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Research Article
Evaluation of Susceptibility and Innate Immune Response to *Candida albicans* in Mice with Sub-health

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Abstract

Background and Objective: Sub-health, also known as yajiankang, is mainly caused by fatigue and an unbalanced diet and has become a serious threat to public health. This study aimed to evaluate the susceptibility and innate immune response to intestinal *Candida albicans* (*C. albicans*) infection in mice induced by fatigue combined with an unbalanced diet as a sub-health state model. **Materials and Methods:** The KM mice were randomly divided into 4 groups: control group (CG), control mice infected with *C. albicans* group (CCG), model group (MG) and model mice infected with *C. albicans* group (MCG). The *C. albicans* content in feces was counted and barrier function analysis was performed on the small intestine. The levels of sIgA, IL-1β and TNF-α were determined by enzyme-linked immunosorbent assay. Expression of lysozyme was determined by Western blot. **Results:** These results indicated that *C. albicans* content was significantly increased, but the sIgA level, phagocytic percentage and index, 50% hemolytic complement (as CH50 level) and expression of lysozyme were significantly decreased in the mice infected with *C. albicans* group (MCG) relative to those in the other groups. The IL-1β and TNF-α levels were significantly decreased in the MCG relative to those in the control mice infected with *C. albicans* group. In the barrier function analysis, inflammatory cells in the mucosa, sparse microvilli and vacuolar phenomenon were observed in the MCG. **Conclusion:** The results indicated that innate immunity was down regulated and susceptibility to *C. albicans* infection was significantly higher in mice in the sub-health state than in control mice.

Key words: Innate immunity, sub-health, imbalance diet, *Candida albicans*, fatigue

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

With rapidly developing economies and a faster pace of life, the number of people with sub-health has also increased. Sub-health, which is also known as yajiankang, is a new concept defined in a previous study that has become a serious threat to public health because a surprisingly large number of people suffer from this condition[1,2]. According to the World Health Organization, approximately 35-50% of people worldwide are in a sub-healthy state[3]. The main risk factors for sub-health are fatigue (such as from lack of relaxation, working extended hours and occupational stress), unbalanced diet, psychological factors and bad habits and behaviors[4,5]. Fatigue and/or an unbalanced diet are considered to be the main factors that cause a sub-health state[6].

Studies have indicated that the immune function can be reduced in persons in the sub-health state[7,8]. However, innate immunity in the sub-health state has not been extensively investigated, only the complement activity of humans in the sub-health state has been researched[9,10]. Furthermore, *Candida albicans* is a major opportunistic fungal pathogen in humans[11]. In a healthy host, *C. albicans* exists as a benign commensal organism in general, but it may cause infections when the body has poor immunity[12,13]. Additionally, *Candida* species have become an ever-increasing problem in immunocompromised patients[14].

The purpose of this study was to investigate the susceptibility and innate immune response to intestinal *C. albicans* in mice induced by fatigue combined with an unbalanced diet as a sub-health state model.

MATERIALS AND METHODS

Reagents and materials: *C. albicans* 03382 was obtained from Peking University First Hospital (Beijing, China). Tacoma Scarlett medium was purchased from Zhengzhou Bosai Biotechnology Co. Ltd. (Zhengzhou, China). The assay kits for secretory immunoglobulin A (sIgA), interleukin-1 Beta (IL-1β) and tumor necrosis factor (TNF)-α were purchased from Uscn Life Science Inc. (Wuhan, China). Sheep red blood cells were purchased from Kowloon Biological Products Co., Ltd. (Zhengzhou, China). Anti-lysozyme was purchased from Abcam (Cambridge, UK). Western blot and immunoprecipitation cell lysates were purchased from Beyotime Biotechnology Co. Ltd. (Jiangsu, China). Protein marker was purchased from Beijing Gold Biotechnology Co. Ltd. (Beijing, China).

Establishment of animal model: Kun Ming (KM) mice, 20±2 g in weight, were purchased from Weitong Lihua Co. (Beijing, China), laboratory animal license, SCXK (Army): 2012-0001. The animals were kept under SPF laboratory conditions (Center for Animal Experiments of the Liaoning University of Traditional Chinese Medicine, Shenyang, China) and received a standard laboratory diet and filtered tap water *ad libitum*. All animal procedures followed the Liaoning Provincial Animal Welfare and Care Guideline in accordance with the National Institute of Health's guidelines regarding the principles of animal care (2004). At the end of the treatment, the rats were sacrificed by cervical dislocation under general anesthesia induced by hydrate anesthesia.

The animal model was established as previously described with minor modification[15-17]. The mice were only fed cabbage and were made to perform exhausting weight-loading swimming exercises on the 1st day. Then, the mice were administered lard intragastrically and received a normal diet on the 2nd day. The experiment was performed for 14 days. This process successfully established the sub-health model.

The KM mice were randomly divided into 4 groups, 30 mice per group: control group (CG), control mice infected with *C. albicans* group (CCG), model group (MG) and model mice infected with *C. albicans* group (MCG). Subsequently, *C. albicans* (2×10^8 CFU mL^{-1}) were intragastrically administered to the mice in the CCG and MCG groups and the mice in the CG and MG groups received the same volume of saline. In most experiments, the feces of the mice from the 4 groups were collected under sterile conditions on the 7th day and then a 0.5 cm section of each small intestinal segment (4 cm from the stomach) was removed and placed into 4% paraformaldehyde solution or 2.5% glutaraldehyde solution for barrier function analysis. In some experiments, a 1.5 cm section of each small intestinal segment (6 cm from the stomach) was removed and the mucosa was scraped gently from the intestines by using a glass slide. Then, the mucosa was immediately immersed into liquid nitrogen and stored at -80 until analysis[18]. In some experiments, the mice were injected intraperitoneally with 1 mL of 5% soluble starch solution on the 4th day. The mice were injected intraperitoneally with 1 mL of 1×10^8 CFU mL^{-1} *C. albicans* at 1 h before being sacrificed on the 7th day. Peritoneal cavities were lavaged by injection of 2 mL of sterile saline followed by gentle massaging of the peritoneal cavity. Peritoneal lavage fluid was then removed by using a pipette inserted into a small incision in the abdominal cavity. In some experiments, the mice were injected intraperitoneally with 1 mL of 5% soluble starch solution on the 4th day. Whole blood was collected from mice through the tail vein on the 7th day. Then, the mice were sacrificed and soaked in 75% ethanol for 10 min. Peritoneal cavities were lavaged by

injection of 2 mL of sterile saline, followed by gentle massaging of the peritoneal cavity. Peritoneal lavage fluid was then removed by using a pipette inserted into a small incision in the abdominal cavity. The peritoneal lavage fluid was ultracentrifuged for 5 min at 2,000 rpm to discard the supernatant and then 1640 medium containing 10% fetal bovine serum was added to culture macrophages.

Enumeration of *C. albicans* in feces: The feces from mice in each group were collected on the 7th day. The feces (0.1 g) from CG and MG were mixed in 1 mL of saline and the feces (0.1 g) from the CCG and MCG were mixed in 10 mL of saline. Approximately 0.1 mL of each homogenized sample was inoculated on the surface of Scarlett Tacoma medium and then maintained in a 37 humidified incubator for 48 h to count the number of *C. albicans*.

Histological analysis: The small intestine specimens were fixed in 4% paraformaldehyde solution and routinely processed as paraffin sections after 24 h. The specimens were stained with hematoxylin, eosin and observed by optical microscopy[19] (BZ-9000; Keyence, Osaka, Japan).

Electron microscopy observation: Samples from the small intestine were fixed with 2.5% paraformaldehyde solution for >2 h and rinsed with 0.1 M phosphate buffer. Then, the samples were fixed with osmic acid fixative, dehydrated, embedded, sectioned and stained with 3% uranyl acetate followed by lead citrate before observation by electron microscopy[18].

Expression of sIgA: Mucosal samples (0.1 g) were mixed in 1 mL of phosphate-buffered saline (PBS). The samples were homogenized and the homogenates were ultracentrifuged for 10 min at 2,000 rpm. The sIgA levels in the supernatant were measured by using enzyme-linked immunosorbent assay (ELISA).

Evaluation of phagocytosis function: One drop of peritoneal lavage fluid was placed in the center of a glass slide. Then, the slide was cultured in a water bath at 37 for 20 min, rinsed with saline 3 times, stained with Wright-Giemsa for 5 min and the number of macrophages and macrophages phagocytosis of *C. albicans* counted. The phagocytic percentage and phagocytic index were also calculated.

Measurement of 50% hemolytic complement (CH50): The whole blood was kept for 1 h at room temperature and then centrifuged for 10 min at 2,000 rpm to obtain serum. Then, the CH50 of the mice in each group was determined as previously described by Costabile[20].

Determination of IL-1β and TNF-α levels: The macrophage cells were maintained in 1640 medium supplemented with 10% fetal bovine serum in a 37 humidified incubator containing 5% CO_2 for 2 h. Then, the old medium was discarded and the cells were cultured in new medium for 24 h and centrifuged to obtain the supernatant. The IL-1β and TNF-α levels of the mice in each group were determined by ELISA.

Expression of lysozyme: The cells were cultured as described above, centrifuged, the supernatant discarded and then the cells were washed with PBS 3 times. The total proteins were extracted as previously described to determine the expression of lysozyme by the Western-blot method[21].

Statistical analysis: All experiments used groups of 10 mice and the results were averaged. For cell staining experiments, all slides were prepared from each animal in duplicate and 10 fields were counted per slide. All values are expressed as Mean±SD. The significance of differences among the groups was determined by one-way ANOVA by using the statistical package for social science program (SPSS 16.0, Chicago, IL, USA). The significance threshold was set at $p<0.05$ for this test.

RESULTS

Counts of *C. albicans* in feces: The *C. albicans* contents were significantly higher in the CCG and MCG than in the CG ($p<0.01$), *C. albicans* content was significantly lower in the MG ($p<0.01$) but higher in the MCG than in the CCG ($p<0.01$), moreover, the *C. albicans* contents were significantly higher in the MCG than in the MG ($p<0.01$) (Fig. 1).

Histological analysis: Histological analysis of the small intestine indicated that the villi were unevenly arranged or missing in the CCG and MCG mice. Moreover, the phenomenon of infiltration by inflammatory cells in the mucosa was observed in the MCG. There was no obvious change in the CG and MG (Fig. 2).

Electron microscopy observation: Different lengths and sparse microvilli, swelling mitochondria and vacuolar phenomenon were observed in the CCG and MCG. Moreover, dilated endoplasmic reticulum and shedding

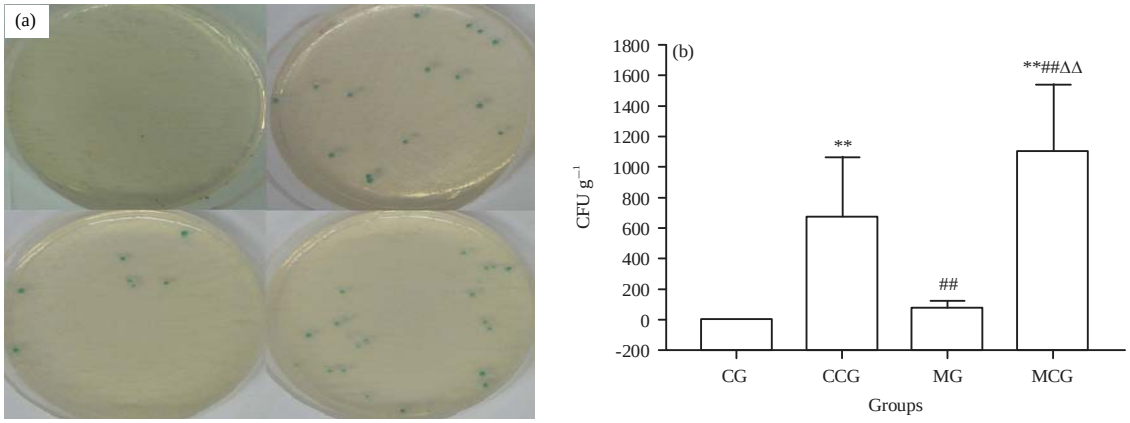

Fig. 1(a-b): Content of *Candida albicans* in feces of mice in each group. **Compared with the CG, p<0.01, ##Compared with the CCG, p<0.01, ΔΔCompared with the MG, p<0.01. CG: Control group, CCG: Control mice infected with *C. albicans* group, MG: Model group, MCG: Model mice infected with *C. albicans* group. Values are expressed as Mean±SD

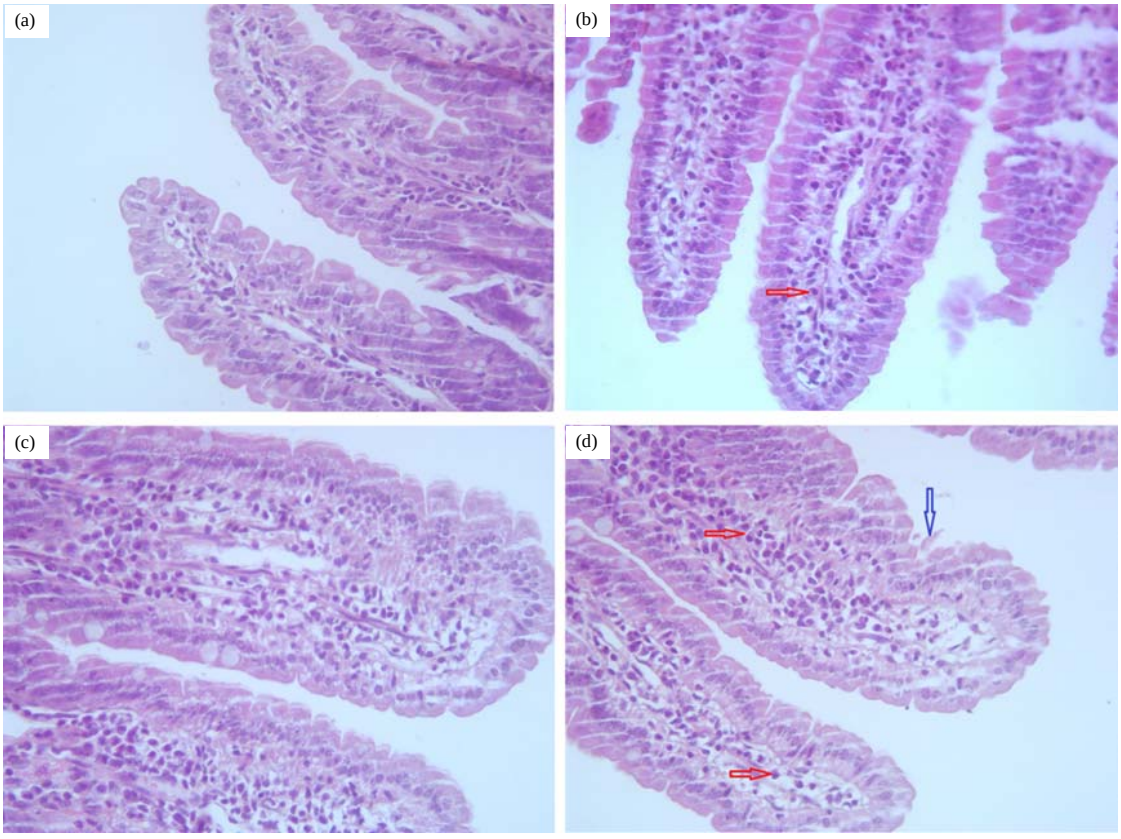

Fig. 2(a-d): Histological analysis of the small intestine in each group (stained with hematoxylin and eosin, magnification: 200 times). (a) Control group (CG), (b) Control mice infected with *C. albicans* group (CCG), (c) Model group (MG) and (d) Model mice infected with *C. albicans* group (MCG). The red arrow indicates inflammatory cells in the mucosa, the blue arrow indicates the damaged villi

ribosome of the surface could be observed in the MCG. There was no obvious change in the CG and MG (Fig. 3).

Expression of sIgA: The sIgA levels were significantly lower in the MG, CCG and MCG than in the CG (p<0.01), the sIgA level

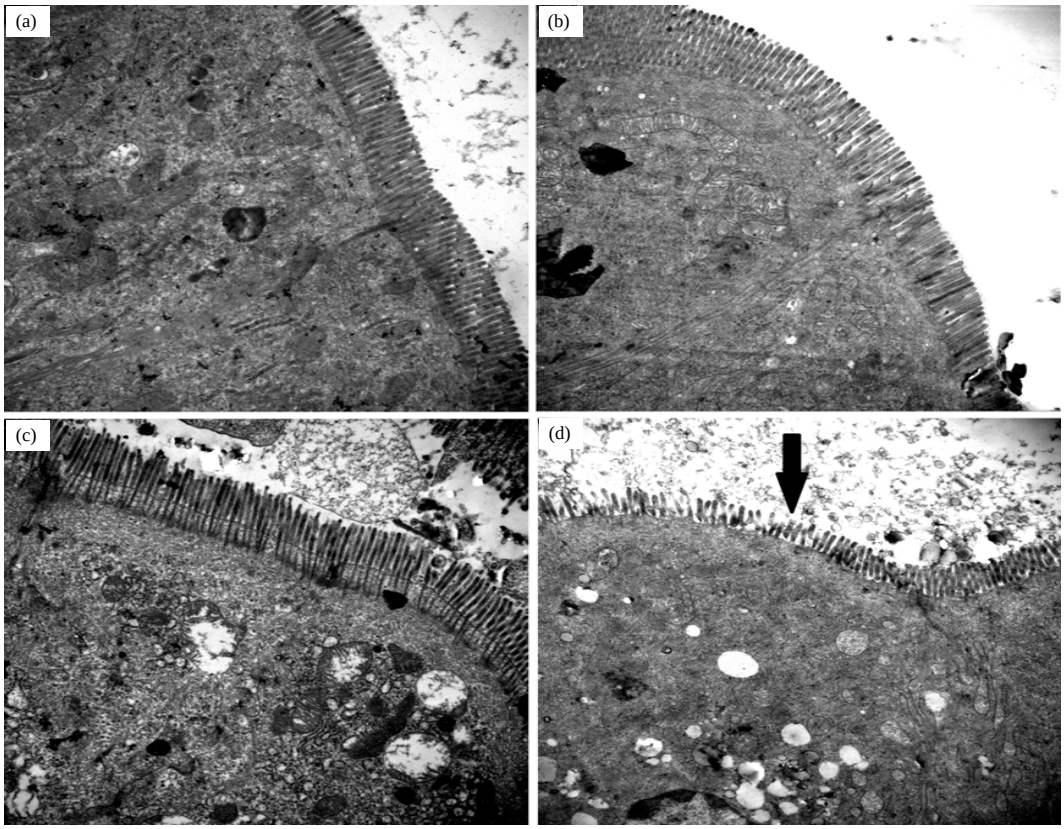

Fig. 3(a-d): Electron microscopy observation of the small intestine in each group (H7650 electron microscope, acceleration voltage: 80 kV, magnification: 15000 times, scale: 2 μm). (a) Control group (CG), (b) Control mice infected with *C. albicans* group (CCG), (c) Model group (MG) and (d) Model mice infected with *C. albicans* group (MCG). The black arrow indicates different lengths and sparse microvilli

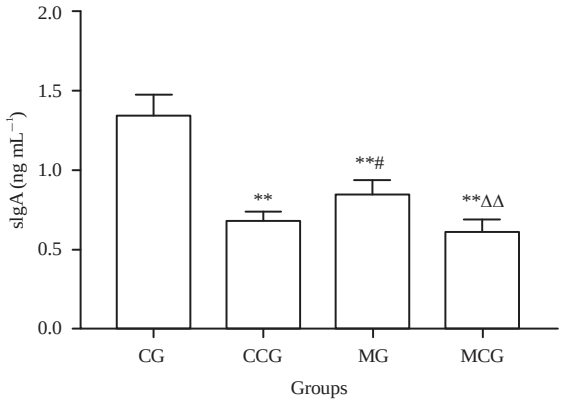

Fig. 4: sIgA level in the intestinal mucosa of mice in each group. **Compared with the CG, p<0.01, #compared with the CCG, p<0.05, ΔΔcompared with the MG, p<0.01. CG: Control group, CCG: Control mice infected with *C. albicans* group, MG: Model group, MCG: Model mice infected with *C. albicans* group. Values are expressed as Mean±SD

was significantly increased in the MG than in the CCG (p<0.01), the sIgA level was significantly decreased in the MCG than in the MG (p<0.01) (Fig. 4).

Evaluation of phagocytosis function: The phagocytic percentage and index were significantly lower in the MG, CCG and MCG than in the CG (p<0.01), the phagocytic percentage was significantly lower in the MG (p<0.01), the phagocytic indexes were significantly decreased in the MG and MCG than in the CCG (p<0.01), the phagocytic percentage and index were significantly decreased in the MCG than in the MG (p<0.01) (Fig. 5).

Measurement of 50% hemolytic complement (CH50): The CH50 values were significantly lower in the MG, CCG and MCG than in the CG (p<0.01), the CH50 was significantly lower in the MCG than in the CCG (p<0.05) and the CH50 was significantly lower in the MCG than in the MG (p<0.05) (Fig. 6).

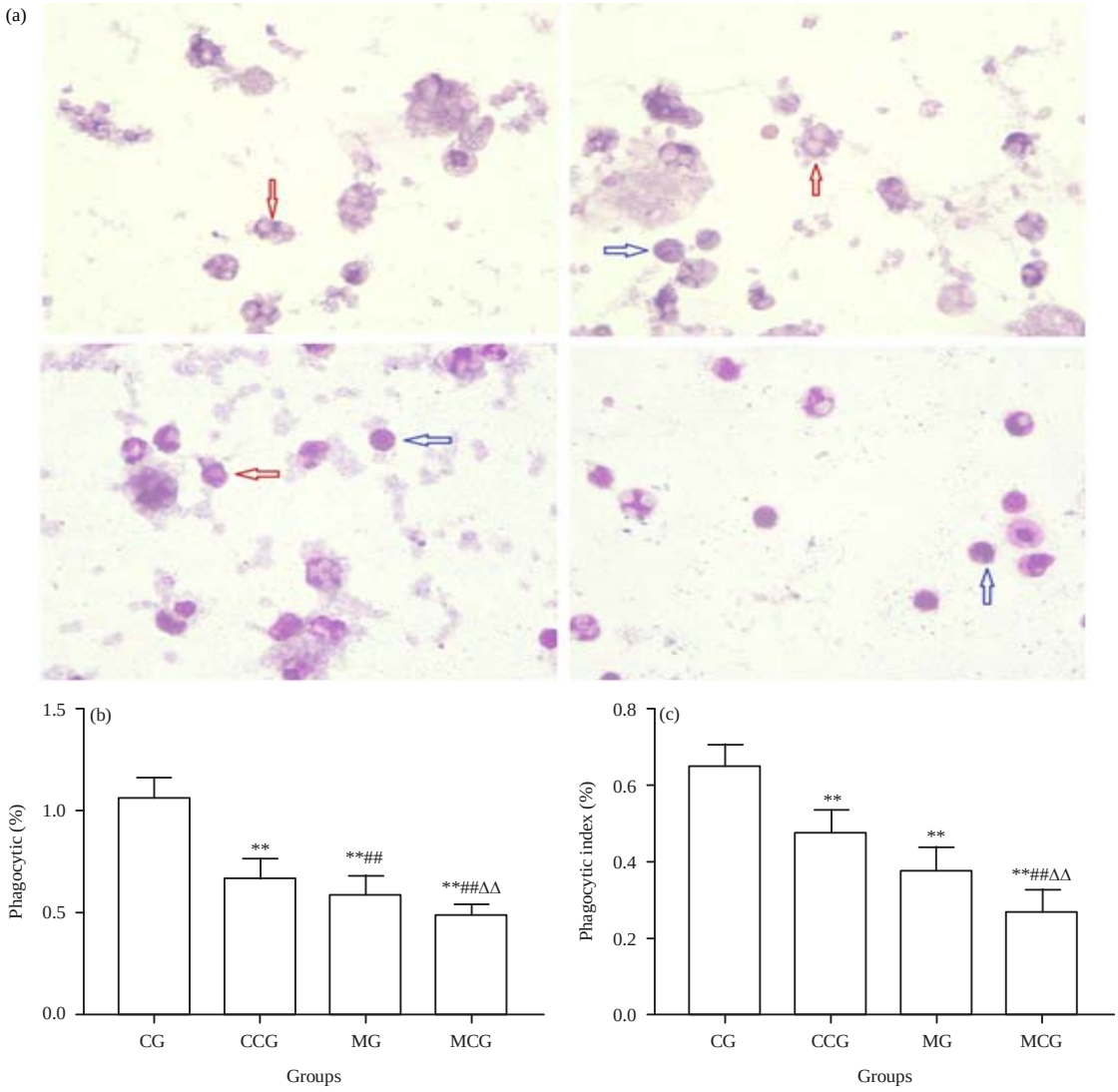

Fig. 5(a-c): Evaluation of macrophage phagocytosis function in each group, (a) Red arrow indicates the macrophagic phagocytosis of *C. albicans*, blue arrow indicates the macrophages without *C. albicans*, (b) Phagocytic percentage and (c) Index of macrophages. **Compared with CG, p<0.01, ##compared with CCG, p<0.01, ΔCompared with MG, p<0.05, ΔΔcompared with MG, p<0.01. CG: Control group, CCG: Control mice infected with *C. albicans* group, MG: Model group, MCG: Model mice infected with *C. albicans* group. Values are expressed as Mean±SD

Determination of IL-1β and TNF-α levels: The IL-1β and TNF-α levels were significantly lower in the CCG and MCG than in the CG (p<0.01), the IL-1β and TNF-α levels were significantly lower in the MCG than in the CCG (p<0.01) and the IL-1β and TNF-α levels were significantly higher in the MCG than in the MG (p<0.01) (Fig. 7).

Expression of lysozyme: Lysozyme was down regulated in the CCG and MCG relative to expression in the CG (p<0.01), lysozyme was down regulated in the MCG relative to expression in the CCG (p<0.05) and lysozyme was down regulated in the MCG relative to expression in the MG (p<0.05) (Fig. 8).

DISCUSSION

The World Health Organization has pointed out that health is not only the absence of disease and weakness, but also the presence of an intact state of physical, psychological and social adaptation. The sub-health state, also known as the "third state" or "gray state," is between a healthy state and a diseased state. The main symptoms of sub-health are fatigue,

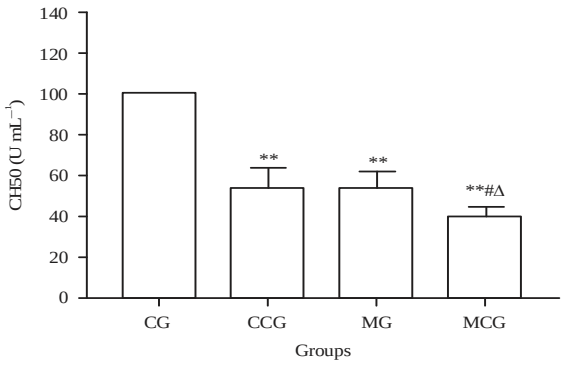

Fig. 6: Measurement of CH50. **Compared with the CG, p<0.01, #compared with the CCG, p<0.05, Δcompared with the MG, p<0.05. CG: Control group, CCG: Control mice infected with *C. albicans* group, MG: Model group, MCG: Model mice infected with *C. albicans* group. Values are expressed as Mean±SD

pain, loss of appetite and abdominal discomfort. The main factors associated with sub-health are fatigue and an unbalanced diet[3]. *C. albicans*, as an opportunistic pathogen, is suitable for exploring the susceptibility to the sub-health state. Therefore, the sub-health mouse model was established by inducing fatigue and providing an unbalanced diet to evaluate the susceptibility to *C. albicans* infection and determine innate immunity. There was no difference between the CG and MG, but the results for the mice infected with *C. albicans* showed that the *C. albicans* content was significantly higher in the MCG than in the CCG, which suggested that the mice in the sub-health state were more susceptible to infection with *C. albicans*. Furthermore, histological analysis of the small intestine also supported this conclusion. The barrier function was slightly damaged (short or missing villi and fewer infiltrations by inflammatory cells) in the MG relative to that in the CG, but it was more severely

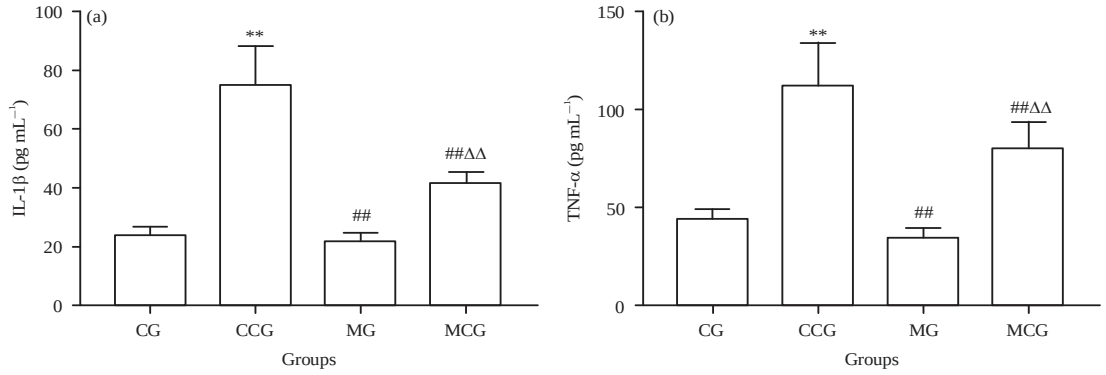

Fig. 7(a-b): Determination of IL-1β and TNF-α levels in each group. **Compared with the CG, p<0.01, ##compared with the CCG, p<0.01, ΔΔcompared with the MG, p<0.01. CG: Control group, CCG: Control mice infected with *C. albicans* group, MG: Model group, MCG: Model mice infected with *C. albicans* group. Values are expressed as Mean±SD

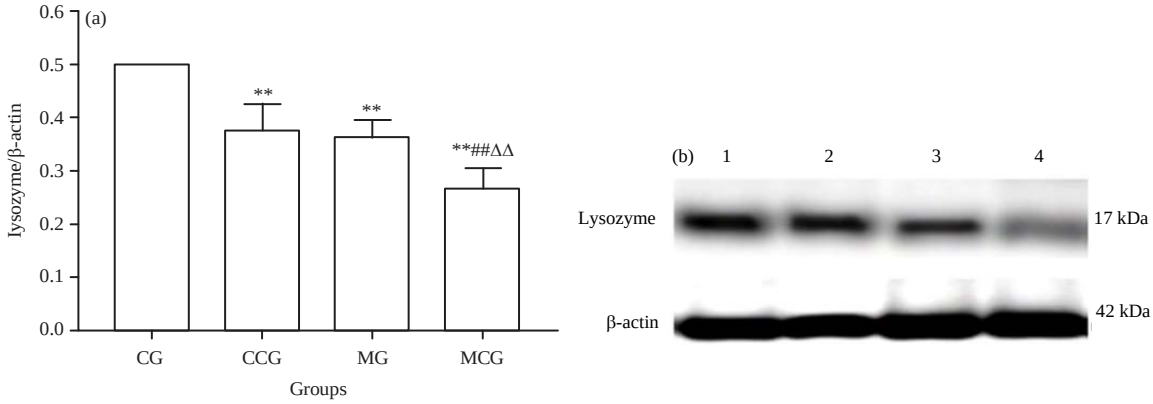

Fig. 8(a-b): Expression of lysozyme/β-actin in each group. **Compared with the CG, p<0.01, #compared with the CCG, p<0.05, ΔΔcompared with the MG, p<0.01. CG: Control group, CCG: Control mice infected with *C. albicans* group, MG: Model group, MCG: Model mice infected with *C. albicans* group. 1: CG, 2: CCG, 3: MG, 4: MCG. Values are expressed as Mean±SD

damaged in the MCG than in the CCG (mice infected with *C. albicans*), which indicated that the sub-health state changed the ecological environment of the intestinal tract and thus could lead to proliferation of *C. albicans* and infection.

sIgA is produced from the intestinal mucosa, is related to intestinal innate immunity[22] and has a principal role in the immune system because it prevents infection at early stages by excluding bacteria and viruses from the gastrointestinal tract[23]. In the present study, the sIgA level was significantly lower in the MG than in the CG, which suggests that mice in the sub-health state can more easily be infected with *C. albicans*, in accordance with previous research[24].

Macrophage phagocytic function is closely related to the innate immune system[25]. The phagocytic percentage and index were significantly decreased in the MG, which indicated that fatigue and an unbalanced diet can decrease the function of macrophages and this effect would be strengthened by concurrent infection with *C. albicans*. Moreover, a previous study showed a positive correlation between CH50 value and the phagocytic effect[26]. The CH50 level was found to be lower in the MG and MCG, which indicated that the sub-health state may reduce immunity through inhibition of the phagocytic effect.

Interleukin-1 (IL-1), which is also known as lymphocyte stimulator, is a master cytokine of local systemic inflammation[27]. In the necrotic area of acute inflammation, dying cells lose membrane integrity, which leads to the release of IL-1α and activation of nearby resident macrophages by binding to the IL-1RI to synthesize more cytokines, including IL-1β[28]. TNF-α is a proinflammatory cytokine produced by macrophages and other cell types. It is well known that inflammation has an essential role in the pathogenesis of infections[29]. An *in vitro* study showed that TNF-α can enhance PMN to inhibit the growth of *C. albicans* by producing reactive oxygen species and releasing lysozyme[30]. The study showed that infection of mice in the sub-health state with *C. albicans* was associated with down regulation of IL-1β, TNF-α and lysozyme, which strongly suggested that severe infection with *C. albicans* is caused by poor phagocytic function of macrophages.

CONCLUSION

It was the first study to investigate the susceptibility and innate immune response to *C. albicans* infection in mice in a sub-health state. Individuals in a sub-health state can be more easily infected with *C. albicans* and experience down regulation of the innate immune indicators (such as IL-1β, TNF-α and sIgA). These results differ from those of previous studies, possibly because this study systematically investigated the innate immune response in mice in the sub-health state. The study results should be useful in further studies of humans in the sub-health state.

SIGNIFICANCE STATEMENT

This study showed that sIgA, IL-1β and TNF-α and expression of lysozyme can be beneficial for evaluating the immune response to mice with sub-health. The study results will be useful for identifying critical areas of susceptibility to infection and innate immune response in the sub-health state that have not been widely explored previously. Future research may lead to a new theory on the innate immune response to sub-health.

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