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Antibiotics and chemical disease-control agents reduce innate disease resistance in crayfish

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ABSTRACT

The aquaculture industry has developed rapidly in recent years, and in China Crayfish *Procambarus clarkii* represent an important aquaculture fishery. However, bacterial and viral diseases are becoming an increasingly serious threat, causing considerable economic losses. Farmers use a large number of drugs and chemicals to destroy pathogenic microorganisms and to purify aquaculture water. The purpose of this study was to assess the effects of such drugs on crayfish immune systems. Five of the most commonly used fishery drugs and water treatment chemicals were analyzed: norfloxacin, calcium hypochlorite, quick lime, povidone iodine and copper sulfate. Crayfish immune activity tests revealed that total hemocytes counts, as well as the activities of phenoloxidase and superoxide dismutase, decreased following exposure to all five treatments. These treatments, especially calcium hypochlorite and norfloxacin, significantly enhanced hemocyte apoptosis in crayfish, regardless of disease status. Calcium hypochlorite, in particular, led to a significant decrease in the survival rates of crayfish infected with white spot syndrome virus or *Vibrio alginolyticus*. Our results indicate that water treatment and disease control compounds commonly used in aquaculture can reduce the innate immunity and therefore disease resistance of crayfish.

1. Introduction

In recent years, the global aquaculture industry has developed rapidly, however disease control remains an ongoing issue,.many species of pathogens regularly found in aquaculture ponds including viruses, bacteria, fungi and parasites [1–3]. A range of antibiotics and chemical control agents are widely applied to effectively prevent and treat diseases in aquaculture systems, particularly in China which is the largest global aquaculture producer. The use of 25 different antibiotics has been reported, however only 13 antibiotics have been authorized for application in Chinese aquaculture with a further 12 in use that have not been authorized [4]. Crayfish are an important component of the aquaculture fishery in China, however, bacterial and viral diseases are an increasingly serious threat, causing considerable economic losses. Due to intensive culturing practices and confined ponds, crayfish aquaculture systems often incur tremendous losses if invaded by pathogens. All crustaceans, including crayfish, lack a highly specific adaptive immune system, instead opposing foreign pathogens primarily via innate immunity [5]. Vibriosis and white spot syndrome virus (WSSV) have caused irreversible damage to the crustacean aquaculture industry worldwide [6], and there are no effective measures have been found to prevent these diseases until now.

As a member of invertebrates, the crayfish has no specific immunity like fish, but only innate immunity. The innate immune mechanism of crayfish is mainly achieved by phagocytosis and some enzymes. The phagocytosis of foreign pathogens is mainly accomplished by hemocytes in crustacean. Therefore, hemocytes play an important role in the innate immunity of crayfish. Superoxide Dismutase (SOD) is an active substance derived from living organisms, which can eliminate harmful substances produced in the process of metabolism. Phenoloxidase, in the presence of oxygen molecules, can oxidize phenolic quinones. Quinones can inhibit microbial infection and protect themselves.

At present, major producers generally utilize sterilization chemicals and anti-viral drugs, such as calcium oxide, norfloxacin, Povidone iodine, Copper sulfate and calcium hypochlorite, for water treatment. The antibacterial principle of copper sulfate inhibits spore germination or mycelial growth of pathogenic bacteria by releasing soluble copper ions. Under acidic conditions, the release of large amounts of copper ions can coagulate the protoplasm of pathogenic bacteria and play a bactericidal role. Norfloxacin acts on DNA gyrase of pathogenic bacteria, hinders DNA replication and inhibits bacteria. Povidone iodine provides affinity to the bacterial membrane by surfactant, and combines the iodine contained in it with the cytoplasm of the bacterial membrane, so as to oxidize the sulfhydryl compounds, peptides,

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proteins, enzymes and lipids to achieve bactericidal purposes. Sodium hypochlorite is the main component of bleaching powder. Hypochlorite ions and some chloride ions are ionized from dissolved water by water molecules. The strong oxidation of hypochlorite makes it possible to kill bacteria and chloride ions have certain sterilization functions. Therefore, bleaching powder has the effect of sterilization and disinfection. The biochemical reaction of quicklime with water releases a great deal of heat and produces calcium hydroxide. In a short period of time, the pH value of the pool water increases rapidly to above 11 and then microorganisms in the water body are killed. However, the effects these treatments can have on the innate immune systems of crayfish is unknown. The objective of this study was to assess crayfish immune activity following exposure to five common drugs and water treatment chemicals employed in the aquaculture industry, in disease-free crayfish and those infected with WSSV and *Vibrio alginolyticus*. Total hemocytes counts (THC), as well as the activities of phenoloxidase (PO) and superoxide dismutase (SOD) were tested.

2. Methods and materials

2.1. Materials

The healthy adult crayfish (approximately 20 g and 8–10 cm each) were obtained from a seafood market of Zhejiang. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang A & F University (Hangzhou, China). Calcium hypochlorite was purchased from Bojie Environmental Protection Technology Co., Ltd. (Ningbo, China). Calcium Oxide, Norfloxacin and Povidone iodine were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Copper sulfate was purchased from Guangdong Fine Chemical Engineering Research and Development Center. The use concentration is the lowest concentration recommended by manufacturers. The specific use concentration is as follows:calcium hypochlorite, 1.5 g/m^3; quick lime, 15 g/m^3; norfloxacin, 3 mg/m^3; povidone iodine, 0.75 mL/m^3, Copper sulfate 0.7 g/m^3.

2.2. Crayfish group and WSSV, Vibrio alginolyticus stock

Crayfish *P. clarkii*, approximately 20 g and 10 cm each, were reared at 25 °C, They were divided into 13 groups. There are 9 crayfish in each group. They were kept in tanks with sand-filtered, ozone-treated and flow-through fresh water and fed with commercial pellet feed at 5% of body weight per day. In each of the five experimental groups, five drugs were added to the water (calcium hypochlorite, 1.5 g/m^3; quick lime, 15 g/m^3; norfloxacin, 3 mg/m^3; povidone iodine, 0.75 mL/m^3, copper sulfate, 0.7 g/m^3). Walking legs from randomly selected individuals were subjected to PCR assays to ensure that the crayfish were WSSV-free before experimental challenge. Then the crayfish was used for the challenge test as the following, WSSV (LD50 = 1.5×10^5 copies/mL, 200 μL) was used for the challenge test in five experimental groups. And in the other five experimental groups *Vibrio alginolyticus* (LD50 = 1.5×10^6 copies/mL, 200 μL) were used for the challenge test. The WSSV and *Vibrio alginolyticus* stock were prepared according to the previous study [7].

2.3. Superoxide dismutase (SOD) assay

SOD activity was determined according to the previous report using nitro blue tetrazolium (NBT) chloride in the presence of riboflavin. Briefly, 100 mL of hemolymph was homogenized in a mechanical homogenizer containing 0.5 mL of phosphate buffer (50 mM, pH 7.8). The homogenate was centrifuged for 5 min at 6000 g at 4 °C and the supernatant recovered was heated for 5 min at 65 °C to obtain a new supernatant after centrifugation (crude extract), which was stored at 20 °C until use. Samples were maintained on ice at all times to avoid protein denaturation. A mixture of NBT, 20 mM of reaction mixture (0.1 mM EDTA, 13 mM Methionine, 0.75 mM NBT, and 20 mM Riboflavin in Phosphate Buffer, 50 mM, pH 7.8) and 0–100 mL of the crude extract were placed under fluorescent light for 2 min or until A560 in the control tubes reached 0.2 to 0.25 OD. The results were expressed as relative enzyme activity. Each group has three repetitions, and the average value is taken as the final value.

2.4. Prophenoloxidase (proPO) assay

PO activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihy-droxyphenyl alanine (L-DOPA) according to the reported method [8]. Briefly, the diluted hemolymph was centrifuged at 800 × *g* at 4 °C for 20 min to collect the pellet which was resuspended gently in cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride (1.10 M Trisodium citrate, pH 7.0). The suspended pellet was centrifuged again and the pellet was resuspended with 100 mL of cacodylate buffer. The resuspended pellet was incubated with 50 mL trypsin (T- 0303, Sigma, 1 mg/mL) at 25 °C for 10 min, which served as an activator; 50 mL L-DOPA was then added followed by 800 mL of cacodylate buffer 5 min later. The optical density at 490 nm was measured using spectrophotometer-117 (Systronics, Shanghai, China). Each group has three repetitions, and the average value is taken as the final value.

2.5. Total hemocyte count (THC) assay

Hemolymph (100 μL) was withdrawn from the ventral sinus of each crayfish into a 1 mL sterile syringe (25 gauge) containing 0.9 mL anticogulant solution (Trisodium Citrate 30 mM, Sodium Chloride 0.34M, Ethylenediaminetetraac-Etic acid (EDTA) 10 mM, pH 7.55). A drop of the anticoagulant-hemolymph mixture (20 μL) was placed on a hemocytometer, and a THC was made under an inverted phase-contrast microscope (Leica DMIL, Germany). Each group has three repetitions, and the average value is taken as the final value.

2.6. Mortality statistics

Crayfish *P. clarkii*, approximately 20 g and 10 cm each, were reared at 25 °C, They were divided into 13 groups. There are 20 crayfish in each group. They were kept in tanks with sand-filtered, ozone-treated and flow-through fresh water and fed with commercial pellet feed at 5% of body weight per day. In each of the five experimental groups, five drugs were added to the water (The same amount as 2.2). Crayfish in the experimental group were infected with WSSV or *V. alginolyticus*, and statistical mortality everyday (15 days).

2.7. Apoptosis analysis by flow cytometry

Apoptosis assays were conducted with BD Phrmingen TM FITC Annexin V Apoptosis Kit (Invitrogen, USA), following to the manufacturer's protocol. The hemolymph was drawn using 2 mL syringe with 20 mM of EDTA at a ratio of 1:1, and hemocytes were collect from the mixture which was centrifuged at 300 g at 4 °C for 5 min. Subsequently, acquired hemocytes were suspended with PBS, counted and adjusted with PBS to a cell density of 5×10^6 cells/mL. And then, The blood cells of 500 μL were mixed with 25 μL FITC labeled WSSV. Place on ice for half an hour, Centrifuge at 300 g, centrifugation at 4 °C for 5 min, Discarding the supernatant, high salt PBS suspension cells. repeat two times. High salt PBS was prepared by immobilized paraformaldehyde at the final concentration of 1% (avoid light preservation in 4 °C), and the percentage of phagocytosis was counted by flow cytometry.

2.8. Data analysis

The mean and standard deviation of three repeated experiments

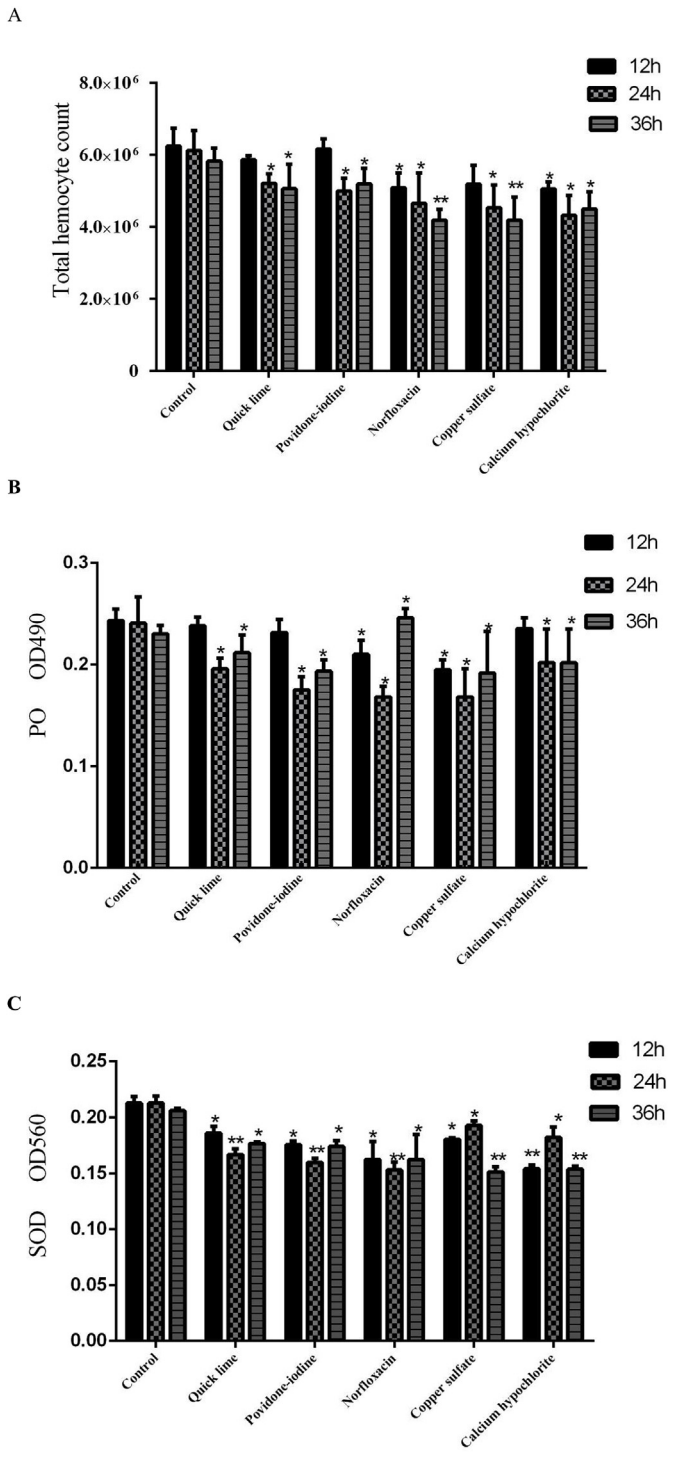

Fig. 1. Total hemocyte count (THC), PO activity, and SOD activity of crayfish in fresh water for five drugs. Data are shown as means ± SD (standard deviation) of three separate individuals in the tissues. Means in the same column sharing a same superscript letter are not significantly different and determined by Tukey's test ($P > 0.05$). Double asterisks indicate a very significant difference ($P < 0.01$) to the control for a given time period. Single asterisks indicate a significant difference ($P < 0.05$) to the control.

were obtained by one-way ANOVA with three independent experimental data. Unidirectional variance analysis was used to estimate statistical differences in accordance with the lowest bit difference (LSD) and Duncan multiple comparisons. All measurements were analyzed using SPSS 19. Probability level 0.01 was used to show statistically significant differences ($P < 0.01$).

3. Results

3.1. Immunological parameters of non-infected crayfish

Disease-free crayfish were exposed to five different treatments:,-norfloxacin, calcium hypochlorite, quick lime, povidone iodine and copper sulfate. Starting from the time of injection of 0 h, the anticoagulant was extracted by syringe and blood cells were extracted according to 1:1 ratio. Each crayfish was sampled three times (12 h, 24 h and 36 h). After 12 h in the treatments, THC in crayfish treated with, norfloxacin and calcium hypochlorite were significantly lower ($P < 0.05$) than those in the control group (Fig. 1A). By 24 h, THC were significantly lower ($P < 0.05$) in crayfish treated with all five compounds than those in the control group. By 36 h, norfloxacin and copper sulfate treatments had led to highly significant decreases ($P < 0.01$) in THC compared with the control group.

PO activities in crayfish treated with norfloxacin and copper sulfate were significantly lower ($P < 0.05$) following 12 h than in crayfish in the control group, while there was no significant difference between the other treatments and the control (Fig. 1B). At 36 h, PO activities in crayfish treated with calcium hypochlorite were significantly higher ($P < 0.05$) than the control, whereas they were significantly lower ($P < 0.05$) than the control in crayfish treated with all four other treatments. PO activity of quick lime, povidone iodine, copper sulfate and calcium hypochlorite groups were decreased significantly ($P < 0.05$) compared with the control group by 24 h.

SOD activities in crayfish treated with all five compounds were significantly lower ($P < 0.05$) following 12 h in the treatments compared with those in the control group (Fig. 1C). By 24 and 36 h, SOD activities in crayfish under all treatments were significantly lower ($P < 0.05$) than those in the control. These differences were highly significant ($P < 0.01$) in crayfish treated with copper sulfate and calcium hypochlorite groups after 36 h. It can be concluded that these commonly used fishery drugs and chemicals can have adverse effects on the immune parameters of cultured crayfish.

3.2. Immunological parameters of WSSV-infected crayfish

Following 24 h exposure to the experimental treatments, crayfish were challenged by WSSV. THC in crayfish treated with norfloxacin and calcium hypochlorite groups were significantly lower ($P < 0.05$) than in the control crayfish group at 12 h post-challenge (Fig. 2A). By 24 and 36 h post-challenge, THC in crayfish from all treatments were significantly lower ($P < 0.05$) than in the control. These differences were highly significant ($P < 0.01$) in crayfish treated with norfloxacin and copper sulfate.

PO activities in crayfish treated with povidone iodine and calcium hypochlorite groups were significantly higher ($P < 0.05$) at 12 h post-challenge than in the control crayfish (Fig. 2B). By 24 h post-challenge, PO activities in crayfish from all treatments, except quick lime, were significantly lower ($P < 0.05$) than in those from the control. By 36 h post-challenge, only the PO activities of crayfish treated with povidone iodine, copper sulfate and calcium hypochlorite were significantly lower ($P < 0.05$) than in the control crayfish.

SOD activities in crayfish treated with all five compounds, except norfloxacin, were significantly lower ($P < 0.05$) at 12 h post-challenge than in the control crayfish (Fig. 2C), and by 24 and 36 h SOD activities in crayfish across all treatments were significantly lower than the control ($P < 0.05$). In crayfish treated with calcium hypochlorite, these differences were highly significant ($P < 0.01$) at 36 h post-challenge. It can be concluded that these commonly used fishery compounds can reduce the immune parameters of cultured crayfish when infected with WSSV.

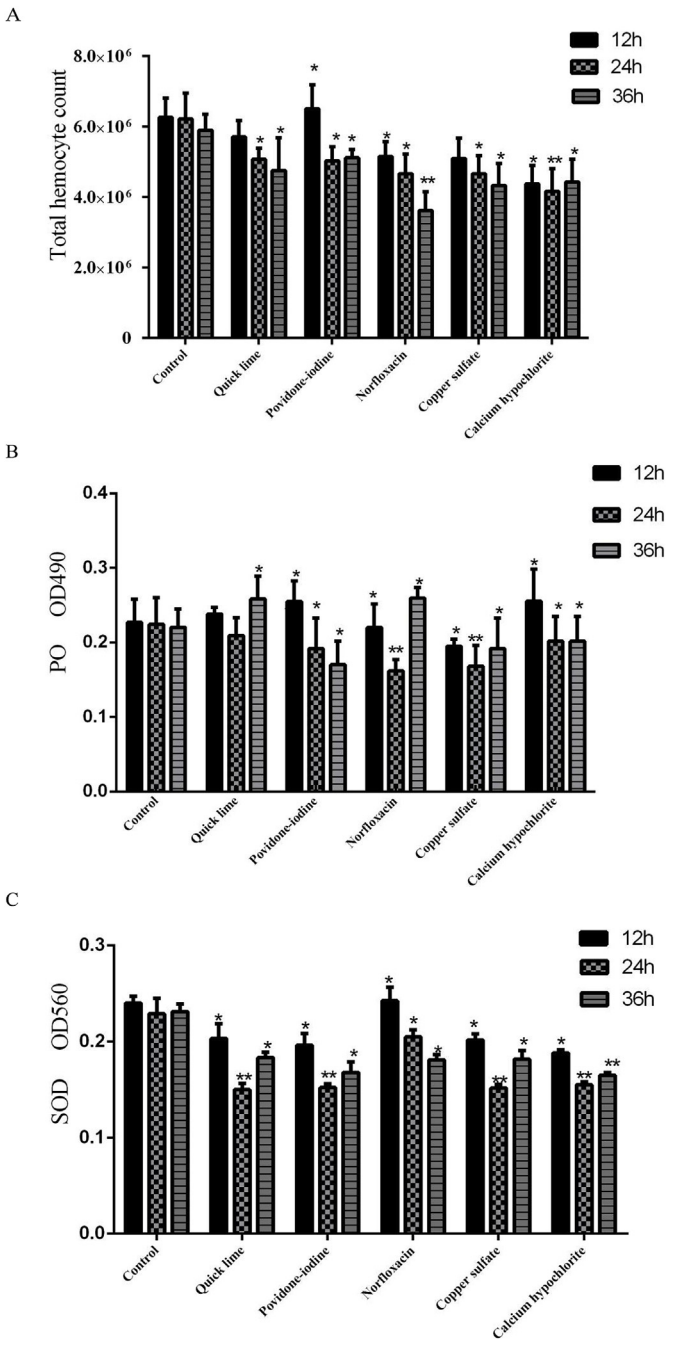

Fig. 2. Total hemocyte count (THC), PO activity, and SOD activity of crayfish in fresh water for five drugs with WSSV infection. Data are shown as means ± SD (standard deviation) of three separate individuals in the tissues. Means in the same column sharing a same superscript letter are not significantly different and determined by Tukey's test ($P > 0.05$). Double asterisks indicate a very significant difference ($P < 0.01$) to the control for a given time period. Single asterisks indicate a significant difference ($P < 0.05$) to the control.

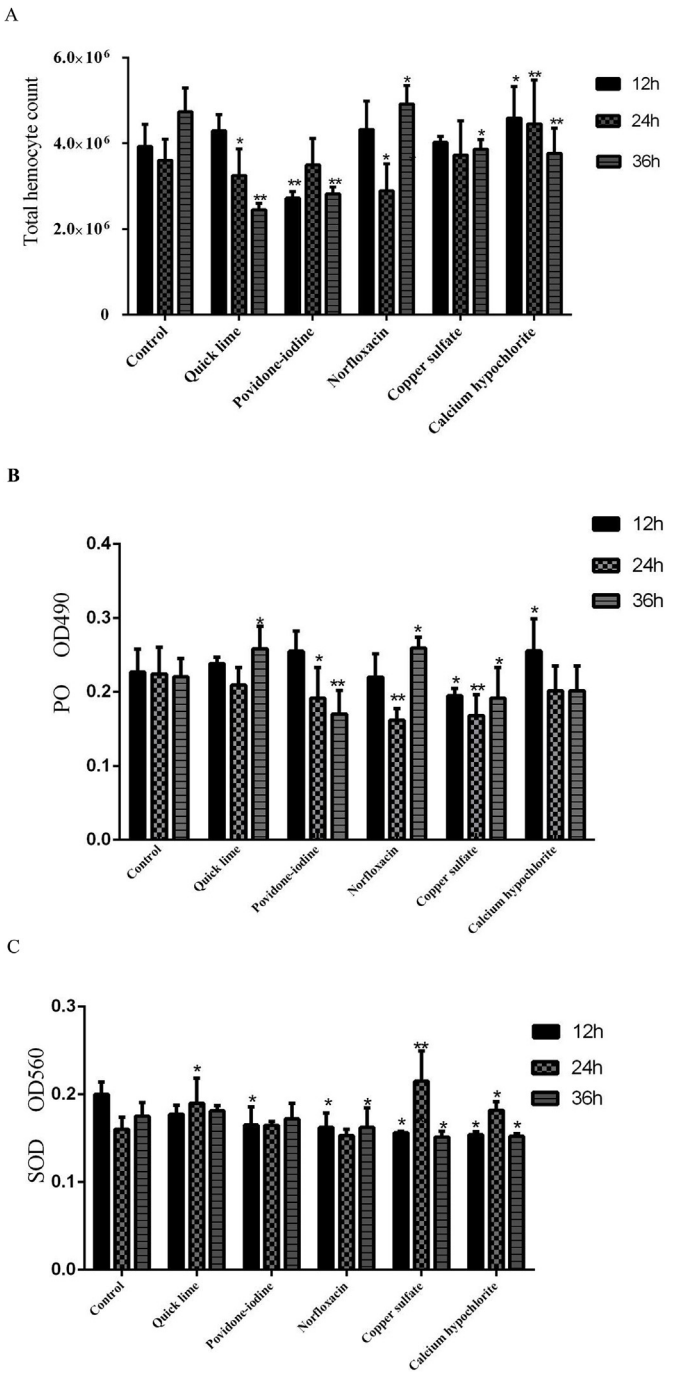

Fig. 3. Total hemocyte count (THC), PO activity, and SOD activity of crayfish in fresh water for five drugs with *Vibrio alginolyticus* infection. Data are shown as means ± SD (standard deviation) of three separate individuals in the tissues. Means in the same column sharing a same superscript letter are not significantly different and determined by Tukey's test ($P > 0.05$). Double asterisks indicate a very significant difference ($P < 0.01$) to the control for a given time period. Single asterisks indicate a significant difference ($P < 0.05$) to the control.

3.3. Immunological parameters of crayfish infected with V. alginolyticus

Following 24 h exposure to the experimental treatments, crayfish were challenged by *V. alginolyticus*. At 12 h post-challenge, THC in crayfish treated with povidone iodine were lower than in control crayfish, and the difference was highly significant ($P < 0.01$) (Fig. 3A). By 24 h post-challenge, THC in crayfish treated with quick lime, and norfloxacin were also significantly lower ($P < 0.05$) than in control crayfish. By 36 h post-challenge, THC in crayfish under all treatments except norfloxacin were significantly lower ($P < 0.05$) than in control crayfish. For crayfish treated with quick lime, povidone iodine and calcium hypochlorite these differences were highly significant ($P < 0.01$).

PO activities in crayfish treated with copper sulfate were significantly lower ($P < 0.05$) at 12 h post-challenge than in control crayfish (Fig. 3B). By 24 h post-challenge, PO activities in crayfish treated with povidone iodine, norfloxacin and copper sulfate were all

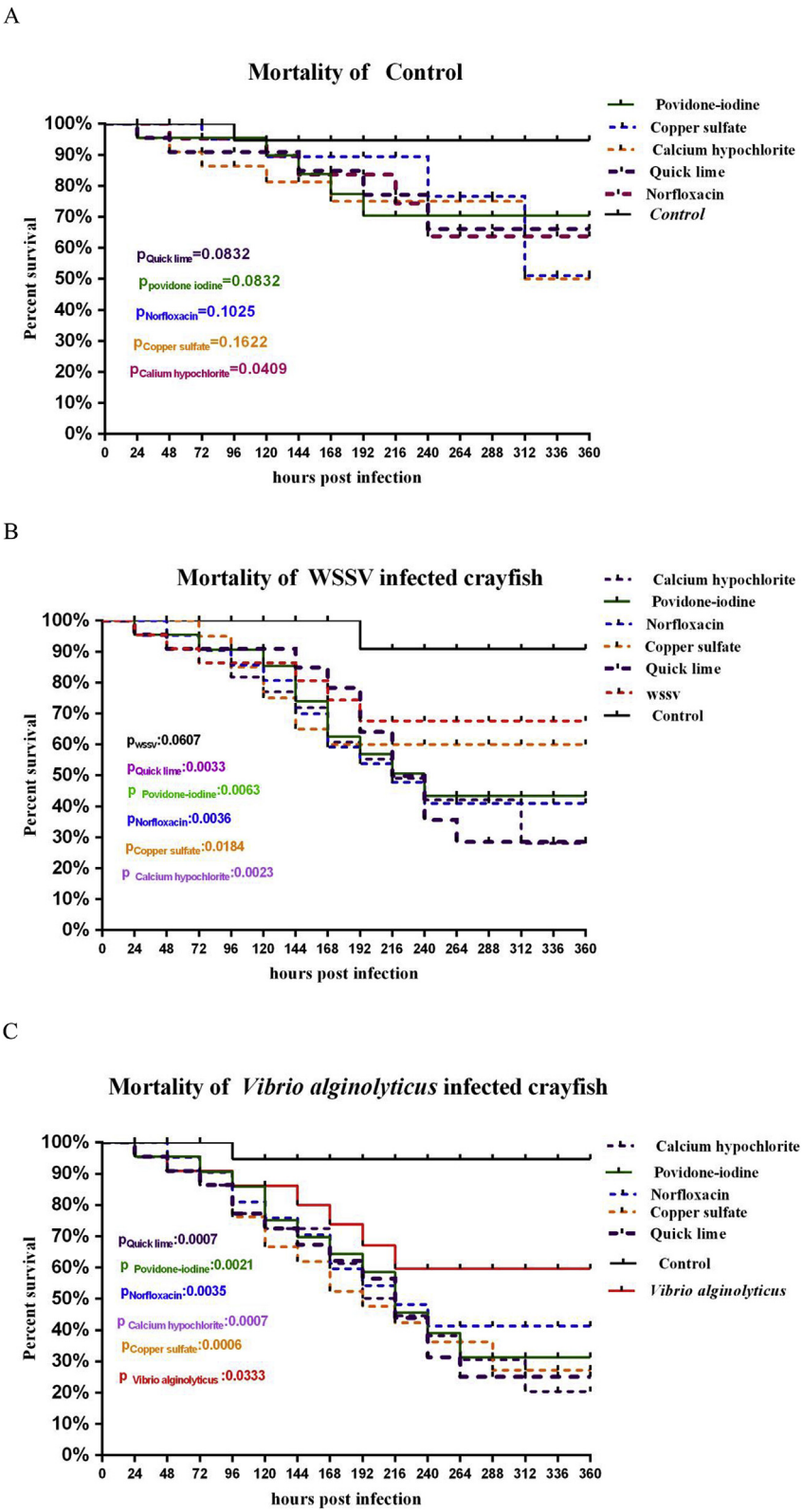

Fig. 4. Cumulative mortality of crayfish infected with white spot syndrome virus (WSSV) and *Vibrio alginolyticus*, and treated with quick lime, povidone iodine, norfloxacin, copper sulfate and calcium hypochlorite. Mortality was monitored continuously and recorded twice daily for 15 days. The mortality rate in each group (20 individuals) is presented as mean ± SD for triplicate independent experiments.

significantly lower ($P < 0.05$) than in control crayfish, and by 36 h these differences were highly significant ($P < 0.01$) in crayfish treated with povidone iodine and copper sulfate. In contrast, PO activities in crayfish treated with quick lime and norfloxacin were significantly higher ($P < 0.05$) than in control crayfish at 36 h post-challenge.

SOD activities in crayfish treated with povidone iodine, norfloxacin, copper sulfate and calcium hypochlorite were significantly lower ($P < 0.05$) at 12 h post-challenge than in control crayfish (Fig. 3C).

However, by 24 h post-challenge, SOD activities in crayfish treated with quick lime, copper sulfate and calcium hypochlorite were significantly higher ($P < 0.05$) than in control crayfish. By 36 h post-challenge, SOD activities in crayfish treated with norfloxacin, copper sulfate and calcium hypochlorite were significantly lower ($P < 0.05$) than in control crayfish. It can be concluded that these commonly used fishery drugs and chemicals can reduce the immune parameters of cultured crayfish when infected with *V. alginolyticus*.

3.4. The mortality of crayfish infected with WSSV or Vibrio alginolyticus

Disease-free crayfish survival rates decreased over time in all experimental treatments (Fig. 4A). Specifically, after 312 h the survival rates of crayfish treated with all five compounds were significantly lower ($P < 0.01$) than in control crayfish. In particular, crayfish treated with copper sulfate and calcium hypochlorite showed the lowest survival rates. Overall, data indicate that the mortality of crayfish can increase significantly when treatments exceed 24 h. However, crayfish survival rates remain equivalent to the control when fresh water is replaced following 24 h of treatment (data not shown).

In WSSV-infected crayfish, fresh water was replaced following 24 h of drug treatments at which time the WSSV challenge occurred. After 312 h the survival rates of crayfish under all treatments except copper sulfate were significantly lower ($P < 0.01$) than in WSSV-infected crayfish (Fig. 4B). In particular, crayfish treated with quick lime and calcium hypochlorite showed the lowest survival rates.

The *V. alginolyticus*-infected crayfish experiment was carried out as per the WSSV challenge outlined above. After 312 h, the survival rates of crayfish under all five treatments were significantly lower ($P < 0.01$) than those infected with *V. alginolyticus* (Fig. 4C). In particular, crayfish treated with calcium hypochlorite showed the lowest survival rates. We can conclude that these commonly used fishery drugs and chemicals can increase the mortality of cultured crayfish that are infected with WSSV or *V. alginolyticus*.

3.5. Hemocyte apoptosis of crayfish

After 24 h in the treatments, the hemocyte apoptosis rates were significantly greater ($P < 0.05$) in disease-free crayfish treated with all five compounds compared to crayfish infected with *V. alginolyticus* (Fig. 5G). No matter the infection of WSSV or *V. alginolyticus,* the apoptotic rate of povidone iodine, norfloxacin, cupric sulfate and calcium hypochlorite was significantly higher than that of the control group (Fig. 6G). In particular, crayfish treated with calcium hypochlorite showed the highest hemocyte apoptosis rate. At 24 h post WSSV-challenge, the hemocyte apoptosis rate was significantly greater ($P < 0.05$) in crayfish in all five treatments, except quick lime, compared to control crayfish (Fig. 6G). In particular, crayfish treated with calcium hypochlorite and norfloxacin showed significantly higher ($P < 0.01$) hemocyte apoptosis rates than other treatments (Fig. 6G). But the highest rate of apoptosis is calcium hypochlorite. At 24 h post *V. alginolyticus*-challenge, the hemocyte apoptosis rate was significantly greater ($P < 0.05$) in all treated crayfish compared with the control crayfish (Fig. 7G). In particular, crayfish treated with calcium hypochlorite showed the highest hemocyte apoptosis rate (Fig. 6G). We concluded that these commonly used aquatic medicines and chemicals can significantly increase the apoptosis rate of blood cells infected with *V. alginolyticus*.

Control + *V. alginolyticus*; (B) Quicklime + *V. alginolyticus*; (C) Povidone-iodine + *V. alginolyticus*; (D) Norfloxacin + *V. alginolyticus*; (E) Copper sulfate + *V. alginolyticus*; (F) Calcium hypochlorite + *V. alginolyticus*; (G) Columnar graph of hemocyte apoptosis. Data are shown as means ± SD (standard deviation) of three separate individuals in the tissues. Means in the same column sharing a same superscript letter are not significantly different and determined by Tukey's test ($P > 0.05$). Double asterisks indicate a very significant difference ($P < 0.01$) to the control for a given time period. Single asterisks indicate a significant difference ($P < 0.05$) to the control.

4. Discussion

Antibiotics and chemical agents have been used to effectively kill pathogens in aquaculture for many years [4]. However, the application of antibiotics or chemicals often only target a single class of pathogenic microorganisms but not all that affect the system. In addition, the effects of antibiotics and chemicals on immunity and disease resistance in aquatic organisms is largely unknown. In the present study, we aimed to investigate the changes in immunity and disease resistance in crayfish *P. clarkii* following treatment with five common drugs and water treatment chemicals used in the aquaculture industry. We found that THC, as well as the activities of PO and SOD, decreased in crayfish following all five treatments. Each drug has a different effect on the crayfish. Quicklime, Povidone-iodine and Copper sulfate whether or not they were also infected with WSSV or vibriosis, THC showed a downward trend, The effect of quicklime on *V. alginolyticus* infection is the most significant. And this result is also reflected in cell apoptosis, *V. alginolyticus* soaked in quicklime group was significantly higher than that in control group and WSSV infection group. After infected with WSSV or *V. alginolyticus*, the THC of the norfloxacin group increased first and then decreased, and the apoptosis rate was also significantly higher than that of the control group. Norfloxacin group also has a high mortality rate in the mortality rate of infected shrimp. The influence on the immune system of the crayfish can not be ignored, Quicklime and Copper sulfate these three drugs have similar effects B These three drugs have little effect on the apoptosis rate of crayfish, but they have a certain effect on mortality. especially calcium hypochlorite, can significantly enhance the hemocyte apoptosis rate in crayfish whether or not they were also infected with WSSV or vibriosis. Calcium hypochlorite also caused higher mortality than the other treatments in infected crayfish. As calcium hypochlorite is widely used for water purification in aquaculture, its influence on the immunity of aquatic animals should not be ignored. Therefore, the infection of WSSV or *V. alginolyticus* to the shrimp should be avoided after water purification in the process of aquaculture, otherwise the loss will be much higher than that of the untreated water.

WSSV, which was first discovered in Taiwan in 1992, has caused mass mortalities and devastating production losses to shrimp farming in many areas [9,10]. WSSV is known to infect many crustacean species, including crayfish [11,12]. Both farmed and wild *P. clarkii* in Louisiana (USA) are natural hosts for WSSV [13], and the disease has also now become a considerable threat to *P. clarkii* culture in China. Vibriosis was initially introduced into crayfish farming via the use of fresh marine food [14,15]. In the present study, WSSV and *V. alginolyticus* infections caused greater mortality in crayfish grown under the five drug and chemical treatments than in those kept in untreated fresh water. Therefore, we can conclude that antibiotics and water treatment chemicals have the potential to negatively impact the immunity of crustaceans, thereby reducing their resistance to viral and bacterial diseases. Increasing levels of mortality are caused by environmental stress in crustacean farming [16,17], and the results of our study implicate these commonly used drugs and water purification chemicals as additional stressors for crustaceans.

A total of 32 antibiotics have been detected in aquatic products, with quinolones and sulfonamides being the dominant residual chemicals [4]. Through the consumption of aquatic products tainted by antibiotics, humans may acquire adverse drug reactions or antibiotic-resistant bacteria. Greater attention is being focused on food safety in modern society, and antibiotics are gradually becoming banned from use in aquaculture systems. Probiotics and natural medicines are increasingly being used instead for the prevention and treatment of aquatic animal diseases [18–21]. Feeding crayfish a prebiotic (xylooligosaccharide) and probiotic (*Enterococcus faecalis*) supplemented diet

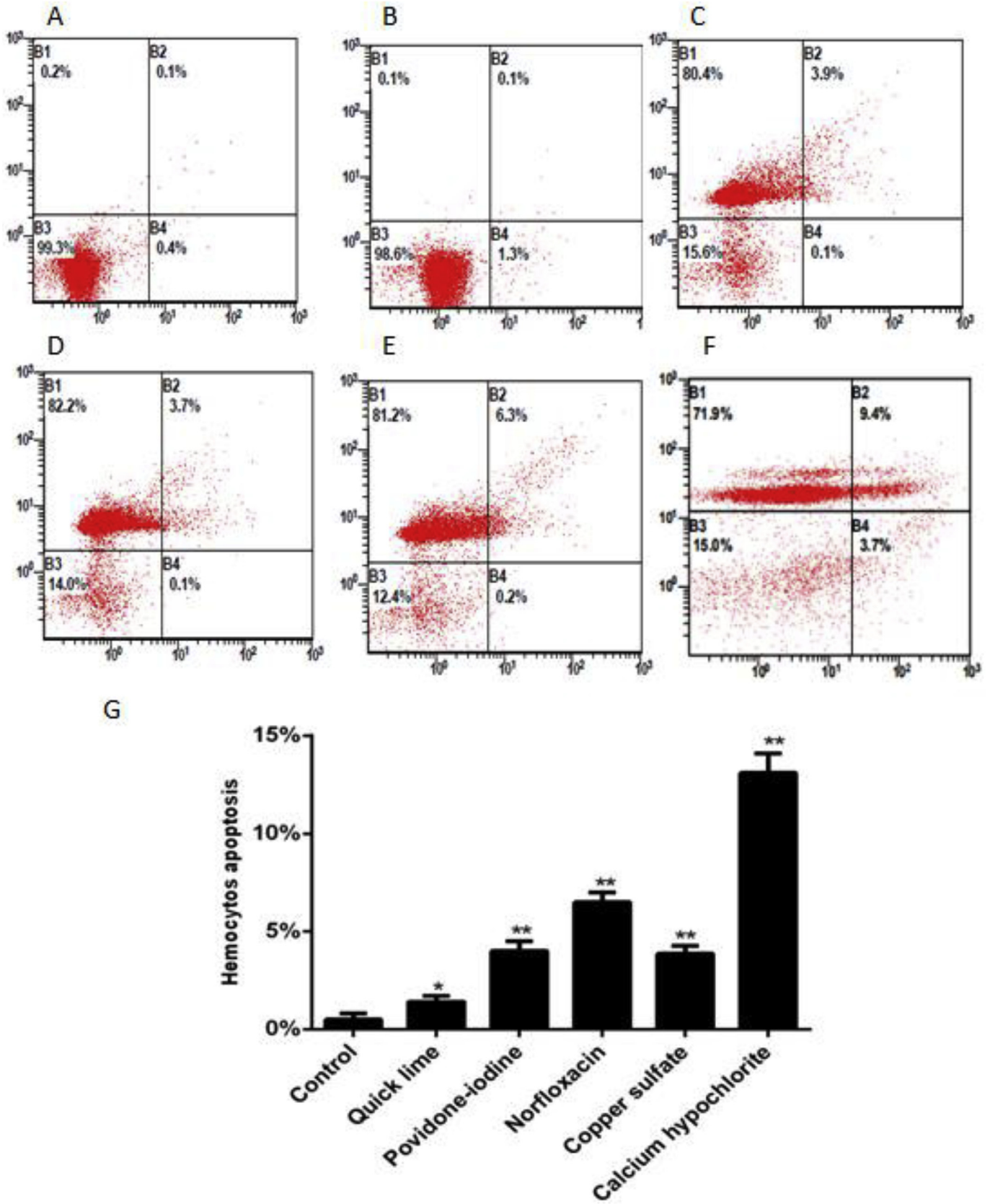

Fig. 5. Flow cytometry detection of hemocyte apoptosis in crayfish. (A) Control; (B) Quicklime; (C) Povidone-iodine;; (D) Norfloxacin; (E) Copper sulfate; (F) Calcium hypochlorite; (G) Columnar graph of hemocyte apoptosis. Data are shown as means ± SD. Columns sharing superscript letters are not significantly different as determined by Tukey's test ($P > 0.05$). Double asterisks indicate a very significant difference ($P < 0.01$) to the control for a given time period. Single asterisks indicate a significant difference ($P < 0.05$) to the control.

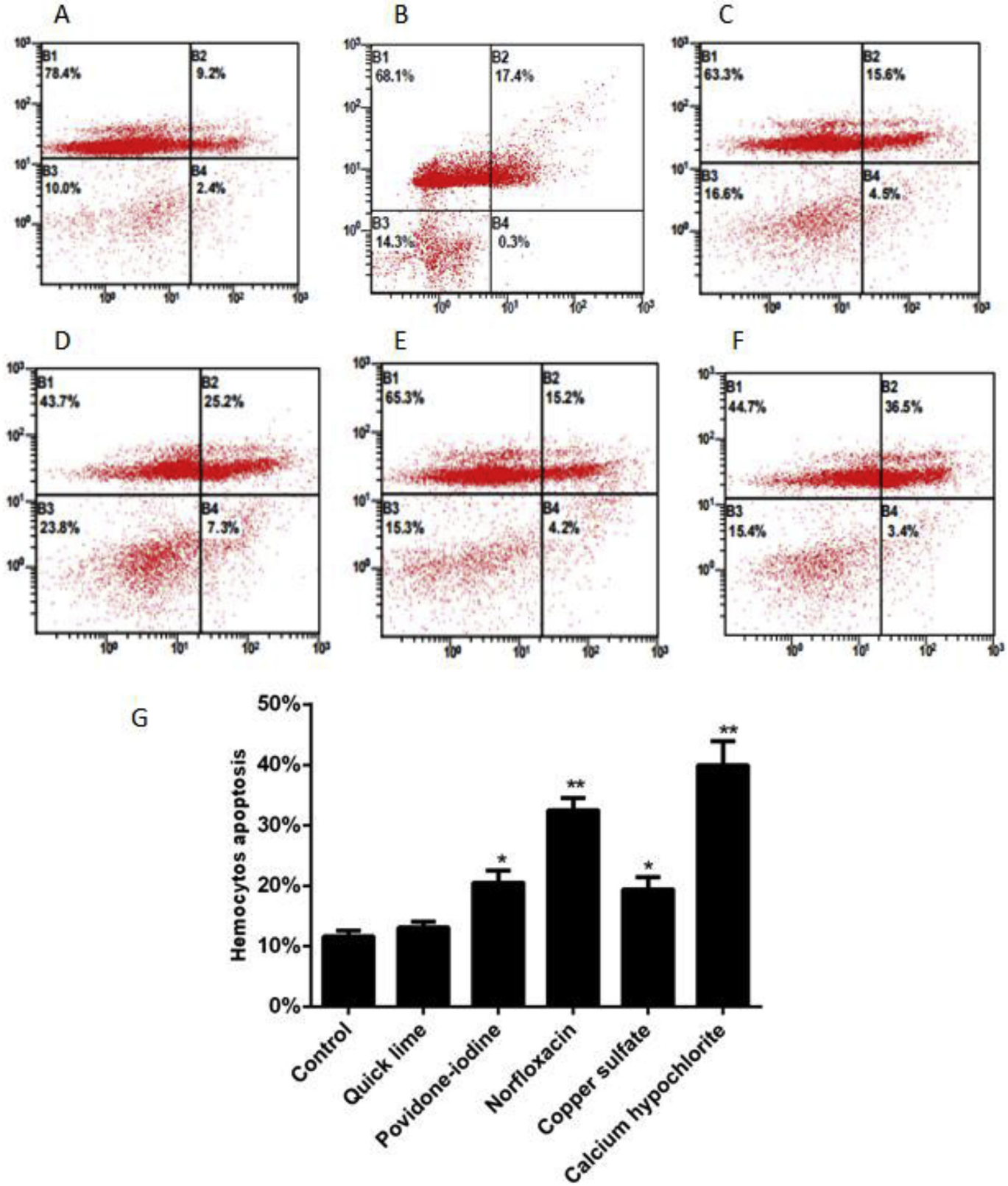

Fig. 6. Flow cytometry detection of hemocyte apoptosis in crayfish with WSSV infection. (A) Control + WSSV; (B) Quicklime + WSSV; (C) Povidone-iodine + WSSV; (D) Norfloxacin + WSSV; (E) Copper sulfate + WSSV; (F) Calcium hypochlorite + WSSV; (G) Columnar graph of hemocyte apoptosis. Data are shown as means ± SD (standard deviation) of three separate individuals in the tissues. Means in the same column sharing a same superscript letter are not significantly different and determined by Tukey's test ($P > 0.05$). Double asterisks indicate a very significant difference ($P < 0.01$) to the control for a given time period. Single asterisks indicate a significant difference ($P < 0.05$) to the control.

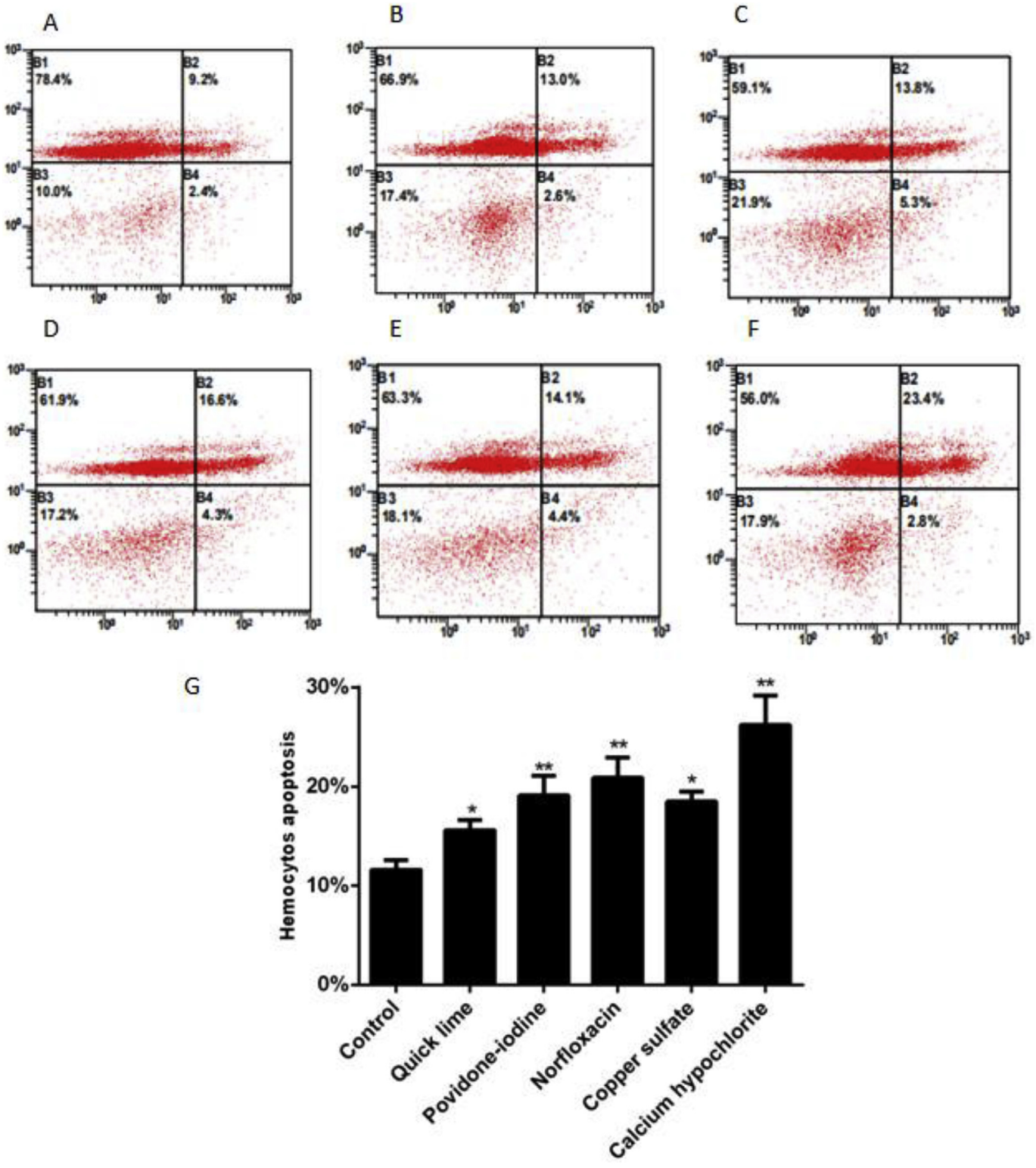

Fig. 7. Flow cytometry detection of hemocyte apoptosis in crayfish with *Vibrio alginolyticus* infection.

has been shown to increase growth rates as well as resistance to *Aeromonas hydrophila* [22]. Therefore, to support optimal food safety and human health, the use of antibiotics and chemicals should be reduced in aquaculture, and the use of probiotics and natural drugs should be vigorously promoted.

Acknowledgments

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