $P < 0.05$  or  $P < 0.01$ ). After anthocyanins treatment, the spleen and thymus indices were significantly increased ( $P < 0.05$ ) compared with the group receiving the same dose of fluoride only (Figures 1A and 1B).

*Anthocyanins inhibit the apoptosis of monocytes and lymphocytes in the blood of fluoride-treated rats:* To some extent, the decrease in immune organ indices indicates a decrease in the number of immune cells. Mononuclear cells in the blood mainly include monocytes and lymphocytes. Monocytes are an important component in the body's defense system, and the number of monocytes change when the body develops inflammation or other diseases.<sup>39,40</sup> Lymphocytes are an important cellular component in the body's immune response function. It has been reported that excessive fluoride reduces the percentage of peripheral blood lymphocytes in experimental animals.<sup>23,41,42</sup> Next, we examined apoptosis of monocytes and

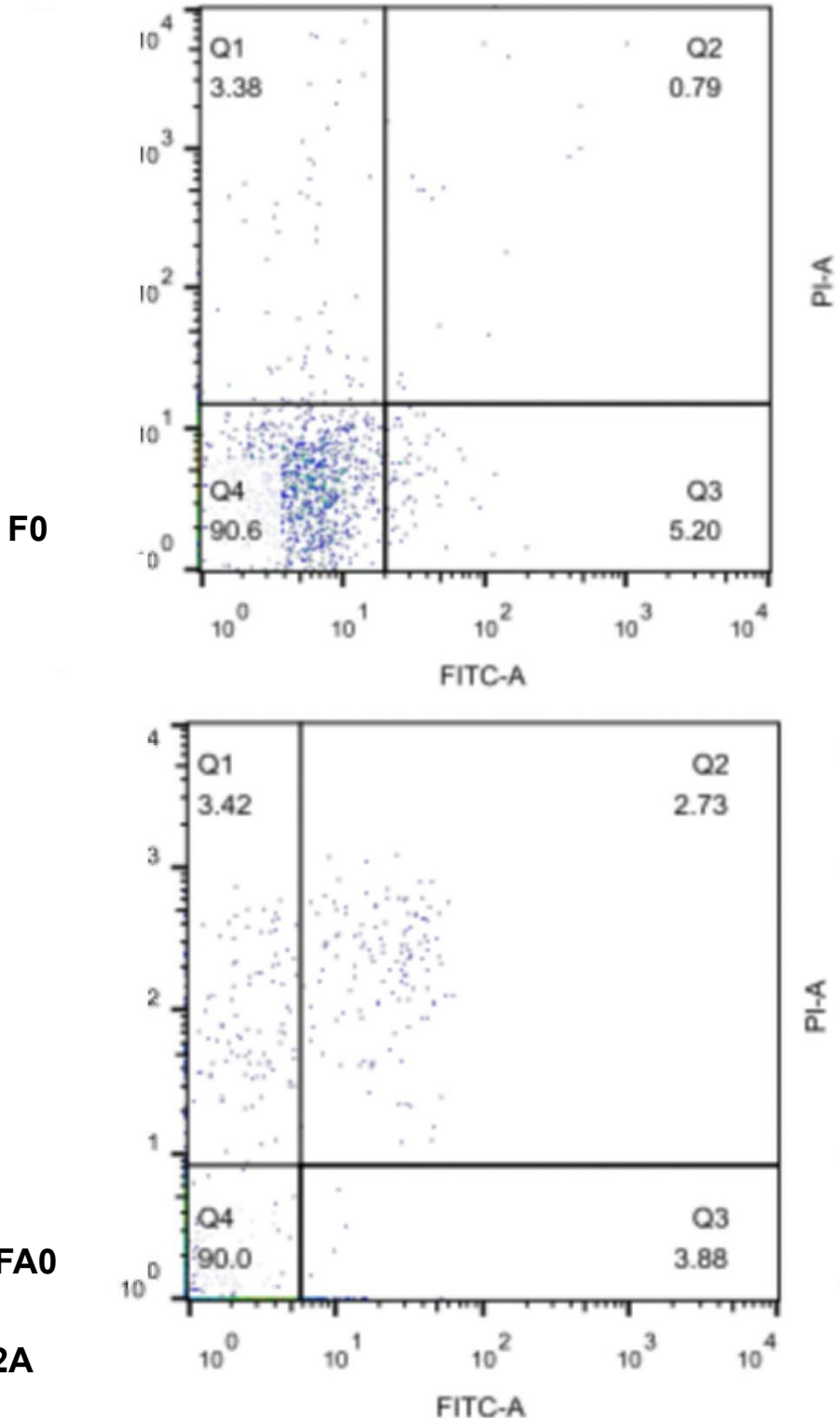
lymphocytes in the blood of rats. The apoptotic rates of blood monocytes and lymphocytes in the fluoride only exposure groups (F1, F2 and F3) both increased in a dose-dependent manner when compared with the normal control group (F0). Monocytes were more sensitive to sodium fluoride. The 50 mg/L, 100 mg/L and 150 mg/L concentrations of F<sup>-</sup> increased apoptosis of monocytes (P<0.01), while apoptosis of lymphocytes occurred at higher concentrations of F<sup>-</sup> (100 mg/L and 150 mg/L) (P<0.05 or P<0.01). Following anthocyanins treatment, the anthocyanin-only group (FA0) showed no difference in apoptosis between the two cell types compared with the normal control group (F0), and the apoptotic rates of both cell types decreased (P<0.05 or P<0.01) when compared with the groups treated with the same dose of fluoride (F1 and FA1, F2 and FA2 and F3 and FA3) (Figures 2A–2J).

*Effect of anthocyanins on immunoglobulins and cytokines in the serum of fluoride-treated rats:* Immunoglobulins and cytokines play an important role in immune response and immune regulation. Therefore, we examined the contents of IgG, IL-1, IL-2 and TNF- $\alpha$  in rat serum. The concentrations of IgG and IL-1 in serum decreased in a dose-dependent manner, and the decline in the medium and high doses was more obvious. The concentrations of these two markers were significantly increased in the anthocyanins treatment groups (FA0, FA1, FA2 and FA3). Serum IL-2 was more sensitive to fluoride exposure. As shown in the figure, the fluoride only exposure groups (F1, F2 and F3) showed a significant decrease in serum IL-2 levels compared with the normal control group (F0). Exposure to the lower dose of fluoride caused a significant decrease in serum IL-2 in rats. Following treatment with anthocyanins, the serum IL-2 content increased significantly. Fluoride exposure had little effect on the content of TNF- $\alpha$  in serum, but treatment with anthocyanins had a marked influence on its content. As shown in the figure, the content of TNF- $\alpha$  in serum showed a downward trend with increased fluoride concentrations, and following treatment with fluoride combined with anthocyanins, the serum levels of TNF- $\alpha$  increased significantly compared with the same dose of fluoride only (Figures 3A–3D).

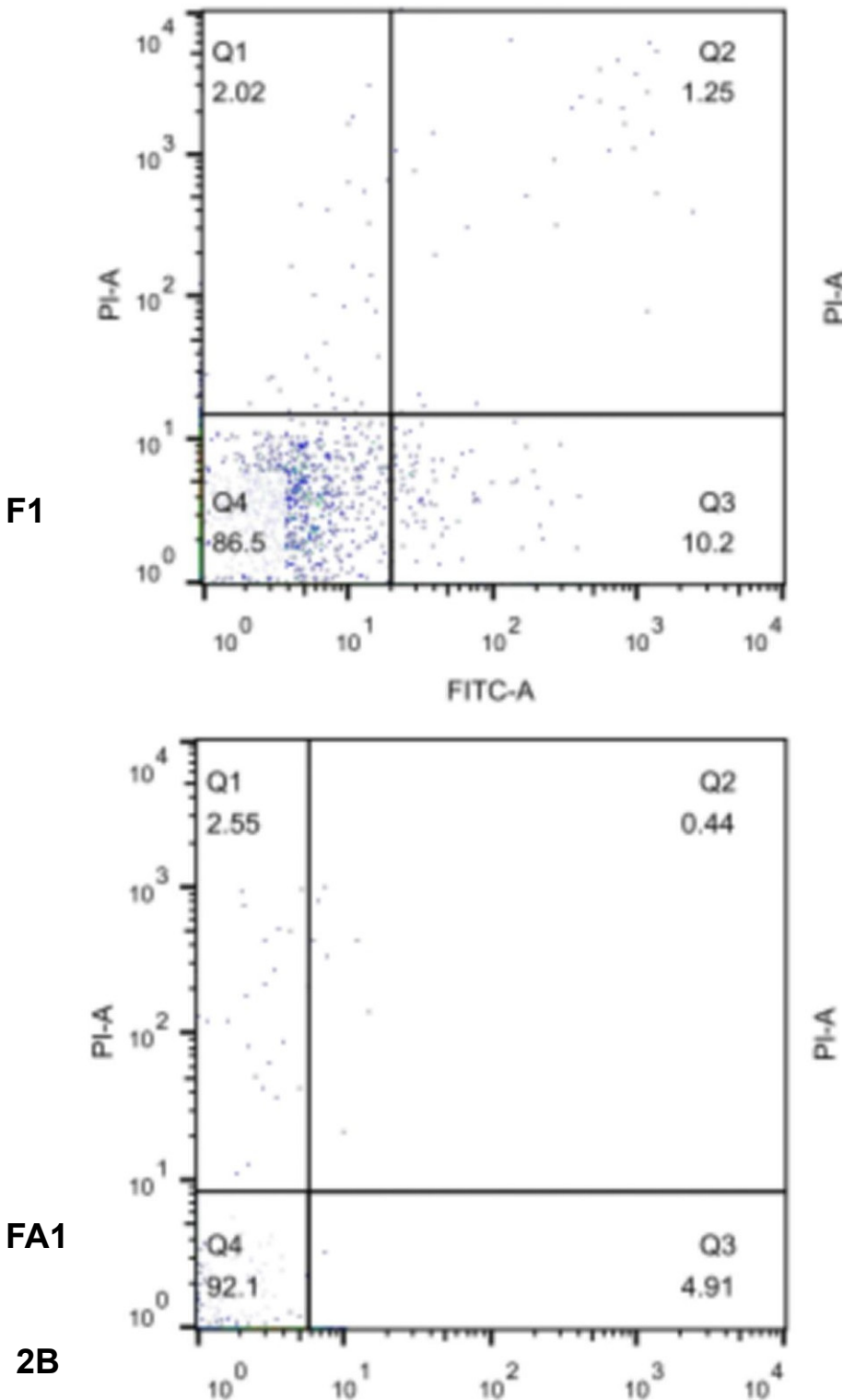
*Anthocyanins treatment improves oxidative stress in rats:* The above studies demonstrated that anthocyanins treatment effectively ameliorated the reduction in immune organ indices, the apoptosis of immune cells and the immune active substances caused by fluoride. Next, we determined whether anthocyanins played a protective role in the body by reducing the level of oxidative stress. The results showed that the activities of SOD and GSH-Px in the serum of fluoride-treated groups were significantly decreased compared with the F0 group. The activities of SOD (P<0.05) and GSH-Px (P<0.01) were significantly increased compared with the same dose of fluoride plus anthocyanins treatment. The content of GSH showed a downward trend with increased doses of fluoride compared with the F0 group. Following the addition of anthocyanins, there was an upward trend in the content of GSH compared with the same dose of fluoride only, and this was more obvious in the high dose fluoride treatment groups. Compared with the F0 group, the content of MDA showed an upward trend in the fluoride only treated groups. Following treatment with anthocyanins, MDA content decreased significantly in the normal control group and the low-dose fluoride group (Figures 4A–4D).



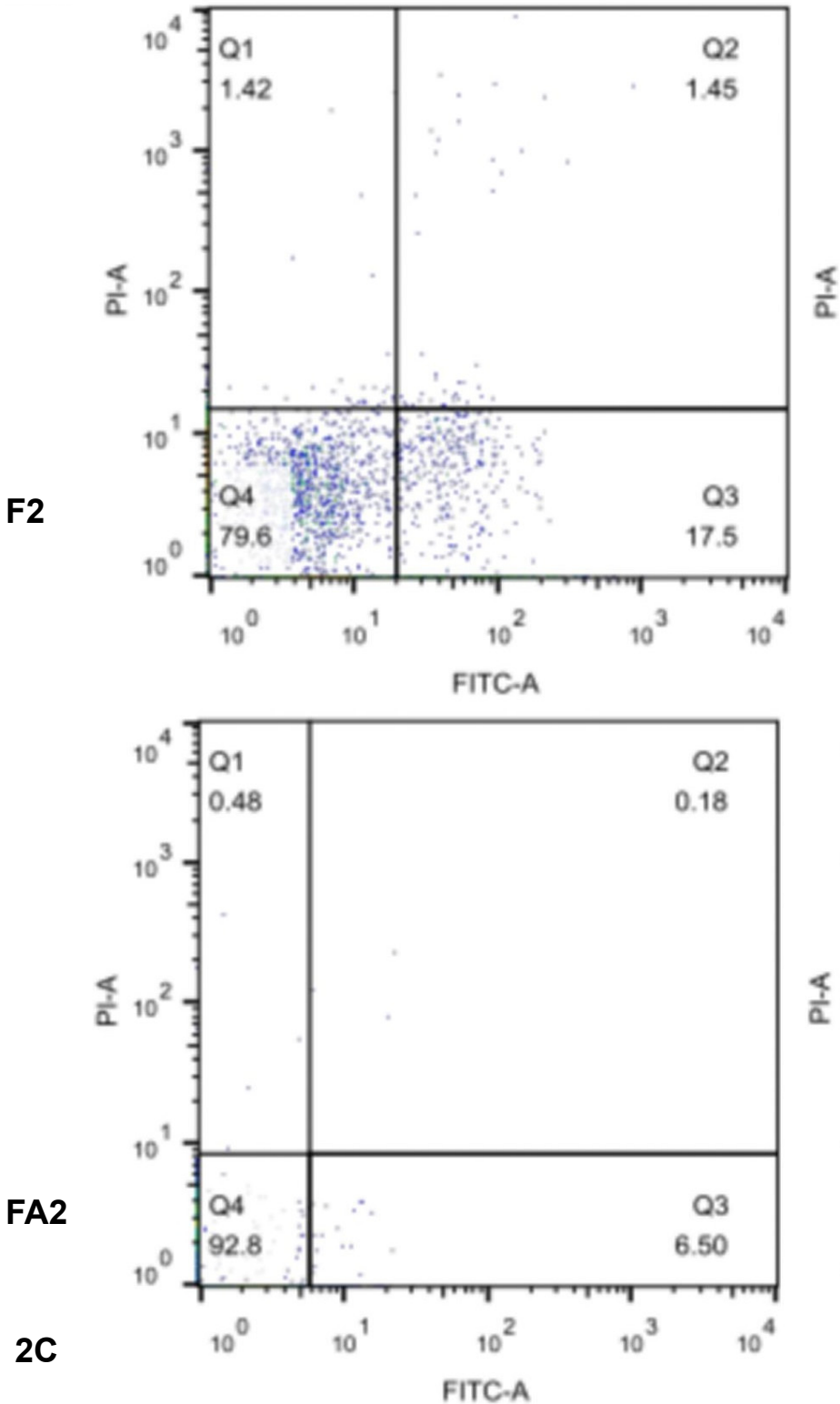
**Figures 1A and 1B.** The effect of anthocyanins (A) on immune organ indices in rats exposed to fluoride (F). 1A: Immune spleen index in rats; 1B: Immune thymus index in rats. Data are presented as the mean  $\pm$  SD (n = 6). \* P < 0.05, compared with the F0 group, #, ¥ and & P < 0.05 for the two groups; \*\* P < 0.01, compared with the F0 group. The grouping and treatment of the experimental animals were as described in the experimental protocol section. F0: distilled water; F1: F=50 mg/L; F2: F=100 mg/L; F3: F=150 mg/L; FA0: distilled water + A; FA1: F 50mg/L + A; FA2: F 100mg/L + A; and FA3: F 150mg/L + A. Anthocyanin dose = 100 mg/kg body weight/day).



**Figure 2A** The effect of anthocyanins (A) on monocyte apoptosis in the blood of rats exposed to fluoride (F). The grouping and treatment of the experimental animals were the same as above (Fig.1). F0: distilled water; FA0: distilled water + A. Anthocyanin dose = 100 mg/kg body weight/day).

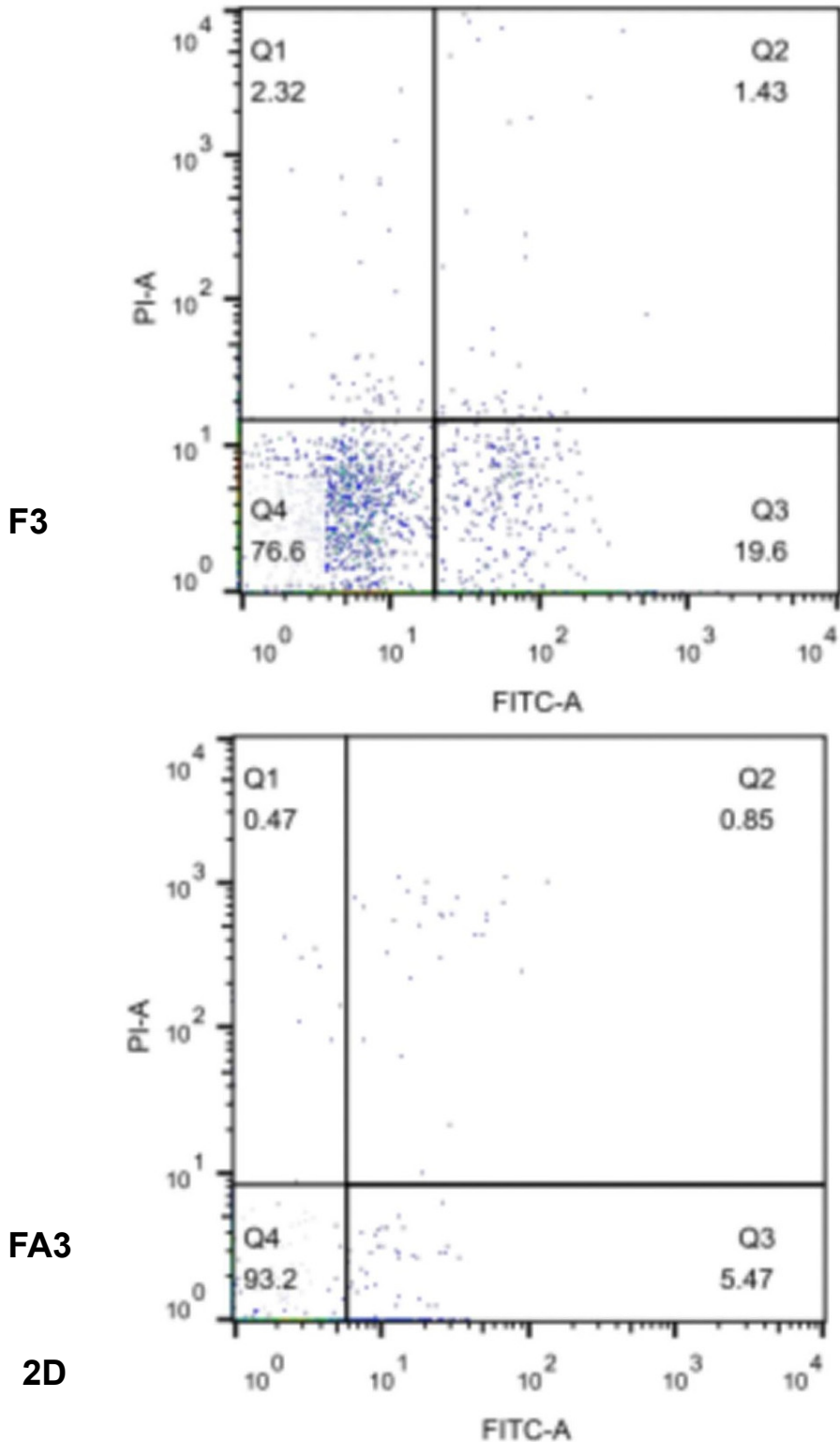


**Figure 2B** The effect of anthocyanins (A) on monocyte apoptosis in the blood of rats exposed to fluoride (F). The grouping and treatment of the experimental animals were the same as above (Fig.1). F1: F= 50 mg/l; FA1: F = 50 mg/L + A. Anthocyanin dose = 100 mg/kg body weight/day).

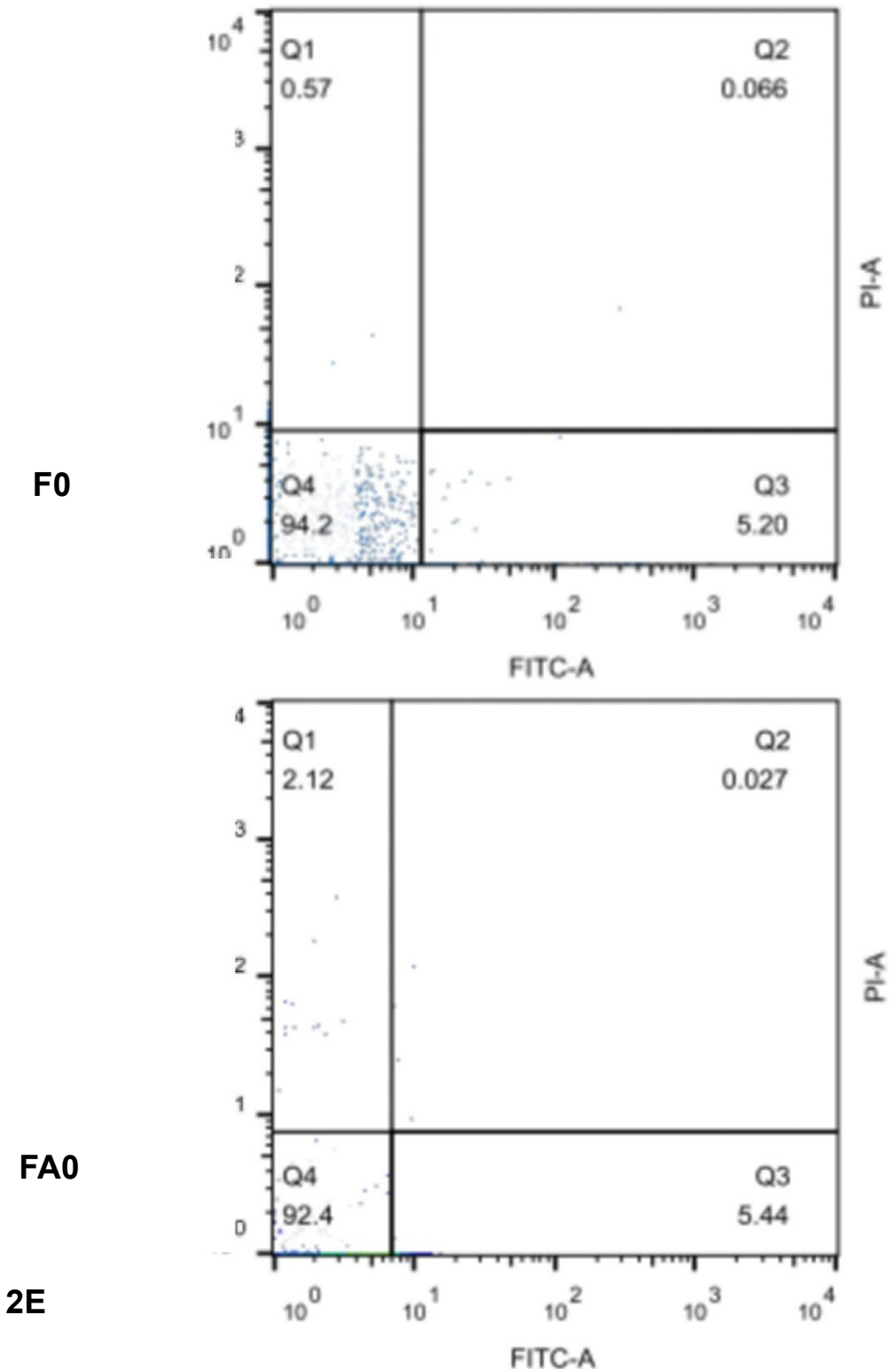


**Figure 2C.** The effect of anthocyanins (A) on monocyte apoptosis in the blood of rats exposed to fluoride (F). The grouping and treatment of the experimental animals were the same as above (Fig.1). F2: F = 100 mg/l; FA2: F = 100 mg/L + A. Anthocyanin dose = 100 mg/kg body weight/day).

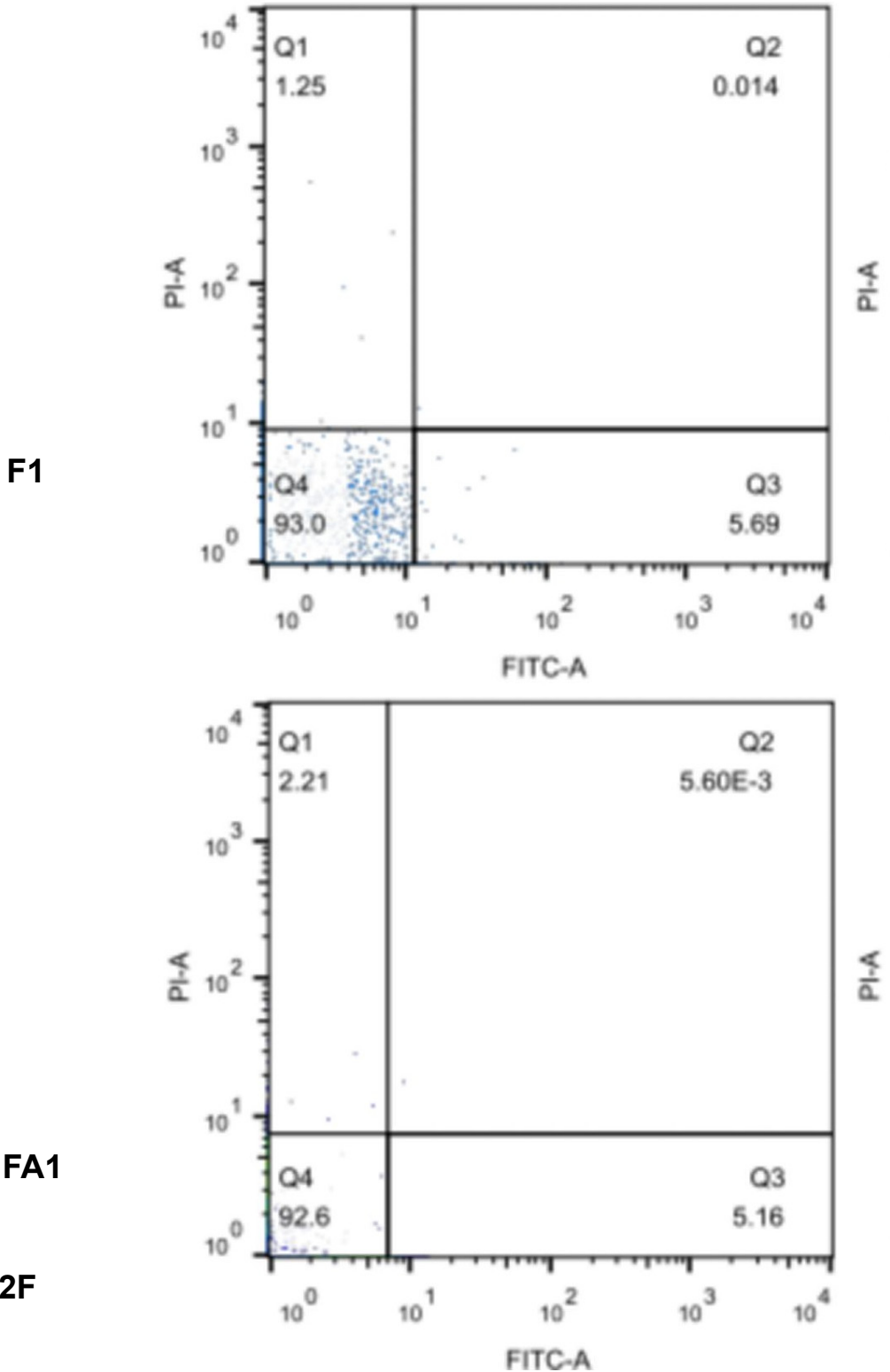




**Figure 2D.** The effect of anthocyanins (A) on monocyte apoptosis in the blood of rats exposed to fluoride (F). The grouping and treatment of the experimental animals were the same as above (Fig.1). F3: F= 150 mg/l; FA3: F= 150 mg/l + A. Anthocyanin dose = 100 mg/kg body weight/day).

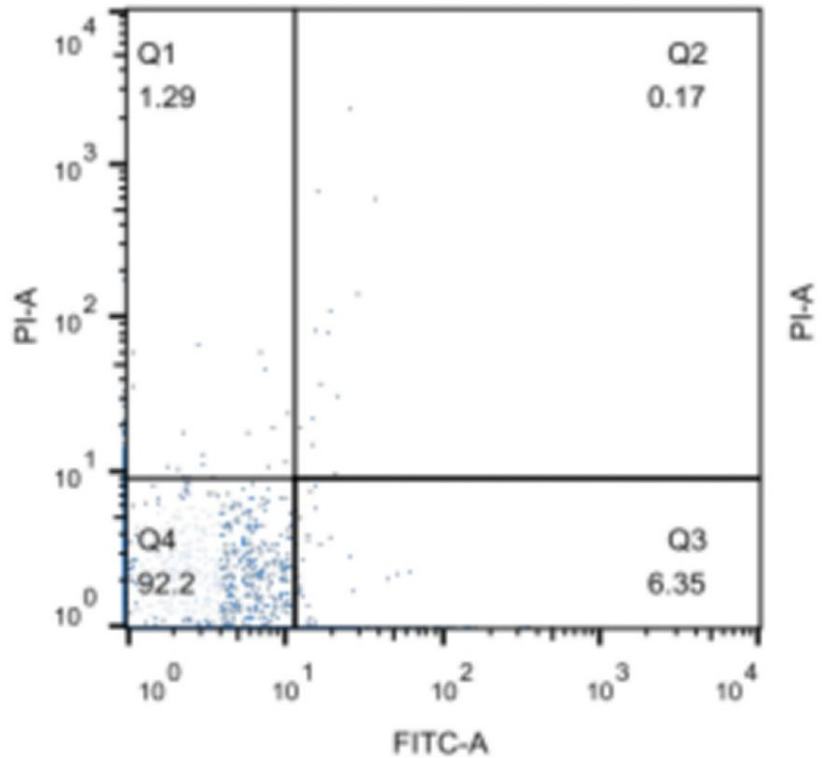


**Figure 2E** The effect of anthocyanins (A) on lymphocyte apoptosis in the blood of rats exposed to fluoride (F). The grouping and treatment of the experimental animals were the same as above (Fig.1). F0: distilled water; FA0: distilled water + A. Anthocyanin dose = 100 mg/kg body weight/day).

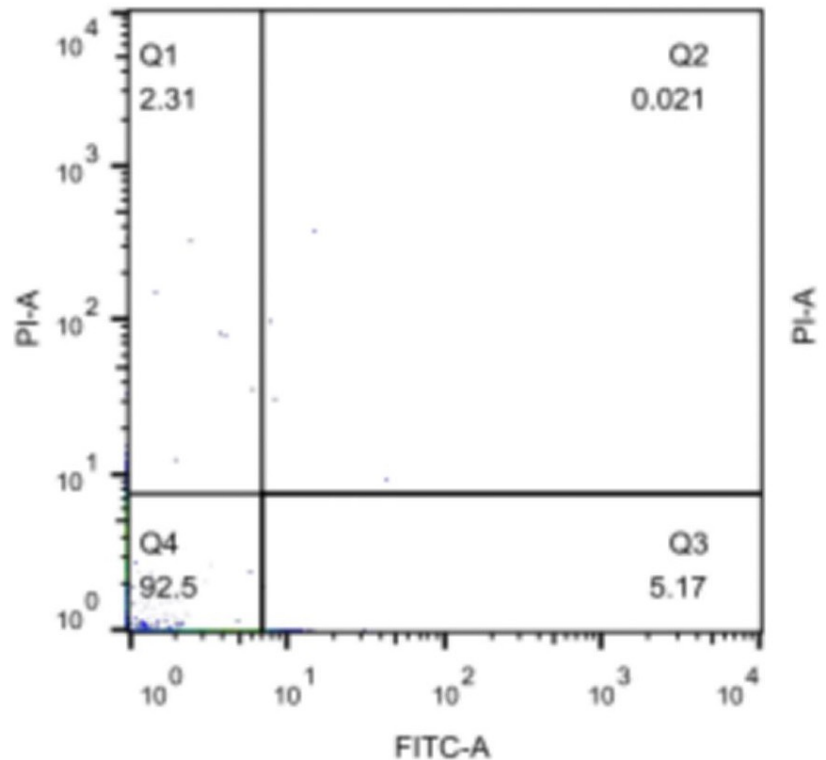


**Figure 2F** The effect of anthocyanins (A) on lymphocyte apoptosis in the blood of rats exposed to fluoride (F). The grouping and treatment of the experimental animals were the same as above (Fig.1). F1: F = 50 mg/L; FA1: F = 50 mg/L + A. Anthocyanin dose = 100 mg/kg body weight/day.

F2



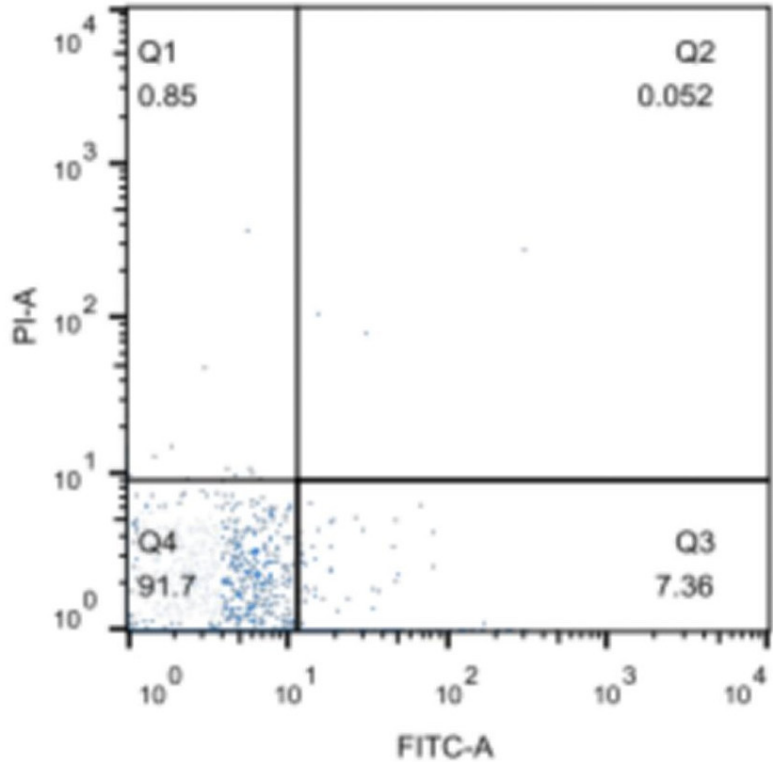
FA2



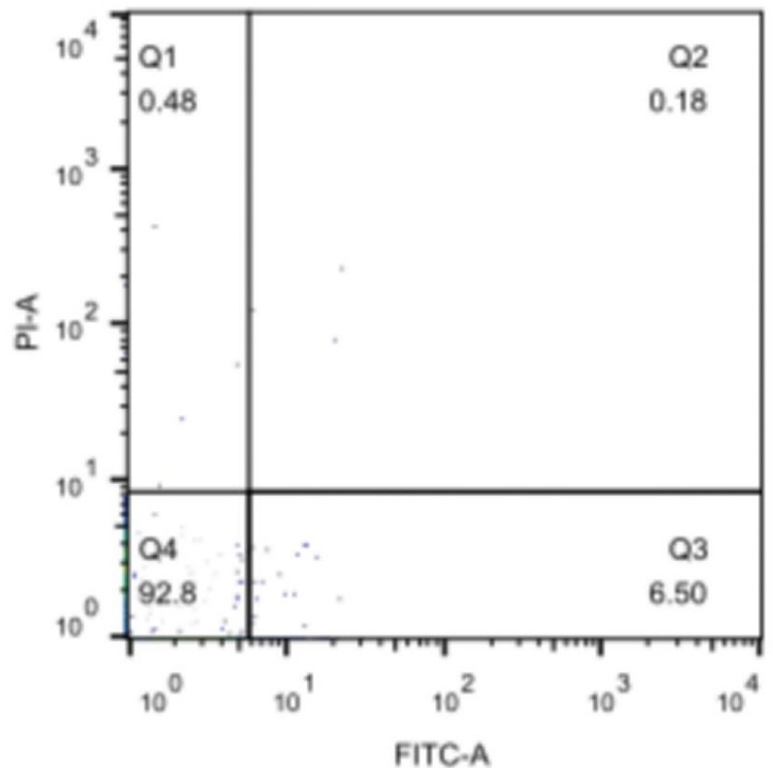
2G

**Figure 2G** The effect of anthocyanins (A) on lymphocyte apoptosis in the blood of rats exposed to fluoride (F). The grouping and treatment of the experimental animals were the same as above (Fig.1). F2: F = 100 mg/L; FA2: F = 100 mg/L + A. Anthocyanin dose = 100 mg/kg body weight/day).

F3

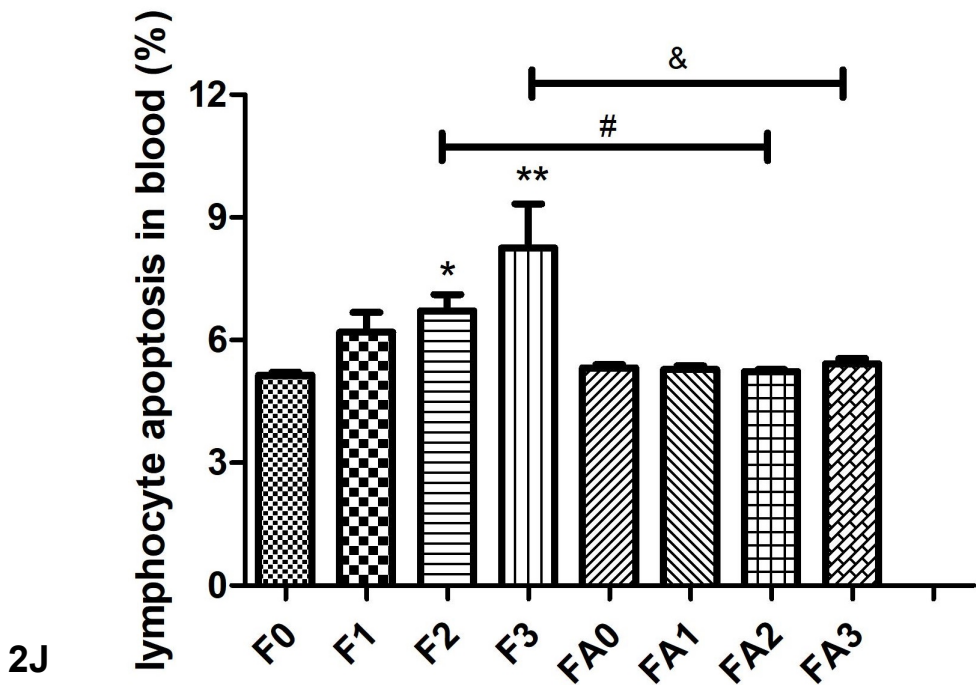
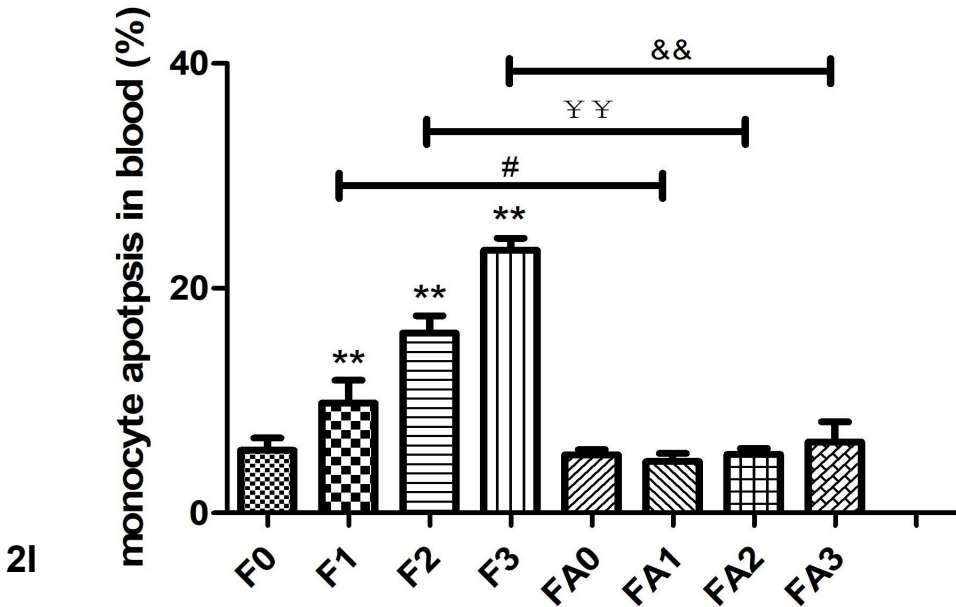


FA3



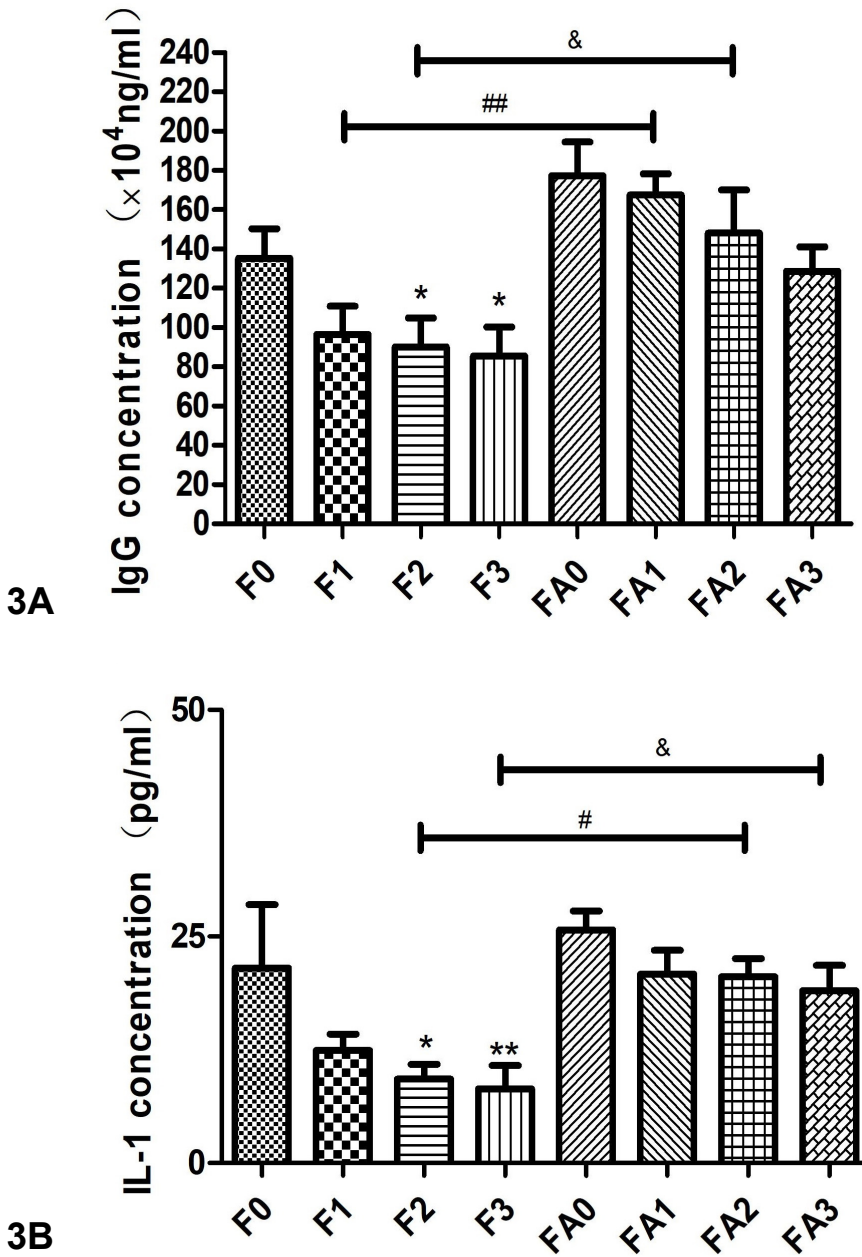
2H

**Figure 2H** The effect of anthocyanins (A) on lymphocyte apoptosis in the blood of rats exposed to fluoride (F). The grouping and treatment of the experimental animals were the same as above (Fig.1). F3: F = 150 mg/L; FA3: F = 150 mg/L + A. Anthocyanin dose = 100 mg/kg body weight/day).

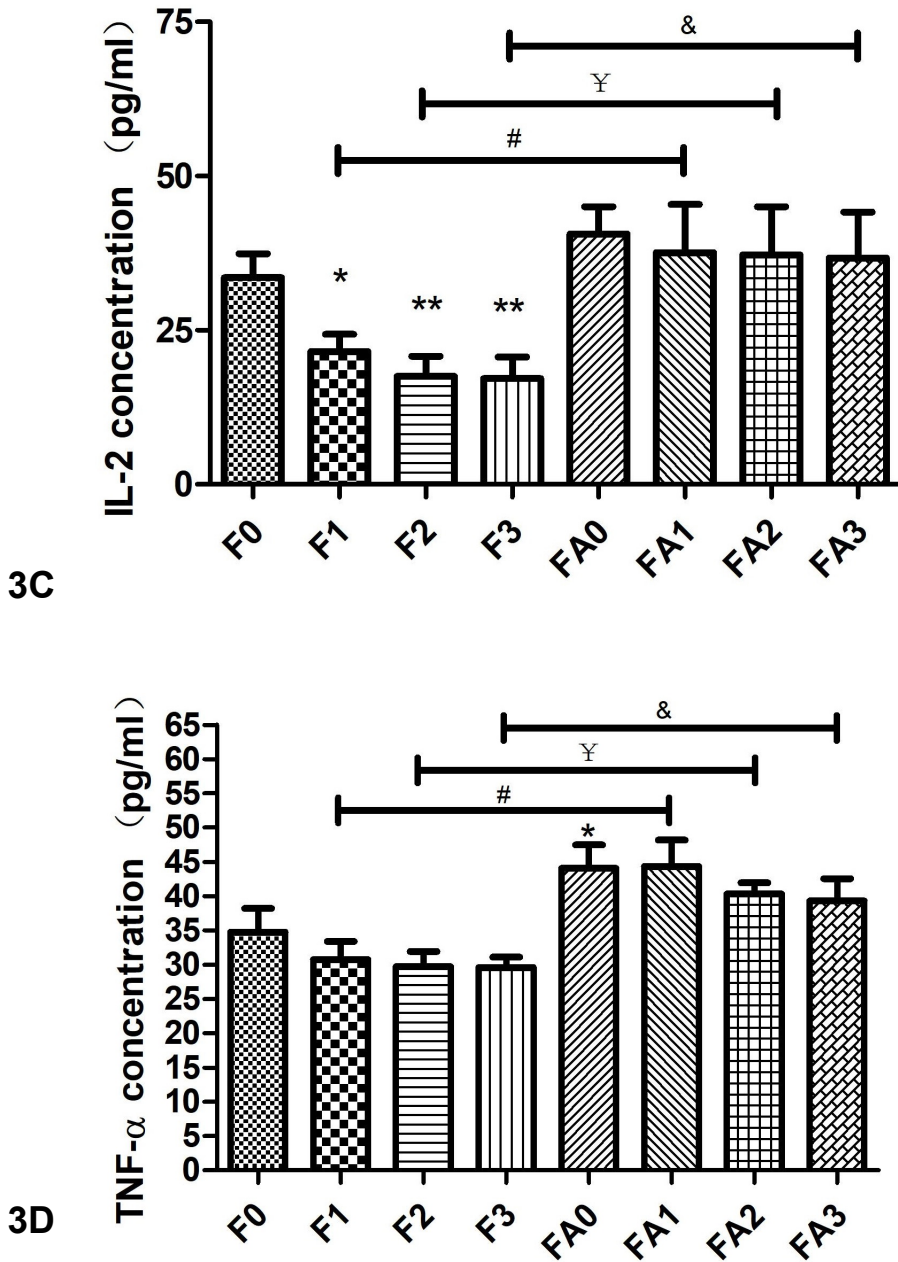


**Figures 2I and 2J.** The effect of anthocyanins (A) on lymphocyte apoptosis in the blood of rats exposed to fluoride (F). The grouping and treatment of the experimental animals were the same as above (Fig.1). F0: distilled water, F1: F = 50 mg/L, F2: F = 100 mg/L, F3: F = 150 mg/L, FA0: distilled water = A, FA1: F = 50 mg/L + A, FA2: F = 100 mg/L + A, FA3: F = 150 mg/L + A. Anthocyanin dose = 100 mg/kg body weight/day. Data are presented as the mean  $\pm$  SD (n = 6). \* P < 0.05, compared with the F0 group; # and & P < 0.05 for the two groups; \*\* P < 0.01, compared with the F0 group; ## and ¥ ¥ P < 0.05 for the two groups.

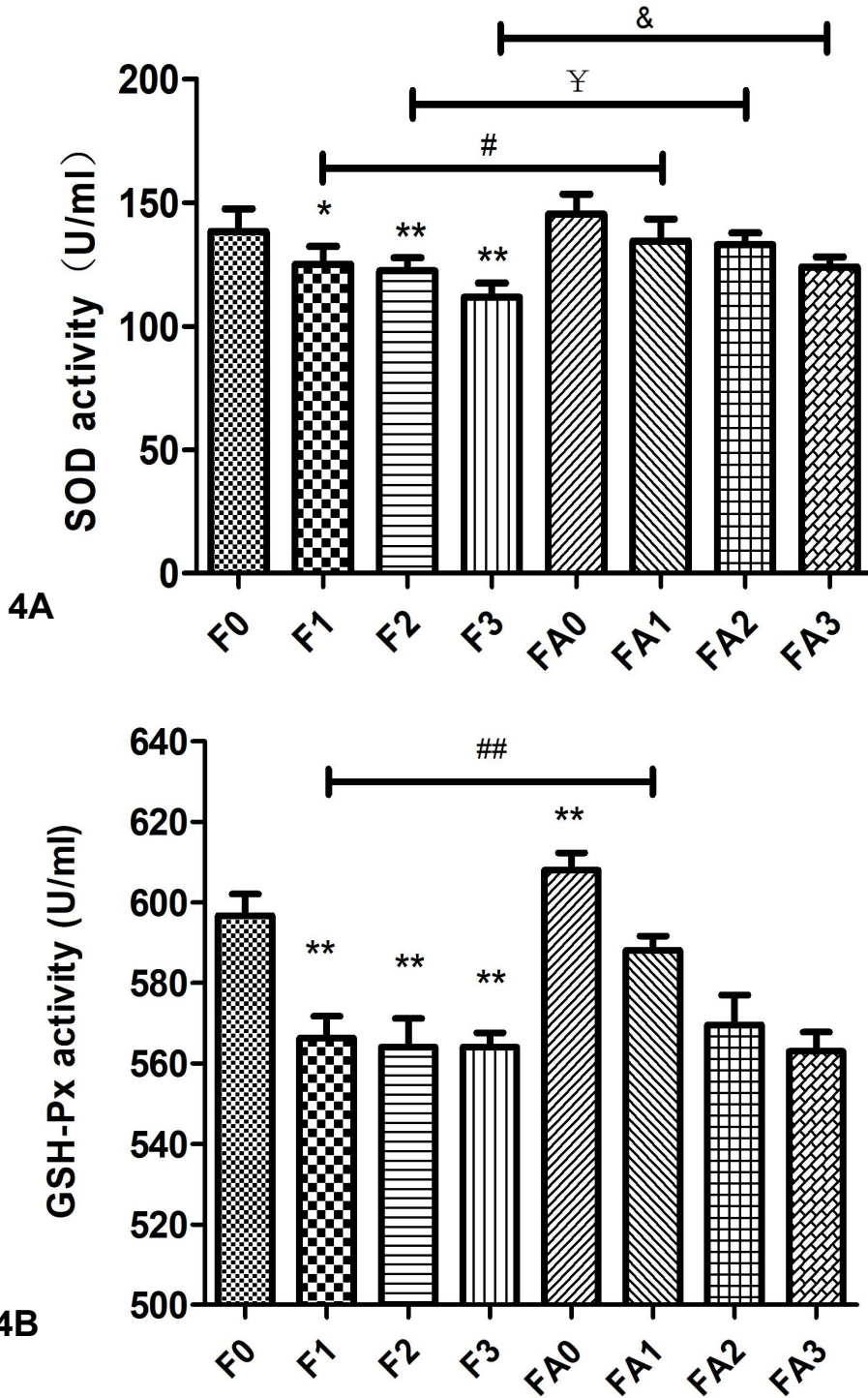




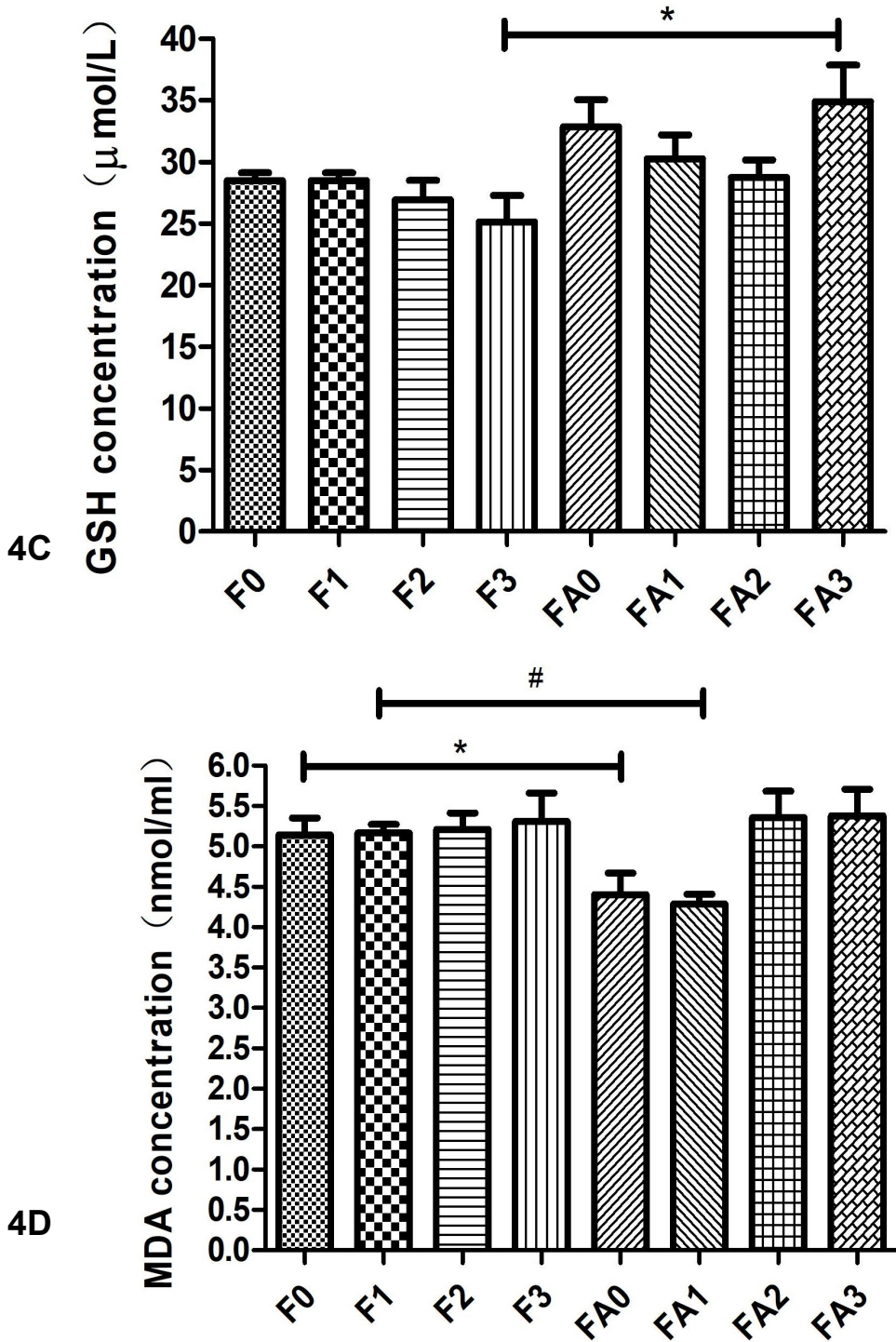
**Figures 3A and 3B.** The effects of anthocyanins on serum IgG and cytokines (IL-1, IL-2 and TNF- $\alpha$ ) in rats exposed to fluoride. The grouping and treatment of the experimental animals were the same as above (Fig.1). 3A: Serum IgG content in rats; 3B: Serum IL-1 content in rats. Data are presented as the mean  $\pm$  SD (n = 6). \* P < 0.05, compared with the F0 group; # and & P < 0.05 for the two groups; \*\* P < 0.01, compared with the F0 group; and ## P < 0.05 for the two groups.



**Figures 3C and 3D.** The effects of anthocyanins on serum IgG and cytokines (IL-1, IL-2 and TNF- $\alpha$ ) in rats exposed to fluoride. The grouping and treatment of the experimental animals were the same as above (Fig.1). 3C: Serum IL-2 content in rats; 3D: Serum TNF- $\alpha$  content in rats. Data are presented as the mean  $\pm$  SD (n = 6). \* P < 0.05, compared with the F0 group; #, ¥, and & P < 0.05 for the two groups; \*\* P < 0.01, compared with the F0 group; and ## P < 0.05 for the two groups.



**Figures 4A and 4B.** The effect of anthocyanins on oxidative stress in rats exposed to fluoride. The grouping and treatment of the experimental animals were the same as above (Fig.1). 4A: Serum SOD activity in rats; 4B: GSH-Px enzyme activity in rat whole blood. Data are presented as the mean  $\pm$  SD (n = 6). \* P < 0.05, compared with the F0 group; #, ¥, and & P < 0.05 for the two groups; \*\* P < 0.01, compared with the F0 group; and ## P < 0.05 for the two groups.



**Figures 4C and 4D.** The effect of anthocyanins on oxidative stress in rats exposed to fluoride. The grouping and treatment of the experimental animals were the same as above (Fig.1). 4C: Serum GSH content in rats; 4D Serum MDA content in rats. Data are presented as the mean  $\pm$  SD (n = 6). \* P < 0.05, compared with the F0 group; #, ¥, and & P < 0.05 for the two groups; \*\* P < 0.01, compared with the F0 group; and ## P < 0.05 for the two groups.

## DISCUSSION

Fluoride exposure can reduce immune organ indices,<sup>36-38</sup> the number of immune cells,<sup>22-28</sup> the content of immunoglobulins and the level of cytokines,<sup>23</sup> thus affecting immune function in the body. The changes in the spleen and thymus indices can reflect the immune function and prognosis of an organism.<sup>43-45</sup> The results showed that the spleen and thymus indices were significantly lower than those in the normal control group (F0) and the rates of monocytes and lymphocytes apoptosis in blood were significantly higher than that in the normal control group (F0) after 90 days exposure to fluoride in drinking water, and the apoptosis rates of these two cell types also increased in a dose-dependent manner, which was consistent with our previous research results.<sup>46</sup>

The decrease in immune cells leads to a decrease in the content of immune active substances. As the most important antibody component in serum, IgG is synthesized and secreted by plasma cells in the spleen and lymph nodes, which plays a protective role in humoral immunity. IL-1 is mainly produced by monocytes and its content can reflect the immune regulation ability of the body as an immunomodulator. IL-2 is mainly secreted by lymphocytes, which can regulate the activity of leukocytes in the immune system and participate in antibody reactions, hematopoiesis, and tumor monitoring. TNF- $\alpha$  is produced by many immune cells and plays an important role in inflammation, apoptosis, and immune system development. Our results showed that the contents of IgG, IL-1, IL-2 and TNF- $\alpha$  in the serum of rats exposed to fluoride showed a downward trend with increased fluoride dose, and the serum content of IL-2 was most affected by fluoride. IL-2 decreased significantly at a lower dose (50 mg/L F<sup>-</sup>), followed by IgG and IL-1 in serum where the contents at the middle and high doses (100 mg/L F<sup>-</sup> and 150 mg/L F<sup>-</sup>) decreased significantly. The above results show that excessive exposure to fluoride can damage immune function in experimental animals, lead to atrophy of immune organs, apoptosis of immune cells, and decrease immune active substances, thus affecting immune function.

Fluoride has immunotoxicity, but the mechanism is unclear. Excessive fluoride disrupts the balance of oxidative stress in organisms which leads to an increase in reactive oxygen species, an accelerated consumption of antioxidant enzymes such as glutathione peroxidase, and an accumulation of lipid peroxidation products.<sup>47,48</sup> Oxidative stress is known to affect both gene regulation and protein expression by damaging DNA, proteins, and lipids. Previous studies have shown that fluoride can induce oxidative stress, reduce the activity of SOD and GSH-Px, decrease the content of GSH, and increase the content of MDA in the blood.<sup>25,26</sup> In this study, blood oxidative stress markers in rats including SOD and GSH-Px activity and the content of GSH and MDA were detected. SOD and GSH-Px activity were significantly reduced, and the concentration of GSH showed a downward trend, while the content of the oxidative stress product MDA showed an upward trend in fluoride-treated rats. As an important antioxidant enzyme *in vivo*, SOD activity indirectly reflects the ability to scavenge free radicals. SOD and GSH-Px are the most important enzyme components in the antioxidant system and the significant decline in their activity



following fluoride exposure indicates that oxidative stress occurred in the body. The above results suggested that fluoride may lead to immune system injury through oxidative stress.

Anthocyanins, which are recognized antioxidants, have been widely used due to their wide availability, non-toxicity, high safety and unique physiological function.<sup>49</sup> As the largest water-soluble pigments in the plant kingdom, they are widely obtained from plants, such as blueberry (also known as cranberry). Experimental studies have quantified the content of anthocyanins in blueberry juice using a conversion factor with the molecular weight of each anthocyanin and found that there was 3.909mg of total anthocyanin per mg of extract.<sup>50</sup> Anthocyanins from blueberry can improve the antioxidant capacity of the body. Anthocyanins play a key role in the treatment of many diseases, such as obesity, intestinal flora imbalance, cardiovascular diseases and so on. They have been demonstrated to alleviate symptoms of gut inflammation through the modulation of pro-inflammatory cytokines and anthocyanin-rich foods such as berries can improve endothelial function, blood pressure and arterial stiffness.<sup>51,52</sup> Anthocyanin-related human studies have shown that the activities of these compounds depend on their activities as reactive oxygen species scavengers, capacity to prevent the activation of NF- $\kappa$ B, and reduce the expression of target genes, including those participating in inflammation.<sup>53</sup> Experimental studies have confirmed that they can inhibit oxidative stress in mammalian endothelial cells and in the human colon cell lines Caco-2 and HT-29,<sup>32,54</sup> reduce the total oxidative state in the liver of experimental animals and enhance its total antioxidant capacity (TAC),<sup>55</sup> reverse the decline in serum TAC,<sup>56</sup> and eventually prevent oxidative damage in the body. In addition, it has been reported that the anthocyanins from blueberry can also regulate the level of cytokines and protect immune system function.<sup>34,35</sup> Therefore, on the basis of systematically expounding the influence of fluoride on the immune system, this study examined whether anthocyanins play a protective role in the immune system, in order to alleviate immune system injury due to fluorosis. The results showed that SOD and GSH-Px activity and GSH content in the fluoride plus anthocyanins groups were higher than those in the group treated with fluoride alone, and that MDA content was significantly reduced after treatment with anthocyanins and low dose fluoride, which showed that anthocyanins improved the oxidative state of rats and alleviated the oxidative stress induced by fluoride. In addition, anthocyanins significantly inhibited the decreases in spleen and thymus indices, the apoptosis of monocytes and lymphocytes in blood, and the decrease in IgG, IL-1, IL-2, and TNF- $\alpha$  in serum. The above results showed good correlation with the results of oxidative stress-related indicators. Taken together, these results indicated that anthocyanins could regulate immune system injury induced by fluoride. Anthocyanins may play a protective role in immune system injury induced by fluoride by inhibiting the oxidative stress response, which suggests that anthocyanins may be used as a dietary antioxidant to alleviate oxidative stress injury induced by fluoride.



## CONCLUSION

This study systematically confirmed the immunotoxicity of fluoride in experimental animals and the possible role of oxidative stress in this process. In addition, the antioxidant value of blueberry anthocyanins in immune function injury induced by fluoride was confirmed for the first time, which indicated their protective effect on the immune system.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest related to the contents of this article.

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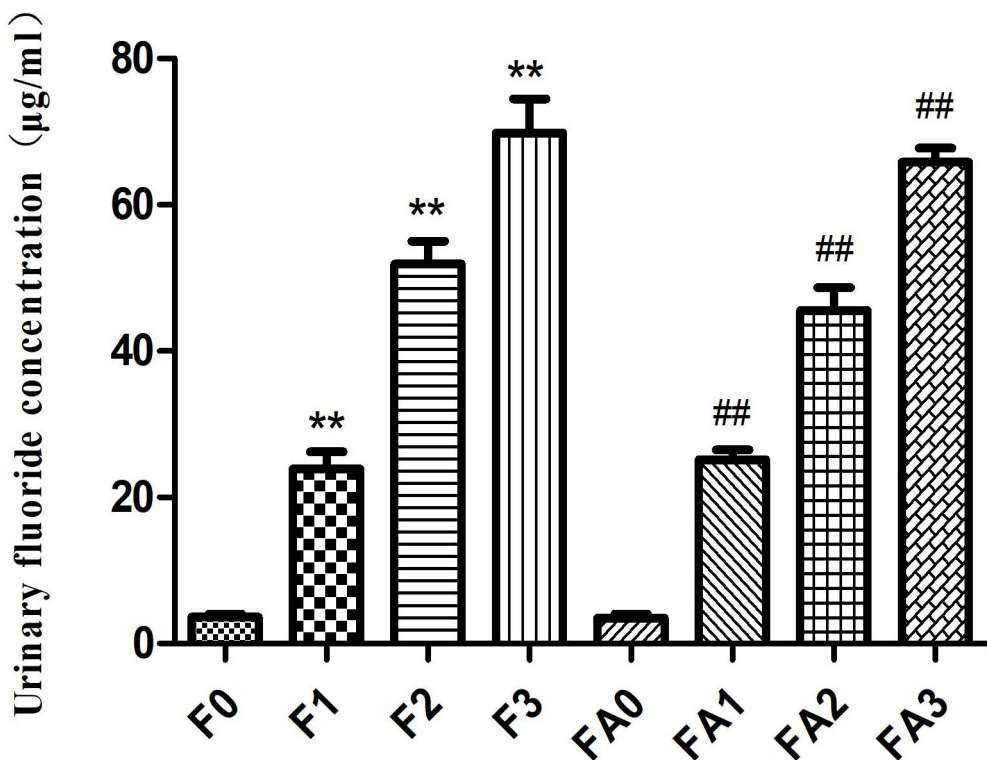
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### SUPPLEMENT



**Figure 5.** The changes in urinary fluoride content in rats. Fluoride groups (F0, F1, F2 and F3) were treated with 0 mg/L F<sup>-</sup>, 50 mg/L F<sup>-</sup>, 100 mg/L F<sup>-</sup>, and 150 mg/L F<sup>-</sup>, respectively. The fluoride combined with anthocyanins groups (FA0, FA1, FA2 and FA3) were treated with the same dose of fluoride as the fluoride groups, plus anthocyanins (100 mg/kg.body weight/day). Data are presented as the mean ± SD (n = 6). \*\* P < 0.01, compared with the F0 group, ## P < 0.01, compared with the FA0 group.