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Original Article

Antibacterial activity and mechanism of berberine on avian *Pasteurella multocida*

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Abstract: In this study, the antibacterial activity and mechanism of berberine against avian *Pasteurella multocida* were investigated by evaluating bacteria growth, observing ultrastructure changes and studying the synthesis of protein and DNA. The antibacterial susceptibility test indicated the minimum inhibition concentration (MIC) of berberine against *P. multocida* was 39 μg/mL and the antibacterial kinetic curves showed that the antibacterial activity of berberine was in a concentration-time-dependent manner. After treatment with berberine (39 μg/mL), the bacteria cells were severely damaged, irregular cell shape, ruptured cell wall and membrane, condensed cytoplasm or lost cytoplasm were observed under the transmission electron microscopy. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) stain assays demonstrated that berberine could inhibit the synthesis of protein and DNA. In conclusion, these results suggested that berberine inhibit the cell function of *P. multocida* by damaging the cell structure and inhibiting synthesis of protein and DNA, resulting in the cell death.

Keywords: Berberine, *Pasteurella multocida*, antibacterial activity, mechanism

Introduction

P. multocida is the causative agent of various animal diseases, such as fowl cholera, bovine and rabbit hemorrhagic septicemia, enzootic pneumonia, swine atropic rhinitis [1]. Fowl cholera, caused by avian *P. multocida*, is a common and widely distributed disease of poultry. The acute form of the disease is mainly present a septicaemic disease with high morbidity and mortality; chronic form is often characterized by chronic airway inflammation and pneumonia [2]. The feed intake of infected poultry is decreased, and the somatic growth slows down or the egg production rate falls sharply. And the mortality rate is usually 20% to 30% or higher. The disease cause huge economic losses to poultry industry [3]. Antibiotic therapy is the main treatment in controling *P. multocida* infection. However, in recent years, antibiotic resistance has developed due to the indiscriminate use of commercial antibiotics in pathogenic bacteria [4]. So there is a great need to develop ecologically sustainable antimicrobial agents with high efficacy and low toxicity to combat this problem.

In our previous study, extract of *Rhizoma coptidis* from Sichuan of China and *Cortex phellodendri* had showed a strong antibacterial activity *in vitro* against *P. multocida* [5]. Berberine, a main active ingredient of *Rhizoma coptidis* and *Cortex phellodendri*, has anti-inflammatory [6, 7], antimicrobial [8, 9], and antiviral effects [10]. In recent years, berberine as a broad-spectrum antimicrobial agent has attracted more and more attention [11, 12]. However, so far there are no available reports about antibacterial activity and mechanism of berberine on *P. multocida*, or continueing in-depth exploration.

The aim of this study was to evaluate the antibacterial activity of berberine and elucidate its action mechanism against *P. multocida*.

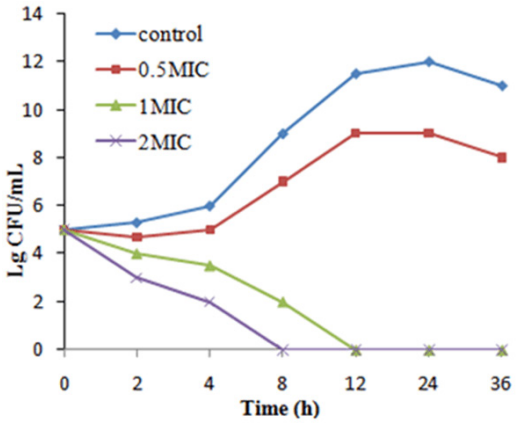


Figure 1. The time-kill curves of berberine against *P. multocida*.

Materials and methods

Microbial strain and chemicals

P. multocida (C481 A:1 strain, obtained from the China veterinary culture collection center, Beijing, China) was cultivated in Meller-Hinton (MH) broth which contained 0.5% calf serum (GIBCO). Inoculum was incubated on Nutrient agar which contained 0.5% calf serum, and then single colony was proliferated in MH broth which contained 0.5% calf serum. Berberine hydrochloride with the purity of ≥98% was obtained from China Control Institute of veterinary bio-products and pharmaceuticals, Beijing. The berberine was dissolved in 6.25% DMSO.

Antibacterial susceptibility test

Minimum inhibition concentration (MIC) value of *P. multocida* was determined by broth dilution method described in the National Committee for Clinical Laboratory Standards [13]. The berberine was added into MH broth to achieve concentrations ranging from 5 mg/mL to 0.039 mg/mL. Then, the bacterial suspension was added into the MH broths to approximately achieve a final concentration of 1×10^7 CFU/mL. 6.25% DMSO was used as a negative control. The OD_{600} values of each tube were measured with a UV spectrophotometer before incubation and measured again after 24 h of incubation at 37°C. Similar values of OD_{600} after 24 h indicated the absence of *P. multocida* growth. MIC is the berberine concentration in the tube [14].

Antibacterial kinetic curves study

Three different concentrations of berberine (0.5 MIC, MIC and 2 MIC) were added into tubes containing bacterial inoculum (roughly 5×10^5 CFU/mL). All samples were incubated at 37°C for 0, 2, 4, 8, 12, 24 and 36 h, then 0.1 mL sample was collected from each tube for colony counting. At least, two replications were performed for each sample.

Transmission electron microscopy (TEM) assay

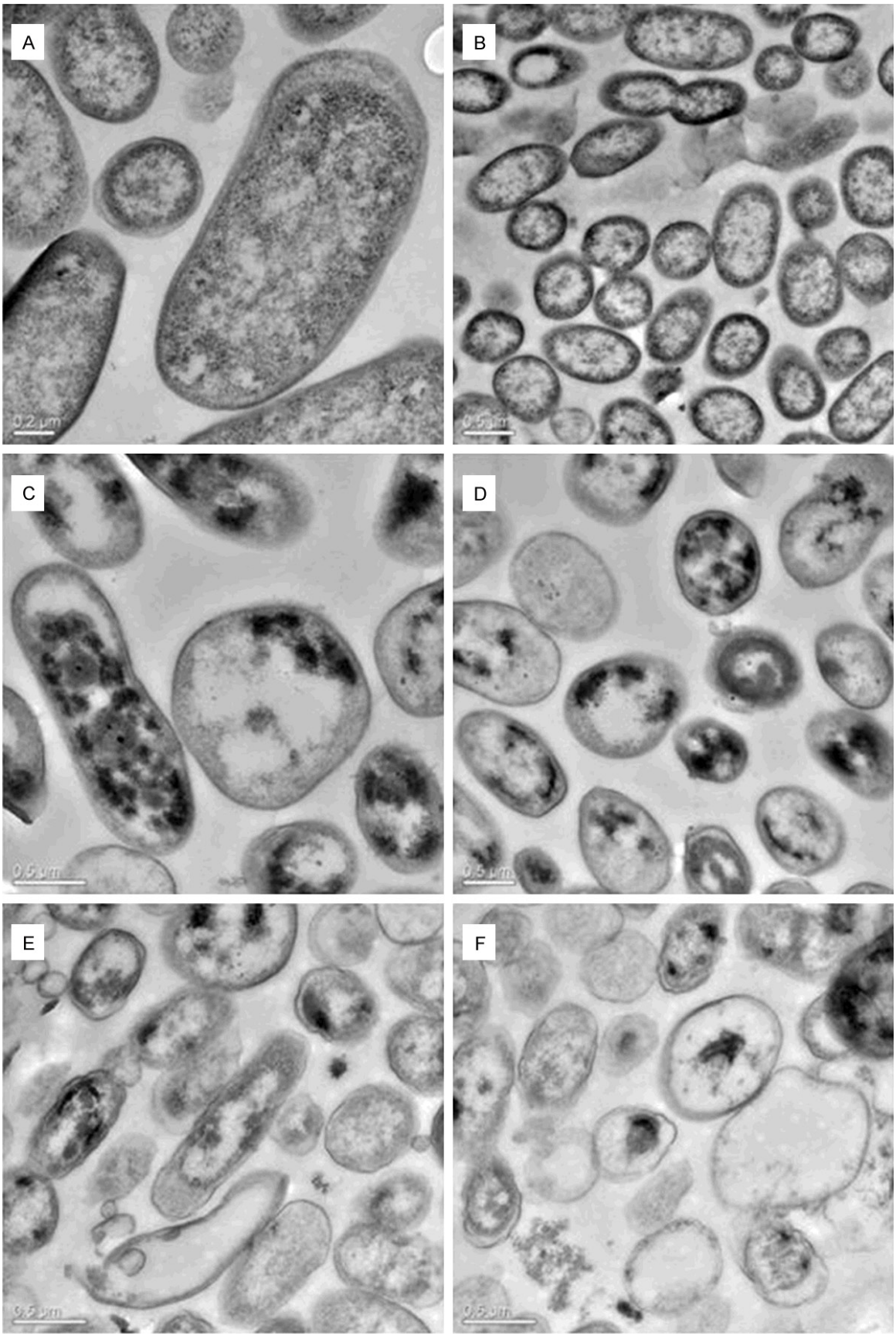
10 mL of *P. multocida* suspensions at the concentration of 10^8 CFU/mL were exposed to MIC concentration of berberine, while bacteria cultured in MH broth without berberine was used as the control. The cultures were incubated at 37°C for 4 h and 8 h, respectively. The *P. multocida* suspensions were centrifuged at 8000 g for 15 min at 4°C, and then bacteria cells were washed by saline for three times. Then the pellet was fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.2) overnight at 4°C. After dehydrated, embedded and stained [15], morphology of the *P. multocida* cells were observed undera transmission electron microscope.

SDS-PAGE assay

P. multocida cells (10^8 CFU/mL) were treated with MIC concentration of berberine. Controlled experiment was conducted in absence of berberine. Samples were collected at different time intervals (1, 2, 4, 8 and 12 h) and centrifuged for 10 min at 6000 g. The number of bacteria in each group was adjusted to the same. Then 150 μL di-distilled water and 50 μL protein loading buffer were added to the pellet. Samples were denatured for 10 min and then 10 μL of each sample was loaded on the gel. Electrophoresis was performed at a constant voltage of 80 V through the stacking gel (5%) and at 120 V through the separation gel (12%). After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 and then decolorized to obtain the separated protein bands.

DAPI staining assay

The berberine was diluted into the concentration of MIC with MH broth and added into the bacteria suspension at final concentration of 10^8 CFU/mL. Physiological saline group was used as control. The cultures were incubated at


A
0.2 μm
B
0.5 μm
C
0.5 μm
D
0.5 μm
E
0.5 μm
F
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Fugure 2. TEM diagrams of *p. multocida* cells treated and untreated with berberine. A and B are untreated cells. C and D are treated cells at concentration of MIC for 4 h. E and F are treated cells at concentration of MIC for 8 h.

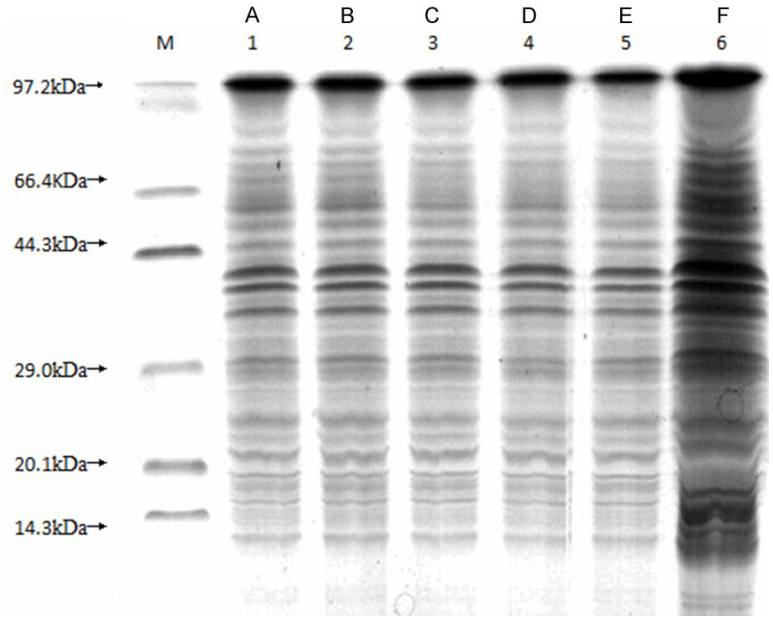

Figure 3. SDS-PAGE profiles of treated and untreated group. Lanes 1-5 were protein patterns of *P.* multocida treated with berberine for 1 h, 2 h, 4 h, 8 h and 12 h, respectively. Lane 6 was protein patterns of normal cells.

37°C with shaking at 150 rpm for 10 h. Then 1.0 mL suspension was mixed with 1 μg/mL DAPI, and incubated at room temperature in the dark for 1 h. A small drop of the mixture was dispersed on the glass slide to observe DNA fluorescence intensity under fluorescence microscope.

Results

Antibacterial activity of berberine

The susceptibility test showed the MIC value of berberine against *P. multocida* was 39 μg/mL, while 6.25% DMSO had no effect on growth of *P. multocida.*

Antibacterial kinetic curves of berberine against P. multocida

Antibacterial kinetic curves of berberine (**Figure 1**) showed that the growth curves of *P. multocida* in the 0.5-MIC treated group had the same integral growth cycle with control group: adjustment phase, logarithmic phase, stable phase and decline phase, except a lower multiplication rate. Whilein the MIC- and 2MIC-treated group, *P. multocida* directly entered into decline phase without adjustment phase, logarithmic phase and stable phase. As shown in **Figure 1**, all of the *P. multocida* cells were killed by berberine at MIC within 12 h and 2 MIC within 8 h, respectively.

Ultrastructural changes of P. multocida

In the TEM graphs, normal *P. multocida* cells showed a typical structure with intact cell wall, smooth membrane, a uniformly distributed cytoplasm and clear nuclear area in the middle of cells (**Figure 2A** and **2B**). While the structure of *P. multocida* cells in the MIC-treated group was different from normal cells. After treatment for 4 h, some cell walls and membranes were dissolved and cytoplasm had lost its even distribution. Besides, the chromatin of some cells partly dissolved and migrated to the cell edge (**Figure 2C** and **2D**). After treatment for 8 h, cells were severely damaged, the shape of most of cells became irregular, plasmolysis appeared in the cell most of cells also had lost cytoplasmic contents, even resulting in cytoplasmic vacuolation (**Figure 2E** and **2F**).

Protein analysis of P. multocida cells treated with berberine

SDS-PAGE protein profiles of untreated and treated *P. multocida* cells were shown in **Figure 3**. As seen in **Figure 3**, the protein profiles of treated bacteria were significantly different from those of the control. Protein bands of treated bacteria cells (**Figure 3A-E**, lanes 1-5) became much fainter, and some even disappeared compared with the untreated cells (**Figure 3F**, lane 6). The protein profiles of treated bacteria for different times were also different slightly. Protein bands of lane 1 were almost the same as lane 2 and 3. But most of protein bands (approximately 29.0 kDa to 97.2 kDa) in

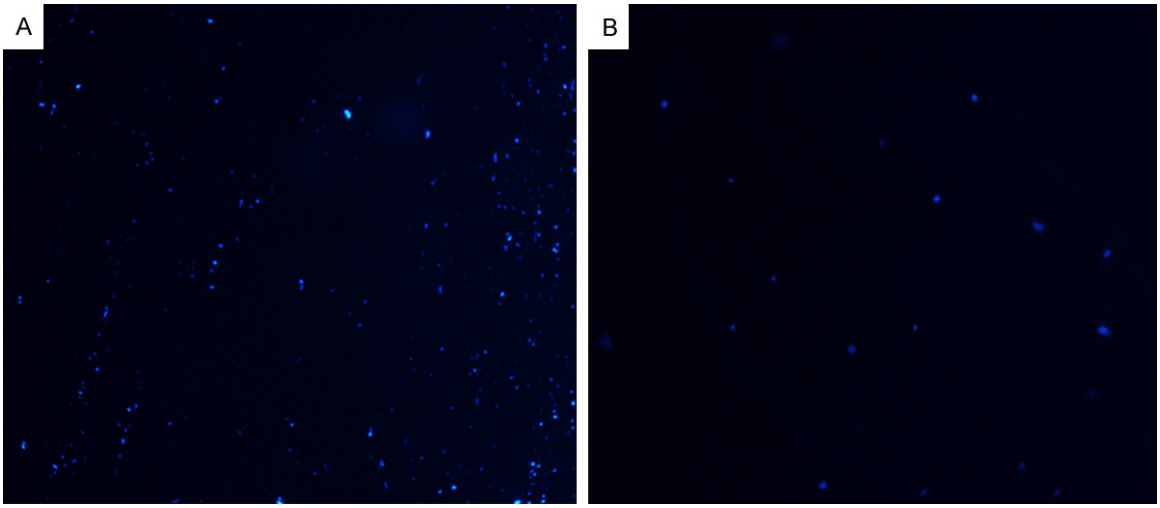

Figure 4. Control group (A): DNA fluorescence intensity of *P.* multocida showing fluorescing bright; the experimental group (B): DNA fluorescence intensity was weaker.

lane 4 and 5 became shallower compared with those in lane 1, 2 and 3. The result showed the protein synthesis of *P. multocida* could be inhibited by berberine.

DAPI staining assay

The fluorescing stain DAPI is a highly specific stain for DNA under a wide range of conditions. It is mostly used as a cytochemical probe for nuclear, mitochondrial, and chloroplast DNA [16]. As shown in **Figure 4**, the DNA fluorescence intensity of untreated *P. multocida* (**Figure 4A**) was obviously stronger than that of the treated cells (**Figure 4B**). It was concluded that berberine has inhibitory effect against DNA synthesis.

Discussion

In the present study, the MIC of berberine against *P. multocida in vitro* was 39 μg/mL. Previous studies have reported the MIC of berberine against *Streptococcus agalactiae* was 78 μg/mL [17] and against *Actinobacillus pleuropneumoniae* was 313 μg/mL [18], compared with these results, berberine exhibited stronger antibacterial activity to *P. multocida*. Meanwhile, the result of antibacterial kinetic curves showed at concentration of 39 μg/mL, all the bacteria cells could be killed within 12 h. Previous studies reported berberine exhibited antibacterial activities against *Escherchia coli* and *Bacilu subtilis*, which under the concentration of 582 μg/mL and 952 μg/mL would cause 50% decrease of growth rate constant of *E. coli* and *B. subtilis*, respectively [4]. Thus it is obvious that berberine against *P. multocida* had a stronger inhibitory effect than most other bacteria. However, the antibacterial kinetic-curves showed berberine at concentration of 0.5 MIC could only inhibit reproduction of *P. multocida*, but after treatment with MIC and 2 MIC berberine, *P. multocida* cells were completely killed within 8 h and 12 h. It indicated berberine inhibit the growth of *P. multocida* mainly dependent on concentration and time. The result provides more rational basis for determining optimal dosage for antimicrobial treatment. Although the antimicrobial activity of berberine was weaker than antibiotics, berberine could not easily get their antimicrobial resistance strain and multiple drug-resistant bacteria [19], thus berberine might be considered to be applied in clinical practice.

Many drugs from herbs achieved its antibacterial effect mainly by acting on the cellular structure and affecting the function of bacteria [20]. Under the transmission electron microscopy, ultrastructure of treated bacteria cells had changed and the cell membrane was distorted and dissolved. The out membrane plays an important role in maintaining the morphology and protecting the cellular contents. Normal metabolism and growth of bacteria could be affected by broken cell membrane and wall [21, 22]. So we speculated the cell death might be concerned with the extensive

loss of intracellular vital contents passed through the damaged cell membrane.

Furthermore, we searched for further possible antibacterial mechanism of berberine against *P. multocida* by investigating the macromolecular synthesis. The SDS-PAGE result showed some protein bands of bacteria treated with berberine became fainter and even disappeared. Studies have shown that the extract of opuntia could inhibit protein synthesis, finally caused the protein bands decrease and color dodge [23, 24]. It was speculated that DNA have blocked the protein synthesis or the synthesis process by antibacterial drugs [23, 24]. It is worth mentioning that in our study, the DNA fluorescence intensity of treated group was weaker than that of the control group. It was illuminated that berberine might be block protein synthesis by inhibiting DNA synthesis. This is consisted with other research results [17, 18].

Although the berberine has good antibacterial effect, it is worth mentioning that berberine has cytotoxicity in some eukaryotic cells, and the cytotoxicity is different in different cells, the cytotoxicity is even presented in dose and time dependent manners [25]. Therefore, it is necessary to ascertain the toxic concentration at first to guide clinical application.

In summarize, berberine exhibited strong antibacterial activity against *P. multocida* by damaging the physical structure, leading to leakage of cell contents and inhibiting synthesis of protein and DNA. These results provide a theoretical basis for clinical practice of berberine. Nevertheless, this study only demonstrated the preliminary mechanism of berberine against *P. multocida*, the further interaction of berberine with *P. multocida* still need to be explored in future research.

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Disclosure of conflict of interest

None.

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