



Baicalin prevents *Candida albicans* infections via increasing its apoptosis rate



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ARTICLE INFO

Article history:

Received 21 June 2014

Available online 24 July 2014

Keywords:

Baicalin
Candida albicans
Drug target
Apoptosis

ABSTRACT

Background: These experiments were employed to explore the mechanisms underlying baicalin action on *Candida albicans*.

Methodology and principal findings: We detected the baicalin inhibition effects on three isotope-labeled precursors of ^3H -UdR, ^3H -TdR and ^3H -leucine incorporation into *C. albicans* using the isotope incorporation technology. The activities of Succinate Dehydrogenase (SDH), cytochrome oxidase (CCO) and Ca^{2+} - Mg^{2+} ATPase, cytosolic Ca^{2+} concentration, the cell cycle and apoptosis, as well as the ultrastructure of *C. albicans* were also tested. We found that baicalin inhibited ^3H -UdR, ^3H -TdR and ^3H -leucine incorporation into *C. albicans* ($P < 0.005$). The activities of the SDH and Ca^{2+} - Mg^{2+} ATPase of *C. albicans* in baicalin groups were lower than those in control group ($P < 0.05$). Ca^{2+} concentrations of *C. albicans* in baicalin groups were much higher than those in control group ($P < 0.05$). The ratio of *C. albicans* at the G0/G1 stage increased in baicalin groups in dose dependent manner ($P < 0.01$). There were a significant differences in the apoptosis rate of *C. albicans* between baicalin and control groups ($P < 0.01$). After 12–48 h incubation with baicalin (1 mg/ml), *C. albicans* shown to be markedly damaged under transmission electron micrographs.

Innovation and significance: Baicalin can increase the apoptosis rate of *C. albicans*. These effects of Baicalin may involved in its inhibiting the activities of the SDH and Ca^{2+} - Mg^{2+} ATPase, increasing cytosolic Ca^{2+} content and damaging the ultrastructure of *C. albicans*.

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1. Introduction

Candida albicans is a human normal typical flora but also a common opportunistic deep-infective fungus. Due to the extensive utilization of radiotherapy, chemotherapy, the immunosuppressive agents, the broad spectrum antibiotics and various indwelling catheters, *C. albicans* has been demonstrated to become the most common fungi in patients with a weakened immune system who had an internal environment disorder and dysbacteriosis [1–4]. Therefore, the treatment of *C. albicans* infection has attracted the interests of the worldwide researchers.

Since amphotericin B was firstly employed as an antifungal agent, it has been over 40 years for human to treat fungal diseases [5–10]. Considering several disadvantages of the western antifungal medicine existed, such as its high price, toxicity and

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<http://dx.doi.org/10.1016/j.bbrc.2014.07.040>

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drug-resistance, etc., many researchers have all the time been to looking for the more broad spectrum, highly effective and non-toxic antifungal agents. Recently, extracting antifungal agents from plants began to be popular. However, most of the works just limited to isolating and extracting the antifungal active components from Chinese medicine, the mechanisms underlying the effects of those antifungal components on *C. albicans* were seldom studied. Roman et al. [11–13] have reported that citral altered the mitochondrial morphology in *aspergillus flavus*. In addition, their studies further suggests that citral can not only reduce the synthesis and utilization of cell energy but also inhibit the synthesis of NADPH, thus, the irreversible inhibition of the synthesis of nucleic acids, proteins, lipids and carbohydrate finally result in cell death. Several literature [14,15] have demonstrated that three effective components of antifungal agents (berberine, eugenol and curcumin) inhibited the cell division of *C. albicans*. Our previous work [5] has also investigated the mechanism underlie the resistance of cinnamaldehyde and citral to *aspergillus* using the isotope-labeled method and scanning electron microscopy, and revealed that these compounds might eliminate fungi by damaging the

structure of cell wall and membrane, as well as interfering the synthesis of DNA, RNA and protein in *aspergillus flavus*.

Huangqin is a widely used, source-plentiful and cheap Chinese herb. Pharmacological studies suggest that Huangqin and its active ingredients baicalin play as anti-inflammatory, antibacterial, antipyretic, antihypertensive antifungal roles [5,16–22]. They are also helpful in diuresis, choleresis, liver protection, immune regulation and antitumor therapy [5,16,19,21,23,24]. There are some papers written in Chinese reporting the effects of baicalein, aglycone of baicalin and baiclin on *Candida*. For example, Dai et al. observed that baicalein treatment induced apoptosis in *C. albicans* cells, which associated with the breakdown of mitochondrial membrane potential. However, the influences of baicalin on *C. albicans* are not fully elucidated. Here our experiments mainly further explored the mechanism underlying the protective effects of baicalin on *C. Albicans* infections by inducing its apoptosis.

2. Materials and methods

2.1. Materials

C. albicans strains (2.2086) were purchased from the Chinese Academy of Preventive Medicine. Baicalin with 98% purity was provided by the Institute of Traditional Chinese Medicine in Jiangxi Province. The isotopes ^3H -UdR with a concentration of 1.0 mCi/ml and a specific radiation activity of 18.0 Ci/mmo1; ^3H -TdR with a concentration of 1.0 mCi/ml and a specific radiation activity of 30.0 Ci/mmo1; ^3H -leucine with a concentration of 0.5 mCi/ml and a specific radiation activity of 60.0 Ci/mmo1) were provided by Shanghai Nuclear Technology Development Co., Ltd. in the Chinese Academy of Sciences. Horse heart cytochrome C and bovine serum albumin (BSA) were from the Sigma, USA; RPM11640 powder purchased from the Gibco Co.USA. SDH and ultramicro ATP enzyme kit were from the Nanjing Jiancheng Co.; Fura-2/AM purchased from Biotium Co. USA.

2.2. Isotope-labeled precursor incorporation test

Baicalin at concentrations of 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml were added in 96-well plate. 20.0 μl 2.5×10^4 cfu/ml fungi strains solution was added to each well prior to 20.0 μl 0.5 $\mu\text{Ci/ml}$ ^3H -UdR, ^3H -TdR and ^3H -leucine. The total volume of each well was 200.0 μl with Sabouraud's liquid medium supplemented. In control group, baicalin was replaced by sterile normal saline (NS). Then the 96-well plate was incubated in a wet box at 35 °C for 24 h. The samples were collected with a multichannel cell collector and absorbed onto 49 type glass fiber filter paper, after dried at 80 °C for 2 h, they were put into a flash bottle. Then, flash liquid added and bathed overnight. Pulses per min (cpmw) in samples were detected with β -Liquid Scintillation Counter. Incorporation inhibition rate was calculated according to the formula [incorporation inhibition rate = (average value of cpm in control group – average value of cpm in test groups)/average value of cpm in control group \times 100%].

2.3. Separation of mitochondria

C. albicans suspensions were treated with different doses of baicalin and incubated at 35 °C for the indicated period. After being washed twice, the suspensions at a concentration of 1×10^7 cfu/ml were obtained. These samples were repeated freeze–thaw 3–5 times and levigated to the homogenate. Afterwards, 4.5 ml of *C. albicans* homogenate was slowly added to cover the surface of 4.5 ml of 0.34 mol/L sugar solution in centrifuge tube. The samples were centrifuged at 1300 g for 5 min and the supernatant collected

and re-centrifuged at 17,000 g for 15 min, removed the supernatant, and the precipitates were re-suspended in buffer to harvest mitochondrial samples.

2.4. Detection of mitochondrial protein contents using the Lowry method

Mitochondrial protein contents were examined using the Lowry method indicated in our previous paper [25].

2.5. Activity analysis of SDH and Ca^{2+} - Mg^{2+} ATPase

Activities of the SDH and the Ca^{2+} - Mg^{2+} ATPase in *C. albicans* mitochondria were detected according to the manufactures' instructions.

2.6. Detection of CCO activity

1.5 ml 100 mmol/L phosphate buffer (pH 7.0) and 0.1 ml mitochondrial suspension (containing 50 μg of mitochondrial protein) were diluted with distilled water to 2.9 ml and incubated at 30 °C for 2 min. After adding 0.1 ml reductive cytochrome C, the oxidation rate of the reductive cytochrome C was examined with a spectrophotometer to calculate the CCO activity.

2.7. Detection of the changes of *C. albicans* cytoplasmic free Ca^{2+} by Fura-2/AM

C. albicans suspensions (1×10^7 cfu/ml) were treated with different doses of baicalin and incubated at 35 °C for the indicated period. The harvested *C. albicans* were re-suspended in phosphate-buffered saline (PBS) to a concentration of 2×10^6 cfu/ml. After adding Fura-2/AM to a final concentration of 5 $\mu\text{mol/L}$, the samples were incubated at 35 °C for 1 h with constantly oscillations in dark. Then, the redundant Fura-2AM was washed away with PBS, *C. albicans* suspensions were absorbed in a quartz colorimetric tube to be detected by a Hitachi-850 fluorescence spectrophotometer at 500 nm wavelength. The excitation spectrum was scanned at a speed of 2000 nm/min from 300 nm to 400 nm. Values of the fluorescence excitation peak at 340 nm and 380 nm were recorded to determine the Fura-2/AM probe load in *C. albicans*. The fluorescence intensity recorded at 340 nm indicates the cytoplasmic free Ca^{2+} concentration of *C. albicans*. Data were expressed as mean \pm standard deviation ($\bar{x} \pm s$) and analyzed with the SPSS.

2.8. Determination of the cell cycle and the apoptosis rate of *C. albicans* by flow cytometry

C. albicans suspensions were treated with different doses of baicalin and incubated at 35 °C for the indicated period. The samples in each group were washed twice, and the precipitates were mixed and re-suspended with PBS. Afterwards, they were undergone 3 times freeze–thaw, the suspension was washed twice with PBS and adjusted to a concentration of 1×10^7 cfu/ml. After adding 1 ml propidium iodide (PI), the suspensions were hold at 4 °C for 24 h then, the PI was washed away, the samples were filtered with 300-well nylon net. Finally, the cell cycle and the apoptosis rate of *C. albicans* were detected by flow cytometry.

2.9. The ultrastructure of *C. albicans* under transmission electron microscope (TEM)

C. albicans were incubated with 1 mg/ml baicalin or NS and for 12 h, 24 h and 48 h at 35 °C respectively. The samples were collected in due time and centrifugated at 1300 g for 5 min. After discarding the supernatant, the precipitate was washed twice with

PBS, pre-fixed with 2.5% glutaraldehyde, then fixed with 1% osmium tetroxide and suffered a gradient of dehydration with ethanol and acetone. Afterwards, the samples were embedded with resin, made into ultrathin sections and double-stained with uranyl acetate-lead nitrate solutions. In the end, these samples were to be examined under TEM.

3. Results

3.1. Influence of baicalin on the biosynthesis of DNA, RNA and polypeptide in *C. albicans*

The decreased cpm values of isotope-labeled precursors ($^3\text{H-UdR}$, $^3\text{H-TdR}$ and $^3\text{H-leucine}$) accompanied with the increased baicalin contents (0.25 mg/ml, 0.5 mg/ml and 1 mg/ml), indicating that the incorporation inhibition rates of isotope-labeled precursors ($^3\text{H-UdR}$, $^3\text{H-TdR}$ and $^3\text{H-leucine}$) were positively linked with the baicalin contents. The incorporation inhibition rate of $^3\text{H-UdR}$ -labeled precursor was higher than that of $^3\text{H-TdR}$ and $^3\text{H-leucine}$ -labeled precursors ($P < 0.005$). However, no significant differences in the incorporation inhibition rate existed between $^3\text{H-TdR}$ and $^3\text{H-leucine}$ -labeled precursors ($P > 0.05$) (Table 1).

3.2. Influence of baicalin on the activities of SDH, Ca^{2+} - Mg^{2+} ATPase and CCO in *C. albicans* mitochondria

The activities of SDH and Ca^{2+} - Mg^{2+} ATPase in *C. albicans* mitochondria in 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml baicalin groups were significantly lower than those of control group ($P < 0.05$), suggesting that these enzymes activities were negatively associated with baicalin doses (Fig. 1). However, the CCO activity was not to be affected by baicalin ($P > 0.05$). In addition, the activities of SDH, Ca^{2+} - Mg^{2+} ATPase and CCO in *C. albicans* appeared not to be influenced by incubation time with baicalin.

3.3. Influence of baicalin on the cytoplasmic free Ca^{2+} concentration in *C. albicans*

the cytoplasmic free Ca^{2+} concentrations in *C. albicans* in 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml baicalin groups were significantly higher than those in control group ($P < 0.05$), implying that the cytoplasmic free Ca^{2+} concentrations were positively related to the baicalin doses. The cytoplasmic free Ca^{2+} concentrations were also gradually increased with baicalin incubation time from 6 h to 24 h ($P < 0.05$) (Table 2).

3.4. Influence of baicalin on the cell cycle and the apoptosis rate of *C. albicans*

In 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml baicalin groups, the *C. albicans* ratio at the G0/G1 phase was significantly higher than that in control group ($P < 0.01$) (Fig. 2). The apoptosis rate of *C. albicans* in control group was zero after 48 h incubation with NS whereas

Table 1
Incorporation inhibition rates of isotope-labeled precursors.

Concentration of baicalin (mg/ml)	Incorporation inhibition rate (%)		
	$^3\text{H-UdR}$	$^3\text{H-TdR}$	$^3\text{H-leucine}$
0.25	57.33 ± 3.25	52.11 ± 3.85 ^a	49.54 ± 6.87 ^{ab}
0.5	80.09 ± 2.28	66.31 ± 5.55 ^a	61.69 ± 7.77 ^{ab}
1	98.74 ± 0.33	72.96 ± 4.93 ^a	71.97 ± 5.22 ^{ab}

^a $P < 0.01$ vs $^3\text{H-UdR}$.

^b $P > 0.05$ vs $^3\text{H-TdR}$.

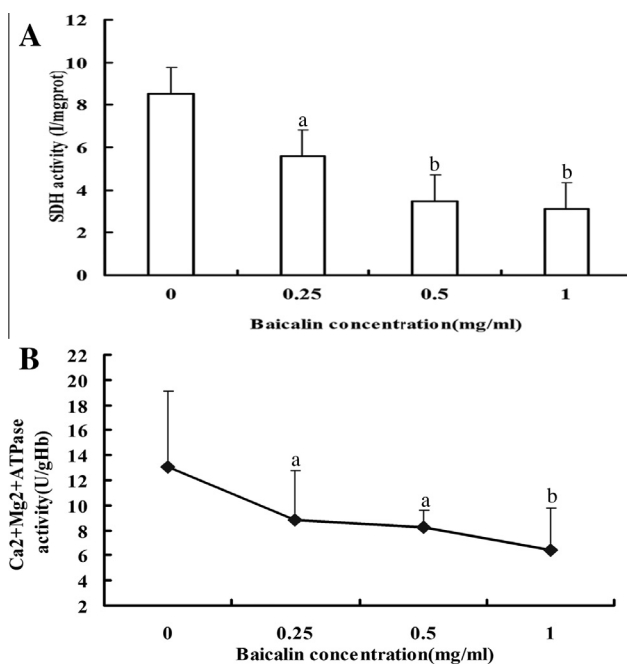


Fig. 1. The activities of SDH (A) and Ca^{2+} - Mg^{2+} ATPase (B) in *Candida albicans* inhibited by baicalin at different doses ($\bar{x} \pm s$). *Candida albicans* fungi suspensions were treated with different concentrations doses of baicalin, and were incubated at 35 °C for the corresponding period. Separation of mitochondria was made as Section 2. Activities of the SDH and Ca^{2+} - Mg^{2+} ATPase in the *Candida albicans* mitochondria were detected according to the manufactures' instructions. Compared to the control, ^a indicate $P < 0.05$, ^b indicates $P < 0.01$.

the apoptosis rate in different baicalin groups were markedly increased. Moreover, these results indicated that the apoptosis rate was positively associated with the baicalin doses (Fig. 3).

3.5. Effects of baicalin on the ultrastructure of *C. albicans*

In control group, 24 h incubation with NS, *C. albicans* become round or oval, its wall and membrane were intact, its nucleus and nucleoli with an intact nuclear membrane can be clearly observed, and its cytoplasm appeared to be homogenous. After 48 h incubation, a slight shrinkage in the wall and membrane of

Table 2
Influence of baicalin on *Candida albicans* cytoplasmic free Ca^{2+} ($\bar{x} \pm s$).

Group	Incubation time (h)			
	6	12	24	48
Control	222.16 ± 0.49	228.72 ± 0.41	233.06 ± 4.79	242.92 ± 0.44
Baicalin (0.25 mg/ml)	243.86 ± 2.31 ^a	268.84 ± 0.42 ^b	340.80 ± 5.51 ^b	299.40 ± 0.91 ^b
Baicalin (0.5 mg/ml)	276.28 ± 2.14 ^b	312.62 ± 0.87 ^b	368.00 ± 1.51 ^b	302.08 ± 3.78 ^b
Baicalin (1.0 mg/ml)	280.84 ± 0.38 ^b	322.88 ± 2.26 ^b	400.94 ± 4.84 ^b	298.30 ± 2.22 ^b

Compared to the control,

^a Indicate $P < 0.05$.

^b Indicates $P < 0.01$.

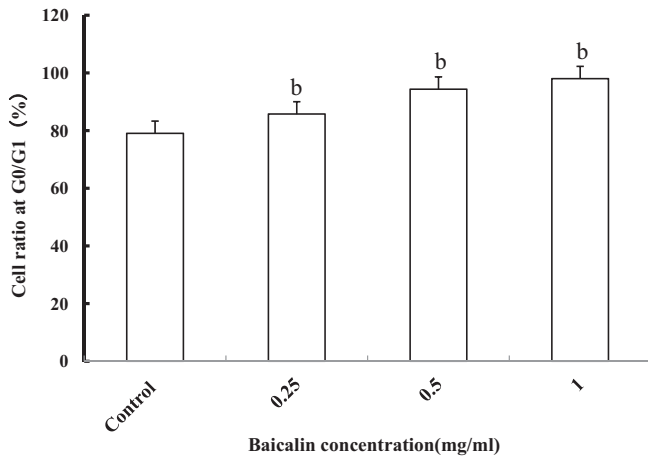


Fig. 2. Influence of baicalin on the cell cycle of *Candida albicans* ($\bar{x} \pm s$). *Candida albicans* fungi suspensions were treated with different doses of baicalin and incubated at 35 °C for the corresponding period. The samples in these groups were treated as Section 2. The cell cycle and the apoptosis rate of *Candida albicans* fungi were detected with flow cytometry. Compared to the control, ^b indicates $P < 0.01$.

C. albicans was observed, and cloud-shape areas with low density of electrons presented in its cytoplasm (Fig. 4). On the other hand, incubated with 1 mg/ml baicalin for 12 h, the wall and membrane of *C. albicans* began to shrink. After 24 h baicalin treatment, the damaged wall, non-homogenous cytoplasm, bubble formation and patchy areas with low electron density were shown in *C. albicans*. Up to 48 h incubation with baicalin, the wall border of *C. albicans* appeared to be blurred and partially dissolved, then nuclear fragments, dissolved organelles and broken cytoplasm were also disclosed. In addition, the walls of some *C. albicans* are clear and intact but several nuclear fragments in the nuclear region were

also observed. Furthermore, the organelles around nucleus disappeared and were replaced by cloud-shape area with low electron density (S1).

4. Discussions

As an opportunistic, deep-infective fungi, *C. albicans* is a primarily infective microbe for the patients with immune dysfunction (e.g., diabetes, tumors, leukemia, organ transplantations and acquired immunodeficiency syndrome (AIDS)) [1–4]. At present, to control *C. albicans* infections clinically using antifungals is quite limited due to its toxicity, high price and drug resistance. Since the 1920s, researchers have been searching for an antifungal drug with higher efficiency and less toxicity. Many Chinese herbs with good antifungal effects were successfully screened in the 1960s. Huangqin and its active component baicalin have been suggested acting as a strong antifungal roles on deep-infective fungi such as *C. albicans* [5,16,19,21,23,24]. Considering its mature extraction methods, its extensive sources, its low toxicity and cheap, baicalin has been proved to be a promising Chinese herb in prevention and treatment of *C. albicans* infections.

After 24 h incubation with baicalin, the damaged cell wall, non-homogenous cytoplasm, bubble formation and patchy areas with low electron density were shown. Up to 48 h incubation, the wall border of *C. albicans* appeared to be blurred and partially dissolved, then nuclear fragments, dissolved organelles and broken cytoplasm were also observed. In addition, the cell walls of some *C. albicans* are clear and intact but several nuclear fragments in the nuclear region were also observed. The organelles around nucleus disappeared and replaced by cloud-shape area with low electron density. These results imply that baicalin may destroy the wall and membrane of *C. albicans*, which may fail to maintain their natural morphological structure and conduct the exchange between

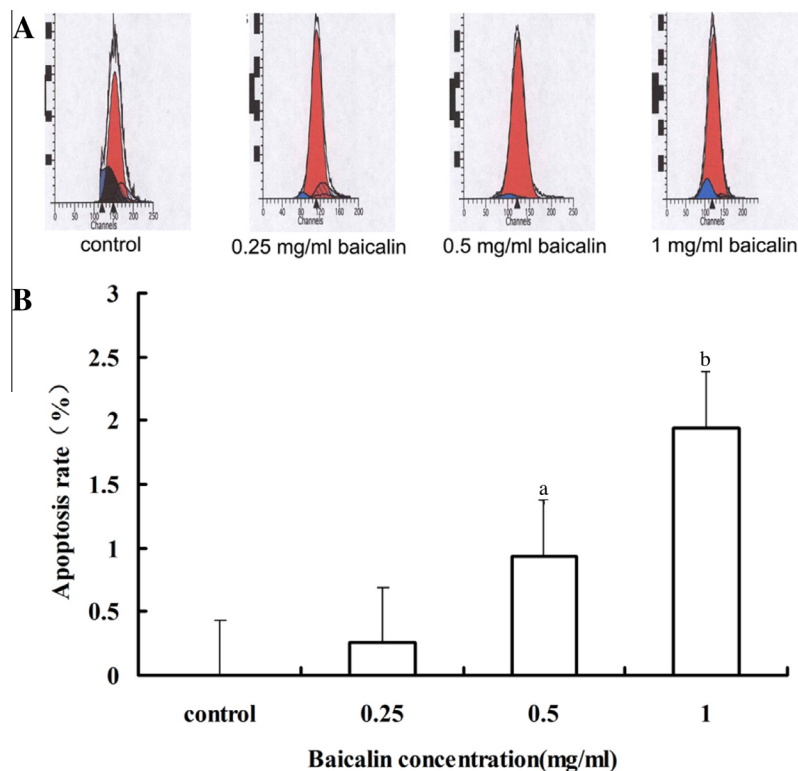


Fig. 3. Influence of baicalin on the cell apoptosis rate of *Candida albicans*. (A) Effect of different baicalin concentrations on the apoptosis rate of *Candida albicans*; (B) quantitative analysis of the influence of baicalin on the cell apoptosis rate of *Candida albicans* ($\bar{x} \pm s$). *Candida albicans* fungi suspensions were treated with different doses of baicalin and incubated at 35 °C for the corresponding period. The samples in these groups were treated as Section 2. The apoptosis rate of *Candida albicans* fungi were detected with flow cytometry. Compared to the control, ^a indicate $P < 0.05$, ^b indicates $P < 0.01$.

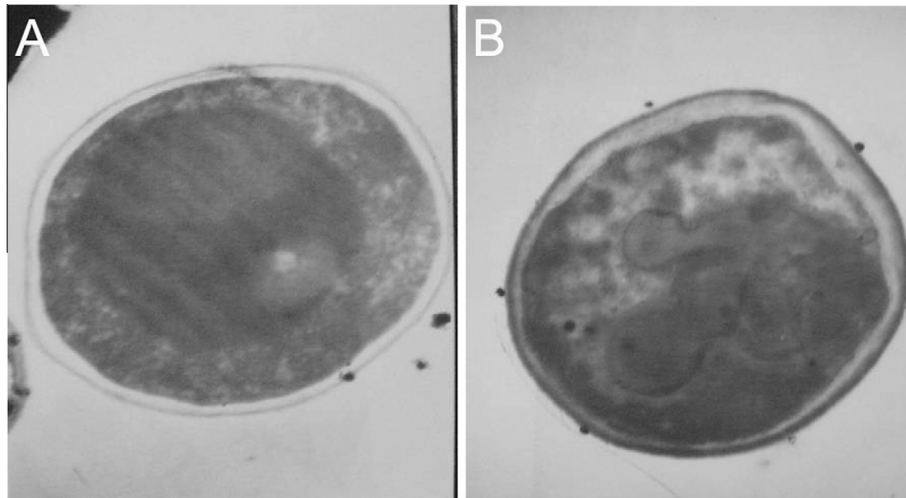


Fig. 4. The ultrastructure of *Candida albicans* without drug (TEM $\times 20,000$, 24 h and 48 h). (A) Cultured after 24 h, (B) cultured after 48 h.

intracellular and extracellular ingredients. Additionally, many enzymes in the cell membrane cannot normally function, thus nutrients absorbed by *C. albicans* decreased and the synthesis of the biological macromolecules reduced, finally leading to these fungal apoptosis even death. These alterations were supported by our observations that baicalin significantly inhibited the infiltration of the three isotope-labeled precursors (^3H -UdR, ^3H -TdR and ^3H -leucine) into *C. albicans*. It suggests that baicalin may cause apoptosis even cell death by inhibiting the synthesis of DNA, RNA and proteins of *C. albicans*.

Mitochondria are a primary organelle responsible for cell respiration and oxidative phosphorylation, it plays an important role in maintaining the normal cellular structure, function and energy metabolism. They correlated with the enzyme activity in the endometrium and matrix. The mitochondria and its enzymes are the targets of many antifungal drugs. To further explore the mechanisms of apoptosis even cell death induced by baicalin, we investigated the influences of baicalin on the activities of SDH, Ca^{2+} - Mg^{2+} ATPase and CCO in *C. albicans* mitochondria. Our works indicated that baicalin decreased the activity of the SDH in *C. albicans* mitochondria, and the higher concentration of baicalin, the lower the SDH activity. However, baicalin failed to affect CCO activity obviously. It has been reported that the alterations in SDH activity accompanied by injured mitochondria simultaneously [21,26]. They also correspond directly to the number of mitochondria and influence the oxidative phosphorylation process. Baicalin, therefore, may damage the mitochondria of *C. albicans* by repressing the SDH activity, then the damaged mitochondria have the respiratory chain and the entire enzymes series inhibited, thus the process of oxidative phosphorylation and the energy coupling system were destroyed, finally, the decrease of energy supplies influenced gene regulation and DNA duplication, leading to cell depression or death.

Ca^{2+} - Mg^{2+} ATPase hydrolyzes ATP and transfers cytoplasmic Ca^{2+} out of the cells to keep a relatively low intracellular Ca^{2+} , which is one of the important mechanisms maintaining cell stability and its functions. Early in 1975, Marriott [27] first reported the existence of *C. albicans* ATPase. In the present study, we observed that the activity of Ca^{2+} - Mg^{2+} ATPase in *C. albicans* was down-regulated by baicalin, and cytoplasmic free Ca^{2+} increased. Moreover, the higher dose of baicalin associated with the lower ATPase activity and the higher cytoplasmic free Ca^{2+} . The lower ATPase activity disturbed the Ca^{2+} pump function and the cytoplasmic Ca^{2+} balance, even leading to cell Ca^{2+} overload and apoptosis. Recent

studies have indicated that the apoptosis-like cell death exists not only in multicellular organisms but also in single-cell fungi [28–34]. In addition, our experiments firstly demonstrated that *C. albicans* incubated with baicalin in vitro underwent apoptosis process; after 48 h baicalin treatment, the morphological changes of *C. albicans* apoptosis were shown under a transmission electron microscopy. However, it is still necessary to further verify whether the increase of cytoplasmic free Ca^{2+} in *C. albicans* is the cause of apoptosis or the result of apoptosis.

Acknowledgment

This study was supported by Natural Science Foundation of Jiangxi Province (#0440040) and National Natural Science Foundation of China (No. 81260504).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2014.07.040>.

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