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Original Article

Edible bird's nest improves hemorheology and immune function in mice with transplanted uterine leiomyomas

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Abstract: Objective: To investigate the effect of edible bird's nest (EBN) on tumor growth, hemorheology and immune function of mice with transplanted uterine myomas. Methods: A subcutaneous tumor model of human uterus myoma was established in mice, and the mice were randomly divided into a model group, EBN group, estradiol receptor (ER) group and ER+EBN group. Body weight and tumor volume were measured at 2 weeks, 4 weeks and 8 weeks after the uterus myoma transplantation. Eight weeks after transplantation, the tumor weight was assessed, the morphology of different organs was observed, and the pathological changes of the uterus myoma was observed. Besides, the levels of ER and progesterone receptor (PR), various hemorheological parameters (including hematocrit, plasma viscosity and whole blood viscosity under different shearing conditions), and immune functions (CD_3^+, CD_4^+ and CD_8^+ cells) were also measured. Enzyme-linked immunosorbent assay (ELISA) was used to detect the levels of tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), nitricoxidesynthase (NOS) and vascular endothelial growth factor (VEGF) in each group. Results: There were no statistical differences in body weight, tumor weight, tumor volume, uterus myoma pathology or the levels of ER and PR between the model group and EBN group, nor between the ER group and ER+EBN group (all $P>0.05$). Similarly, no notable morphological differences were observed in the heart, liver, spleen, lung, kidney, stomach, intestines and uterus among different groups (all $P>0.05$). However, in contrast to the model group, the EBN group exhibited significant reductions in hemorheology indicators, the proportion of CD_8^+ cells, as well as the levels of TNF-α, NOS and VEGF (all $P<0.05$). Conversely, the proportion of CD_3^+ and CD_4^+ cells, the CD_4^+/CD_8^+ ratio and the level of IL-2 in the EBN group were obviously increased (all $P<0.05$). Compared with the ER group, the proportion of CD_8^+ cells, the levels of TNF-α, NOS and VEGF in the ER+EBN group were significantly decreased while the proportion of CD_3^+ and CD_4^+ cells, the CD_4^+/CD_8^+ ratio and the level of IL-2 in the ER+EBN group were obviously increased. Conclusion: For mice with uterine myoma transplantation, EBN does not influence tumor growth but significantly regulates hemorheology and enhances immune function.

Keywords: Edible bird's nest, uterine myoma, hemorheology, tumor growth, immune function

Introduction

Edible bird's nest (EBN), a well-known Chinese dish, is a salivary secretion produced by swiftlets. *Collocalia, Aerodramus and Hydrochous,* which are the three genera of swiftlets known to produce the valuable EBN [1]. Rich in protein, which constitutes 50% of its dry weight, EBN provides an abundant source of amino acids and glycoproteins [2]. It also contains essential calcium, sodium, potassium, carbohydrates, sialic acid, etc. These nests are highly valued and often referred to as the "Caviar of the East", a title that dates back to the Tang Dynasty. Traditionally valued for both its nutritional content and medicinal properties [3], recent studies have highlighted the neuroprotective and antiviral capabilities of edible bird's nest [4, 5]. It has been shown to have beauty benefits and to enhance immune function [6, 7]. Additionally, research indicates that it can alleviate various ailments of the respiratory and digestive system [8]. Further benefits include bone strengthening, antioxidant, and anti-inflammatory

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properties [9]. Despite its widespread culinary and medicinal use, there remains limited information on the effects of edible bird's nest supplementation on the development of uterine myoma.

Uterine myoma is the most common benign tumor in the female reproductive system [10]. The growth of these tumors is heavily influenced by ovarian sex hormones [11], such as estrogen and progesterone. The actions of these hormones on target cells and tissues are partially mediated by various local cytokines, which regulate cell transformation, growth, cell hypertrophy, angiogenesis and extracellular matrix formation, leading to the formation and growth of myoma [12]. Study has demonstrated that estrogen receptors (ER) play diverse roles in different types of tumors [13]. For instance, in breast cancer, prostate cancer, endometrial cancer, the estrogen receptor pathway promotes tumor growth, while in cancers such as colon cancer, liver cancer and gastric cancer, the estrogen receptor pathway plays an anti-tumor role [14]. Some scholar advise against the consumption of EBN by patients with uterine myoma, citing concerns that its active ingredients may alter the levels of estrogen or progesterone, thus affecting the occurrence and development of uterine myoma [15]. However, to date, research on the effect of EBN on the development of uterine myoma is scarce.

To fill this gap, this study was designed to explore whether EBN has an ameliorating effect on the development of uterine myoma in an experimental mouse model.

Material and methods

Animal model

This study was approved by the animal ethics Committee of Qingdao Hiser Hospital Affiliated of Qingdao University (Approval No. 2023-111), and it was performed according to the guidelines issued by the Chinese Association for Laboratory Animal Sciences. Fifty female NOD/SCID mice, aged 8 weeks, weight 20-25 g were purchased from Animal Centre of Qingdao University. These experimental animals were housed under controlled conditions with a room temperature maintained at 22±1°C and relative humidity ranging from 45-75%. They were subjected to a 12 h light-dark cycle. Additionally, the mice had ad libitum access to food and water.

The subcutaneous tumor mouse model of human uterus myoma was established according to previous studies [16]. Tissues from the center of human uterine fibroids with better vitality were selected and immersed into the 4°C sterile physiological saline for thorough rinsing. Ophthalmic scissors were used to cut the tissues into small pieces approximately 3 mm × 3 mm. Pentobarbital sodium (0.15 ml/10 g) was injected intraperitoneally to anesthetize SCID mice. After the mice were anesthetized, they were kept on the operating board. Then, the armpit area was disinfected with alcohol, and a 0.8 cm incision was made in the underarm skin using sterile small curved scissors. Two prepared tissue specimens were placed under the subcutaneous area on the right side of SCID's armpit and fixed well. Finally, the incision was sutured.

A total of 40 female mice were assigned into the model group, EBN group, ER group and EBN+ER group. Mice in the model group were orally administered an equal amount of 0.9% sodium chloride every day. Mice in the EBN group were orally administered 1.5 g/kg EBN every day. Mice in the ER group were intraperitoneally injected with 0.5 mg/kg of estradiol benzoate three times per week. Mice in the ER+EBN group were orally administered 1.5 g/kg EBN every day and injected with 0.5 mg/kg of estradiol benzoate three times per week. Mice in the control group didn't undergo human uterus myoma transplantation and were orally administered an equal amount of 0.9% sodium chloride.

Mice were euthanized using the method of 0.5 ml/10 g of pentobarbital sodium injection at different time points. The observed indicators were as follows: (1) At 2 weeks, 4 weeks and 8 weeks after the uterus myoma transplantation, the body weight and the tumor volume [(length × width)^2/2] were observed and compared among groups. (2) At 8 weeks after the uterus myoma transplantation, the tumor weight was obtained and compared among groups. (3) At 8 weeks after the uterus myoma transplantation, the organs including heart, liver, spleen, lung, kidney, stomach, intestines and uterus were obtained and compared among groups, with reference to those in the mice from the control group.

Hematoxylin-eosin (HE) staining

Transplanted uterine myoma tissue samples from mice were processed for HE staining

according to routine procedures. Each HE-stained section was observed in four high-magnification fields. The process involved washing the tissues in water, followed by dehydration, paraffin embedding, and sectioning.

Immunohistochemistry of estrogen receptor (ER) and progesterone receptor (PR) expression

The sections were gradually dehydrated, and antigen retrieval was performed in boiling sodium citrate buffer for 5 min. After washing with phosphate buffer saline (PBS) at room temperature, sections were incubated in 3% hydrogen peroxide in PBS for 8 minutes at room temperature, followed by another PBS wash. Next, the sections were then incubated overnight at 4°C with rabbit anti-Estrogen receptor (1:500, Cat# P2300Rb, ElAab, USA) or rabbit anti-progesterone receptor (1:500, Cat# SAB5600247, Sigma-Aldrich, USA). The next day, secondary antibody (1:1000, Cat# A3812, Sigma-Aldrich, USA) was added and incubated at 37°C for 20 min. The images were developed using DAB (Lot number: D5637, Sigma-Aldrich, USA) and counterstained with hematoxylin (Lot number: H9627, Sigma-Aldrich, USA). The sections were observed under an optical microscope. Quantitative analysis of ER and PR expressions was based on staining intensity and percentage of positive cells: 0 (blue), 1 (light yellow), 2 (brown yellow), and 3 (brown). The positive cells under the microscope were counted, with 0 points for <5% (negative), 1 point for 6%-15%, 2 points for 16%-30%, and 3 points for >31%. The final score for each section was the sum of the intensity and percentage scores.

Detection of hemorheological indicators

To evaluate hemorheological parameters, 3 ml of blood was drawn from the abdominal aorta into a test tube containing heparin as anticoagulant. The plasma viscosity and hematocrit value were measured using an LBY-N6 Compact fully automated blood rheometer (Beijing Precil Instrument Co., LTD.), and the whole blood viscosity was measured at low, medium, and high shear rates, respectively.

Flow cytometry

For flow cytometry analysis, 100 μL of anticoagulant venous blood was placed in a flow cytometry tube. According to the instructions in the CD45/CD4/CD8/CD3 detective Kits (Lot number: V273698, Beckman Coulter, USA), 5 μL of anti-mouse CD3, CD4 or CD8 antibodies was added, respectively. The mixture was incubated in the dark for 15 minutes. Then, 2 mL of red blood cell lysate was added. After mixing well, it was incubated for 10 minutes followed by centrifugation. Afterward, 2 mL of PBS buffer was added, the tube was shaken well, and centrifugation was repeated to remove the supernatant. The cell pellet was then resuspended in 0.5 mL of PBS. The percentages of CD_3^+, CD_4^+, and CD_8^+ cells were finally quantified using flow cytometry.

ELISA

The serum of mice from different groups was collected through centrifugation at 3500 rpm for 10 minutes. The levels of tumor necrosis factor-α (TNF-α) (Lot number: PT512, Shanghai Beyotime Biotech. Inc., China), interleukin-2 (IL-2) (Lot number: PI575, Shanghai Beyotime Biotech. Inc., China), nitricoxidesynthase (NOS) (Lot number: ZY1235EM, Shanghai Zeye Biotech. Inc., China) and vascular endothelial growth factor (VEGF) (Lot number: PV957, Shanghai Beyotime Biotech. Inc., China) in serum were detected by Enzyme-linked immunosorbent assay (ELISA) according to the protocols of the manufacturer. The optical density was examined at 450 nm by the microplate reader.

Statistical analysis

All data in this study were analyzed with SPSS 22.0. The measurement data were presented by mean ± standard deviation ($\bar{x} \pm SD$), and the independent sample t-test was used for comparison between the two groups. Repeated measures ANOVA or one-way ANOVA followed by post hoc Bonferroni test was performed for analysis among multiple groups. The count data was presented as percentages/cases. The comparison among groups was performed using χ^2 test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Comparison of body weight, tumor weight and tumor volume among different groups

The body weight of mice showed a descending trend after the transplantation of uterine myoma, except for the EBN group, where a temporary weight gain was observed at 4 weeks after the uterine myoma transplantation, as seen in **Figure 1A**. In contrast to the model group, the uterine tumor weight of the ER

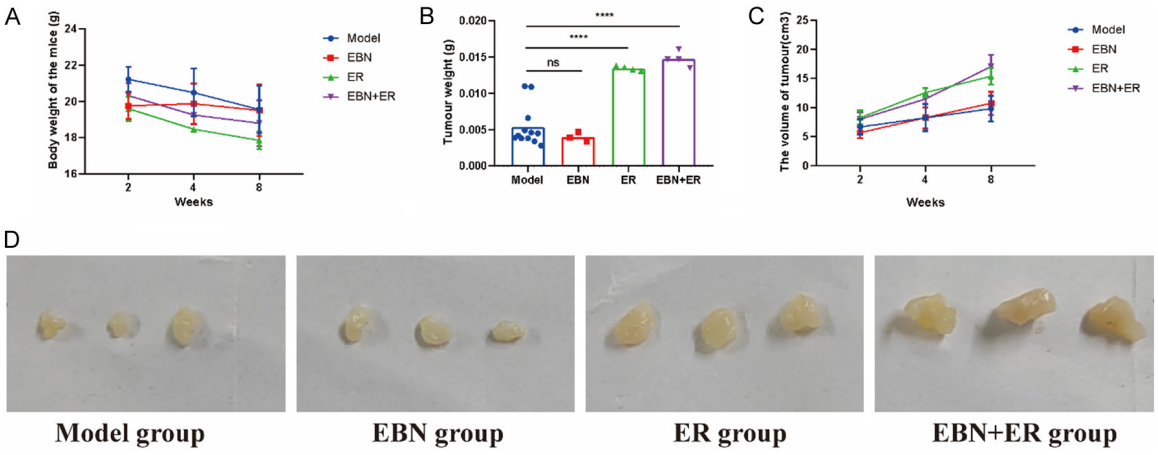

Figure 1. The comparison of body weight, as well as weight and volume of tumors among model group, EBN group, ER group and EBN+ER group. ****P<0.001 vs model group. A: Body weight of the mice at the different time points in different groups. B: Tumor weight in the different groups. C: The volume of tumor at the different time points in different groups. D: The observation for size of tumor in different groups. Note: EBN: Edible bird's nest; ER: Estradiol receptor.

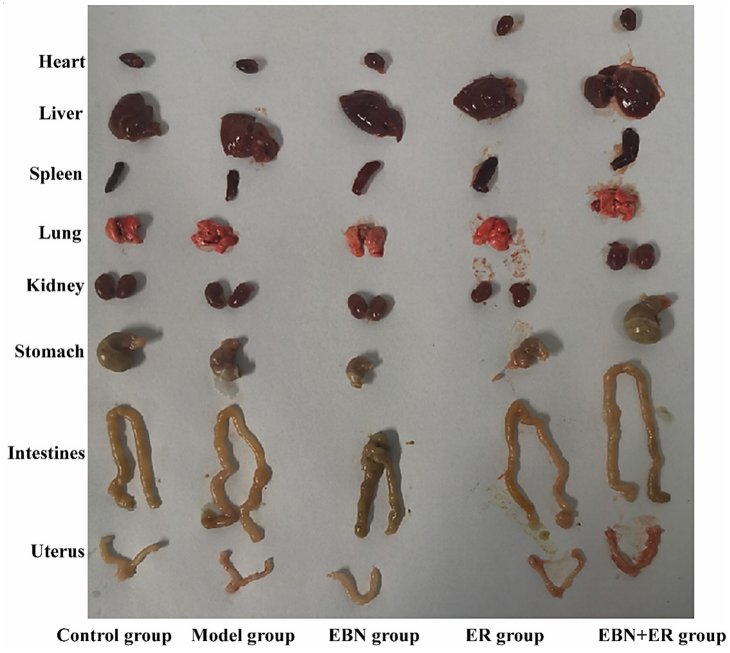

Figure 2. The observation of various organs from mice in the five groups. Note: EBN: Edible bird's nest; ER: Estradiol receptor.

group and EBN+ER group was significantly increased (all P<0.001), while there was no significant difference for tumor weight between the model group and EBN group, as shown in **Figure 1B**.

As seen in **Figure 1C** and **1D**, after the transplantation of uterine fibroids, each group showed a certain growth trend over time. However, the growth rate of tumors from the model group and the EBN group were slow, and the tumor volume was relatively small. In contrast, the uterine fibroids from the ER group and the ER+EBN group had a faster growth rate and large tumor volume. At 8 weeks after the transplantation of uterine fibroids, there was no statistical difference in tumor volume between the model group and EBN group, while the tumor volume of the ER group and ER+EBN group were significantly larger than that of the model group (all P<0.05).

Comparison of morphology of different organs among various groups

At 8 weeks after transplantation, the examination of various organs (heart, liver, spleen, lungs, kidneys, stomach, intestines, and uterus) revealed no significant differences in morphology among the different groups, as indicated in **Figure 2**.

Comparison of HE staining of uterine myoma tissues among different groups

As shown in **Figure 3A**, HE staining revealed proliferation, thickening and significant infiltration of inflammatory cells in uterine fibroids of each group. The changes observed in the EBN

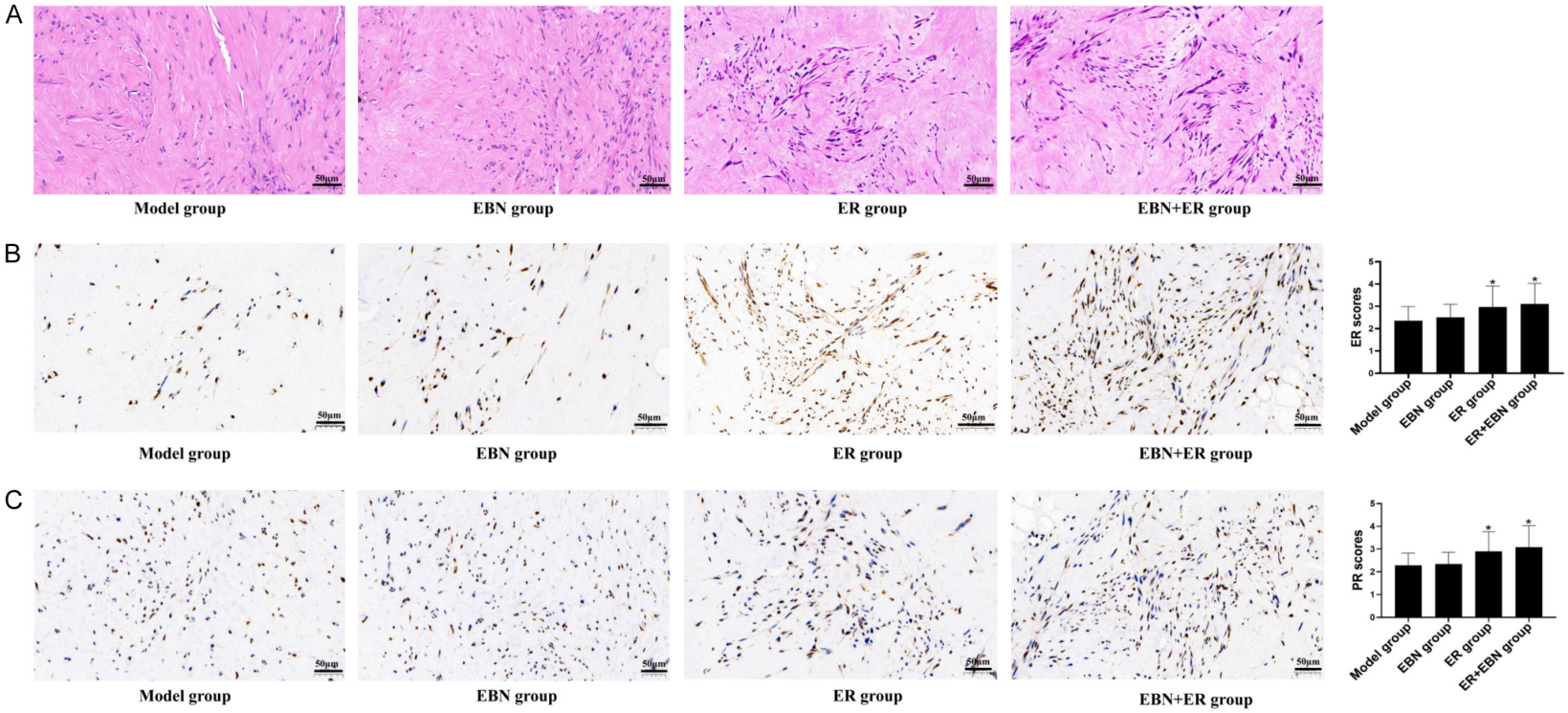

Figure 3. HE staining of uterine myoma tissues and immunohistochemistry detection of ER and PR expression. *P<0.05 vs model group. A: HE staining of uterine myoma tissues. B: Estrogen receptor (ER) expression. C: Progesterone receptor (PR) expression. Note: EBN: Edible bird's nest; HE: Hematoxylin-eosin.

Table 1. Comparison of hemorheology across different groups

Groups	Hematocrit value (%)	Plasma viscosity (mPa·s)	Whole blood viscosity (mPa·s)		
			Low shear	Middle shear	High Shear
Model group	55.81±4.12	1.53±0.06	27.90±3.19	7.75±0.39	5.86±0.44
EBN group	46.38±2.35*	1.35±0.05*	24.38±2.87*	6.17±0.42*	5.17±0.39*
ER group	60.22±3.18*	1.71±0.07*	31.16±3.36*	8.92±0.51*	6.42±0.47*
ER+EBN group	56.45±3.46Δ	1.62±0.05Δ	28.25±2.90Δ	7.98±0.58Δ	5.91±0.40Δ

Note: *P<0.05 vs Model group; ΔP<0.05 vs ER group. EBN: Edible bird's nest; ER: Estradiol receptor.

group were similar to those in the model group, whereas the alterations in the ER group and ER+EBN group were obviously more severe than those in the model group.

Comparison of ER and PR expression among different groups

As seen in **Figure 3B** and **3C**, the ER and PR expression in the model group were similar with those in the EBN group, both displaying relatively low expression of ER and PR. In contrast, ER and PR expression in the ER group and ER+EBN group were significantly higher than those in the model group, respectively (all P<0.05). In addition, there were no significant differences in ER and PR expression between the ER group and ER+EBN group.

Comparison of hemorheological indicators among groups

Compared to the model group, the hematocrit value, plasma viscosity and whole blood viscosity of the EBN group were significantly decreased, while those in the ER group were obviously increased (all P<0.05). Compared with the ER group, the hematocrit value, plasma viscosity and whole blood viscosity of the ER+EBN group were remarkably reduced (all P<0.05), as seen in **Table 1**.

Comparison of T cell subsets among different groups

As shown in **Figure 4**, compared to the model group, the proportions of CD_3^+ and CD_4^+ cells in the EBN group significantly increased, while the proportions of CD_8^+ cells obviously decreased (all P<0.05). The CD_4^+/CD_8^+ cell ratio in the EBN group were significantly higher than that in the model group (P<0.05). Conversely, the proportions of CD_3^+ and CD_4^+ cells, as well as the CD_4^+/CD_8^+ ratio in the ER group were significantly lower than those in the model group, while the proportion of CD_8^+ cell in the ER group was significantly higher than that in the model group (all P<0.05). In addition, compared with the ER group, the proportions of CD_3^+ and CD_4^+ cells, as well as the ration of CD_4^+ to CD_8^+ in the ER+EBN group were remarkably increased and the proportion of CD_8^+ cells was obviously lower (all P<0.05).

Comparison of TNF-α and IL-2 levels among different groups

As seen in **Figure 5**, compared with the model group, the level of TNF-α in the EBN group was significantly reduced, while the level of IL-2 in the EBN group was obviously increased (all P<0.05). In contrast, compared to the model group, the level of TNF-α in the ER group was significantly increased, while the level of IL-2 in the ER group was remarkably reduced (all P<0.05). Moreover, the level of TNF-α in the ER+EBN group was significantly lower than that in the ER group, while the level of IL-2 in the ER+EBN group was higher than that in the ER group (all P<0.05).

Comparison of NOS and VEGF levels among different groups

Compared to the model group, the NOS and VEGF levels in the EBN group were significantly decreased, while the NOS and VEGF levels of the ER group were obviously increased (all P<0.05). Moreover, in contrast to the ER group, the NOS and VEGF levels in the ER+EBN group were remarkably decreased (all P<0.05), as seen in **Figure 6**.

Discussion

In recent years, the incidence of uterine fibroids has been increasing annually, moreover, increasingly affecting younger women and significantly impacting their physical and mental health. The specific cause of uterine fibroids is not fully understood yet. However, they are widely recognized as a sex hormone-dependent tumor disease. The development of uterine

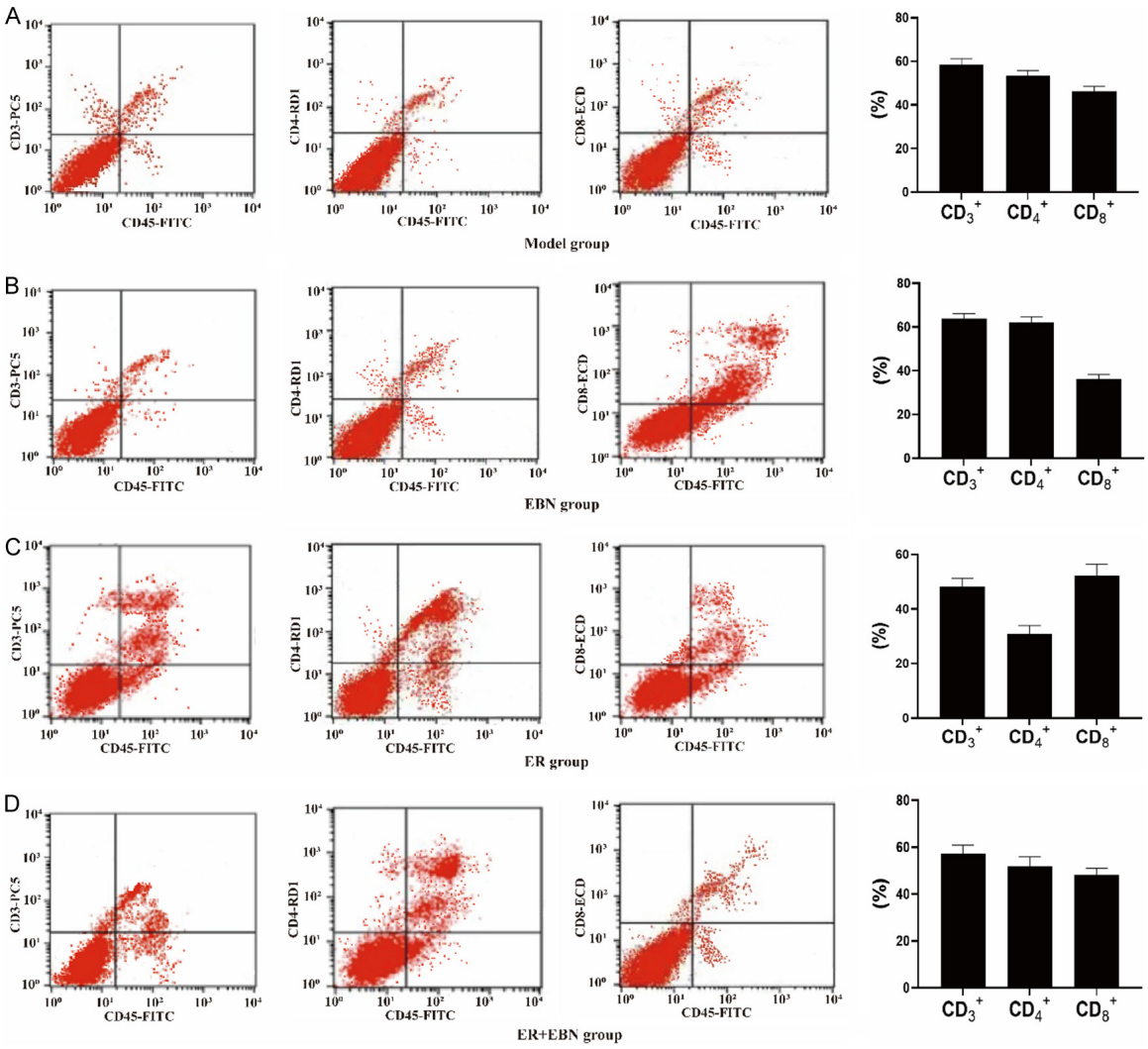

Figure 4. The proportion of CD_3^+, CD_4^+, CD_8^+ cells was detected by flow cytometry. A: The proportion of CD_3^+, CD_4^+, CD_8^+ cells in model group. B: The proportion of CD_3^+, CD_4^+, CD_8^+ cells in EBN group. C: The proportion of CD_3^+, CD_4^+, CD_8^+ cells in ER group. D: The proportion of CD_3^+, CD_4^+, CD_8^+ cells in ER+EBN group. Note: EBN: Edible bird's nest; ER: Estradiol receptor.

fibroids involves a complex biological process with multiple interacting factors and stages [17]. This includes changes in the local microenvironment, mediated by a variety of elements such as growth factors and sex hormones [18]. Research indicates that estrogen could promote angiogenesis, which is crucial for the growth and development of uterine fibroids, and during their progression, estrogen receptors in uterine fibroid tissues are usually excessively increased and expressed [19]. Furthermore, the most active form of estradiol in serum levels tends to enhance estrogenic activity. This increase is supported by the endogenous production of estrogen, which occurs through the biosynthesis of compounds like dehydroepiandrosterone, androstenediol, 27-hydroxycholesterol, and androstenedione [20, 21], and other metabolites with estrogen-like activity have also been reported [22], underscoring the similarity of their effects to those of ovarian estrogen.

In this study, we investigated the effects of EBN on the growth of uterine fibroids by monitoring the changes in body weight, weight and volume of uterine fibroid in mice. The results showed that the growth of uterine fibroids was closely related to estrogen levels in mice, which is in accordance with previous studies [23].

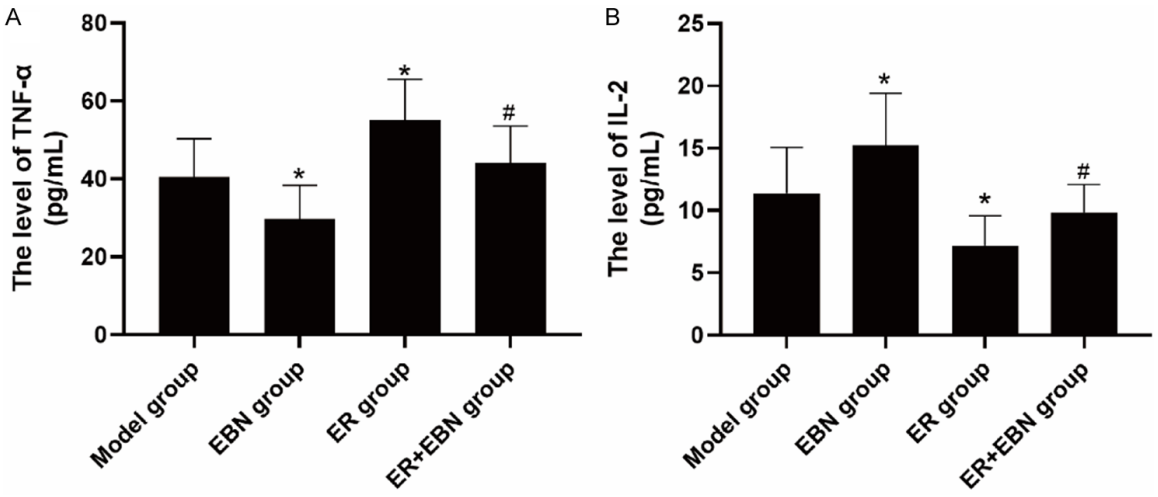

Figure 5. Comparison of inflammatory factors among different groups. A: The level of TNF-α. B: The level of IL-2. *P<0.05 vs model group; #P<0.05 vs ER group. Note: TNF-α: tumor necrosis factor-α; IL-2: interleukin-2; EBN: Edible bird's nest; ER: Estradiol receptor.

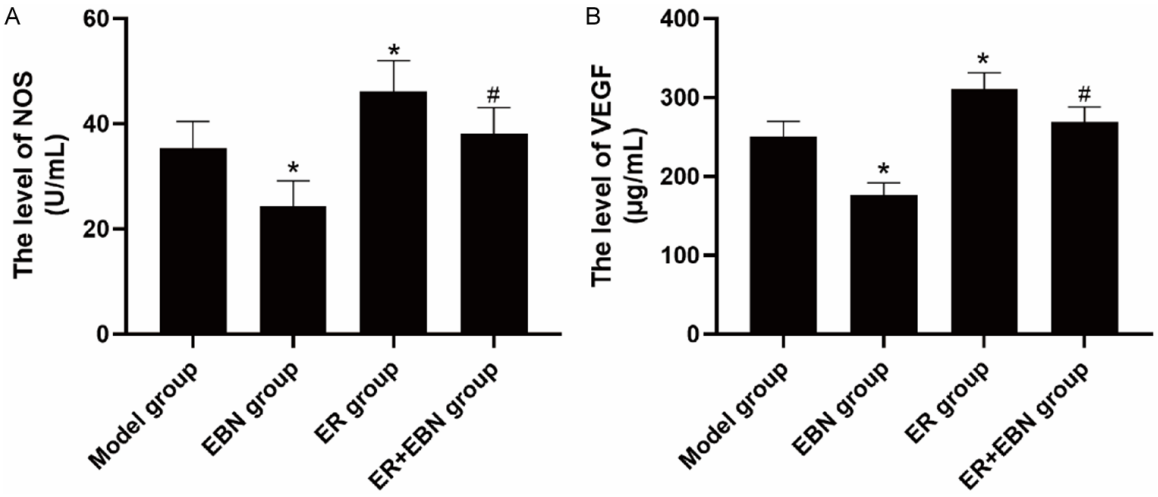

Figure 6. Comparison of the levels of NOS and VEGF among different groups. A: The level of NOS. B: The level of VEGF. *P<0.05 vs model group; #P<0.05 vs ER group. Note: NOS: nitricoxidesynthase; VEGF: vascular endothelial growth factor; EBN: Edible bird's nest; ER: Estradiol receptor.

However, there was no significant growth of uterine fibroids in the EBN group, indicating EBN did not promote the growth of uterine fibroid. However, these results were different from the report by Hou et al. [24], who reported increased estrogen levels in ovariectomized rats that received EBN, attributed to enhanced biosynthesis from non-ovarian sources. The discrepancy could stem from different experimental subjects regarding body weight, all groups experienced a decline, likely due to the progressive deterioration in physical fitness following uterine fibroid transplantation. However, the body weight of mice in EBN group was slightly increased at the fourth week after uterine fibroids transplantation, which might be attributed to the nutritional benefits of EBN. This suggests that while individual differences were notable, they could serve as a reference for further studies. Further in-depth research is needed to explain the specific mechanism. However, the results suggest that EBN does not promote fibroid growth, potentially providing a reliable basis for refining the guidelines for its use and targeting its consumer base more accurately.

Estrogen and progesterone exert their effects through their receptors, ER (Estrogen Receptor) and PR (Progesterone Receptor). Upon binding, estrogen and progesterone, to ER and PR regulate gene expression after binding to DNA. Therefore, the effects of estrogen and progesterone are highly dependent on the presence of the ER and PR. Increased serum levels of estrogen and progesterone typically lead to a positive feedback increase in their receptors. Xia et al. found that the expression levels of ER and PR in human uterine myoma smooth muscle cells were significantly influenced in a time- and dose-dependent manner [25]. Pier et al. further confirmed that the ER and PR proteins in patients with uterine fibroids were significantly increased, suggesting that high expression levels of ER and PR could provide references for clinical diagnosis and treatment of uterine fibroids [26]. Consistent with these findings, our study also reported similar results. Immunohistochemistry analysis revealed that in contrast to the model group, ER and PR expression in uterine fibroids from the ER group were significantly increased, while there were no statistical differences between the EBN group and model group. This indicates that EBN doesn't affect ER and PR expression in uterine fibroids.

Previous study revealed that patients with uterine fibroids had higher blood viscosity, plasma viscosity, hematocrit, red blood cell aggregation indices compared to normal individuals [27]. Study has also demonstrated that blood in patients with uterine fibroids tends to be in a more viscous, less fluid state, indicative of blood stasis [28]. Moreover, the involvement of NOS and the VEGF pathway in tumor angiogenesis, and the association of TNF-α and IL-2 levels with the development of uterine fibroids have also been documented [29-31]. In this study, the findings corroborate these observations, showing that hemorheological indicators and levels of TNF-α, NOS, and VEGF were significantly lower in the EBN group compared to the model group. Additionally, compared to the ER group, these indicators were markedly reduced in the ER+EBN group. This suggests that EBN could reduce the blood viscosity of uterine fibroids patients with blood stasis syndrome, which was similar with other reports [32, 33]. However, the exact mechanism is not clear yet.

Increasing evidence suggests that EBN can regulate immune function. Fan et al. found that EBN significantly enhanced the activation of CD_3^+ T cells in a mouse model [7]. Beyond its immunomodulatory properties, EBN has also demonstrated immune-enhancing capabilities through its antiviral activity [34]. Moreover, EBN plays a significant role in maintaining fetal-maternal immune homeostasis during pregnancy, underlining its broad-spectrum immunological impacts [35]. Immune functions are closely associated with the development of uterine fibroids. In this study, it was found that EBN could significantly increase the levels of CD_3^+ and CD_4^+ cells, and the CD_4^+/CD_8^+ ratio, while decrease the level of CD_8^+ cells, compared with model group. The immune functions in the ER+EBN group were significantly improved in contrast to the ER group. These findings are consistent with previous research [33] and underscore EBN's potential as a therapeutic agent in enhancing immune responses.

The present study, while insightful, is subject to several limitations that should be noted. First, the composition of edible bird's nest obtained from different regions varies, resulting in the limitation in generalizability of current results. Second, a single dose of EBN was investigated in this study, so it is unknown whether the effects of EBN on uterine fibroids are concentration dependent. Third, methodologies employed were relatively basic; more sophisticated techniques are needed to elucidate the precise mechanisms by which EBN influences uterine fibroids. Fourth, This study only uses fresh stewed bird's nest at 95°C for the experiment, and it is uncertain whether bird's nest with different stewing processes would affect uterine fibroids.

In summary, EBN does not promote the growth of uterine fibroids and may significantly influence hemorheology and enhance immune functions in mice with transplanted uterine fibroids. These findings suggest the potential evidence for the oral administration of EBN in patients with uterine fibroids. However, further research involving multicenter trials and human subjects is essential to validate these results and fully understand the therapeutic potential and limitations of EBN.

Disclosure of conflict of interest

None.

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