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Original Article

Analysis of the Mechanism of Action of *Atractylodes macrocephala* Koidz Polysaccharide on Breast Cancer-Related Depression Based on the TLR4/MyD88/NF-κB Signaling Pathway

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ABSTRACT

Objectives: In the study, we analyzed the effect of *Atractylodes macrocephala* Koidz Polysaccharide (AMKP) on Breast Cancer-Related Depression (BCRD) to provide new references for future treatment of BCRD. **Materials and Methods:** First, after the liver toxicity test to determine the concentration of AMKP, we established a mouse model of BCRD and divided it into model, fluoxetine and AMKP groups treated with saline, fluoxetine and AMKP gavage, respectively. In addition, a group of normal control mice was set up. We subjected mice to the tail suspension test, forced swimming test and sugar water preference test. After completion, the mice were executed and blood and brain tissues were collected to detect inflammatory factors, immune cells and neurotransmitters by Enzyme Linked Immunosorbent Assay (ELISA), flow-through cell counting and chromatography. Finally, Western blot was performed to detect the changes of TLR4/MyD88/NF-κB signaling pathway in the brain tissues of mice in each group BCRD. **Results:** Compared with the model group, mice in the AMKP and fluoxetine groups had a lower rate of sugar-water consumption, while the resting time in the tail-suspension test and forced-swimming test was prolonged ($p<0.05$). In addition, although the neurotransmitter and synaptic functions of the AMKP group were not as excellent as those of the fluoxetine group, the AMKP group had lower inflammatory factors and better immune functions ($p<0.05$). **Conclusion:** AMKP improves neurological function and depressive behavior in BCRD mice by inhibiting the TLR4/MyD88/NF-κB pathway.

Keywords: *Atractylodes macrocephala* Koidz Polysaccharide, Breast cancer-related depression, Immune function, Neurological function, TLR4/MyD88/NF-κB.

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INTRODUCTION

According to the 2020 global cancer statistics data report released by the International Agency for Research on Cancer (IARC), there were 19.3 million new cases of malignancies globally in 2020, among which female Breast Cancer (BC) ranked first in incidence, accounting for 11.7% of all new cases.[1] As the most common female malignancy, BC has the characteristics of complex tumor microenvironment, invasive behavior, heterogeneity, high proliferation rate and resistance to treatment, with most patients requiring complete mastectomy for treatment, resulting in concurrent BC-Related Depression (BCRD) in such patients.[2] Statistics indicate that approximately 60% of BC patients develop BCRD, which not only increases the risk of adverse events but may also seriously compromise clinical treatment and eventually lead to a poor prognosis.[3] The pathogenesis of BCRD is complex, possibly involving neuroimmune factors, neuroendocrine, DNA oxidative damage and synaptic plasticity.[4] However, clinical research on this disease is in its initial stage, which seriously hampers the development of clinical prevention and treatment.

Atractylodes macrocephala Koidz Polysaccharide (AMKP) is a neurotherapeutic drug in traditional Chinese medicine, mainly composed of *Atractylodes macrocephala* (Compositae) and various saccharides (glucose, mannose, arabinose, etc.,).[5] AMKP has been indicated to exhibit excellent neuro-enhancing effects on Alzheimer's disease, cerebral ischemia injury diseases, etc., by increasing the levels of monoamine neurotransmitters in the brain and regulating neuroplasticity.[6,7] Moreover, for

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gastrointestinal digestive diseases, AMKP has also been verified to effectively regulate the structure of the intestinal flora and improve the body's immune-inflammatory response.[8] In addition, the Toll-Like Receptor 4 (TLR4)/Myeloid Differential protein-88 (MyD88)/Nuclear Factor kappa-B (NF-κB) signaling pathway is repeatedly mentioned in the research on AMKP. For example, AMKP inhibits LPS-induced hepatitis in mice via the TLR4/MyD88/NF-κB signaling pathway,[9] or inhibits the release of inflammatory factors in the spleen via TLR4/MyD88/NF-κB[10] For BCRD, TLR4/MyD88/NF-κB is also an important pathogenic pathway, Luo W and other studies have mentioned that Resatorvid inactivates hippocampal microglia through the TLR4/NF-κB/NLRP3 signaling pathway to alleviate BCRD.[11] Therefore, we speculate that the effect of AMKP on BCRD may also be related to TLR4/MyD88/NF-κB, but there are currently few reports on AMKP treatment for BCRD.

In this regard, this study will analyze the influence of AMKP on BCRD and explore whether its action pathway is related to the TLR4/MyD88/NF-κB pathway, so as to provide new references and guidance for the future treatment of BCRD.

MATERIALS AND METHODS

Animal data

Forty-eight SPF-grade BALB/c female mice were provided by Beijing Novo Nordisk Pharmaceuticals Science and Technology Co., Ltd. (Animal Use Permit Number: SYXK (Beijing) 2023-0029). The animals, weighing 18-24 g, were 6-8 weeks old and kept in an environment with a temperature of 23-25°C and a humidity of 35-45% under a 12-hr light/12-hr dark cycle. The breeding process strictly adhered to the standards formulated by the National Regulations on the Administration of Laboratory Animals, All the experimental procedures involving animals were conducted in accordance with ARRIVE guidelines and approved by the Animal Ethics Committee of Affiliated Hospital of Hebei University (No.m2024015), The mice were randomly divided into 8 groups (n=6), Figure 1 illustrates the flow of this study.

AMKP toxicity testing

Four groups of mice were selected for toxicity testing. Referring to the research by Zhu Q *et al.*,[12] 1×10^6 4T1 inflammatory BC cells were injected into the armpit of mice after 3 days of adaptive feeding and subcutaneous tumorigenesis was observed 7 days later. When the subcutaneous tumor volume reached about 80 mm^3, a corticosterone suspension (30 mg/kg) was injected subcutaneously into the back of mice for 21 consecutive days to induce the BCRD model. These four groups of BCRD mice were labeled as blank, low-dose, medium-dose and high-dose groups, respectively. Among them, the mice in the low-, medium- and high-dose groups were given intragastric administration of AMKP dissolved in 0.9% sodium chloride solution at doses of 10.0, 20.0 and 30.0 g/kg, respectively (5 mL/kg) for 7 days. The mice in the blank group were given intragastric administration of pure sodium chloride solution. After the completion, the mice were anesthetized with an overdose of sodium pentobarbital, the abdominal aortic blood was collected, and the brain tissues were separated for testing.

Liver and kidney function testing

The abdominal aortic blood was collected in a coagulation-promoting tube, left to stand at room temperature for 30 min and then centrifuged to isolate the serum. Liver and kidney function indicators were detected using an automatic biochemical analyzer, including Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Total Bilirubin (TBIL), Alkaline Phosphatase (ALP), Serum creatinine (Scr), Blood Urea Nitrogen (BUN), Uric Acid (UA) and Cystatin C (CysC).

Western blot detection of TLR4/MyD88/NF-κB pathway expression

The mouse brain tissue was cut into pieces, homogenized, fully lysed and centrifuged at 12,000 r/min for 10 min to collect the supernatant. A 5× protein gel electrophoresis loading buffer was added to the protein supernatant, fully mixed evenly, denatured in a 95°C environment for 10 min and electrophoresed at a voltage of 80 V. Then, the proteins were transferred to a membrane under 60 V voltage, added with a Tris-Borate-Sodium Tween (TBST) sealing solution (containing 5% skimmed milk powder) for 1 hr of sealing at room temperature and immersed in diluted TLR4, MyD88, NF-κB and β-actin primary antibodies (1:1000) for overnight reaction at 4°C. Subsequently, it was incubated in a secondary antibody working solution (1:2000) for 60 min in a dark, room-temperature environment. The membrane was developed by the electrochemiluminescence method, and the gray value was analyzed by Image J software.

AMKP intervention

The remaining four groups of mice were marked as normal, model, fluoxetine and AMKP groups, respectively. First, BC tumor-bearing mice were constructed using the above-mentioned method. The normal group mice were fed normally without treatment. Model, fluoxetine and AMKP groups were injected with corticosterone to induce BCRD. After the induction, the AMKP group was given AMKP at the corresponding concentration according to the above toxicity test results, the fluoxetine group was treated with fluoxetine (19.5 g/kg) and the model group was intervened with the same amount of normal saline, all by intragastric administration.

Behavior detection

Tail-suspension test

The mice were treated with fasting but not water-deprivation for 24 hr. Then, 1/3 of the mouse's tail was fixed to the bracket

with tape to put the mouse in a suspended state, ensuring that the mouse's head was about 60 cm from the ground. After 30 sec of adaptation, the immobility time of the mouse's limbs within 3 min was recorded.

Forced swim test

A transparent cylindrical tube with a height of 50 cm and a diameter of 20 cm was filled with water at a depth of about 30 cm. Then, mice were placed in it and allowed to swim for 1 min. After that, the immobility time of the mice within 5 min was recorded and photographed.

Sucrose preference test

Each mouse was housed in individual cages. After being fasted and deprived of water for 12 hr, 1 bottle of 1% sucrose water and one bottle of distilled water with their masses weighed in advance were placed. 1 hr later, the 2 bottles were taken out to measure the masses of the remaining sucrose water and distilled water. Sugared water consumption rate=sugared water consumption/(distilled water consumption+sugared water consumption)×100%.

Inflammatory reaction monitoring

After the behavior tests were completed, the mice were euthanized with excessive anesthesia and the abdominal aortic blood and complete brain tissue of the mice were collected for subsequent detection. Serum was separated from the aortic blood by centrifugation to determine the levels of inflammatory factors Interleukin-1β/6 (IL-1β/6) and Tumor Necrosis Factor-α (TNF-α) by Enzyme-Linked Immunosorbent Assay (ELISA).

Immune function testing

2 mL of abdominal aortic blood of the mice was collected to detect the proportion of immune cells by flow cytometry. The proportion of Treg cells was determined by intranuclear staining and the proportions of Th1, Th2 and Th17 cells were measured by intracellular staining.

Neurotransmitter Detection

After the behavior tests were completed, the mice were euthanized by excessive anesthesia to obtain the complete brain tissue. The mouse cerebral cortex was taken, weighed and added with 50 μL of methanol: acetonitrile (1:1) solution per 10 mg of tissue for 8 min of homogenization at 4°C. Then 100 μL of the homogenate was taken to add with 10 μL of the internal standard solution isoproterenol (10 μg/mg). After mixing vortex, the supernatant was obtained by centrifugation and 100 μL was used for chromatographic analysis to measure the contents of neurotransmitters 5-Hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), Dopamine (DA) and Norepinephrine (NE).

Immunohistochemistry (IHC)

The mouse brain tissue was fixed in 4% paraformaldehyde solution and paraffin-embedded to make paraffin sections, which were then dewaxed, hydrated, antigen-repaired, endogenous peroxidase blocked and BSA-blocked successively. Synaptophysin (SYN) and Postsynaptic Density protein 95 (PSD95) primary antibodies (1:50) as well as the corresponding secondary antibodies (HRP-labeled) were then added. Next, color development with DAB was carried out, the nucleus was restained, gradient ethanol dehydration was performed and transparentization and mounting were carried out. The positive rates of SYN and PSD95 were observed under a microscope.

Detection of TLR4/MyD88/NF-κB pathway expression

The expression of the TLR4/MyD88/NF-κB pathway in the mouse brain was detected by the same method as described above (Western blot).

Statistical methods

Statistical analysis was conducted using SPSS23.0 software. All data in this study were confirmed to follow a normal distribution after the Shapiro-Wilk test and recorded as (±s). Repeated measures analysis of variance and Least-Significant Difference intra-group test were employed for comparison among multiple groups. A minimum significance threshold of $p<0.05$ was used.

RESULTS

AMKP toxicity test results

After testing, there were no notable differences in ALT, ALP, Scr, BUN, UA and CysC among the blank, low-dose, medium-dose and high-dose groups ($p>0.05$). In addition, the blank, low-dose and medium-dose groups also showed similar AST and TBIL levels ($p>0.05$), lower compared to the high-dose group ($p<0.05$), indicating that high-dose AMKP may impose a burden on mouse liver function. In terms of the expression of the TLR4/MyD88/NF-κB pathway, it was found that TLR4, MyD88 and NF-κB protein levels were lower in the low-, medium- and high-dose groups compared with the blank group ($p<0.05$), with the lowest levels observed in the high-dose group, followed by the medium-dose group ($p<0.05$). Based on the above results, we chose AMKP with a concentration of 20.0 g/kg for follow-up tests (Figure 2).

Effect of AMKP on the behavior of BCRD mice

Compared with the control group, the sugared water consumption rates of the model, fluoxetine and AMKP groups were all decreased, while the immobility time in the tail suspension and forced swim tests was prolonged ($p<0.05$). Among them, the fluoxetine and AMKP groups showed no marked differences in behavior test results ($p>0.05$), with a higher sugared water consumption rate and less immobility time in the tail suspension

and forced swim tests compared to the model group (p<0.05) (Figure 3).

Impact of AMKP on inflammatory reaction and immune function in BCRD mice

Model, fluoxetine, AMKP groups had higher IL-1β, IL-6, TNF-α concentration, Th1, Th2 and Th17 cell percentage than control group (p<0.05), while Treg cell percentage was lower than control group (p<0.05). Additionally, the AMKP group showed reduced concentrations of IL-1β, IL-6 and TNF-α, decreased proportions of Th1, Th2 and Th17 cells and an elevated proportion of Treg cells than the fluoxetine and model groups (p<0.05) (Figure 4).

Effects of AMKP on neurotransmitters and synapses in BCRD mice

5-HT, DA and NE levels were lower in the model, fluoxetine and AMKP groups than in the control group, while 5-HIAA was higher (p<0.05). Among the models, fluoxetine and AMKP groups, 5-HT, DA and NE were higher in the fluoxetine and AMKP groups compared to the model group (p<0.05); the 5-HIAA levels in the fluoxetine and AMKP groups were lower compared with the model group (p<0.05). 5-HT, DA, NE was higher in the fluoxetine group than in the AMKP group and 5-HIAA was lower than in the AMKP group (p<0.05). IHC staining indicated no significant difference in the positive rates of SYN and PSD95 between the fluoxetine group and the AMKP group (p>0.05), higher than the model group but lower than the control group (p<0.05) (Figure 5).

Effects of AMKP on TLR4/MyD88/NF-κB pathway in BCRD mice

Finally, the detection of TLR4/MyD88/NF-κB pathway expression showed markedly elevated TLR4, MyD88 and NF-κB protein expression in the model group compared with the control group (p<0.05); however, there was no statistically significant difference in TLR4, MyD88 and NF-κB protein levels between the fluoxetine group and the AMKP group (p>0.05), which were lower in comparison with the model and control groups (p<0.05) (Figure 6).

DISCUSSION

BC patients endure tremendous psychological pressure during the diagnosis and treatment process and the uncertainties about the future and concerns about body image contribute to the frequent occurrence of BCRD.[13] Effective controls of depressive symptoms in BCRD patients can help improve medication adherence and encourage active participation in treatment, ultimately improving patient outcomes. In this study, we discovered that AMKP had

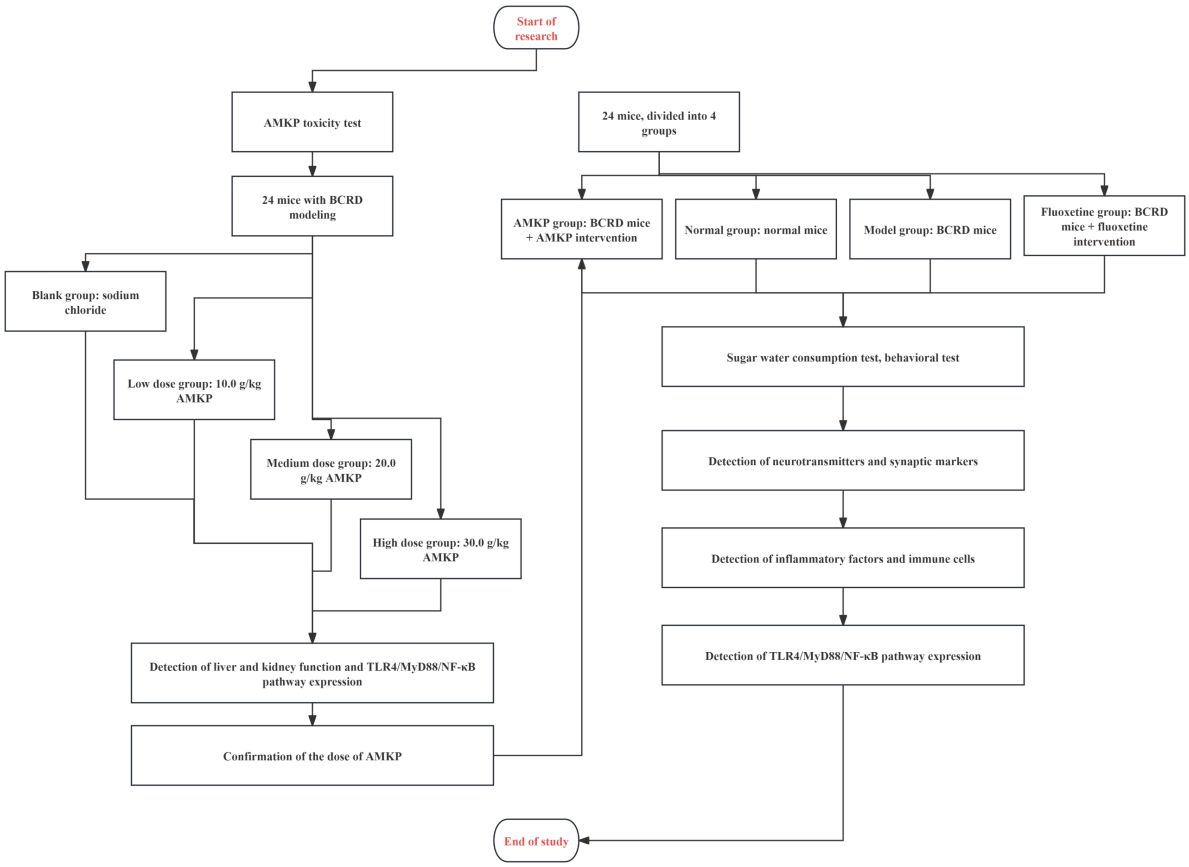

Figure 1: Flowchart of this study.

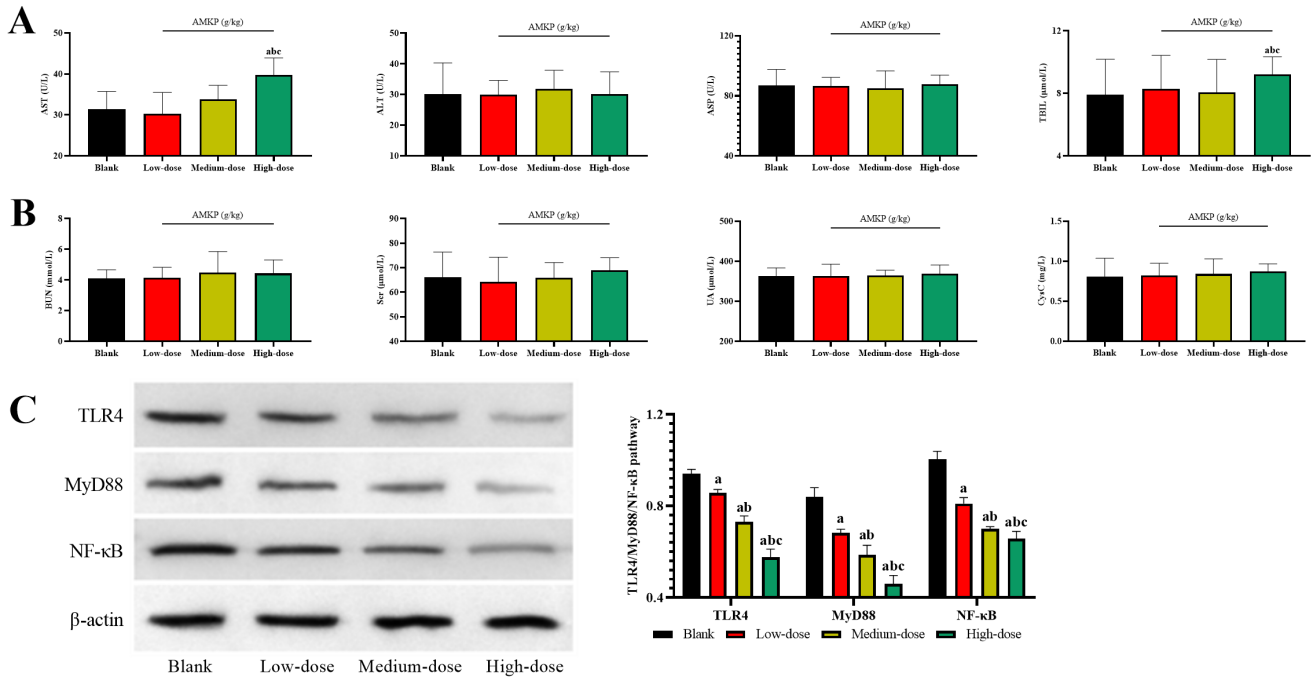

Figure 2: AMKP toxicity test results. (A) Effects of AMKP on liver function indicators AST, ALT, ASP and TBIL in BCRD mice. (B) Effects of AMKP on BUN, Scr, UA and CysC, indicators of renal function in BCRD mice. *vs* blank group [a]*p*<0.05, *vs* low-dose group [b]*p*<0.05, *vs* medium-dose group [c]*p*<0.05.

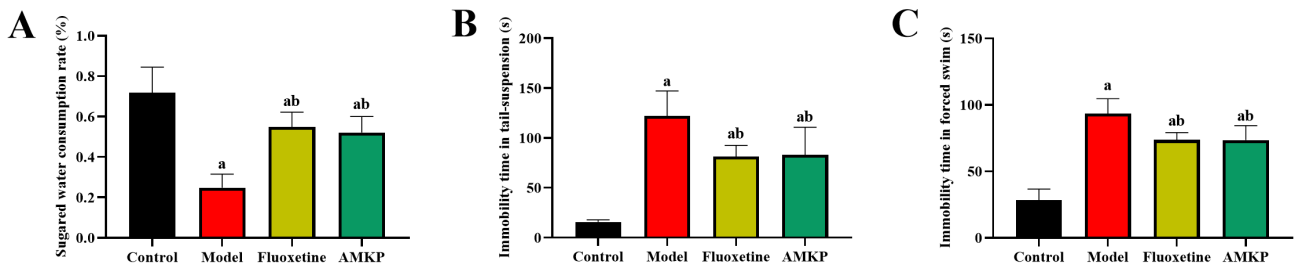

Figure 3: Effect of AMKP on the behavior of BCRD mice. (A) Effect of AMKP on sugar-water consumption rate in BCRD mice. (B) Comparison of immobility time in tail-suspension. (C) Comparison of immobility time in forced swim.

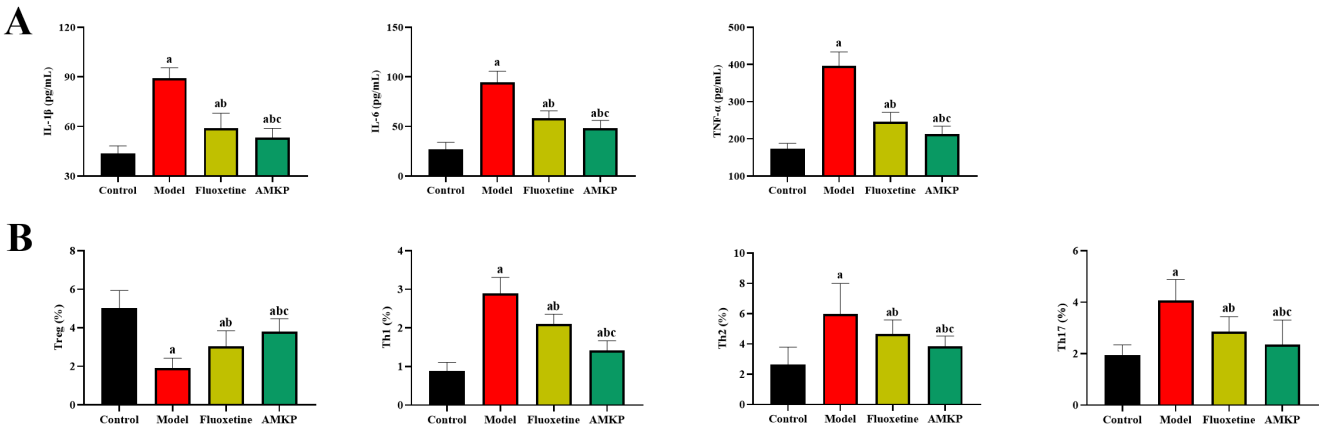

Figure 4: Impact of AMKP on inflammatory reaction and immune function in BCRD mice. (A) Effect of AMKP on inflammatory factors IL-1β, IL-6 and TNF-α in BCRD mice. (B) Effect of AMKP on the percentage of immune cells Treg, Th1, Th2 and Th12 in BCRD mice. *vs* control group [a]*p*<0.05, *vs* model group [b]*p*<0.05, *vs* fluoxetine group [c]*p*<0.05.

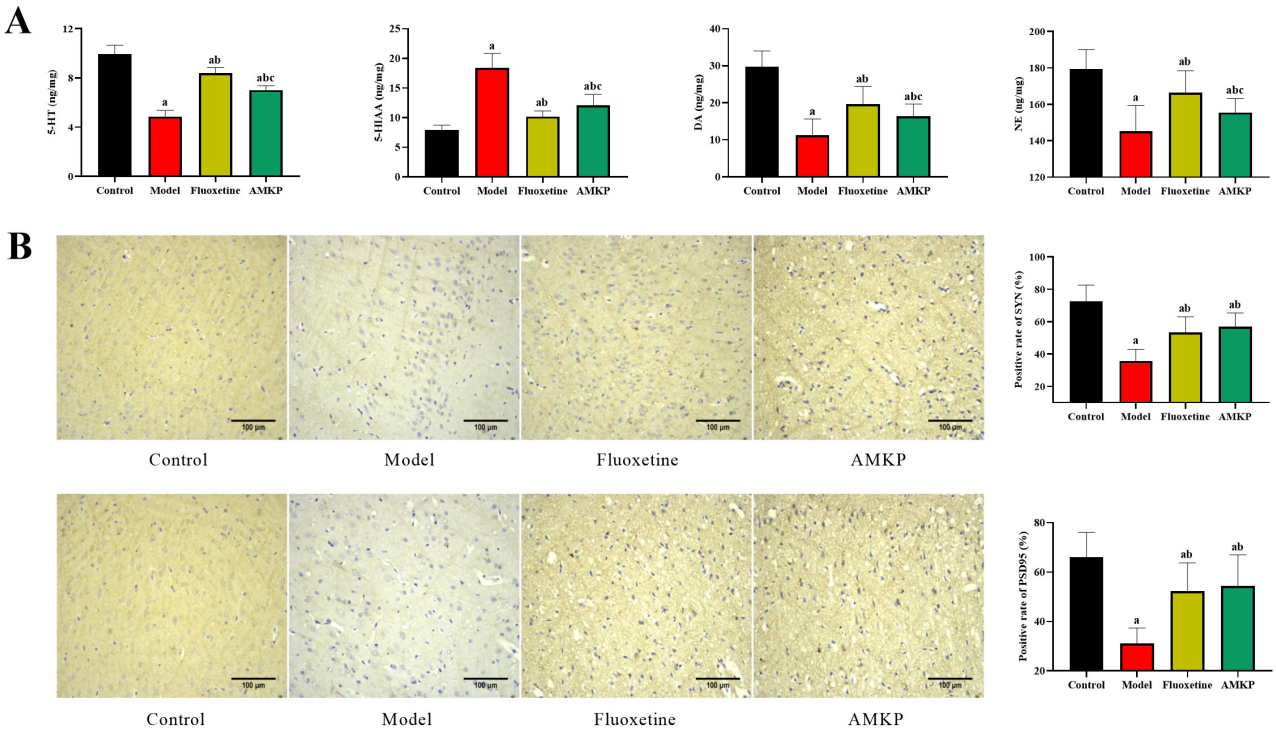

Figure 5: Effects of AMKP on neurotransmitters and synapses in BCRD mice. (A) Effects of AMKP on neurotransmitter indices 5-HT, 5-HIAA, DA, NE in BCRD mice. (B) Effect of AMKP on synaptic indicators SYN, PSD95 in BCRD mice (IHC, 200×). *vs* control group $^{a}p<0.05$, *vs* model group $^{b}p<0.05$, *vs* fluoxetine group $^{c}p<0.05$.

an outstanding therapeutic effect on BCRD, which lays a reliable foundation for the future clinical application of AMKP.

First of all, we induced a BCRD mouse model by 4T1 and corticosterone, which is also the most commonly used BCRD modeling modality in clinical research, with an extremely high success rate.[14] AMKP, rich in a variety of carbohydrates, is highly likely to cause a burden on liver and kidney metabolism when used in excessive doses, affecting human health.[15] Although the clinical application of AMKP is common at present, its impact on BCRD is still rarely reported. Therefore, we first need to determine the dosage of AMKP used in BCRD. Through the toxicity test, it was found that there was no difference in renal function among blank, low-dose, medium-dose and high-dose groups, but AST and TBIL in the high-dose group were increased. The concentration of AMKP at 30.0 g/kg may cause abnormal liver function in mice. The TLR4/MyD88/NF-κB pathway expression results also showed an obvious reduction in TLR4, MyD88 and NF-κB protein levels in the low-, medium- and high-dose groups, preliminarily confirming the influence of AMKP on the TLR4/MyD88/NF-κB pathway. Based on these results, we selected 20 g/kg, which had no burden on liver and kidney function in mice and a significant effect on the TLR4/MyD88/NF-κB pathway, as the dose of AMKP in the follow-up study.

In addition, fluoxetine is the most commonly used drug for the clinical treatment of depression and its effect has been verified many times.[16,17] Therefore, we set up a fluoxetine group to represent the effect of conventional treatment of BCRD in the clinic, which can be compared with the effect of BCRD. After the BCRD model was established again, we found that the sugared water consumption rate of the model, fluoxetine and AMKP groups was lower than that of the control group, while the immobility time in the tail suspension and forced swim tests was longer, which is in line with the typical animal behavior of BCRD,[18] once again corroborating the successful modeling. Among them, the behavior test results of the fluoxetine and AMKP groups were significantly improved compared with the model group, suggesting that both AMKP and fluoxetine can ameliorate the depressive behavior of BCRD mice. Among them, fluoxetine, as a commonly used clinical drug for depression, has been repeatedly validated for its efficacy, so the behavior test results of this group of mice can also be expected. The absence of statistical significance in behavior detection results between the AMKP and fluoxetine groups demonstrates the excellent effect of AMKP on alleviating BCRD. In the research by Choi NR *et al.*, AMKP was found to regulate the stress behavior of mice with intestinal stress syndrome,[19] which can also support our research results. Similarly, depression is also considered a psychological stress reaction, which is caused by the comprehensive influence of various factors such as the external environment and endocrine.[20] Therefore, we believe that the occurrence of BCRD may also be potentially associated with immune and inflammatory changes caused by BC in the human body. In this study, we observed more significantly relieved inflammatory responses and enhanced

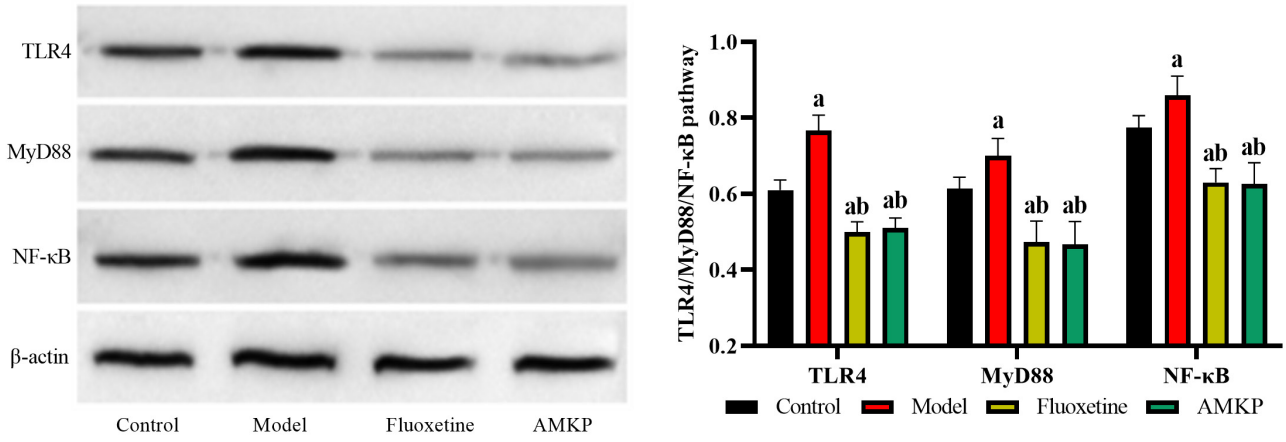

Figure 6: Effects of AMKP on TLR4/MyD88/NF-κB pathway in BCRD mice. vs control group [a]$p<0.05$, vs model group [b]$p<0.05$.

immune function in the AMKP group than in the control, model and fluoxetine groups, which also proves the excellent role of AMKP in regulating human immune function. In terms of neurotransmitters and synapses, although the AMKP group did not perform as excellent as the fluoxetine group, it showed more significant improvements compared with the model group. It indicates that AMKP also has an excellent function of nourishing nerves, which may also be one of the mechanisms by which AMKP effectively alleviates BCRD. Chen L *et al.*, found in an animal study that AMKP affected the nervous function of mice through glucose metabolism, gut microbiota and energy metabolism,[21] consistent with our findings. Combined with the fact that AMKP has better inflammatory and immune improvement effects than fluoxetine mentioned earlier, AMKP may be an excellent and safe clinical treatment for BCRD in the future.

Finally, we reaffirmed the effect of AMKP on the TLR4/MyD88/NF-κB pathway. Compared with the control group, the TLR4/MyD88/NF-κB pathway was significantly activated in the model group, while the protein levels of TLR4, MyD88 and NF-κB were notably reduced in the AMKP and fluoxetine groups, indicating that these two drugs have significant inhibitory effects on the TLR4/MyD88/NF-κB pathway. This is because the TLR4/MyD88/NF-κB pathway is a signal transduction pathway closely associated with neurological function and has been shown to affect cerebral ischemia/reperfusion injury or modulate neuroinflammation in diabetic peripheral neuropathy by affecting the gut microbiota.[22,23] And precisely because of the excellent neuromodulation of AMKP and fluoxetine, the TLR4/MyD88/NF-κB of these two groups of mice were significantly changed. These results not only reaffirmed the relationship between TLR4/MyD88/NF-κB and BCRD but also re-emphasized the excellent nerve improvement effect of AMKP.

Of course, more experiments, such as HE staining of brain tissue and observation of the morphology of neuron cells, are needed to verify the influence mechanism of AMKP on BCRD. In addition, we also need to carry out cell assays to confirm the biological behavior of AMKP on nerve cells, to provide a more comprehensive reference for clinical practice.

CONCLUSION

AMKP effectively ameliorates the neurological function of BCRD mice and alleviates their depressive behaviors while playing a role of anti-inflammation and optimizing immune function, possibly by inhibiting the expression of the TLR4/MyD88/NF-κB pathway. In the future, AMKP will probably be an excellent treatment option for BCRD.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

AMKP: *Atractylodes macrocephala* koidz polysaccharide; **BCRD:** Breast cancer-related depression; **TLR4:** Toll-like receptor 4; **MyD88:** Myeloid differential protein-88; **NF-κB:** Nuclear factor kappa-B; **BC:** Breast cancer; **ALT:** Alanine aminotransferase; **AST:** Aspartate aminotransferase; **TBIL:** Total bilirubin; **ALP:** Alkaline phosphatase; **Scr:** Serum creatinine; **BUN:** Blood urea nitrogen; **UA:** Uric acid; **CysC:** Cystatin C; **TBST:** Tris-borate-sodium tween; **IL-1β/6:** Inflammatory factors interleukin-1β/6; **TNF-α:** Tumor necrosis factor-α; **ELISA:** Enzyme-linked immunosorbent assay; **5-HT:** 5-hydroxytryptamine; **5-HIAA:** 5-hydroxyindoleacetic acid; **DA:** Dopamine; **NE:** Norepinephrine; **SYN:** Synaptophysin; **PSD95:** Postsynaptic density protein 95.

ETHICAL STATEMENT

The study protocol was approved by the Animal Ethics Committee of Affiliated Hospital of Hebei University (No. m2024015).

AUTHOR CONTRIBUTIONS

SS.C designed the studies, XF L and C.F drafted and modified the manuscript. SS.T and J.Y performed the statistical analysis and collecting data. XN.S participated in acquisition, analysis, or interpretation of data. XF.L and C.F made equal contributions in this work as co-first authors. All authors read and approved of the final manuscript.

SUMMARY

In this study, a mouse model of Breast Cancer-Related Depression (BCRD) was induced by 4T1 and corticosterone to clarify the application value of *Atractylodes macrocephala* Koidz Polysaccharide (AMKP). Compared with simple BC mice, BCRD mice exhibited typical depressive behaviors, with intensified inflammatory responses and deteriorated immune functions. The two groups of BCRD mice, intervened with AMKP and fluoxetine, respectively, had decreased sugared water consumption rates ($p<0.05$) and prolonged immobility time in the tail-suspension and forced swim tests ($p<0.05$), confirming that AMKP can effectively ameliorate the depressive behavior of BCRD mice. Besides, although the improvement of AMKP on neurotransmitters and nerve synapses was not as remarkable as that of fluoxetine, it could more effectively suppress the inflammatory response of BCRD mice and improve their immune function ($p<0.05$), suggesting that AMKP has excellent therapeutic potential for BCRD.

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